Inhibition of Acylcoenzyme A:Cholesterol Acyltransferase Activity by PD128042: Effect on Cholesterol Metabolism and Secretion in CaCo-2 Cells

F. Jeffrey Field*, Ella Albright and Satya Mathur
Department of Internal Medicine, University of Iowa, Iowa City, Iowa 52242

The regulation of cholesterol uptake and secretion by acylcoenzyme A:cholesterol acyltransferase (ACAT) was investigated in the human intestinal cell line, CaCo-2. A new ACAT inhibitor, PD128042 (CI-976), was first characterized. The addition of the fatty acid anilide to membranes prepared from CaCo-2 cells inhibited ACAT activity without altering the activities of HMG-CoA reductase, fatty acid Co-A hydrolase, or triglyceride synthetase. PD128042 was a competitive inhibitor of ACAT with 50% inhibition occurring at a concentration of 0.2 μg/mL. When added to the medium of CaCo-2 cells at a concentration of 5 μg/mL, PD128042 inhibited oleate incorporation into cholesteryl oleate by 92% and increased oleate incorporation into triglycerides and phospholipids by 51% and 38%, respectively. After incubating CaCo-2 cells with the ACAT inhibitor, the rate of newly synthesized cholesterol decreased by 75% and membranes prepared from these cells contained significantly less HMG-CoA reductase activity. PD128042 significantly decreased the basolateral secretion of newly synthesized cholesteryl esters without affecting the secretion of newly synthesized triglycerides or phospholipids. The inhibitor decreased the esterification of labeled exogenous cholesterol which was taken up by the cell from bile salt micelles. Moreover, after 16 hr of ACAT inhibition, less labeled unesterified micellar cholesterol was associated with the cell. The esterification of cholesterol in CaCo-2 cells plays an integral role in the uptake of cholesterol through the apical membrane and its eventual secretion at the basolateral membrane.
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The esterification of cholesterol by the small intestinal absorptive cell is believed to be an important step in the transport of cholesterol from the lumen to its eventual secretion into the lymphatics as part of a triglyceride-rich lipoprotein particle (1). The intracellular enzyme which catalyzes this reaction is acylcoenzyme A:cholesterol acyltransferase (ACAT) (2). This enzyme has been found in the intestines of many animal models, including humans (3-6). It is an enzyme which is regulated by a variety of factors including dietary cholesterol and fats, bile salts, hormones, such as progesterone and thyroxine, oxygenated sterols, and changes in membrane fatty acid saturation (3,4,7-10).

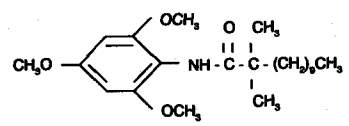
Previous studies have demonstrated a decrease in cholesterol absorption after inhibiting ACAT activity in the small intestine. Bennett-Clark and Tercyak (11) used Sandoz compound 58-035 and demonstrated that less cholesteryl esters were secreted into the lymph of lymph-fistula rats, an observation which is consistent with the inhibition of ACAT activity. Heider, Pickens, and Kelly (12) showed in cholesterol-fed rabbits that cholesterol absorption could be inhibited by 46% utilizing a related ACAT inhibitor, 57-118. Another ACAT inhibitor, DL-melinamide, was used by Fukushima *et al.* (13) and Natori *et al.* (14). These investigators observed a significant decrease in the transport of cholesterol and particularly cholesteryl esters into the lymph of lymph-fistula rats following the administration of DL-melinamide. The data suggest that intestinal ACAT has a regulatory role in the absorption of cholesterol.

The present study was undertaken to investigate the effect of ACAT inhibition on the regulation of cholesteryl ester transport in CaCo-2 cells. A new ACAT inhibitor, PD128042 (CI-976, Parke-Davis Pharmaceutical Research Division, Warner-Lambert Co., Ann Arbor, MI), a fatty acid trimethoxyanilide (see Scheme 1), whose synthesis has been recently described (15), was used in this study. The present results suggest that the secretion of newly synthesized cholesteryl esters is decreased following the inhibition of ACAT. Moreover, after chronic inhibition of ACAT activity, less unesterified cholesterol is taken up from bile salt micelles at the apical membrane.

MATERIALS AND METHODS

Materials. [9,10-^{3}H]Oleic acid, [2-^{14}C]acetate, [4-^{14}C]cholesterol, [1-^{14}C]oleoyl Coenzyme A, [5-^{3}H]mevalonic acid, 3-hydroxy-3-methyl [3-^{14}C]glutaryl Coenzyme A, and [1,2-^{3}H]cholesterol were purchased from New England Nuclear (Boston, MA). Oleic acid, oleoyl coenzyme A, glucose-6-phosphate, glucose-6-phosphate dehydrogenase and nucleotide adenine diphosphate were purchased from Sigma (St. Louis, MO). HMG-CoA was from P-L Biochemicals, Inc. (Milwaukee, WI). PD128042 was a generous gift from Brian Krause, Parke-Davis Pharmaceutical Research Division, Warner-Lambert Co. All other chemicals were reagent grade.

Cell culture. Cells were grown in T-75 plastic flasks



SCHEME 1. Structure of PD128042.

*To whom correspondence should be addressed.

Abbreviations: ACAT, acylcoenzyme A:cholesterol acyltransferase; DMEM, Dulbecco's modified Eagle minimum essential medium.

(Corning Glassworks, Corning, NY) in Dulbecco's modified Eagle minimum essential medium (DMEM, Gibco, Grand Island, NY) containing 4.5 g/L glucose and 1% nonessential amino acids, supplemented with 20% fetal calf serum (Hyclone Laboratories, Inc., Logan, UT), 4 mM glutamine, 10 μg/mL insulin, 50 μg/mL penicillin, and 50 μg/mL streptomycin. Prior to confluency, the cells were split according to the method of Mohrmann *et al.* (16). Monolayers were grown on 60 mm plastic Petri dishes in 5 mL of supplemented DMEM. Fresh medium was added every 2 days. Cultures were used only after dome formation, usually 11–13 days after plating. Experiments were performed in medium-199/Earle's (Gibco) containing 1 mM HEPES, pH 7.4. Since the amount of cellular protein per dish did not vary, 5.50 ± 0.03 mg/dish, n=12, the data are expressed as per dish. For experiments performed on micropore filters, CaCo-2 cells were aliquoted on fibronectin-treated micropore polycarbonate filters (Transwells-3412; 0.4 μm pore size; Costar, Cambridge, MA) separating an upper and lower well. Medium in the upper (1.5 mL) and lower (2.6 mL) wells was changed every other day. The cells were used 10 or 11 days after plating and by this time had formed tight monolayers as assessed by electron microscopy. Tight junctions were also present as demonstrated by freeze fracture studies. To estimate monolayer "tightness," transport of albumin from the top well to the bottom well was measured as described by Shasby and Shasby (17). The cells were not used if more than 2.0% of the albumin was found in the lower well after 60 min. Cell viability was monitored by trypan blue exclusion, cell protein, and light microscopy. PD128042 had no significant effect on CaCo-2 cell viability at any of the concentrations tested.

Measurement of lipid synthesis. The rates of cholesteryl ester, triglyceride, and phospholipid synthesis were estimated by oleic acid incorporation into the respective lipid class as previously described (10). Cholesterol synthesis was measured by [^{14}C]acetate incorporation into unesterified and esterified cholesterol as described (18).

In experiments using the transwells, medium containing fetal bovine serum was removed from the bottom well and was replaced with M199 containing 10 mM HEPES, without fetal bovine serum, pH 7.4. The medium from the top well was also removed and the cells were washed five times with 1 mL of M199 buffer. PD128042, at the concentrations indicated, was added to 1.5 mL of M199 in 0.01 mL of dimethylsulfoxide. After incubation with the ACAT inhibitor, the medium was removed and replaced by M199 containing 1 mM taurocholate, 50 μM cholesterol, and 25 μM [^{3}H]oleic acid (spec act, 30,000 dpm/nmol). After six hr, media from the upper and lower wells were removed and centrifuged at 2,000 rpm for 15 min to remove any intact cells or debris. The supernatants from the media were extracted with chloroform/methanol (2:1, v/v). The chloroform phase was taken to dryness under a stream of nitrogen. The residue was taken up in 0.125 mL of chloroform and applied to Silica Gel G thin-layer plates. The plates were developed with hexane/diethyl ether/methanol/acetic acid (70:30:1:1, by vol) and the lipids were visualized with iodine vapor. The bands corresponding to cholesteryl ester, triglycerides, and phospholipids were scraped into 4 mL of liquid scintillation fluid and counted as described previously (10). The cells on the micropore filter were washed three times with 1.0 mL of ice-cold phosphate-buffer saline. The cells were scraped carefully from the filters in 1.0 mL of 0.2 N NaOH. After neutralizing the alkali with HCl, lipids were extracted from the cell digests and separated as described above.

Cholesterol uptake and esterification. Cells were cultured on plastic dishes and micropore filters as described above. After a preincubation with or without PD128042, 50 μM [^{14}C]cholesterol (spec act, 5,000 dpm/nmol), solubilized in M199 containing 1 mM taurocholate and 30 μM monoolein was added to the apical medium. At the end of six hr, cells were analyzed as described above except that bands corresponding to unesterified and esterified cholesterol were scraped from the plates and counted.

Enzyme assays. Acylcoenzyme A:cholesterol acyltransferase activity was measured in a total volume of 0.2 mL containing 0.100–0.150 mg of membrane protein, 0.5 mg of fatty acid-poor bovine serum albumin, and 8.6 nmol of [^{14}C]oleoylCoA (spec act, 23,000 dpm/nmol). The assay mixture was incubated at 37°C for 5 min before the addition of the substrate to start the reaction. The reaction was stopped after 5 min with the addition of chloroform/methanol (2:1, v/v). [^{3}H]Cholesteryl oleate was added as an internal standard and the samples were processed as previously described (19). In one experiment, cholesterol, solubilized in egg phosphatidylcholine (2:1, mol/mol) was added to the assays prior to determining ACAT activity (9). 3-Hydroxy-3-methylglutaryl coenzyme A reductase activity was measured in a total volume of 0.170 mL containing 0.100–0.150 mg of protein, 25 mM glucose-6-phosphate, 3 mM NADP, 70 mM NaCl, 0.174 units of glucose-6-phosphate dehydrogenase, and 15 mM dithiothreitol. The remaining volume was made up by buffer containing 0.04 M KH_2PO_4, 0.05 M KCl, 0.25 M sucrose, and 0.03 M EDTA, pH 7.2. The mixture was incubated for 10 min before starting the assay with 15 nmol of [^{14}C]HMG-CoA (spec act, 22,500 dpm/nmol). Assays were stopped after 20 min by the addition of 0.025 mL of concentrated HCl. D,L-[5-^{3}H]mevalonic acid (20,000 dpm) was added as an internal standard and the assays were processed as previously described (20). Fatty acylcoenzyme A hydrolase and triglyceride synthetase activity were determined as described for the ACAT assay except bands corresponding to free fatty acids and triglycerides were scraped from the plates and counted. Activities were measured in total membranes which were prepared by centrifuging the cellular whole homogenates at 100,000 × *g* for 1 hr. The resulting membrane pellets were resuspended in the sucrose buffer described above and recentrifuged at 100,000 × *g*. The total membrane pellets were diluted with buffer and used immediately for the assays (21).

Chemical and statistical analysis. Protein was determined according to the method of Lowry *et al.* (22), and the unpaired Student's *t*-test was used to determine significance.

RESULTS

Effect of PD128042 on the activities of HMG-CoA reductase, fatty acyl-CoA hydrolase, triglyceride synthetase, and ACAT in CaCo-2 cell membranes. To determine the specificity of PD128042, total membranes were prepared from CaCo-2 cells and incubated with increasing concentrations of the inhibitor solubilized in dimethylsulfoxide. Control assays contained the same amount of dimethylsulfoxide but without the inhibitor. After 15 min, the respective assays were performed as described in Materials and Methods. The activities which were determined in control assays represented 100% and were as follows: HMG-CoA reductase, 1.31 nmol/mg/30 min; fatty acyl Co-A hydrolase, 6.17 nmol/mg/5 min; triglyceride synthetase, 1.70 nmol/mg/5 min; and ACAT, 139 pmol/mg/5 min.

As shown in Figure 1, PD128042 inhibited the activity of ACAT only. Its effect was dose-dependent with 50% of the activity remaining at a concentration of 0.2 μg/mL of PD128042. Maximal inhibition occurred between 2 and 5 μg/mL. Activities of HMG-CoA reductase, triglyceride synthetase, and fatty acyl CoA-hydrolase were not affected by PD128042 even at the highest concentration used (10 μg/mL).

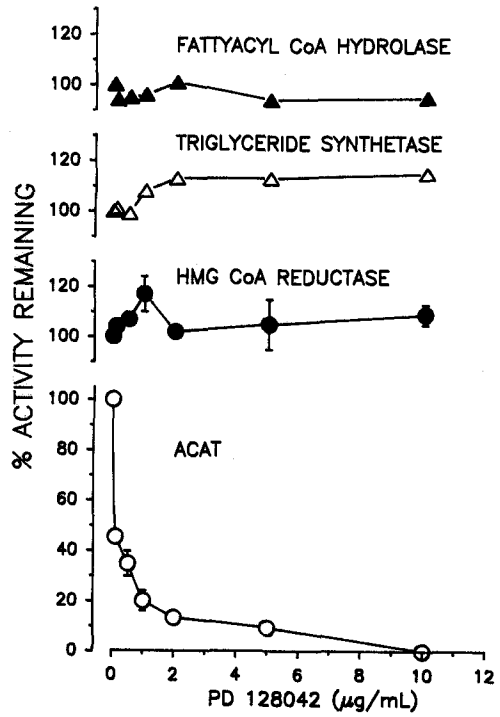


FIG. 1. Effect of PD128042 on the activities of HMG-CoA reductase, fatty acyl Co-A hydrolase, triglyceride synthetase, and ACAT. Total membranes were prepared from CaCo-2 cells as described in Materials and Methods. The membranes, 100-150 μg protein, were incubated for 15 min with PD128042 solubilized in 0.01 mL of dimethylsulfoxide at the concentrations shown. Control assays contained the same amount of dimethylsulfoxide but without the inhibitor. The assays were initiated by adding the respective labeled substrates. Control assays were designated as 100% and the values are given in the text. The points represent the mean ± SE of three to six assays performed at each concentration of PD128042.

Effect of PD128042 on ACAT activity: Competition by fatty acyl-CoA and cholesterol. To determine if the inhibition of ACAT activity by PD128042 was competitive, the inhibitor was added to CaCo-2 cell membranes at a concentration of 0.2 μg/mL. ACAT activity was then measured at increasing concentrations of its fatty acyl-CoA substrate. In each assay, the ratio of oleoyl coenzyme A to bovine serum albumin was kept constant. Figure 2 shows these results. In this experiment, 0.2 μg/mL of PD128042 inhibited ACAT activity more than what was observed in the previous experiment. In the insert, the double reciprocal plot of the data is shown. The results of this experiment suggest that PD128042 is a competitive inhibitor of ACAT activity.

To investigate the effects of cholesterol on the inhibition of ACAT activity by PD128042, CaCo-2 cell membranes were incubated for 1 hr with liposomes containing egg phosphatidylcholine and increasing concentrations of cholesterol (cholesterol/phospholipid ratio of 2:1, mol/mol). PD128042, 0.2 μg/mL, was added and the mixture was incubated for a further 30 min. ACAT activity was then measured by adding labeled oleoyl CoA. The results in Figure 3 show that in control membranes without PD128042, ACAT activity progressively increased as more exogenous cholesterol was added to the assay. At 100 μM cholesterol, ACAT activity increased by 66%. In contrast, in membranes incubated with PD128042, ACAT activity did not increase with the addition of increasing concentrations of exogenous cholesterol.

Effect of PD128042 on the incorporation of [^{3}H]*oleate into cellular lipids.* CaCo-2 cells were preincubated for 2 hr with increasing concentrations of PD128042. At the end of this time, the medium containing the in-

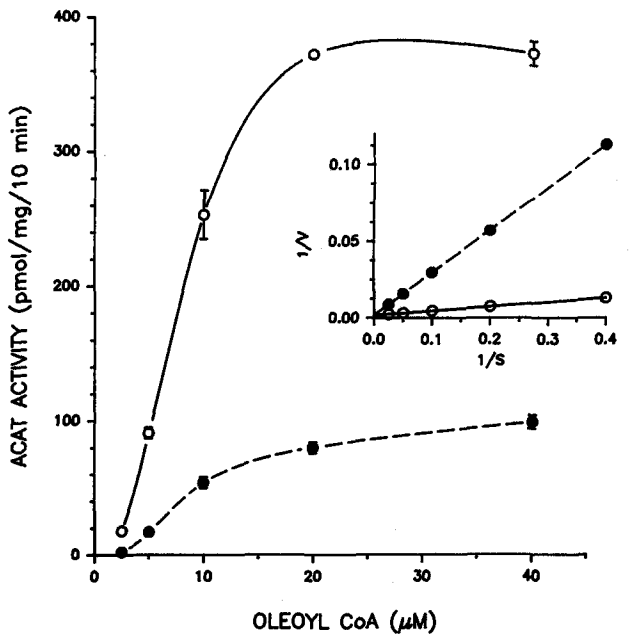


FIG. 2. Effect of PD128042 on membrane ACAT activity. The membranes were incubated for 15 min with or without 0.2 μg/mL of PD128042. The reaction was started by adding labeled oleoyl CoA at the concentrations shown. The albumin to substrate ratio was kept constant. The points represent the mean of duplicate or triplicate assays at each concentration. ○, Control; ●, PD128042.

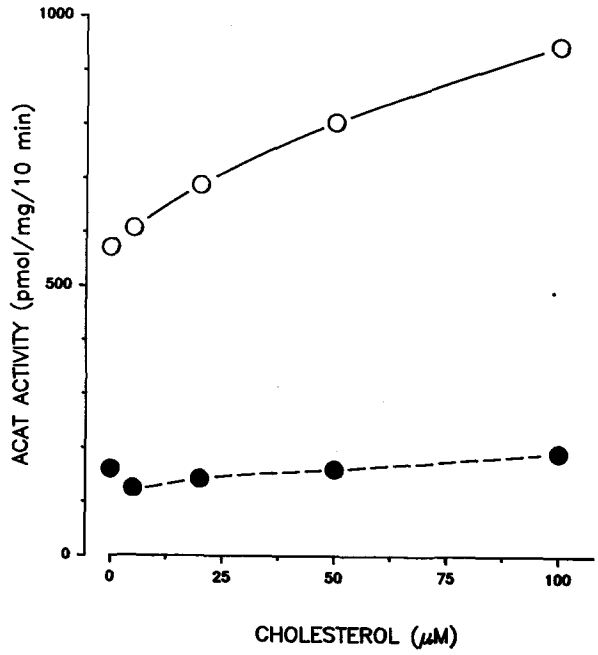


FIG. 3. Effect of PD128042 on membrane ACAT activity. CaCo-2 cell membranes were incubated for 1 hr with liposomes containing egg phosphatidylcholine and increasing concentrations of cholesterol (cholesterol/PC, 2:1, mol/mol). PD128042, 0.2 μg/mL, was added and 30 min later ACAT activity was determined. The points represent the mean ± SE of triplicate assays. ○, Control; ●, PD128042.

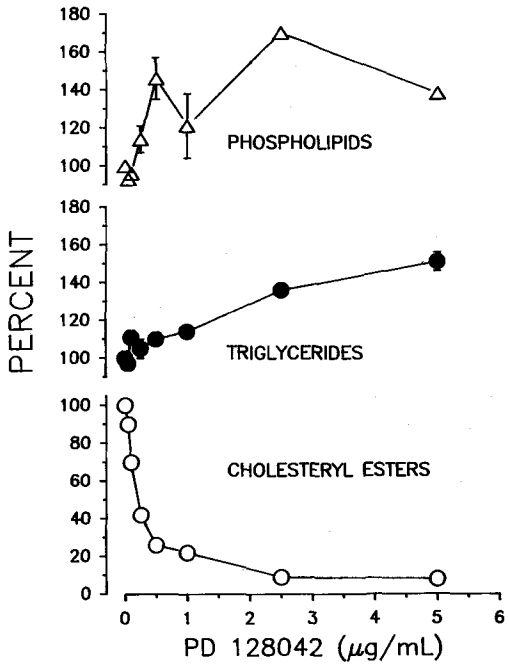


FIG. 4. Effect of PD128042 on [^{3}H]oleate incorporation into cellular lipids. PD128042 was added in dimethylsulfoxide (0.01 mL) to CaCo-2 cells cultured on plastic dishes at the concentrations shown. Control dishes received the vehicle alone. After 2 hr, the medium containing the inhibitor was removed and labeled oleate, 50 μM (spec act, 13,500 dpm/nmol), attached to albumin (3:1, mol/mol) was added for 30 min. The cells were washed with M199 and scraped from the dishes. Lipids were extracted and separated as described in Materials and Methods. Control values were designated as 100% and are given in the text. The values represent the mean ± SE of three dishes at each concentration.

hibitor was removed and the monolayer was washed three times with 1 mL of M199. Labeled oleate attached to albumin, 50 μM, was added and thirty min later, the lipids were extracted from the cell digests and separated (Fig. 4). Control cells, which were incubated with the vehicle alone, incorporated [^{3}H]oleate into cholesteryl esters, triglycerides, and phospholipids at rates of 74 pmol/30 min, 3.40 nmol/30 min, and 2.55 nmol/30 min, respectively. The effect of PD128042 on the incorporation of oleate into cholesteryl oleate was dramatic, reaching maximal inhibition between 1.0 and 2.5 μg/mL. The rate of oleate incorporation into triglycerides was stimulated, particularly so at the higher concentrations of PD128042. The estimated rate of phospholipid synthesis was unaffected until concentrations of the ACAT inhibitor reached 0.5 μg/mL. At this concentration and above, the incorporation of oleate into phospholipids was increased.

Effect of PD128042 on cholesterol synthesis. The results shown in Figure 1 suggested that when PD128042 was added to membranes, it had no affect on HMG-CoA reductase activity. Decreasing the cells ability to esterify cholesterol by PD128042, however, might lead to an expansion of a pool of unesterified cholesterol, which, in turn, could regulate intracellular cholesterol synthesis. To investigate if PD128042 could have a secondary effect on cholesterol synthesis, CaCo-2 cells were incubated for 4 hr in the presence or absence of increasing concentrations of PD128042. Labeled acetate was added during the last hour and the incorporation of acetate into cholesterol was determined (Fig. 5A). In the presence of the ACAT inhibitor, there was a concentration-dependent decrease in the rate of acetate incorporation into unesterified cholesterol. Results shown in Figure 5B also demonstrate that the effect of PD128042 on the rate of newly synthesized cholesterol was time-dependent. Acetate incorporation into total lipids was not reduced by PD128042.

A substantial decrease in the synthesis of new cholesterol by PD128042 was unexpected. To further support this observation, CaCo-2 cells were incubated for 4 hr in the presence or absence of 10 μg/mL of PD128042. The monolayers were washed and total membranes were prepared. In membranes prepared from cells incubated with PD128042, HMG-CoA reductase activity was 298 pmol/mg/30 min as compared to 628 pmol/mg/30 min in membranes prepared from control cells (Fig. 6). The inhibition of ACAT activity by PD128042 in intact cells, therefore, resulted in the inhibition of HMG-CoA reductase activity. In these same membranes, the activities of ACAT were 20 pmol/mg/5 min *vs* 263 pmol/mg/5 min in cells incubated with or without the inhibitor, respectively (Fig. 6).

Effect of PD128042 on the basolateral secretion of lipids by CaCo-2 cells. To investigate the effect of

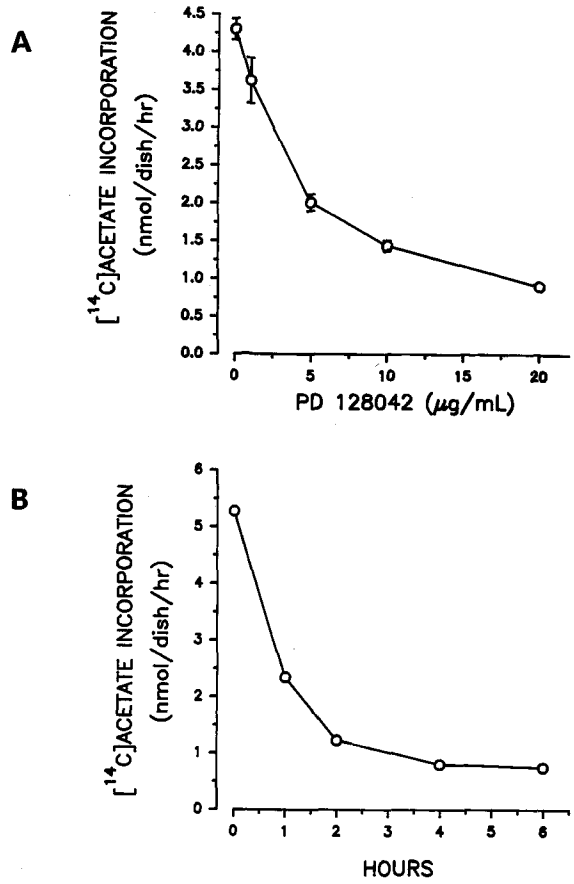


FIG. 5. Effect of PD128042 on [^{14}C]acetate incorporation into cholesterol. A: CaCo-2 cells were incubated for 4 hr with increasing concentrations of PD128042. During the final hour, [^{14}C]acetate, 5 mM (spec act, 100 dpm/nmol), was added. The monolayer was washed and scraped from the plates. Lipids were extracted and separated by thin-layer chromatography as described in Materials and Methods. B: At the indicated time, PD128042, 20 μg/mL, was added to the medium of CaCo-2 cells. During the last hour of incubation, [^{14}C]acetate incorporation into cholesterol was determined as described above. The data points represent the mean ± SE of 3-6 dishes.

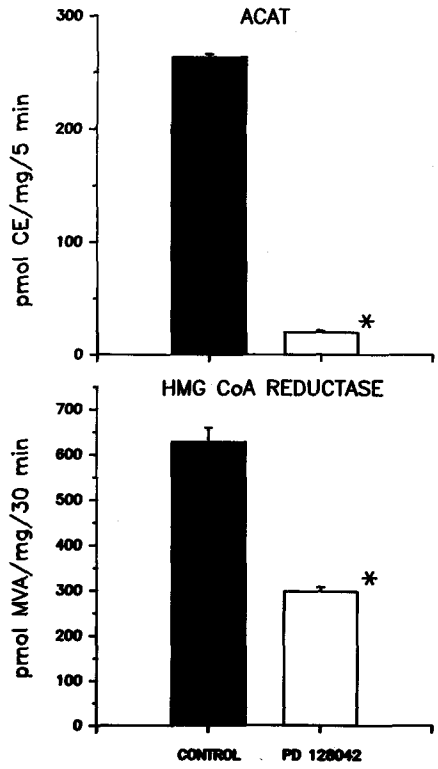


FIG. 6. Effect of PD128042 on membrane ACAT and HMG-CoA reductase activities. CaCo-2 cells were incubated for 4 hr with 10 μg/mL of PD128042. Following the incubation, the cells were washed and total membranes were prepared. The activities of HMG-CoA reductase and ACAT were determined. The data represent the mean and SE from two experiments with assays performed in triplicate, *, $p<0.001$ *vs* control.

PD128042 on the secretion of newly synthesized lipids, CaCo-2 cells were grown on polycarbonate filters separating an upper and lower well. PD128042 or its vehicle was added for 30 min. Following the preincubation, the medium containing the inhibitor was removed and 25 μM of labeled oleate solubilized in a solution containing 1 mM taurocholate and 50 μM of cholesterol was added to the apical medium. After 3 and 6 hr, the incorporation of labeled oleate into cellular lipids and lipids secreted into the apical medium and basal wells was determined. Figure 7 shows the data from the 6 hr time point. The results of the 3 hr time point were similar except the values were 50% of those at 6 hr. As expected, in cells incubated with the ACAT inhibitor, the incorporation of oleate into cellular cholesteryl oleate was dramatically decreased. Because of the decrease in newly synthesized cholesteryl esters, there was a significant decrease in the amount of labeled cholesteryl esters found in the basal well. In contrast, PD128042 did not alter the incorporation of labeled oleate into cellular triglycerides or phospholipids or the amount of these labeled lipids found in the basal well. The secretion of labeled cholesteryl esters and triglycerides was basally oriented. For reasons which are not clear, phospholipid secretion was not polarized as similar amounts of labeled phospholipids were observed in both the apical and basal wells.

Effect of PD128042 on cholesterol uptake and esterification by CaCo-2 cells. Results from previous studies have suggested that cholesterol absorption is decreased after inhibiting cholesterol esterification in the gut (11,12). To investigate the effect of PD128042 on cholesterol absorption and esterification, CaCo-2 cells were preincubated for 30 min with PD128042. After removing the medium containing the inhibitor, 50 μM of labeled cholesterol solubilized in a solution containing 1 mM taurocholate and 30 μM monoolein was added to the apical surface. At 1, 2, and 4 hr, the amount of labeled free and esterified cholesterol within cells was determined. As shown in Figure 8, the amount of labeled unesterified cholesterol within cells was not altered by the ACAT inhibitor. No detectable esterification of the absorbed cholesterol was observed in CaCo-2 cells preincubated with PD128042. At 1, 2, and 4 hr, the amount of labeled unesterified cholesterol secreted into the basal well was similar. The amount of labeled cholesteryl esters secreted into the basal well in both

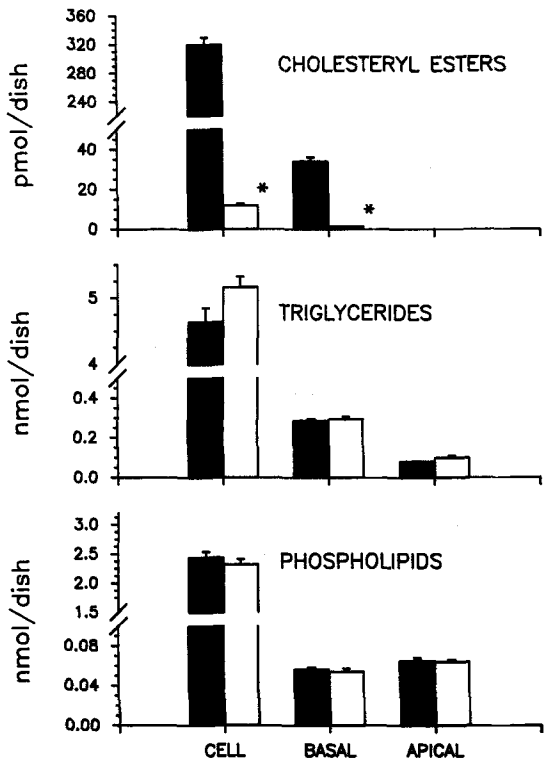


FIG. 7. Effect of PD128042 on the incorporation of [^{3}H]oleate into lipids released at the basolateral membrane. CaCo-2 cells were cultured on micropore polycarbonate filters. After washing the monolayer, M199 containing 5 μg/mL of PD128042 was added to the apical surface for 30 min. The medium containing the inhibitor was removed and a solution containing 1 mM taurocholate, 50 μM cholesterol, and 25 μM [^{3}H]oleic acid (spec act, 30,000 dpm/nmol), was added to the upper well. After six hr, lipids were extracted from the upper and lower wells and separated as described in Materials and Methods. The cells were washed three times with 1 mL of M199 and scraped from the filters. Cellular lipids were extracted and separated. The results are the mean ± SE of four control and 20 experimental wells. Lower well, basal; upper well, apical. ■, Control; □, PD128042.

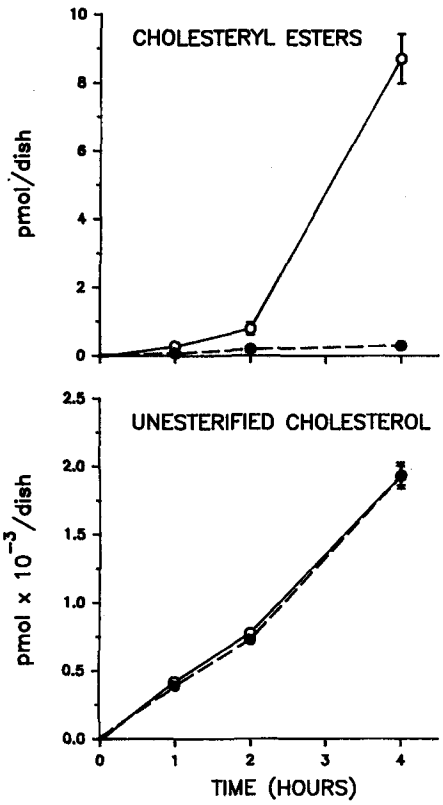


FIG. 8. Acute effect of PD128042 on the uptake and esterification of [^{14}C]cholesterol. Cells were cultured and ACAT activity inhibited as described in Figure 7. [^{14}C]Cholesterol, 50 μM (spec act 5,000 dpm/nmol), was added to the apical medium in a solution containing 1 mM taurocholate and 30 μM monoolein. After 6 hr, the lipids from the lower and upper wells were extracted and separated. The cells were washed and scraped from the filters. Lipids were extracted and separated. The results are the mean of four control and eight experimental wells. ○, Control; ●, PD128042.

control and ACAT-inhibited cells was too low for detection (data not shown).

If CaCo-2 cells were preincubated overnight with the ACAT inhibitor, there was a consistent decrease in the amount of labeled unesterified cholesterol associated with the cell following a 1, 3, and 6 hr incubation with labeled micellar cholesterol (Fig. 9). In control cells, the cholesterol which was taken up was esterified. By contrast, no detectable esterification occurred in cells incubated overnight with PD128042. Labeled cholesteryl esters secreted into the basal wells were too low for detection (data not shown).

DISCUSSION

PD128042 is a potent inhibitor of human intestinal ACAT activity with an IC_{50} of approximately 0.2 μg/mL. PD128042 had no direct effect on HMG-CoA reductase, triglyceride synthetase, or fatty acyl CoA hydrolase activities when added to CaCo-2 cell membranes. PD128042 is a fatty acid anilide and competes for ACAT with the fatty acyl-CoA substrate much like the fatty acylamides 57-118 and 58-035 (12). It is clear from the present data, however, that the inhibition of ACAT activity by PD128042 is not affected by increased amounts of exogenously added cholesterol. The inhibitory effect of PD128042 on ACAT activity, therefore, will not be minimized by an increase in cholesterol flux through the intestine.

Inhibition of ACAT activity by PD128042 in CaCo-2 cells resulted in a significant decrease in the rate of newly synthesized cholesterol and a corresponding decrease in HMG-CoA reductase activity. A decrease in cellular reductase activity occurs when there is excess unesterified cholesterol available to the cell, either from newly synthesized or lipoprotein cholesterol (23,24). The present data suggest, therefore, that in intestinal cells in which ACAT activity is inhibited by PD128042, unesterified cholesterol, which would otherwise be esterified, enters other intracellular pools of cholesterol which may, in turn, regulate *de novo* cholesterol synthesis.

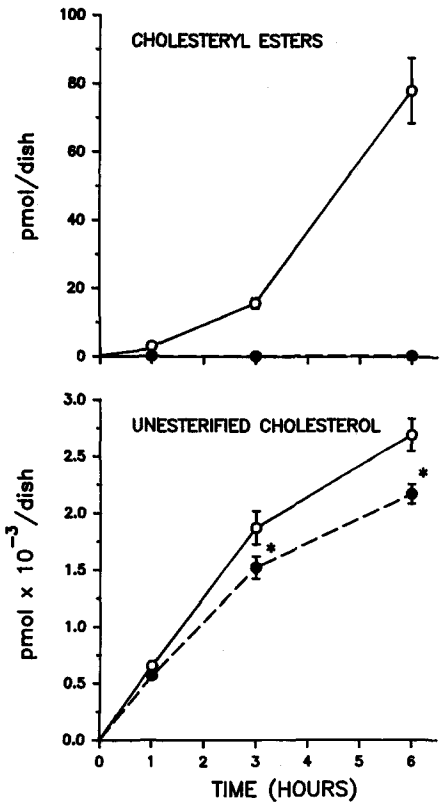


FIG. 9. Chronic effect of PD128042 on the uptake and esterification of [^{14}C]cholesterol. CaCo-2 cells were incubated overnight with 5 μg/mL of PD128042. The next morning [^{14}C]cholesterol, solubilized in a solution containing 1 mM taurocholate and 30 μM monoolein, was added as described in Figure 8. After 6 hr, the lipids from the cells and both lower and upper wells were extracted and separated. The results are the mean ± SE of four control and four experimental wells. ○, Control; ●, PD128042; *, $p<0.05$ *vs* control.

Long-term effects of inhibiting both cholesterol synthesis and esterification in the intestine may be beneficial. Cholesterol synthesized in the intestine contributes directly to the plasma cholesterol compartment (25). Since the rates of cholesterol synthesis in the intestine are second only to the rates observed in the liver (26), a decrease in the contribution made by the intestine could lead to a reduction in plasma cholesterol levels. In the liver, newly synthesized cholesterol has been shown to be important for the assembly and secretion of triglyceride-rich lipoproteins (27). Chronic ACAT inhibition in the intestine could result in the depletion of both surface and core components of the triglyceride-rich lipoprotein by inhibiting the synthesis of unesterified and esterified cholesterol, respectively. Thus, normal lipoprotein assembly may be altered, resulting in a decrease in lipoprotein secretion. It has been previously demonstrated that the composition of triglyceride-rich lipoproteins secreted by CaCo-2 cells can be modified by ACAT inhibition (28). The regulation of cholesterol synthesis by PD128042 occurred in the absence of gross changes in intracellular cholesterol mass. Unesterified and esterified cholesterol contents were similar in cells incubated for 4 hr with the inhibitor. It is not surprising that small changes occurring in intracellular cholesterol pools, which are sufficient to cause changes in HMG-CoA reductase activity, would not be detected by gross measurements of cellular cholesterol mass after 4 hr of ACAT inhibition.

The data also demonstrate that ACAT is responsible for the esterification of cholesterol, which is absorbed from micelles at the apical membrane as PD128042 significantly inhibited this process. As evidenced by the significant decrease in the basolateral secretion of newly synthesized cholesteryl esters (Fig. 7), PD128042 will likely cause a decrease in the transport of cholesteryl esters by the intestine. This supports previous data in lymph fistula rats which demonstrated a significant decrease in the amount of cholesteryl esters transported in lymph chylomicrons following the inhibition of ACAT activity (11).

In a previous study in CaCo-2 cells, the inhibition of ACAT by progesterone resulted in a decrease in the uptake of unesterified cholesterol (10). In the acute situation, despite a significant inhibition of cholesterol esterification by PD128042, the amount of labeled unesterified cholesterol taken up by CaCo-2 cells was similar as compared to the amount taken up by control cells with normal ACAT activity. In a more chronic situation, however, after ACAT activity had been inhibited for 16 hr, there was a modest decrease in the uptake of unesterified cholesterol. Perhaps an expansion of an unesterified cholesterol pool within the intestinal cell is necessary to disrupt the cholesterol gradient across the cell impairing the uptake of the sterol. Further studies will obviously be necessary to define a mechanism for this effect.

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Lipid and Fatty Acid Composition is Altered in Plaque Tissue from Multiple Sclerosis Brain Compared with Normal Brain White Matter

Robert Wilson[a,*] and **Douglas R. Tocher**[b]

[a]Department of Biological and Molecular Sciences and [b]NERC Unit of Aquatic Biochemistry, School of Natural Sciences, University of Stirling, Stirling FK9 4LA, Scotland, U.K.

Plaques and white matter from brains of multiple sclerosis (MS) patients were analyzed for lipid content, class composition, and fatty acid composition of total lipid, together with the fatty acid composition of plaque glycerophospholipids, and the results were compared with white matter from normal brain. Plaques contained less than 30% of the lipid present in normal white matter. Plaque lipid was characterized by significantly increased proportions of glycerophospholipids and decreased cerebrosides and sulfatides. In addition, a subacute plaque contained approximately 10 times the proportion of steryl esters observed in chronic plaques or normal white matter. Total lipid from all the MS plaques showed significantly increased percentages of saturated fatty acids, n-6, n-3 and total polyunsaturated fatty acids and decreased percentages of monoenes and alk-1-enyl ethers in comparison with normal brains. These results were consistent with increased cellularity and astrogliosis associated with MS plaques. However, analysis of plaque glycerophospholipids showed that the fatty acid changes observed in total lipid were not simply due to the increased proportion of glycerophospholipids and decreased myelin lipids, but that the fatty acid composition of the individual glycerophospholipids was different. *Lipids 26,* **9–15 (1991).**

Multiple sclerosis (MS) is a disease of the central nervous system which is characterized by areas of demyelination. Despite considerable effort, the pathogenesis of the disease is unknown. However, it has been recognized that both cell mediated and humoral immunological responses occur (1) although the search for a common infectious (viral) agent has been unsuccessful (2). During the course of demyelination, both myelin and oligodendrocytes are destroyed. This process results in the formation of disseminated plaques characterized by infiltration of fibrous type-2 astrocytes (1,3).

It has long been suggested that there is a relationship between dietary fat and MS (4,5). As a result of the early findings (4), much of the subsequent research into the primary defect in MS was centered on changes in the lipid and fatty acid compositions of macroscopically normal-appearing white matter or myelin from brains of MS patients *post-mortem.* There is contradictory evidence of changes in lipid composition of MS brain (see ref. 6). In general, there is a decrease in cerebrosides, sulfatides and myelin phospholipid, particularly ethanolamine alk-1-enyl phospholipid, and often an increase in cholesteryl ester levels (6–9). The latter has been related to a reduction in myelin cholesterol esterase activity (10). Although there is a lower yield of myelin from MS brain there is little change in the lipid composition of the myelin (7,11). However, changes in the lipid class composition, similar to those in normal-appearing white matter from MS brain, have been noted in a few studies of MS plaques (6,7).

Consistent patterns of change in the fatty acid composition of normal-appearing white matter in MS have been difficult to obtain. There are reports of changes in the fatty acid composition of total lipid (8,9,11) and individual lipid classes (6,8,9,12) from normal-appearing white matter (8,9,11) and myelin (8,11) in MS. Overall, there appears to be increased levels of saturated fatty acids, and decreased levels of monounsaturated fatty acids and polyunsaturated fatty acids (PUFA) in normal-appearing white matter (13). However, other studies have noted fatty acid profiles in MS similar to those of control brain (7,14). In contrast, MS plaques were commonly thought to be not as useful as white matter in elucidating the pathogenesis of MS, and so the fatty acid compositions of plaque lipids have not been thoroughly studied. However, histochemical and immunochemical studies have shown that plaques are active in both demyelination and remyelination (1,15). Furthermore, serial magnetic resonance imaging studies have shown both the appearance and disappearance of MS lesions (16). These studies suggest plaques are in a biochemically dynamic situation and may provide pathogenetic information.

In the present study, we have investigated the lipid and fatty acid composition of MS plaque and white matter in comparison with normal brain white matter in order to determine the major differences between these tissues. Of particular interest was the occurrence and extent of changes in the PUFA composition of plaque tissue.

MATERIALS AND METHODS

Tissue samples. Samples of tissues from MS and normal brains were obtained from The Multiple Sclerosis Society Brain Bank, Institute of Neurology, London. Chronic MS plaques were dissected from associated white matter from the parietal (2 samples) and occipital (1 sample) ventricle regions from different patients (see ref. 17). A subacute plaque was dissected from the occipital ventricle region. Macroscopically normal white matter was obtained from the MS brains from regions adjacent to the chronic plaques. Normal brain white matter from the region of the parietal ventricle was obtained from patients of similar age who died from cardiac failure. All samples were collected and frozen at −70°C within 10 to 44 hr after death of the patient.

Lipid extraction and quantification. Total lipid was extracted from 200–250 mg of brain tissue according to the method of Folch *et al.* (18). After homogenization in approximately 10 volumes of ice-cold chloroform/methanol (2:1, v/v), the homogenates were filtered and 0.25 vol of

*To whom correspondence should be addressed.

Abbreviations: BHT, butylated hydroxytoluene; Cer, cerebroside; CerOH, hydroxycerebroside; CL, cardiolipin; DMA, dimethylacetal; FFA, free fatty acids; MS, multiple sclerosis; PA, phosphatidic acid; PC, phosphatidylcholine; PE, phosphatidylethanolamine; PI, phosphatidylinositol; PS, phosphatidylserine; PUFA, polyunsaturated fatty acid; SE, steryl ester; SM, sphingomyelin; Sulf, sulfatide; SulfOH, hydroxysulfatide; TAG, triacylglycerol; VLCFA, very long chain fatty acid.

0.88% KCl added and the solutions mixed. After separation, the chloroform layer was removed and refiltered, and the solvent removed under nitrogen. The lipid was dried in a vacuum desiccator before weighing. The weighed lipid extracts were redissolved in chloroform/methanol (2:1, v/v) at a final concentration of 10 mg lipid/mL and were stored at −20°C between procedures. All solvents contained 0.01% butylated hydroxytoluene (BHT) as an antioxidant. Protein content was determined in the residue after lipid extraction by sodium hydroxide/sodium dodecylsulfate solubilization, essentially according to Lowry *et al.* (19).

Lipid class analysis. Lipid classes were separated by a one-dimentional double development HPTLC method (20). Approximately 5–20 μg of total lipid was spotted on HPTLC plates that had been pre-run in hexane/diethyl ether (1:1, v/v) and activated at 110°C for 30 min. The plates were developed for 6 cm in methyl acetate/isopropanol/chloroform/methanol/0.25% aqueous KCl (25:25:25:10:9, by vol) to separate phospholipid classes, sulfatides and cerebrosides, with neutral lipids running at the solvent front (21). After drying, the plates were developed fully in hexane/diethyl ether/acetic acid (80:20:2, v/v/v) to separate the neutral lipids and cholesterol. Lipids were stained by charring at 160°C for 20 min after spraying with 3% copper acetate/8% phosphoric acid and identified by comparison with pure, commercial standards. Lipid classes were quantified by calibrated densitometry as described by Olsen and Henderson (20) using a Shimadzu CS-9000 (Shimadzu, Columbia, MD) dual wavelength flying-spot TLC scanner and DR-13 recording integrator.

Separation of lipid classes. Total neutral lipids were separated from total polar lipids by TLC on 20 × 20 cm plates using hexane/diethyl ether/acetic acid (80:20:2, v/v/v) as developing solvent essentially as described previously (22). Individual phospholipid classes, sulfatides and cerebrosides were separated by TLC as above using methyl acetate/isopropanol/chloroform/methanol/0.25% aqueous KCl (25:25:25:10:9, by vol) as developing solvent. The lipids were visualized by spraying with 2′,7′-dichlorofluorescein. The separated phospholipid bands were scraped off and eluted with chloroform/methanol/water (10:10:1, v/v/v). Solvent was removed from the samples as detailed above, with the purified phospholipids redissolved in chloroform/methanol (2:1, v/v) and stored at −20°C between procedures. All solvents were supplemented with 0.01% BHT.

Fatty acid analyses. Fatty acid methyl esters were prepared from samples by acid-catalyzed transmethylation overnight at 50°C, according to Christie (23). Aldehydes released from alk-1-enyl phospholipids formed dimethyl acetal derivatives (DMA) during transmethylation (23). After extraction, the methyl esters and DMA were purified by TLC using hexane/diethyl ether/acetic acid (90:10:1, v/v/v) as solvent. The methyl esters and DMA were redissolved in hexane containing 0.01% BHT and analyzed in a Packard 436 gas chromatograph equipped with either CP Wax 52CB or CP Sil5CB chemically bonded fused silica capillary columns (both 50 m × 0.34 mm i.d.) (Chrompack U.K., Ltd.), on-column injection and using H_2 as carrier gas with a thermal gradient from 50°C to 225°C (CP Wax) or 260°C (CP Sil). Individual methyl esters and DMA were identified by comparison with known standards and a well-characterized fish oil and also by reference to published data (24–26). The identities of all fatty acid methyl esters and DMA were confirmed by GLC/mass spectrometry courtesy of Dr. W. W. Christie, The Hannah Institute, Ayr, Scotland, U.K. Quantification was performed using a Shimadzu CR-3A recording integrator.

Statistical analysis. The four MS plaques, three MS white matter and three normal brain white matter samples were analyzed separately and not pooled. For the lipid class composition and total lipid fatty acid composition each sample was analyzed in triplicate and the results are the means ± SD of each respective group. However, for the fatty acid compositions of individual glycerophospholipid classes, each sample was analyzed once with the data expressed as the means of the three normal brain and four MS plaque samples ± SD. Significance of differences between MS tissues and normal white matter was determined by the Student's *t*-test.

Materials. BHT and pure lipid class standards were from Sigma Chemical Co. Ltd. (Poole, U.K.). HPTLC plates (10 × 10 cm × 0.15 mm) and TLC plates (20 × 20 cm × 0.25 mm) precoated with silica gel 60 (without fluorescent indicator) were obtained from Merck. All solvents were HPLC grade and were obtained from Rathburn Chemicals (Walkerburn, U.K.).

RESULTS

There was no difference between the protein contents of the MS tissue (average 63 mg/g wet wt.) and those of normal brain tissue (average 64 mg/g wet wt.) (Table 1). However, the mean total lipid content of all MS tissue samples was considerably lower than that of normal brain tissue. This resulted in lipid/protein ratios being two-fold, 3.3-fold and 4.3-fold lower for MS white matter, chronic plaques and the subacute plaque, respectively, as compared to normal white matter (Table 1).

The one-dimensional double-development HPTLC system effectively separated all the major lipid classes with the exception of PA and CL (Fig. 1). However, the presence of CL in all MS tissue was confirmed by two-dimensional HPTLC. Other than the quantitatively minor components CL and FFA, there were no significant differences between MS white matter and normal white matter (Table 2). There is a significant loss of cerebrosides ($p<0.01$) and sulfatides ($p<0.05$), together with a significant increase in PE ($p<0.01$), PI ($p<0.02$) and CL ($p<0.002$)

TABLE 1

Lipid and Protein Contents of Multiple Sclerosis White Matter, Multiple Sclerosis Plaque and White Matter from Normal Brain[a]

		Multiple sclerosis		
	Normal WM	WM	Chronic	Subacute
Lipid (mg/g)	173 ± 28	91 ± 39	53 ± 14	40
Protein (mg/g)	64 ± 16	62 ± 15	63 ± 11	64
Lipid/protein ratio	2.9 ± 1.0	1.4 ± 0.6	0.8 ± 0.1	0.6

[a]Total lipid was extracted by the method of Folch *et al.* (18) and determined gravimetrically. Protein content was determined after the lipid extraction by sodium hydroxide/sodium dodecylsulfate solubilization essentially according to Lowry *et al.* (19). WM, white matter.

TABLE 2

Lipid Class Composition of Multiple Sclerosis White Matter, Multiple Sclerosis Plaque and White Matter from Normal Brain[a]

Lipid class	Normal WM	Multiple sclerosis		
		WM	Chronic	Subacute
		% total lipid		
PC	4.7 ± 2.0	5.6 ± 1.7	8.5 ± 1.6	7.7 ± 2.1
PE	16.0 ± 1.2	15.1 ± 0.5	20.6 ± 1.6*	20.1 ± 1.6*
PS	5.6 ± 1.3	5.1 ± 1.1	7.5 ± 0.4	6.3 ± 1.2
PI	1.2 ± 0.3	1.3 ± 0.3	2.1 ± 0.2*	1.8 ± 0.3*
PA/CL	0.1 ± 0.1	0.7 ± 0.2**	1.1 ± 0.2**	0.9 ± 0.2**
SM	5.2 ± 1.1	6.3 ± 0.6	5.4 ± 0.8	4.8 ± 1.1
Total PL	32.9 ± 5.4	34.1 ± 3.8	45.5 ± 2.7*	41.7 ± 5.6
SulfOH	3.2 ± 0.6	3.5 ± 0.3	3.0 ± 0.5	1.1 ± 0.2**#
Sulf	6.2 ± 0.7	6.0 ± 0.8	4.0 ± 0.7**	3.5 ± 0.2**
Total Sulf	9.4 ± 0.7	9.5 ± 0.9	7.0 ± 1.1*	4.6 ± 0.2**#
CerOH	14.9 ± 1.6	13.8 ± 1.0	11.8 ± 0.8*	5.3 ± 0.7**#
Cer	12.4 ± 1.5	11.6 ± 1.4	7.2 ± 0.8**	6.9 ± 0.5**
Total Cer	27.3 ± 2.4	25.4 ± 2.3	19.0 ± 1.4**	12.2 ± 1.1**#
Chol	27.1 ± 2.8	27.4 ± 2.5	25.7 ± 2.6	24.8 ± 2.1
FFA	0.1 ± 0.1	1.2 ± 0.6*	0.6 ± 0.3	0.4 ± 0.2
TAG	1.9 ± 2.4	0.8 ± 0.5	0.7 ± 0.4	1.7 ± 1.2
SE	1.4 ± 0.4	1.6 ± 0.3	1.6 ± 0.4	14.9 ± 4.0**#

[a]Values are means ± S.D. for triplicate analysis and of three samples/group, except for the single subacute plaque. Where indicated (*), values are significantly different to normal brain white matter *($p<0.05$), **$p<0.02$). Where indicated (#), values for the subacute plaque are significantly different to chronic plaques #($p<0.05$). PL, phospholipid; and WM, white matter.

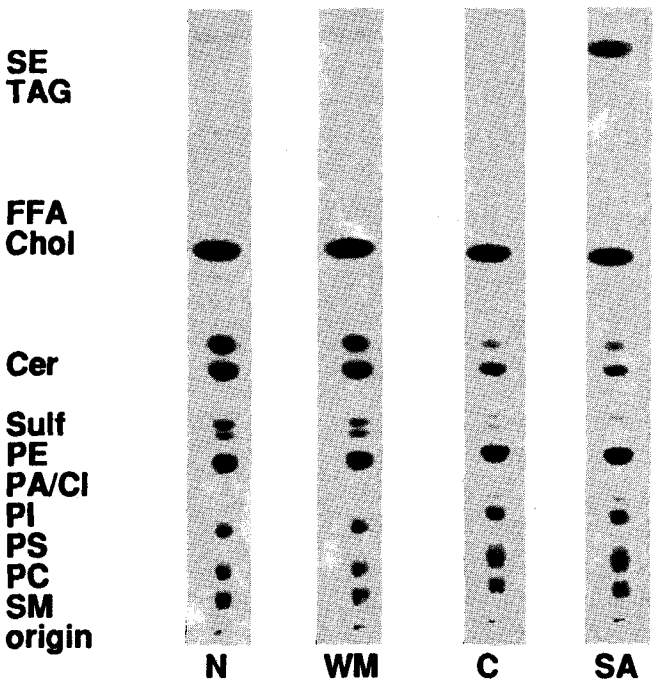


FIG. 1. Separation of lipid classes by one dimensional double development HPTLC (20). Samples are total lipid from N, normal brain white matter; WM, MS, white matter; C, MS chronic plaque; SA, MS subacute plaque. The cerebrosides and sulfatides were resolved into two components with the hydroxy-derivative having the lesser R_f value. Cer, cerebrosides; Chol, cholesterol; CL, cardiolipin; FFA, free fatty acid; PA, phosphatidic acid, PC, phosphatidylcholine; PE, phosphatidylethanolamine; PI, phosphatidylinositol; PS, phosphatidylserine; SE, steryl ester; SM, sphingomyelin; Sulf, sulfatide; and TAG, triacylglycerol.

in both chronic and subacute plaques (Table 2). The percentage of cholesterol was generally lower and FFA was increased in MS plaques, but the differences were not significant.

There were some significant differences between the subacute plaque and the chronic plaques. In the subacute plaque both hydroxycerebrosides and hydroxysulfatides were decreased 2.9-fold, whereas in chronic plaques the decreases were larger in the non-hydroxylated classes (Table 2). Furthermore, the subacute plaque contained almost 15% SE.

The total lipid from the subacute and chronic plaques showed significantly increased percentages of total saturated fatty acids ($p<0.01$ or less), n-6 PUFA ($p<0.05$ or less), n-3 PUFA ($p<0.05$ or less) and total PUFA ($p<0.01$ or less) and decreased percentages of total monoenes ($p<0.002$ or less) and DMA ($p<0.01$ or less) in comparison with normal white matter (Table 3). The increased levels of saturated fatty acids were primarily due to increased 16:0 and, to a lesser extent, 18:0. The decrease in monoenes was more general, including 18:1 and 20:1 isomers and 24:1. The overall decrease in DMA was primarily due to decreased 18:1 DMA. Increases in 20:4n-6 and 22:6n-3 were primarily responsible for the increased PUFA in MS plaques, and n-3 PUFA increased proportionaly more as evinced by the increased n-3/n-6 ratios in the MS plaques (Table 3). MS white matter displayed the same trends as described for plaque tissues but, with the exception of total n-6 PUFA, the differences between MS white matter and normal white matter were non-significant (Table 3).

The presence of very long chain fatty acids (VLCFA, C_{24} and greater) was investigated using a CP Sil5CB

TABLE 3

Fatty Acid and Aldehyde Composition of Total Lipid from Multiple Sclerosis White Matter, Multiple Sclerosis Plaque and White Matter From Normal Brain[a]

Fatty acid and aldehyde	Normal WM	Multiple sclerosis		
		WM	Chronic	Subacute
	% of Total fatty acids and aldehydes			
14:0	0.4 ± 0.1	0.4 ± 0.1	0.5 ± 0.1	0.5 ± 0.1
16:0	9.8 ± 1.3	12.0 ± 1.7	15.8 ± 0.7**	17.2 ± 0.6**
17:0	0.2 ± 0.0	0.2 ± 0.1	0.3 ± 0.1	0.3 ± 0.1
18:0	14.9 ± 1.2	14.6 ± 1.4	18.4 ± 0.7**	16.0 ± 1.1
20:0	0.3 ± 0.0	0.3 ± 0.1	0.2 ± 0.0	0.2 ± 0.0
22:0	0.3 ± 0.1	0.3 ± 0.1	0.1 ± 0.1	0.2 ± 0.0
23:0	0.9 ± 0.1	1.0 ± 0.2	0.6 ± 0.2	0.7 ± 0.2
24:0	1.0 ± 0.3	0.8 ± 0.2	0.5 ± 0.1	0.5 ± 0.1
25:0	0.4 ± 0.1	0.3 ± 0.1	0.1 ± 0.1	0.2 ± 0.1
26:0	0.2 ± 0.0	0.2 ± 0.1	0.1 ± 0.0	0.1 ± 0.0
Total saturated FA	27.4 ± 2.2	30.3 ± 1.3	36.6 ± 1.5**	35.2 ± 1.5**
16:1[b]	1.1 ± 0.1	1.0 ± 0.1	1.2 ± 0.1	2.2 ± 0.1
18:1n-9	24.6 ± 0.8	23.9 ± 1.7	18.0 ± 1.5**	19.5 ± 0.7**
18:1n-7	4.7 ± 0.5	3.9 ± 0.7	3.7 ± 0.3*	4.9 ± 0.5
20:1n-9	3.5 ± 0.8	2.2 ± 0.6	1.2 ± 0.1**	1.1 ± 0.1**
20:1n-7	1.1 ± 0.0	0.8 ± 0.2	0.5 ± 0.1**	0.4 ± 0.1**
22:1	0.2 ± 0.1	0.3 ± 0.1	0.1 ± 0.1	0.2 ± 0.1
24:1	3.8 ± 0.6	2.8 ± 0.6	1.4 ± 0.3**	1.3 ± 0.1**
25:1	0.8 ± 0.1	0.5 ± 0.2	0.4 ± 0.0**	0.3 ± 0.1**
26:1	0.8 ± 0.2	0.8 ± 0.2	0.5 ± 0.0	0.4 ± 0.1
Total Monoenoic FA	40.6 ± 2.5	36.2 ± 1.9	27.0 ± 2.0**	30.3 ± 0.4**
18:2n-6	0.2 ± 0.1	0.7 ± 0.4	0.8 ± 0.6	0.3 ± 0.1
20:2n-6[c]	0.5 ± 0.1	0.7 ± 0.1	0.5 ± 0.5	0.8 ± 0.1
20:3n-6	0.6 ± 0.1	1.0 ± 0.5	0.8 ± 0.3	1.0 ± 0.1
20:4n-6	3.7 ± 0.5	4.9 ± 0.9	6.5 ± 0.3**	7.5 ± 0.3**
22:4n-6	4.6 ± 0.5	4.4 ± 0.7	4.5 ± 0.2	3.5 ± 0.3*
22:5n-6	0.4 ± 0.1	0.5 ± 0.3	0.8 ± 0.4	0.9 ± 0.3
24:4n-6	0.8 ± 0.3	1.3 ± 0.3	0.6 ± 0.2	0.4 ± 0.1
Total n-6PUFA	10.7 ± 1.0	13.5 ± 1.2*	14.5 ± 0.9**	14.6 ± 0.5**
22:5n-3	0.2 ± 0.1	T	0.3 ± 0.1	0.2 ± 0.0
22:6n-3	2.1 ± 1.0	2.8 ± 0.7	8.4 ± 1.9**	6.9 ± 1.2**
Total n-3PUFA	2.5 ± 1.1	2.8 ± 0.7	8.9 ± 1.7**	7.3 ± 1.3**
Total PUFA[d]	13.8 ± 1.7	15.2 ± 0.6	23.7 ± 1.5**	22.9 ± 1.8**
n-3/n-6	0.22 ± 0.08	0.22 ± 0.04	0.62 ± 0.16**	0.49 ± 0.07**
16:0	4.2 ± 0.3	4.4 ± 0.4	3.7 ± 0.3	2.6 ± 0.1
18:0	3.0 ± 0.2	3.0 ± 0.2	4.0 ± 0.2	3.7 ± 0.2
18:1	9.3 ± 1.0	7.3 ± 1.0	4.3 ± 1.0**	3.6 ± 0.3**
Total Aldehyde	16.5 ± 1.3	14.7 ± 1.6	11.1 ± 1.1**	9.9 ± 0.4**
Unidentified	3.8 ± 1.6	1.7 ± 0.5	2.0 ± 1.4	2.6 ± 2.2

[a]Values are means ± S.D. for triplicate analysis of three samples/group except for the single subacute plaque. Where indicated (*), values are significantly different to normal brain white matter *($p<0.05$), **($p<0.02$).

[b]Predominately n-7 isomer.

[c]Contains 20:3n-9.

[d]Including n-9PUFA; FA, fatty acid: T, trace; and WM, white matter.

column. This showed that 25:0/26:0 totalled 0.6% and 0.2%, on average, and 25:1/26:1 totalled 1.8% and 0.8%, on average, in total lipid from normal white matter and MS plaques, respectively (Table 3). The major very long chain PUFA was 24:4n-6 present at 0.8% and 0.6% of the total lipid fatty acids in normal white matter and MS plaques, respectively (Table 3). The 25:0, 25:1, 26:0 and 26:1 were confined mainly to cerebrosides, sulfatides and sphingomyelin with 0.1% 26:0 and 0.6% 26:1 in PC. However, 24:4n-6 was found predominantly in PE and PS (data not shown).

The glycerophospholipids showed the characteristic distribution of fatty acids associated with neural tissues (see ref. 27). PC had high 16:0 and relatively low PUFA levels and PE had low saturated fatty acids and relatively high PUFA levels (Table 4). PS had high 18:0 and a high C_{22}/C_{18}–C_{20} PUFA ratio, whereas PI had high 18:0 and 20:4n-6 (Table 5). This distribution was maintained in MS plaques.

The changes in fatty acid composition observed with total lipid were also reflected in the individual glycerophospholipids (Tables 4 and 5). Therefore, the

TABLE 4

Fatty Acid and Aldehyde Composition of Phosphatidylcholine and Phosphatidylethanolamine from Multiple Sclerosis Plaque and White Matter from Normal Brain[a]

Fatty acid and aldehyde	Phosphatidylcholine		Phosphatidylethanolamine	
	Normal WM	Plaque	Normal WM	Plaque
	% of Total fatty acids and aldehydes			
14:0	0.7 ± 0.1	0.7 ± 0.1	0.1 ± 0.0	0.1 ± 0.0
15:0	0.2 ± 0.1	0.4 ± 0.2	—	—
16:0	28.7 ± 1.1	36.5 ± 3.5**	2.7 ± 0.3	3.5 ± 0.4
17:0	0.5 ± 0.1	0.5 ± 0.1	0.2 ± 0.1	0.1 ± 0.0
18:0	12.2 ± 0.3	11.4 ± 1.6	4.1 ± 1.6	14.0 ± 1.8**
20:0	0.2 ± 0.1	—	0.1 ± 0.1	0.1 ± 0.0
Total saturated FA	42.5 ± 1.1	49.5 ± 2.2**	7.2 ± 1.5	17.8 ± 1.9**
16:1[b]	1.9 ± 0.2	2.1 ± 0.71	0.4 ± 0.0	0.5 ± 0.1
18:1n-9	36.0 ± 1.4	27.3 ± 1.4**	20.8 ± 2.4	10.9 ± 1.0**
18:1n-7	6.7 ± 0.2	6.6 ± 0.5	3.2 ± 0.2	2.3 ± 0.2**
20:1n-9	1.4 ± 0.4	0.6 ± 0.1**	4.5 ± 0.7	1.6 ± 0.2**
20:1n-7	0.9 ± 0.1	0.4 ± 0.1**	1.4 ± 0.2	0.5 ± 0.1**
24:1	0.2 ± 0.1	—	—	—
Total Monoenoic FA	47.1 ± 2.0	37.0 ± 0.9**	30.3 ± 2.9	15.8 ± 1.6**
18:2n-6	0.6 ± 0.0	0.6 ± 0.2	0.1 ± 0.0	0.3 ± 0.2
20:2n-6[c]	—	0.2 ± 0.2	0.3 ± 0.1	0.5 ± 0.2
20:3n-6	0.4 ± 0.1	0.8 ± 0.2*	0.9 ± 0.4	0.8 ± 0.2
20:4n-6	1.8 ± 0.6	4.3 ± 0.5**	4.2 ± 0.7	8.9 ± 1.2**
22:2n-6	—	—	0.7 ± 0.3	0.8 ± 0.3
22:4n-6	0.5 ± 0.2	0.7 ± 0.1	8.5 ± 0.9	8.6 ± 0.8
22:5n-6	—	0.5 ± 0.2**	0.4 ± 0.1	1.4 ± 0.5*
Total n-6PUFA	3.3 ± 0.6	7.1 ± 1.1**	15.1 ± 1.8	21.3 ± 1.2**
20:5n-3	—	—	T	0.1 ± 0.0
22:5n-3	—	0.1 ± 0.0	0.3 ± 0.1	0.5 ± 0.2
22:6n-3	0.8 ± 0.3	2.6 ± 1.3*	3.2 ± 1.7	13.6 ± 2.4**
Total n-3PUFA	0.8 ± 0.3	2.7 ± 1.3*	3.5 ± 1.7	14.2 ± 2.2**
Total PUFA[d]	4.1 ± 1.0	9.8 ± 2.0**	19.4 ± 3.9	36.0 ± 2.2**
n-3/n-6	0.24 ± 0.04	0.38 ± 0.18	0.23 ± 0.09	0.67 ± 0.12**
16:0	0.5 ± 0.4	0.2 ± 0.1	8.6 ± 0.7	6.1 ± 0.7**
17:0	0.1 ± 0.1	0.1 ± 0.1	0.7 ± 0.1	0.6 ± 0.1
18:0	0.1 ± 0.1	0.1 ± 0.0	6.2 ± 0.6	9.8 ± 0.9**
18:1	0.4 ± 0.3	0.2 ± 0.1	20.1 ± 0.8	11.0 ± 1.0**
Total Aldehyde	1.1 ± 1.0	0.6 ± 0.1	35.6 ± 1.4	27.5 ± 1.7**
Unidentified	5.2 ± 0.2	3.1 ± 0.8	7.5 ± 1.9*	2.9 ± 0.8

[a]Values are means ± S.D. of three normal/group and four MS plaque/group. Where indicated (*), values for MS plaque are significantly different to normal brain white matter *($p<0.05$), **($p<0.02$).

[b]Predominately n-7 isomer.

[c]Contains 20:3n-9.

[d]Including n-9 PUFA; FA, fatty acid; T, trace; WM, white matter.

glycerophospholipids in MS plaques had significantly increased percentages of saturated fatty acids, n-6, n-3 and total PUFA and decreased percentages of monoenes superimposed on the distributions described above. The major increase in 16:0 in MS plaque was in PC, and increased 18:0 was primarily in PE and PI. As with total lipid, the decreased monoenes in MS plaque was more evenly spread, but 18:1n-9 accounted for the largest decrease in all glycerophospholipids. The largest increases in 22:6n-3 were observed in PE and PS, whereas 20:4n-6 increased to the greatest extent in PI in MS plaque. The decreased percentages of DMA derived from MS plaque was primarily due to decreased 18:1 DMA from PE (Table 4).

DISCUSSION

The lower lipid contents and lower lipid/protein ratios and the reduced percentages of cerebrosides, sulfatides and ethanolamine plasmalogens in the MS plaques are all classical indicators of demyelination that had occurred or was occurring in the plaques (28). It is difficult to compare the lipid class composition data with much of the earlier work due to the many different ways of expressing the data that have been used. Where comparison is possible there is generally good agreement between the data for normal brain white matter and MS white matter and plaques obtained in the present study and data from earlier studies (7,28). However, the percentages of

TABLE 5

Fatty Acid Composition of Phosphatidylserine and Phosphatidylinositol from Multiple Sclerosis Plaque and White Matter from Normal Brain[a]

Fatty acid	Phosphatidylserine		Phosphatidylinositol	
	Normal WM	Plaque	Normal WM	Plaque
14:0	0.1 ± 0.1	0.1 ± 0.0	0.5 ± 0.2	0.3 ± 0.1
16:0	1.7 ± 0.6	1.3 ± 0.2	18.6 ± 1.1	9.1 ± 1.1**
18:0	38.7 ± 0.7	40.7 ± 1.3*	19.2 ± 1.3	32.6 ± 3.0**
20:0	0.2 ± 0.1	0.3 ± 0.1	0.6 ± 0.1	0.2 ± 0.1**
Total saturated FA	40.7 ± 0.6	42.4 ± 1.0*	38.9 ± 0.5	42.2 ± 0.6**
16:1[b]	0.3 ± 0.1	0.4 ± 0.3	0.7 ± 0.1	0.9 ± 0.7
18:1n-9	33.0 ± 1.7	20.8 ± 4.6**	28.0 ± 1.0	9.3 ± 2.4**
18:1n-7	4.7 ± 0.7	2.9 ± 0.5	3.1 ± 0.2	3.2 ± 1.0
20:1n-9	4.9 ± 1.1	2.3 ± 0.2**	2.1 ± 0.3	0.9 ± 0.4**
20:1n-7	0.8 ± 0.2	0.4 ± 0.1**	1.6 ± 0.1	0.4 ± 0.4**
24:1	—	—	—	0.2 ± 0.1
Total Monoenoic FA	43.7 ± 2.7	26.8 ± 5.1**	35.5 ± 1.1	14.9 ± 3.6**
18:2n-6	0.2 ± 0.0	0.3 ± 0.2	1.1 ± 0.4	1.2 ± 0.5
20:2n-6[c]	0.3 ± 0.1	0.5 ± 0.2	0.6 ± 0.2	1.2 ± 0.5
20:3n-6	0.4 ± 0.1	0.9 ± 0.3*	1.1 ± 0.2	1.9 ± 0.6
20:4n-6	1.2 ± 0.3	2.2 ± 0.4**	8.9 ± 0.7	25.1 ± 3.2**
22:2n-6	1.1 ± 0.3	1.0 ± 0.4	0.5 ± 0.1	0.7 ± 0.3
22:4n-6	2.0 ± 0.5	3.4 ± 0.4**	1.7 ± 0.2	2.6 ± 0.8
22:5n-6	T	1.7 ± 0.7**	0.2 ± 0.1	1.3 ± 0.6*
Total n-6PUFA	5.2 ± 0.9	10.0 ± 1.1**	14.1 ± 0.2	34.0 ± 1.9**
22:5n-3	0.1 ± 0.0	0.3 ± 0.1**	—	0.3 ± 0.1**
22:6n-3	2.2 ± 1.2	14.6 ± 4.0**	1.6 ± 0.4	6.7 ± 3.8**
Total n-3PUFA	2.4 ± 1.2	14.9 ± 4.0**	1.6 ± 0.4	7.0 ± 3.7**
Total PUFA[d]	8.7 ± 2.0	26.1 ± 4.8**	16.2 ± 0.5	41.1 ± 4.3**
n-3/n-6	0.46 ± 0.16	1.49 ± 0.29	0.11 ± 0.03	0.21 ± 0.14
Unidentified	6.9 ± 1.3	4.7 ± 1.0	9.4 ± 1.1*	1.5 ± 0.6

[a]Values are means ± S.D. of three normal/group and four MS plaque/group. Where indicated (*), values for MS plaque are significantly different to normal brain white matter *($p<0.05$), **($p<0.02$).

[b]Predominately n-7 isomer.

[c]Contains 20:3n-9.

[d]Including n-9 PUFA; FA, fatty acid; T, trace; WM, white matter.

PC are lower in the present study when compared with those in earlier studies.

It was noteworthy that our results also showed a significant increase in the proportion of total glycerophospholipids in chronic plaques with significant increases in PE, PI and PA/CL. The significant increases in the proportions of total glycerophospholipids and CL would be consistent with increased cellularity in the plaques. Overall, the qualitative changes in the lipid class composition of the plaques were similar to those previously reported for normal-appearing white matter in MS (6–9). The lipid class compositional changes in normal-appearing white matter in MS are probably an indication of the presence of micro-plaques not observed on macroscopic examination.

The increased saturated fatty acid and decreased monounsaturated fatty acid percentages are consistent with the loss of monoenoic-rich myelin lipids (13). However, whereas increased saturated fatty acids and decreased monoenes are features consistently reported for white matter and myelin in MS, the changes in PUFA levels have been variable. For instance, 18:2n-6 and 20:4n-6 have been reported as decreased in PC in apparently normal white matter in MS (11,29) and myelin (11,30), although other studies have found little difference in PUFA levels (6,14). However, the data presented here for MS plaques were very consistent between samples from different patients, different brain regions and different stages and clearly show a general increase in the proportion of total PUFA. Therefore, the present data are an important new observation in MS, but are consistent with increased cellularity in the plaques, especially chronic plaques (1,3).

The increased PUFA in total lipid in plaques was almost entirely due to an approximate doubling of 20:4n-6 and a three-fold increase in 22:6n-3. All the other PUFA, including 22:4n-6, are far less affected. Therefore, astrogliosis not only increased the total PUFA levels but also altered the PUFA balance and, consequently, the eicosanoid status in the plaques. Furthermore, the increased PUFA was not simply the result of an effective exchange of relatively monoene-rich myelin lipid for relatively PUFA-rich cellular lipids. This was confirmed by the analysis of the glycerophospholipids, which showed that their composition was also different with predominantly increased proportions of 20:4n-6 and 22:6n-3 in the MS plaques. This suggests that the glycerophospholipids of the proliferating and invading astrocytes have a higher PUFA content than glycerophospholipids from tissue

composed predominadntly of myelin. However, it is not clear if these alterations in PUFA balance play any role in the progression of the plaques. It may be that these changes are entirely secondary, but it is also possible that eicosanoids are involved in the disease process (31).

Several other studies reported low levels of certain PUFA, especially 18:2n-6, in serum, platelets and erythrocytes in MS (32,33). Some later studies have confirmed this (34,35), but others have failed to find reduced serum PUFA (36-38). Overall, these studies modified the early idea that an excess consumption of saturated fatty acids was a causal factor in MS (4) to one of a relative deficiency in essential fatty acids (33,39). This hypothesis has resulted in several clinical trials (5,40), with some mild improvement observed with both n-6 and n-3 dietary supplementation (40). However, the mechanisms underlying the effects of essential fatty acids in MS are unclear and probably complex (40). The present results show an alteration in PUFA balance in plaques that may be involved in plaque development.

It is noteworthy that VLCFA were not found in the MS plaques or MS white matter in significant amounts. Indeed, levels were slightly lower than in normal brain white matter. Saturated VLCFA are increased due to impaired peroxisomal β-oxidation and thought to play a major role in the pathology of adrenoleukodystrophy, a disease also characterized by demyelination (41). Although it is not fully understood how VLCFA influence demyelination in adrenoleukodystrophy it is clear from our results that they are not involved in the pathology of MS.

Some differences in the lipid composition between a subacute plaque and chronic plaques were apparent from the data, although it should be noted that only one subacute plaque was analyzed. The subacute plaque had a lower lipid content (40 mg/g tissue wet wt.) than on average for the chronic plaques (53 mg/g tissue wet wt.) and also the lowest lipid/protein ratio. The percentages of cerebrosides, sulfatides and especially their hydroxylated derivatives were also lower in the subacute plaque. These results were perhaps unexpected as myelin lipid loss should be greatest in the chronic plaques and so may indicate some remyelination in the chronic plaques. However, sterol esters were ten-fold higher in the subacute plaque, as would be expected for an actively demyelinating sample. In contrast to class composition, there were no significant differences in the fatty acid composition of total lipid between the subacute and chronic plaques.

This study has provided important data on the lipid and, especially, the fatty acid composition of MS plaque tissue. The consistently increased PUFA in the plaques, due to increased proportions of 20:4n-6 and 22:6n-3, and the low levels of VLCFA were of particular interest. The results were generally consistent with increased cellularity, probably due to astrogliosis, known to be associated with MS plaques. However, the increased proportion of PUFA in the plaques was not simply the result of an increased proportion of glycerophospholipids as the percentages of PUFA in the individual glycerophospholipids was also increased in the plaques.

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Regulation of Polyunsaturated Fatty Acid Metabolism in Tissue Phospholipids of Obese (fa/fa) and Lean (Fa/−) Zucker Rats. 1. Effect of Dietary Lipids on Cardiac Tissue

Klaus W.J. Wahle*, Lesley Milne and **Gwen McIntosh**
Rowett Research Institute, Bucksburn, Aberdeen AB2 9SB, Scotland, U.K.

The present investigation addressed three questions: (i) Does the obese syndrome alter the fatty acid composition of cardiac tissue and membrane phospholipids in obese (fa/fa) rats? (ii) Are changes, if they occur, similar to those reported for tissues of the genetically obese (ob/ob) mouse? (iii) Can cardiac tissue phospholipids and their component fatty acids be modified by dietary lipids and if so does this occur to the same extent in both fa/fa and lean (Fa/−) rats? Proportions of polyunsaturated fatty acids (PUFA) in cardiac total phospholipids of fa/fa rats differed significantly from those of Fa/− rats and from those reported for ob/ob mice. Increased 18:2n-6 and decreased 20:4n-6 and 22:6n-3 in fa/fa rats indicated impaired PUFA metabolism, possibly reduced Δ6 and/or Δ5 desaturase activity, compared with Fa/− rats. No differences in hepatic Δ6 and Δ5 desaturase activity between fa/fa and Fa/− were found but enhanced activity of Δ9 desaturase activity in fa/fa as compared to Fa/− was evident. Inclusion of sunflower oil (SO) or triolein (TO) at 5% and 20% by weight in the diet elicited marked changes in the fatty acyl composition of cardiac phospholipids in both fa/fa and Fa/− rats when compared with animals fed the control Oxoid diet alone. Supplementation with triolein was most effective, reducing 18:2n-6 and increasing 20:4n-6 proportions in fa/fa rats so that they resembled those in Fa/− rats fed the control Oxoid diet. The type of fat rather than the amount of its dietary intake appears to be the main determinant of the observed changes in phospholipid composition. Pair feeding hyperphagic fa/fa rats to the lower intakes of Fa/− rats did not abolish the characteristic differences in the proportions of PUFA in cardiac phospholipids between the two phenotypes. The effect of triolein on cardiac phospholipid fatty acid composition in the present studies is intriguing and may be relevant in understanding the beneficial role of dietary olive oil in ameliorating cardiovascular disease in man.
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Genetically obese Zucker rats (fa/fa) are characterized by a variety of endocrine and metabolic abnormalities when compared to lean rats (Fa/−) of the same strain. In particular hyperinsulinaemia, hyperphagia, increased energetic efficiency and elevated lipogenesis result in excessive accretion of triacylglycerols in adipose tissue giving the obese (fa/fa) rats their characteristic corpulent appearance (1-8). Increased lipogenesis in fa/fa rats occurs mainly in the liver which also exhibits enhanced Δ9 desaturase activity (9). Low proportions of polyunsaturated fatty acids (PUFA) in tissue lipids of fa/fa compared with Fa/− rats (4-9) are probably a reflection of increased synthesis *de novo* of saturated and monounsaturated fatty acids in these animals. The extent to which these abnormalities in fatty acid metabolism in fa/fa rats are also reflected in the fatty acid composition, particularly the essential PUFA, of their tissue and membrane phospholipids is not known.

Changes in the availability of essential fatty acids (EFA) in membrane phospholipids of cells and tissues are potentially of major physiological significance. EFA are important determinants of both the physicochemical and functional properties of cell membranes. They have a structural role as components of phospholipids in membrane bilayers and, more importantly, they are the precursors of the eicosanoids which are involved in the regulation of cellular and tissue metabolism (8-14). Many membrane-bound enzymes, receptors, ion gates and transport proteins exhibit a requirement for specific phospholipid or PUFA (15-18). Decreased proportions of EFA in tissue phospholipids, resulting from endocrine abnormalities and/or dietary deficiencies, could conceivably impair tissue metabolism and consequently reduce tissue function (10,12-14). This is emphasized by the observations that differences in the fatty acid composition of membrane phospholipids from various tissues of genetically obese mice (ob/ob), when compared to lean phenotypes, correlate with adverse changes in physicochemical and functional properties (5,19-22). Defects in membrane function similar to those found in ob/ob mice also occur in tissues of fa/fa rats, particularly in interscapular brown adipose tissue (IBAT) (5,19). This suggests that similar changes in membrane phospholipids to those observed in ob/ob mice may occur in fa/fa rats. However, sufficient physiological differences exist between ob/ob and fa/fa animals (23) to suggest that obesity may not have the same etiology in these animals and may consequently not elicit the same lipid compositional and functional changes in membranes and tissues from both phenotypes.

The present investigation addressed three questions: (i) Does the obese syndrome in fa/fa rats, apart from eliciting excessive triacylglycerol accretion in adipose tissue, also result in changes in phospholipid fatty acid composition of cardiac tissue and membranes when compared with those from Fa/− rats)? (ii) If differences in phospholipid fatty acid composition occur between rat phenotypes, are they similar to those reported for ob/ob and lean mice? (iii) Can the fatty acid composition of tissue/membrane phospholipids in fa/fa and Fa/− rats be modified by dietary lipids and, if so, does this occur to the same extent in both phenotypes.

*To whom correspondence should be addressed at Rowett Research Institute, Greenburn Road, Aberdeen AB2 9SB, Scotland, U.K.

Abbreviations: BHT, butylated hydroxytoluene; EFA, essential fatty acids; Fa/−, lean; fa/fa, obese; GLC, gas-liquid chromatography; HSO, high sunflower oil; HTO, high triolein; IBAT, interscapular brown adipose tissue; LSO, low sunflower oil; LTO, low triolein; ob/ob, genetically obese; PBS, phosphate buffered saline; PC, phosphatidylcholine; PE, phosphatidylethanolamine; PI, phosphatidylinositol; PS, phosphatidylserine; PUFA, polyunsaturated fatty acids; SO, sunflower oil; TLC, thin-layer chromatography; TO, triolein; WCOT, wall-coated open tubular.

The present studies comprise part of a program of research which seeks to determine the extent to which membrane phospholipids in different organs and tissues of obese and lean Zucker rats can be modified by dietary and endocrine manipulations and whether such modifications are deleterious or beneficial to organ and tissue function. Changes in cardiac phospholipid fatty acid composition due to obesity *per se* and/or dietary and/or endocrine manipulations may also help to elucidate the interaction of these factors in the etiology of cardiovascular disease in man. Although not prone to atherosclerosis, rats will exhibit cardiac arrhythmias with certain dietary fats (12).

Preliminary findings have been published in abstract form (24,25).

MATERIALS AND METHODS

Chemicals were purchased from B.D.H. or Sigma Chemicals Co. (both at Poole, Dorset, U.K.). All organic solvents were ANALAR grade with 0.005% butylated hydroxytoluene (BHT) added as antioxidant. Radiolabelled [1-^{14}C]stearic (18:0), [1-^{14}C]linoleic (18:2n-6) and [1-^{14}C]-dihomo-γ-linolenic (20:3n-6) acid were purchased from Amersham (Amersham, U.K.). High performance silica gel thin-layer chromatography (TLC) plates (LK5) were purchased from Whatman (Maidstone, U.K.). Capillary columns were purchased from Chrompack U.K. Ltd. (London, U.K.).

Animals, diets and tissue sampling. Cardiac tissues were obtained from groups of 35–40-day-old lean (Fa/−) and obese (fa/fa) Zucker rats taken from the Rowett Research Institute's colony (Bucksburn, Aberdeen, Scotland, U.K.). Unless otherwise stated, the rats were fed to appetite for 14–16 days from weaning with free access to water. Animals were individually housed in plastic cages with stainless-steel grid flooring in rooms with a 12-hr light/dark cycle and controlled temperature of 22°C.

The control diet consisted of the standard pelleted laboratory diet (Oxoid, Herbert C. Styles, Bewdley, Worcs, U.K.). Experimental diets were based on the Oxoid diet with 5% and 20% (w/w) supplementation of sunflower oil (SO), low sunflower oil (LSO) and high sunflower oil (HSO), respectively, or triolein (TO), low triolein (LTO) and high triolein (HTO), respectively. Energy content, fat content and fatty acid composition (wt %) of the diets are given in Table 1. The commercial diet also contained 17.3% digestible crude protein, 1.2% digestible fiber, 46.7% digestible carbohydrate, adequate minerals, and vitamins.

During pair-feeding, fa/fa rats (4) were housed individually and fed the amount of control diet that age and sex matched Fa/− rats (4) had consumed the previous day. A group of 3 fa/fa rats fed to appetite were included as a comparison. Desaturase assays were carried out on liver microsomal preparations from age and sex matched fa/fa and Fa/− rats which had been fed to appetite on the control Oxoid diet. The proportions of phospholipid species in cardiac tissues were investigated in age and sex matched fa/fa and Fa/− rats fed to appetite in groups of 3 on the control diet, HTO or LTO diets. Animals were killed by stunning and bleeding. Tissues were excised, washed in ice-cold phosphate buffered saline (PBS), placed in snap-seal polythene bags, frozen in liquid nitrogen and stored at −20°C until analyzed (usually within 2–3 wk).

TABLE 1

Energy Content and Fatty Acid Composition of Rat Diets*

Diet	Energy (k cal/g)	% Fat (w/w)	Fatty acid composition (wt%) 16:0	16:1n-7	18:0	18:1n-9	18:2n-6
HSO[a]	4.4	20	7.4	tr	4.4	19.7	69.4
LSO[b]	3.9	5	8.8	tr	5.2	23.6	59.7
OXOID[c] (control)	3.9	3.0	15.1	1.5	3.6	26.8	44.8
LTO[d]	4.0	5.0	5	6.6	7.3	1.8	61.8
HTO[e]	4.7	20	6.1	7.4	1.4	69.4	10.4

[a]20% sunflower oil; [b]5% sunflower oil; [c]control diet, non-supplemented; [d]5% triolein; [e]20% triolein. Only traces of 18:3n-3, 20:4n-6, 20:n-3, 22:5n-3 and 22:6n-3 detected in experimental diets: Oxoid diet contained 1.2, 1.0 and 1.9 (wt%) of 20:4n-6, 22:5n-3 and 22:6n-3, respectively. Mean of two determinations given.

Analytical methods. Tissue lipids were extracted according to the method of Bligh and Dyer (26), separated into neutral and phospholipids on silica cartridges under vacuum (27). Separation of total phospholipids into individual phospholipid classes was achieved by one-dimensional (28) or two-dimensional (29) thin-layer chromatography (TLC). Phospholipid content was determined by phosphorus estimation according to the method of Bartlett (30). Methyl esters of phospholipid fatty acids were formed by a standard transmethylation procedure with methanolic-HCl (31) and analyzed by gas-liquid chromatography (GLC) on 25 M wall-coated open tubular (WCOT) glass capillary columns containing SIL5 or SIL88 (Chrompack U.K., Ltd., Millharbour, London, U.K.) as the liquid phase in a PU4500 Chromatograph (Pye Unicam Ltd., Cambridge, U.K.) at 195°C and 1.5 kg/cm^2 helium pressure. Peaks were plotted and analyzed on an SP4270 plotter/integrator (Spectra-Physics, San Jose, CA) and identified from retention times of authentic standards on the polar and nonpolar liquid phases.

Microsomal and mitochondrial preparations were obtained by homogenization and ultracentrifugation, and their purity checked by methods essentially as described by Hübscher *et al.* (32). The method for determining Δ9 desaturase activity has been described in detail elsewhere (33). The activity of Δ6 and Δ5 desaturase was assayed according to the method of de Gomez-Dumm *et al.* (34) using [1-^{14}C]labelled 18:2n-6 and 20:3n-6 acids (0.1 μCi/μmole) as the respective substrates. Methyl esters of fatty acids were separated and identified using silica gel G plates impregnated with 10% $AgNO_3$ (35) rather than radio-gas chromatography. Radioactivity determinations were done as described previously (33).

Statics. Significant differences between means of the experimental diets and the control diet were assessed by analysis of variance and Student's t-test. Results are expressed as means ± S.D.

RESULTS

Heart weights and lipid content. Weights and lipid content of hearts from rats fed the control, sunflower oil and triolein diets are given in Table 2. No marked effect of diet on heart weights, total lipid, phospholipid or neutral

TABLE 2

Total Lipid, Phospholipid and Neutral Lipid Content of Hearts from Obese and Lean Zucker Rats Fed on Oxoid, 5% Triolein (LTO), 5% Sunflower oil (LSO), 20% Triolein (HTO) or 20% Sunflower oil (HSO)[a]

Phenotype	Diets	Heart weights (mg)	Total lipid (mg/g tissue)	Phospholipid (mg/g tissue)	Neutral lipid (mg/g tissue)
Obese	HSO (10)	409.6 ± 46.4	41.5 ± 1.3	22.0 ± 0.7	21.5 ± 3.9
	LSO (7)	379.8 ± 37.7	46.0 ± 4.8	22.7 ± 3.0	15.9 ± 3.1
	Control (7)	414.3 ± 97.3	42.9 ± 2.9	21.4 ± 1.7	18.6 ± 0.5
	LTO (8)	473.8 ± 60.0	42.6 ± 2.3	22.5 ± 1.9	19.8 ± 3.0
	HTO (9)	451.0 ± 64.0	44.8 ± 4.3	21.6 ± 2.9	20.9 ± 3.1
Lean	HSO (11)	404.2 ± 80.2	38.5 ± 1.2^{d}	22.2 ± 1.5	18.6 ± 3.6^{b,c}
	LSO (9)	461.5 ± 64.4^{d}	39.6 ± 4.4^{d}	20.7 ± 3.6	14.3 ± 3.5^{b,d}
	Control (6)	419.0 ± 91.0^{b}	37.5 ± 4.5^{d}	22.7 ± 5.1	25.0 ± 3.9^{d}
	LTO (8)	517.0 ± 73.0	35.6 ± 3.6^{d}	18.5 ± 2.0^{b,d}	15.6 ± 2.9^{b,d}
	HTO (10)	462.0 ± 55.0	37.9 ± 3.4^{d}	20.4 ± 2.5	14.8 ± 2.5^{b,d}

[a]Means ± SD are given with the number of animals used in parenthesis. Differences between control and experimental diets within each phenotype are denoted by superscript b = P<0.05 or lesser. Differences between phenotypes fed the same diet, including the control diet, are denoted by superscript d = P<0.05 or lesser. Differences between high and low (H,L) diets denoted by superscript c = P<0.05 or lesser. More precise P values given in text.

lipid was evident within the obese phenotype (fa/fa). Both sunflower oil and triolein diets significantly reduced the neutral lipid content of the lean (Fa/−) rats but did not influence any other parameters studied when compared with the control diet.

Cardiac phospholipids from fa/fa animals fed on the control diet had significantly higher (P<0.001) proportions of 18:2n-6 and lower (P<0.001) proportions of both 20:4n-6 and 22:6n-3 than those from Fa/− rats fed this diet (Table 3). The fatty acid analyses showed a significantly greater proportion of 18:2n-6 (P<0.001) than 20:4n-6 in fa/fa rats and a lower proportion of 18:2n-6 (P<0.01) than 20:4n-6 in Fa/− rats. This pattern of PUFA content is reflected in the greater ratio of 18:2n-6/20:4n-6 in fa/fa compared with Fa/− rats.

Triolein diets significantly reduced 18:2n-6 and increased 20:4n-6 in fa/fa and Fa/− rats (P<0.001 or P<0.01) when compared with the control group. The changes elicited by triolein diets were such that the pattern of 18:2n-6 to 20:4n-6 in fa/fa resembled that of Fa/− rats fed on the control diet; this was reflected in reduced 18:2n-6/20:4n-6 ratios in fa/fa rats. Proportions of 22:6n-3 increased significantly in fa/fa fed HTO (P<0.001) but decreased in Fa/− rats fed either of the triolein diets (P<0.001 and P<0.01 for LTO and HTO, respectively) when compared with rats of each phenotype fed the control diet. Triolein diets, as expected, increased the 18:1n-9 content of cardiac phospholipids in both phenotypes.

Sunflower oil diets also reduced 22:6n-3 in fa/fa (P<0.001 for HSO) and in Fa/− rats (P<0.001 for LSO and HSO). These diets also increased 20:4n-6 proportions by a significant but to a lesser extent than triolein diets in fa/fa (P<0.01 and P<0.05 for LSO and HSO, respectively). Neither the pattern of 18:2n-6 relative to 20:4n-6 nor the 18:2n-6/20:4n-6 ratio was altered toward that observed in Fa/− rats fed on the control diet when fa/fa rats were fed on the sunflower oil diets. This contrasts with the marked changes in these parameters elicited when fa/fa rats were fed on the triolein diets. There appeared to be an inverse relationship between dietary 18:2n-6 concentration and cardiac phospholipid 22:6n-3 proportions. Sunflower oil diets elicited a reduction in the 18:1n-9 proportions of cardiac phospholipids (P<0.001 for LSO and HSO, respectively) in fa/fa rats but LSO produced an increase in 18:1n-9 in the Fa/− phenotypes (P<0.05).

Cardiac subcellular preparations. The characteristic differences in the pattern of PUFA in cardiac phospholipids between fa/fa and Fa/− rats were also found in cardiac microsomal and mitochondrial preparations derived from cardiac tissue of the two phenotypes which had been fed the control diet (Fig. 1). The characteristically lower 22:6n-3 in cardiac phospholipids from fa/fa compared with those from Fa/− was also observed in microsomal but not mitochondrial preparations.

Cardiac phospholipid classes. The effect of the HTO diet on the proportions of the major PUFA in phosphatidylcholine (PC) and phosphatidylethanolamine (PE) of cardiac tissue from the two phenotypes is shown in Figure 2. The distinctive differences in the fatty acid pattern of cardiac total phospholipids between phenotypes fed the control diet were more accentuated, particularly in the PC fraction of fa/fa rats. This was again reflected in the 18:2n-6/20:4n-6 ratios (3.7 and 0.6 for fa/fa and FA/−, respectively). In the cardiac PE fractions from rats fed the control diet, the proportion of 18:2n-6 was significantly higher (P<0.001) in fa/fa compared to Fa/− rats, although the proportion of 20:4n-6 was similar; this was again reflected in a higher 18:2n-6 to 20:4n-6 ratio in fa/fa than Fa/− rats (0.9 and 0.4, respectively). The

TABLE 3

Fatty Acid Composition (wt%) of Cardiac Total Phospholipids from Obese and Lean Zucker Rats Fed Various Diets[a]

Phenotype	Diet	Fatty acids							Ratios	
		16:0	16:1	18:0	18:1	18:2	20:4	22:5	22:6	18:0/18:1	18:2/20:4
Obese	HSO (6)	11.6 ± 0.8^{c}	0.3 ± 0.05^{b,c}	24.6 ± 0.6^{b,c}	8.1 ± 0.2^{b,c}	26.7 ± 0.9^{b,b}	14.9 ± 0.12^{b}	2.6 ± 0.1^{b}	3.8 ± 0.3^{b}	3.04	1.79
	LSO (6)	13.2 ± 0.4	0.7 ± 0.05	21.9 ± 0.4^{b}	10.1 ± 0.3^{b}	22.4 ± 0.9^{b}	15.3 ± 0.5^{b}	2.8 ± 0.7	5.2 ± 0.8	2.17	1.46
	Control (7)	12.8 ± 1.7	0.6 ± 0.007	19.0 ± 0.5	12.1 ± 0.5	25.8 ± 1.8	13.2 ± 1.5	2.7 ± 0.2	6.4 ± 1.2	1.60	1.90
	LTO (6)	12.8 ± 0.8	1.6 ± 0.11^{b}	20.6 ± 0.4^{b}	16.2 ± 1.0^{b}	14.3 ± 1.1^{b}	15.8 ± 0.8^{b}	2.2 ± 0.5^{b}	7.4 ± 1.5	1.27	0.91
	HTO (6)	10.3 ± 0.4^{b,c}	0.8 ± 0.1^{b,c}	17.5 ± 0.7^{b,c}	15.6 ± 1.0^{b}	12.5 ± 1.6^{b,c}	18.9 ± 1.1^{b,c}	2.4 ± 0.1^{b}	10.7 ± 0.9^{b,c}	1.12	0.66
Lean	HSO (6)	12.8 ± 3.1^{c}	0.2 ± 0.08^{c}	26.4 ± 1.5^{b,d,c}	8.4 ± 1.2	20.8 ± 1.6^{b,d,c}	19.3 ± 0.3^{b,d,c}	2.6 ± 0.2^{c}	4.0 ± 1.3^{b,c}	3.14	1.10
	LSO (6)	14.0 ± 0.7	0.5 ± 0.08^{b,d}	25.3 ± 0.3^{b,d}	9.2 ± 0.2^{b}	15.9 ± 1.6^{d}	20.9 ± 1.4^{b,d}	1.9 ± 0.5^{b,d}	7.1 ± 0.9^{b,d}	2.75	0.76
	Control (6)	12.7 ± 1.1	0.3 ± 0.05^{d}	23.2 ± 0.9^{d}	8.3 ± 0.8^{d}	16.1 ± 0.4^{d}	18.1 ± 1.3^{d}	2.8 ± 0.26	11.8 ± 1.2^{d}	2.80	0.88
	LTO (6)	12.8 ± 0.5	1.05 ± 0.11^{b,d}	21.2 ± 0.5^{b}	15.9 ± 0.9^{b}	12.0 ± 1.3^{b,d}	20.6 ± 1.3^{b,d}	1.95 ± 0.15^{b,d}	6.7 ± 1.4^{b,d}	1.33	0.58
	HTO (6)	9.6 ± 0.7^{b,c}	0.7 ± 0.0^{b,c}	18.8 ± 0.6^{b,c}	16.3 ± 0.7^{b}	10.8 ± 3.3^{b,d}	22.8 ± 1.6^{b,d,c}	1.9 ± 0.2^{b}	7.9 ± 2.6^{b,d}	1.15	0.47

[a]Means ±SD are given with the number of animals used in parenthesis. Differences between control and experimental diets within each phenotype are denoted by superscript b = P<0.05 or lesser. Differences between phenotypes fed the same diet, including the control diet, are denoted by superscript d = P<0.05 or lesser. Differences between high (H) and low (L) diets within phenotypes are denoted by c = P<0.05 or lesser. Ratios derived from mean values. More precise P values given in text.

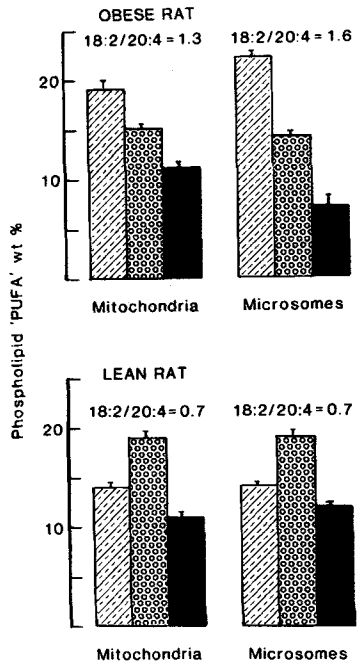


FIG. 1. Proportions of the major PUFA (wt%) in total phospholipids of cardiac mitochondrial and microsomal membranes from fa/fa and Fa/— rats fed on Oxoid diet. Diagonal, stipled hatching, 18:2n-6; circled hatching, 20:4n-6; closed hatching, 22:6n-3. Means and ranges of 2 experiments given. 18:2n-6/20:4n-6 ratios given.

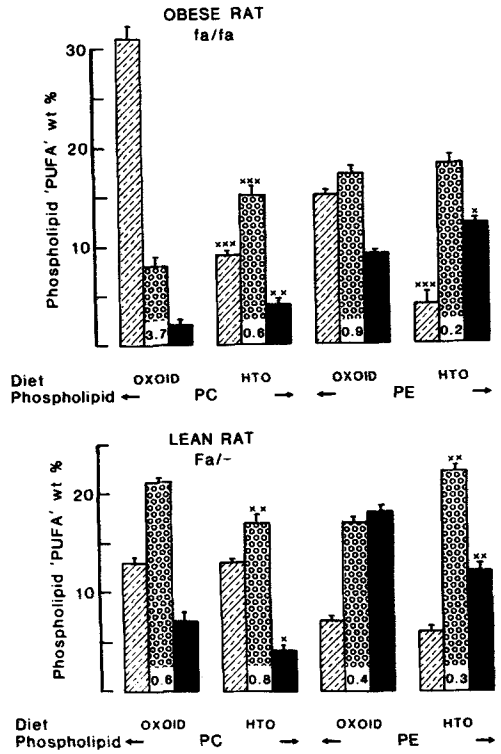


FIG. 2. Proportion of the major PUFA (wt%) in the main phospholipid classes (PC and PE) of cardiac tissue from fa/fa and Fa/— rats fed the control (Oxoid) or triolein (HTO) diets. Diagonal, stipled hatching, 18:2n-6; circled hatching, 20:4n-6; closed hatching, 22:6n-3. Means ± S.D. of 3–5 animals/group are given. P values * = <0.05; ** = <0.01, *** = <0.001 when compared with control diet. Ratios of 18:2n-6/20:4n-6 given in brackets in the 20:4n-6 column.

proportion of 22:6n-3 was significantly lower (P<0.001) in the PE fractions from fa/fa as compared to Fa/− rats on the control diets.

The HTO diet altered the pattern of PUFA in the PC fraction from fa/fa rats to resemble that of the PC fraction from Fa/− rats fed the control diet; this is reflected in the identical 18:2n-6/20:4n-6 ratios (0.6 for both fa/fa − HTO and Fa/− control groups). The altered pattern of PUFA in cardiac PC from fa/fa rats on the HTO diet was due to decreased 18:2n-6 (P<0.001) and increased 20:4n-6 (P<0.001) proportions when compared with fa/fa fed the control diet. HTO also increased the proportion of 22:6n-3 (P<0.01) in PC from fa/fa rats. In Fa/− rats, however, 18:2n-6 proportions in cardiac PC remained unchanged with HTO diet and both 20:4n-6 and 22:6n-6 proportions were reduced (P<0.01 and P<0.05, respectively). The effect of HTO diet on the PC composition was greatly attenuated in Fa/− rats when compared to fa/fa rats.

In cardiac PE fractions the HTO diet reduced 18:2n-6 (P<0.001) and increased 22:6n-3 (P<0.05) in fa/fa rats and increased 20:4n-6 (P<0.01) but decreased 22:6n-3 (P<0.01) in Fa/− rats when compared with values found in the respective phenotypes fed the control diet.

The LTO and HTO diets also altered the proportions of individual phospholipid classes in cardiac tissue from both phenotypes (Table 4). HTO significantly increased PC (P<0.01) and decreased PE (P<0.01) in fa/fa rats. HTO also decreased PE (P<0.01) without affecting PC and decreased PI (P<0.05) in Fa/− rat tissue. LTO diets decreased phosphatidylserine (PS) (P<0.02) and phosphatidylinositol (PI) (P<0.02) in cardiac tissue of fa/fa rats. Unfortunately, the effect of LTO on Fa/− rat cardiac tissue was not determined.

Food intake, phospholipid fatty acids and desaturases. Reducing the food intake of the normally hyperphagic fa/fa rats by feeding them the same amount of food as age and sex matched Fa/− rats (pair-feeding) did not alter the pattern or proportions of fatty acids in their cardiac total phospholipids (data not shown).

No differences in hepatic Δ6 and Δ5 desaturase activity were found between fa/fa and Fa/− rats fed the control diet (data not shown). The Δ9 desaturase activity of hepatic microsomal preparations was significantly greater in fa/fa (15.0±2.1 nmol/30min/mg protein) as compared to Fa/− rats (4.0±1.2 nmol/30min/mg protein) (P<0.001).

DISCUSSION

The significant reductions in the neutral lipid content of hearts from Fa/− rats fed on the sunflower oil and triolein diets were surprising and no simple explanation is apparent. Whether these high fat diets induce a greater rate of mobilization and/or oxidation of fat in cardiac tissue or a reduction in its uptake into the tissue is not clear and would require further investigation.

The characteristic differences observed between phenotypes in the pattern of PUFA in their cardiac total phospholipids, namely increased 18:2n-6 and decreased 20:4n-6 proportions in fa/fa, indicated an impairment in essential PUFA metabolism in the fa/fa compared with Fa/− rats. This was highlighted by the marked differences in 18:2n-6/20:4n-6 ratios between phenotypes on the Oxoid diet which, although not definitive, are considered as indicators of the activity of the Δ6 and Δ5 desaturase enzymes responsible for PUFA metabolism *in situ.* The proportions and ratios of 18:0 and 18:1 indicated enhanced conversion of 18:0 to 18:1 in fa/fa cardiac tissue which is a characteristic feature of fatty acid metabolism in fa/fa animals (9). The findings that the characteristic pattern of PUFA for the two phenotypes persisted in both mitochondrial and microsomal membrane preparations and to a greater or lesser extent in the two major phospholipid classes of cardiac membranes are indicative of their fundamental nature and presumably a reflection of the metabolic and endocrine abnormalities in the fa/fa rats *(vide supra).* In contrast, only minor PUFA compositional changes were observed in the phospholipid classes of hepatic (21) and adipocyte (20) plasma membranes from lean and ob/ob mice. This suggests that the aetiology of obesity *per se* may vary between species and that it does not influence membrane phospholipid composition in a uniform manner in all species. This is not surprising considering the differences that exist between ob/ob and fa/fa animals in certain endocrine and metabolic abnormalities (5). Alternatively, membrane phospholipids from different tissues within an individual animal from a certain species and phenotype may be affected differently by the endocrine and metabolic changes characteristic of a particular form of obesity. Unfortunately, information on the fatty acid composition of cardiac membrane phospholipids in other forms of obesity, particularly in the ob/ob mouse, is

TABLE 4

Effect of Diet on the Proportions of Phospholipid Classes in Cardiac Tissues of Obese and Lean Zucker Rats[a]

Diet	Phenotype	PS	PA	PI	PC	PE	CL	PE/PC
Control	Obese	6.7 ± 0.3	2.4 ± 0.6	6.5 ± 0.8	40.2 ± 1.4	36.0 ± 1.2	5.6 ± 0.8	0.90 ± 0.03
	Lean	5.7 ± 0.7	2.1 ± 0.2	5.0 ± 0.1	42.6 ± 1.0	38.0 ± 0.2	4.4 ± 0.1	0.90 ± 0.02
LTO	Obese	5.6 ± 0.3[b]	2.1 ± 0.2	4.0 ± 0.6[b]	40.5 ± 0.8	35.4 ± 1.1	5.4 ± 0.3	0.88 ± 0.03
	Lean	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
HTO	Obese	6.1 ± 0.2	2.8 ± 0.4[c]	5.1 ± 0.6[c]	44.3 ± 0.7[b,c]	30.4 ± 1.0[b,c]	4.3 ± 1.4	0.69 ± 0.03
	Lean	5.9 ± 1.2	2.1 ± 1.1	3.8 ± 0.7[b]	42.1 ± 3.1	34.5 ± 1.0[b]	5.4 ± 0.9	0.82 ± 0.04

[a]Values are means ± SD of 3 animals/group. Values significantly different from control/diet are indicated by superscript b = P<0.05 or less. Differences between HTO and LTO diets in obese rats denoted by superscript c = P<0.05 or less. Control diet is standard Oxoid laboratory diet; LTO, low triolein diet (5%); HTO, high triolein diet (20%); n.d., not determined; PS, phosphatidylserine; PA, phosphatidic acid; PI, phosphatidylinositol; PC, phosphatidylcholine; PE, phosphatidylethanolamine; CL, cardiolipin.

lacking. Unpublished observations from our laboratory support the view that membrane phospholipids from various tissues in the fa/fa rat, not surprisingly, differ in composition but they also respond differently, in both magnitude and extent, to dietary and endocrine manipulations. Despite the similarity in membrane phospholipid fatty acid composition of the same tissues from lean and ob/ob mice, marked differences in composition of hepatic and adipocyte membranes from ob/ob mice have also been observed (20,22).

The effect of the triolein diets on the proportions and pattern of the major PUFA in cardiac total phospholipids of fa/fa rats was quite dramatic. They elicited what might be described as a 'normalizing' effect with a marked decrease in 18:2n-6 and increases in 20:4n-6 and/or 22:6n-3 so that the fa/fa pattern resembled that of the Oxoid-fed Fa/− 'control' rats in both total phospholipid and in the individual PC and PE fractions. These effects of triolein diets could be interpreted as an induction of the Δ6/Δ5 desaturase pathway, which is supported by marked changes in 18:2n-6/20:4n-6 ratios so that they resembled those found in Oxoid-fed Fa/− rats. An induction of Δ5 desaturase by diets high in oleic acid has been reported (33) but this was attributed to a general deficiency of 18:2n-6 and other essential PUFA since increased proportions of 20:3n-9, a fatty acid which is considered to be a good indicator of such a deficiency, were found (32). No such increases in 20:3n-9 were observed while feeding the present diets (data not given) suggesting that essential PUFA levels in the triolein diets were adequate but low. The precise mechanism by which low or deficient dietary 18:2n-6 levels regulate Δ6/Δ5 desaturase activity has not been elucidated and warrants further investigation. A simple substrate inhibition of enzyme activity seems an unlikely explanation since the LSO and HSO diets in the present investigations gave no indication of such a mechanism as judged by the increased 20:4n-6 proportions in cardiac phospholipids from both fa/fa and Fa/− rats fed these diets.

That fa/fa rats may have impaired PUFA metabolism when compared with Fa/− rats is suggested by the marked differences in the proportions of PUFA in their tissues, namely high 18:2n-6 relative to 20:4n-6 in the fa/fa (9,10,24,33). In this respect, fa/fa rats resemble primates and man (12,36). Although the hepatic activity of the Δ6 and Δ5 desaturases did not differ between fa/fa and Fa/− rats, and this is considered the major quantitative site for PUFA metabolism in rats, cardiac activity of these enzymes, which was not determined, may be sufficiently impaired in fa/fa rats to account for the observed differences in PUFA profiles between phenotypes. This point requires further elucidation.

The response of total phospholipids and individual phospholipid classes of Fa/− rat heart to dietary lipid was less pronounced than those of fa/fa rats. This may be indicative of a greater responsiveness of 'abnormal' fa/fa tissues to modification of their lipid composition by dietary means. This point was illustrated by the marked effect of the HTO diet on cardiac phosphatidylcholine composition in fa/fa compared with Fa/− rats. The phospholipid class distribution in tissues and membranes is generally not amenable to dietary modulation (37–39). However, the present studies show significant changes in PS, PI, PC and PE content of cardiac tissues from fa/fa and Fa/− rats fed the triolein as compared with the control Oxoid diet. Dietary modulations of PE/PC ratios have been observed in cardiac mitochondria, brain and intestine of rats (40–42). The significant decrease in PE and increase in PC observed in cardiac phospholipids of fa/fa rats fed the HTO as compared with the control diet could be indicative of an enhanced methylation of PE to PC elicited by dietary triolein in these animals. This requires verification. Methylation of PE to PC has been postulated as an endocrine-signal transduction mechanism in cell membranes (43) but not without question (44). Increasing the proportion of PC relative to PE in cell membranes can lead to increased membrane viscosity (decreased fluidity) similar to that observed when the saturation index of the phospholipid acyl chains is increased (10,11). Changes in the physicochemical characteristics of cell membranes due to modifications of phospholipid polar head groups or fatty acyl composition have been shown to alter membrane-bound functional proteins in such cells and tissues (10–12). The decrease in the proportion of PI in fa/fa and Fa/− rats fed triolein diets could also have functional implications since PI plays a major role in endocrine signal transductions across cell membranes (45). Investigation of certain functional changes in relation to membrane lipid modification will be determined in future studies.

The observed changes in phospholipid PUFA composition of cardiac tissues elicited by dietary lipid reflect the importance of the type of fatty acid in the lipid rather than the amount of lipid. This is supported by the pair-feeding experiments where fa/fa rats were given the same amount of feed as Fa/− rats, *i.e.* a food restriction regime, without any changes in the fatty acid composition of cardiac phospholipids being evident.

Clearly, the type of dietary fats, in this case particularly triolein diets, can significantly alter the proportions of phospholipids and their fatty acyl constituents in cardiac tissues of fa/fa and to a lesser extent Fa/− rats. The precise relevance of the observed changes in tissue phospholipids to the physiological function of the tissue is not clear at present and requires further investigation. Similarly, the mechanism(s) by which the triolein diets exert their influence on tissue phospholipid PUFA composition, whether by a direct effect on desaturase enzymes or through modification of endocrine stimulation, is not clear and warrants further study.

Ascertaining the relevance of these observations to the understanding of the role of dietary lipids in cardiac function and dysfunction in man would be speculative. However, it is interesting to note the apparent beneficial effect of dietary olive oil with its high content of oleic acid on the incidence of heart disease in Mediterranean populations (46).

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The Presence of Oxidative Polymeric Materials in Encapsulated Fish Oils

Vijai K.S. Shukla[a] and **Edward G. Perkins**[b,*]

[a]Karlshams Research and Development Center, P.O. Box 68, DK8520, Lystrup, Denmark and [b]Department of Food Science, University of Illinois, Burnsides Research Laboratory, Urbana, Illinois 61801

Encapsulated health food oils such as fish oils are readily available in health food stores, pharmacies, and supermarkets. They are popular in the United States as well as in the European countries. However, such oils, because of their high degree of unsaturation, are easily oxidized and form complex mixtures of high molecular weight oxidation products. The present work reports the application of high-performance size exclusion chromatography to the determination of these materials in encapsulated fish oils. Of the six samples studied, five showed from 1–10% of dimeric triacylglycerols and one contained 6.3% trimeric triacylglycerols and 3.1% oligomeric triacylglycerols. Further investigation of this sample with silicic acid chromatography indicated that it contained a total of 36.3% polar material.

Lipids 26, 23–26 (1991).

Fish oils have been extensively studied in recent years due to their antiatherosclerotic (1–3) and hypotriglyceridemic effects (4). Recent reports have discussed the oxidative deterioration of fish oils and have suggested that ingestion of unstabilized fish oils entails a risk of exposure to potentially toxic products of n-3 fatty acid peroxidation. The resultant oxidation products may play a role in carcinogenesis (5,6), inhibit prostacyclin production (7) and may exert other adverse biological effects (8).

Unless extreme care is taken, fish oils, which contain a high percentage of long-chain polyunsaturated fatty acids such as eicosapentaenoic and docosahexaenoic acids in triacylglycerol form, are very susceptible to autoxidation (9,10). Such autoxidation will initially form hydroperoxy free radicals which become stabilized by addition of hydrogen to hydroperoxides or by reaction with triacylglycerols to form dimeric and trimeric triacylglycerols linked *via* peroxidic-type bonds (10).

Encapsulated fish oils are now readily available for purchase. It would be useful to ascertain the presence or absence of such oxidation products and high molecular weight materials in such oils. The technique of high-performance size exclusion chromatography (HPSEC) is well established and has been employed repeatedly to separate triacylglycerols from higher molecular weight oxidation products such as the dimeric, trimeric, and oligomeric materials formed as a result of lipid oxidation (10–20).

It was the purpose of this work to examine several different brands of encapsulated fish oils in order to ascertain the presence or absence of higher molecular weight oxidation products.

*To whom correspondence should be addressed at the Department of Food Science, University of Illinois, Burnsides Research Laboratory, 1208 West Pennsylvania Avenue, Urbana, IL 61801.

Abbreviations: DG, diacylglycerol; FFA, free fatty acids; HPLC, high-performance liquid chromatography; HPSEC, high-performance size exclusion chromatography; MG, monoacylglycerol; TG, triacylglycerol; THF, tetrahydrofuran.

EXPERIMENTAL

Various brands of encapsulated fish oils were purchased locally. Three capsules of fish oil from each of different bottles from different batches were opened. The oil from each capsule was individually taken up into tetrahydrofuran (THF) to make a 20% solution by weight of oil. This solution was injected directly into each of the high-performance liquid chromatography (HPLC) column systems described below. Two different sets of columns were used as follows: Set one was a series of connected polystyrene-divinyl benzene polymer packed columns, in the order 500 Å, 100 Å, and 100 Å. Each column was 30 cm × 4.7 mm stainless steel (PL Laboratories, Shropshire, England) and the packing was 5 microns in diameter. Column set two was a series connected G 2000, to a G 1000 column, prepared with the same general type of polymeric packing as above, 5 microns diameter, 30 cm × 7 mm diameter (Supelco Inc., Bellefonte, PA). Detectors used were the Waters Model 410 differential refractometer and a Du Pont infrared detector set at 1735 cm^{-1} to monitor the ester carbonyl absorption. The remainder of the HPLC system and data handling system was as previously described (21). In both column systems, tetrahydrofuran (THF) was used as the eluting solvent at a flow rate of 1 mL/min. The THF used as mobile phase was kept under an atmosphere of nitrogen gas, and contained 0.027% butylated hydroxytoluene to minimize peroxide formation. This did not interfere with the HPSEC since it eluted in the low molecular weight exclusion volume peak at the end of the run. Polymeric standards as well as lipid standards were obtained from Supelco Inc.

Fractionation of oils into polar and nonpolar components was carried out according to the International Union of Pure and Applied Chemistry (IUPAC) method for the determination of polar components in fats using silicic acid column chromatography (22). The determination of peroxide, iodine, and anisidine values was carried out according to the IUPAC methods (21). Each sample was analyzed in triplicate and the range of values obtained reported. The fatty acid composition of the fish oils was determined according to the procedure of Ackman *et al.* (23). Tocopherols were determined according to the method described by Shukla (24).

RESULTS AND DISCUSSION

Data for six encapsulated oil samples which were analyzed are shown in Table 1. A considerable range of quality is shown as evidenced by the peroxide value and anisidine value. The range of peroxide values found as well as the anisidine values, which are a measure of aldehyde content of a fat, indicate that both hydroperoxides and other types of peroxide linkages as well as their decomposition products are present in these oils. The fatty acid composition of the encapsulated material is given in

TABLE 1

Analytical Values of Encapsulated Fish Oils[a]

Product	Peroxide value	Anisidine value	Iodine value
A[a]	3.2-3.2	29.8-30.5	190.5-190.4
B[b]	2.7-2.6	14.2-14.4	196.2-196.9
C[c]	3.2-2.6	16.9-17.2	203.9-204.8
D[d]	2.2-2.2	29.8-28.6	197.2-201.7
E[e]	20.6-20.9	17.4-18.3	242.4-241.0
F[f]	1.6-1.5	27.5-27.6	289.8-290.6

[a]Range of values from analyses of three different capsules' contents. Expiration date: [a]10/89, [b]11/89, [c]7/91, [d]9/89, [e]10/89, [f]9/90. Analyzed: 3/1/89-5/1/89.

TABLE 2

Fatty Acid Composition of Various Encapsulated Oils

Fatty acid	A	B	C	D	E	F
14:0	7.0	5.8	5.7	6.4	1.0	1.0
15:0	0.6	0.5	0.6	0.8	0.1	0.1
16:0	15.8	15.6	14.3	16.4	2.6	2.7
16:1n-7	9.8	8.8	8.1	8.7	1.5	5.3
17:0	0.4	0.4	0.7	0.6	0.2	0.6
18:0	4.4	4.1	3.8	4.5	2.5	0.5
18:1n-9	15.6	14.9	14.4	13.0	10.7	9.7
18:2n-6	3.7	4.3	4.0	3.4	1.5	3.7
18:3n-3	1.0	1.5	1.2	1.1	1.1	1.2
18:3n-6	—	—	—	—	—	—
18:4n-3	2.4	3.5	4.0	2.7	2.7	5.8
20:0	—	0.6	—	0.6	0.6	—
20:1n-9	2.2	3.3	3.3	2.1	12.7	3.2
20:2n-6	—	—	—	—	—	—
20:4n-3	0.8	1.1	1.3	0.9	1.8	1.6
20:4n-6	0.9	0.7	0.8	1.1	1.2	1.5
20:5n-3	17.3	17.3	18.9	16.2	24.2	33.7
22:0	—	0.1	—	0.2	0.1	—
22:1n-9	—	—	—	—	—	—
22:1n-11	1.7	2.3	3.0	0.9	10.5	1.4
22:4n-3	0.7	0.7	0.7	0.6	1.2	1.3
22:5n-3	2.8	1.9	2.0	3.1	4.1	3.7
22:6n-3	12.3	11.9	12.6	15.8	18.9	22.8
24:0	—	0.1	—	0.1	Tr.	—
24:1n-9	—	—	—	—	—	—
24:1n-11	0.5	0.5	0.6	0.7	0.7	0.1

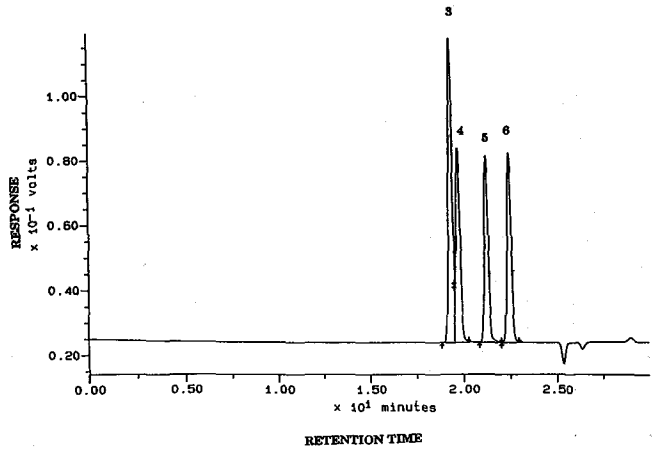


FIG. 1. High-performance size exclusion chromatography of lipid standards. Peak Identity: 3, triacylglycerols; 4, diacylglycerols; 5, monoacylglycerols; 6, free fatty acids.

TABLE 3

Composition of Encapsulated Fish Oils[a]

Sample	FFA[b]	MG[c]	DG[d]	TG[e]	Dimer TG[f]	Trimer TG[g]	Oligomers[h]
A	0.8	0.8	2.9	94.5	2.0	Tr	—
B	0.6	1.2	4.5	92.4	1.3	Tr	—
C	—	0.5	—	98.4	1.0	Tr	—
D	0.8	0.4	1.2	96.3	1.3	Tr	—
E	80.7[i]	9.3	—	10.0	—	—	—
F	0.9	1.1	14.6	64.1	10.0	6.3	3.1

[a]Wt % determined by HPSEC using weighed mixtures of standards to determine response factors.
[b]FFA, free fatty acids, mol wt *ca.* 313 dalton (Da).
[c]MG, monoacylglycerol, mol wt *ca.* 402 Da.
[d]DG, diacylglycerol, mol wt *ca.* 715 Da.
[e]TG, triacylglycerol, mol wt *ca.* 1028 Da.
[f]Dimer TG, dimeric triacylglycerol, mol wt *ca.* 2056 Da.
[g]Trimer TG, trimeric triacylglycerol, mol wt *ca.* 3084.
[h]Oligomers, polymers of triacylglycerols with multiple mol wts of $(1028)_{x+3}$ mol wt calc'd using 22:5 as average chain length and unsaturation (313 Da).
[i]Ethyl esters; the sample is a concentrate of fatty acid ethyl esters.

Table 2. The values appear to be typical of those obtained from fresh oils (23).

Further analysis of each of the encapsulated oils by high-performance size exclusion chromatography (HPSEC) with two different column systems and comparison with standard mixtures of lipids (Fig. 1) indicated that all of the samples contained varying percentages of mono- and diacylglycerols as well as dimeric and higher molecular weight triacylglycerols (Table 3). The appearance of free fatty acids, mono- and diacylglycerols may be a result of enzyme action on the oils prior to encapsulation. As an example of the separation obtained, a chromatogram of sample F obtained from one of the chromatographic systems is shown in Figure 2. High molecular weight thermal and oxidative products may have been formed as a result of thermal treatment of the oil such as in deodorization or as a result of autooxidation prior to encapsulation. High molecular weight oxidation products may have also been formed in the oils after encapsulation. It has been shown previously that soft gelatin capsules are somewhat permeable to oxygen and that this is dependent upon the plasticizer content as well as storage conditions (25). Therefore a certain amount of autooxidation may have taken place within the capsule, at a more increased rate considering the increased susceptibility of fish oils toward oxidation as indicated by Cho *et al.* (26). These authors indicated that the oxygen uptake of eicosapentaenoic and docosahexaenoic acid was 5.2 and 8.5 times faster, respectively, than of ethyl linolenate.

The oils examined in the present study contained from 164 to 7,965 ppm of tocopherols (Table 4), presumably added to stabilize the oils toward oxidation. However, tocopherols are known to be rather poor antioxidants

TABLE 4

Tocopherol Composition of Encapsulated Fish Oils

Product	Tocopherols (total) ppm	Alpha T[a] ppm	Gamma T[b] ppm	Delta T[c] ppm	Alpha TT[d] ppm	Gamma TT[e] ppm
A	164	141	—	—	23	—
B	7965	1380	4349	2236	—	—
C	2475	173	1391	911	—	—
D	859	851	8	—	—	—
E	1744	630	737	376	—	1
F	4399	492	2346	1558	—	3

[a]Alpha tocopherol. [b]Gamma tocopherol. [c]Delta tocopherol. [d]Alpha tocotrienol. [e]Gamma tocotrienol.

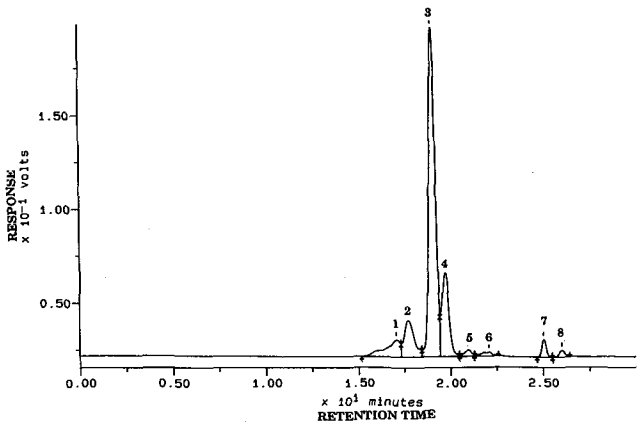


FIG. 2. High-performance size exclusion chromatogram of encapsulated oil (sample F). 1, Triacylglycerol trimer and oligomers; 2, triacylglycerol dimer. Peaks 3–6 are identified in Figure 1. Peaks 7 and 8 are residual solvent and short-chain materials of mol wt *ca.* 100, at the low mol wt exclusion limit of the columns.

when used in edible oils. They are even less efficient when used with oils containing polyunsaturated fatty acids (27). Furthermore, depending upon their level in an oil, tocopherols can exhibit prooxidant effects. In soybean oil containing 1,500 ppm of mixed tocopherols, optimum stability was found when the tocopherol levels were reduced to 400–600 ppm (27). The products examined in this work contained, with the exception of one sample, high levels of tocopherols (Table 4). In view of the prooxidant effects of high levels of tocopherols, and the known permeability of gelatin capsules toward oxygen (25), autooxidation may be expected to continue within an encapsulated oil.

In order to determine the general types of materials found as dimeric, trimeric and oligomeric triacylglycerols, further chromatography was carried out. Oils can contain materials resulting from both thermal and oxidative stress. Thermal stress, occurring in the absence of oxygen produces a variety of high molecular weight materials which elute from HPSEC columns with the same elution volumes as those materials shown in Figures 1 and 2. These materials, because of their elution volumes, can also be described as dimers, trimers and oligomers of triacylglycerols. Ratnayake *et al.* (13) have recently indicated that there were significant levels of materials defined as "polymers" in 10 of 16 samples of encapsulated fish oils they examined. However these authors did not further define the composition of the "polymeric" material. Furthermore the same types of materials may

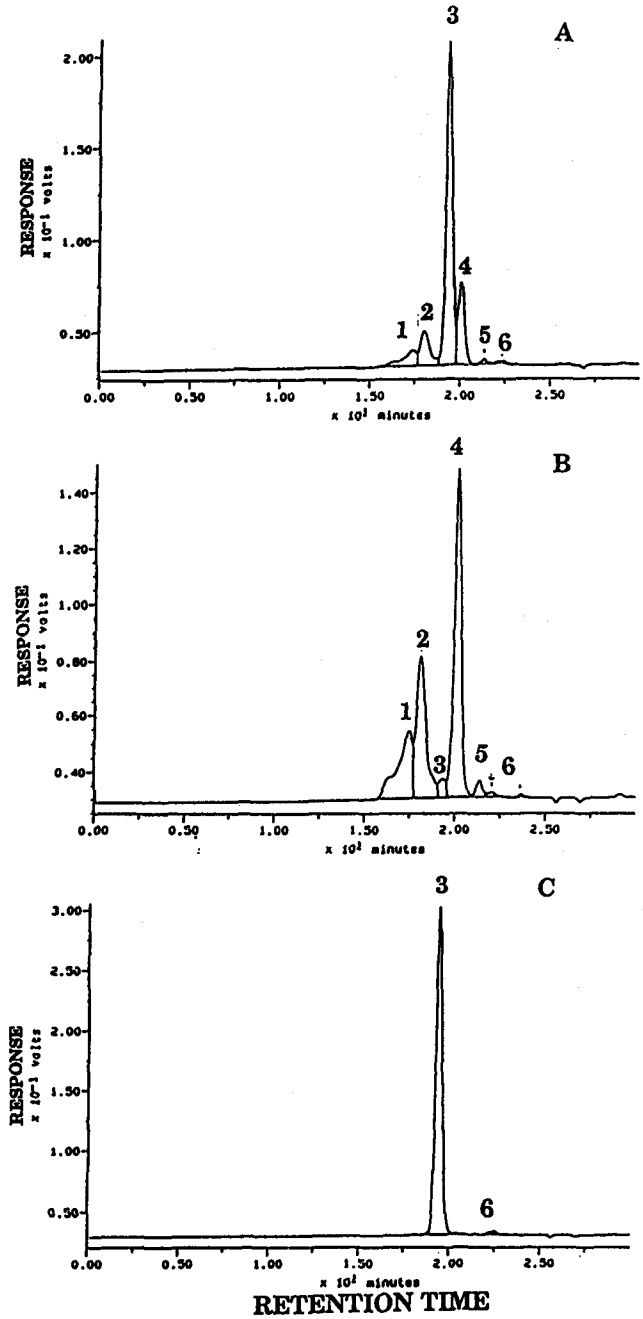


FIG. 3. High-performance size exclusion chromatogram of the polar (B) and nonpolar (C) fractions resulting from silicic acid chromatography of encapsulated oil sample F (A). Peak identity as in Figures 1 and 2.

be formed as a result of oxidative stress and usually contain peroxide linked triglyceride moieties. These materials also exhibit the same elution volumes on the HPSEC columns since their resolution is such that high molecular weight materials differing in molecular weight by one or more extra oxygen atoms cannot be separated. However, it is possible to separate oils which may contain high molecular weight compounds with the aid of adsorption chromatography on silicic acid. The oils can be easily separated into a fraction containing the nonpolar components of the oil and another containing polar and polymeric material (22). Separation of the oil sample F into its nonpolar and polar fraction with the aid of a silica column using the method specified in the IUPAC manual (22) indicated that it contained 36.3 wt % of polar material (by gravimetric determination of the eluted fractions from the IUPAC method). Subsequent HPSEC analysis of both the nonpolar and polar fractions is shown in Figure 3. HPSEC indicated that the polar fraction consisted of materials corresponding to the molecular weights and agreeing with the elution volumes of dimeric and higher polymeric materials, mono- and diacylglycerols and free fatty acids. The nonpolar fraction appeared to contain only triacylglycerol. The data indicated that the higher molecular weight material present is likely to be composed of triacylglycerol bonded *via* peroxy bridges. Heating this oil will lead to the destruction of the peroxidic linkages and the formation of both volatile and nonvolatile degradation products.

Further research on the chemical structure of the products formed in these oils as well as on their nutritional and physiological effects is currently under way in our laboratories.

It appears that because of the unknown health effects of the oxidative polymeric materials, and their high level in some encapsulated oils, caution may be advised when ingesting fish oil capsules on a regular basis.

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Effects of Oleic, Arachidonic and 5,8,11,14-Nonadecatetraenoic Acids on Lipid Secretion and Ketogenesis in Perfused Rat Liver

Ikuo Ikeda[a,*], Jun Murakami[a], Takayuki Oka[a], Michihiro Sugano[a], Hideaki Yamada[b], Sakayu Shimizu[b], Hiroshi Kawashima[c], Yoshifumi Shinmen[c] and Teruo Amachi[c]

[a]Laboratory of Nutrition Chemistry, Kyushu University School of Agriculture 46-09, Higashi-ku, Fukuoka 812, [b]Department of Agricultural Chemistry, Faculty of Agriculture, Kyoto University, Sakyo-ku, Kyoto 606 and [c]Laboratory of Microbial Science, Institute for Fundamental Research, Suntory Ltd., Shimamoto, Osaka 618, Japan

The effects of perfused oleic (18:1n-9), arachidonic (20:4n-6) and 5,8,11,14-nonadecatetraenoic (19:4n-5) acids on triglyceride and cholesterol secretion and ketone body production were studied in isolated rat liver. As compared to oleic and 19:4n-5 acids, both ketone body production and triglyceride secretion were significantly lowered when arachidonic acid was perfused. The concentration of triglyceride in the post-perfused liver was lower upon perfusion with arachidonic acid than upon perfusion with oleic acid or 19:4n-5 acid. Cholesterol secretion in the liver perfused with arachidonic acid or 19:4n-5 acid was significantly higher than with oleic acid. The concentration of cholesterol in the post-perfused liver was slightly but significantly higher with 19:4n-5 acid than with the other fatty acids. The results suggest that 19:4n-5 acid when compared with arachidonic acid affects lipid metabolism in liver differently.
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Polyunsaturated n-6 fatty acids (PUFAs) and particularly linoleic acid exert a hypocholesterolemic effect in experimental animals and humans (1,2). Although several mechanisms have been proposed for this cholesterol-lowering effect (2), the precise mechanism has not been elucidated as yet. Fatty acids are secreted from the liver to varying degrees in very low density lipoprotein (VLDL) and are subsequently metabolized in the blood stream to form intermediate and low density lipoproteins. A decrease in VLDL secretion may thus result in a reduction of serum cholesterol concentration. Beynen and Katan (3) suggested that PUFAs, when compared to saturated fatty acids, are more readily oxidized to ketone bodies in the liver rather than secreted as VLDL-triglyceride.

Studies *in vitro* showed that the rate of oxidation of individual PUFAs depends on the number of double bond and chain length of the PUFA (4-6). In order to gain insights into the effect of various fatty acids on lipid metabolism, it is, therefore, important to study the effect of PUFAs on the secretion of lipids and the production of ketone bodies in the liver. Although arachidonic acid has been shown to have a more pronounced hypocholesterolemic effect than linoleic acid (7), only limited information is available on the metabolic fate of 20:4n-6 in the liver.

In the present study, the effect of infused arachidonic acid on lipid metabolism was compared with that of oleic acid in isolated perfused rat liver. The metabolic effect of 5,8,11,14-nonadecatetraenoic (19:4n-5) acid was also examined because it is of interest to establish structure-function relationships of PUFAs as regulators of lipid metabolism.

*To whom correspondence should be addressed at the Laboratory of Nutrition Chemistry, Kyushu University School of Agriculture 46-09, 6-10-1 Hakozaki, Higashi-ku, Fukuoka 812, Japan.

Abbreviations: 19:4n-5, 5,8,11,14-nonadecatetraenoic acid; 5-HETE, 5-hydroxyeicosatetraenoic acid; 5,12-HETE, 5,12-dihydroxyeicosatetraenoic acid; PUFAs, polyunsaturated fatty acids; VLDL, very low density lipoprotein.

MATERIALS AND METHODS

Materials. Oleic acid (99% purity) was purchased from Nacalai Tesque, Inc. (Kyoto, Japan). Arachidonic acid (99% purity) and 5,8,11,14-nonadecatetraenoic acid (99% purity) were microbial products (8). Bovine serum albumin fraction V and 3-hydroxybutyrate dehydrogenase were obtained from Boehringer Mannheim GmbH (Germany).

Animals and diets. Male Sprague-Dawley rats (Seiwa Experimental Animals, Fukuoka, Japan) weighing 300 g-330 g were housed in a temperature-controlled room and fed commercial rat chow (type CRF-1, Japan Charles River, Kanagawa, Japan) *ad libitum.* Liver was isolated under sodium pentobarbital anesthesia and perfused with recirculating 120 mL Krebs-Henseleit buffer (pH 7.4) containing 0.1% glucose, 1.5% bovine serum albumin and 25% washed bovine erythrocytes at a rate of 20 mL/min at 37°C. The procedure was described in detail previously (9). At the beginning of circulation, 5 mL of 20 mM fatty acid sodium salt (100 μmole) was added and the solution was continuously infused at the rate of 5 mL/hr (100 μmole/hr). At 1-hr intervals, 15 mL perfusate was removed for analysis of ketone bodies and lipids. The perfusion was continued for 4 hr.

Analysis of ketone body. Acetoacetate and β-hydroxybutyrate were measured enzymatically in a deproteinized sample of the perfusate as described previously (9).

Analysis of lipids. Lipids in perfusate and post-perfused liver were extracted and purified by the method of Folch *et al.* (10). Triglycerides (11) and cholesterol (12) were analyzed by procedures described elsewhere. Triglyceride in the perfusate and liver was separated by silica gel G thin-layer chromatography (petroleum ether/diethyl ether/acetic acid, 82:18:1, v/v/v). The triglyceride fraction was visualized with fluorescein, scraped off the plate, and treated with 14% boron trifluoride/methanol (1:1, v/v) (13). Fatty acid methyl esters were extracted with hexane and analyzed by gas-liquid chromatography on a 10% SILAR 10C column (13).

Statistical analysis. Data were analyzed by one-way analysis of variance followed by Duncan's new multiple range test to evaluate the significant difference between a pair of means (14).

RESULTS

Ketone body production in perfused rat liver. Ketone body production in liver perfused with arachidonic acid was significantly lower than in liver perfused with either oleic

acid or 19:4n-5 acid (Fig. 1). Infusion of 19:4n-5 acid slightly decreased ketone body production compared to oleic acid infusion, although the difference was not significant.

Secretion of lipids by perfused rat liver. As in ketone body production, secretion of triglycerides by liver perfused with arachidonic acid was significantly lower than by liver perfused with either oleic acid or 19:4n-5 acid (Fig. 2). Triglyceride secretion was the same in the latter two fatty acids. As shown in Figure 3, liver perfused with 19:4n-5 acid, when compared to oleic acid, secreted two times more cholesterol. Infusion of arachidonic acid resulted in a slight decrease in secretion of cholesterol compared to 19:4n-5 acid infusion, but was significantly higher than in oleic acid infusion at 3 and 4 hr.

Lipid concentration in post-perfused liver. The concentration of cholesterol and triglyceride in post-perfused rat liver is shown in Table 1. The concentration of liver triglyceride was lowest in arachidonic acid infusion, highest in 19:4n-5 acid infusion, and intermediate in oleic acid infusion. The difference between arachidonic acid and 19:4n-5 acid was significant. The concentration of liver cholesterol was slightly but significantly higher with infusion of 19:4n-5 acid than with oleic acid or arachidonic acid.

Fatty acid composition of perfusate and liver triglyceride. The fatty acid composition of triglyceride in perfusate and post-perfused rat liver is shown in Tables 2 and 3, respectively. In each instance, triglyceride in perfusate was enriched with the infused fatty acids. The proportion of the elongated metabolites of 19:4n-5 acid and arachidonic acid also increased in the perfusate triglyceride upon infusion of these PUFAs. When 19:4n-5 acid was perfused, the proportion of the fatty acids tentatively identified as arachidonic acid and 21:4n-5 increased significantly. In post-perfused liver, the proportion of each fatty acid perfused was also increased, but the extent of the increase was moderate compared to that in the perfusate. The extent of the enrichment in liver triglyceride of respective fatty acids infused was relatively higher in arachidonic acid than in 19:4n-5 acid and oleic acid.

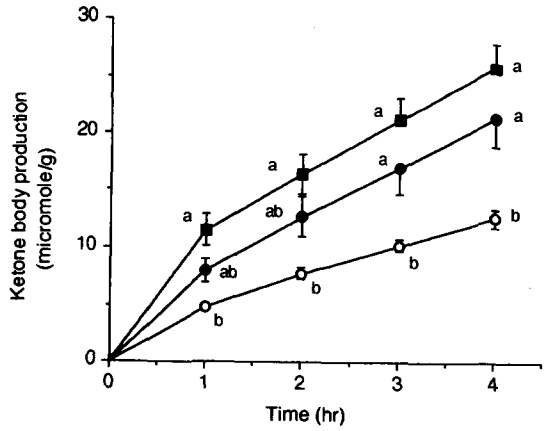


FIG. 1. Ketone body production in perfused rat liver. Rat liver was perfused in the presence of oleic acid (■), arachidonic acid (○) or 19:4n-5 acid (●) for 4 hr. Each point represents the mean ± SE of 6 rats. [a,b] Means not sharing a common superscript letter are significantly different at P<0.05.

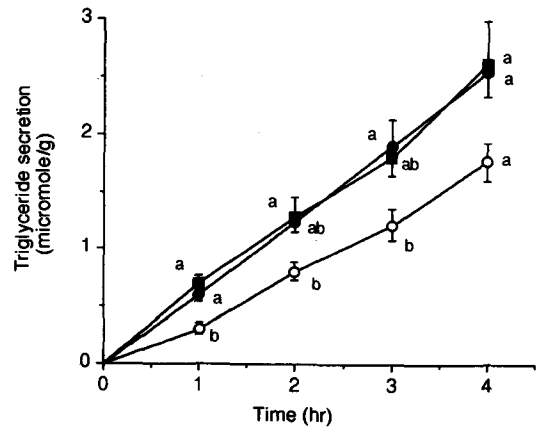


FIG. 2. Triglyceride secretion in perfused rat liver. Rat liver was perfused in the presence of oleic acid (■), arachidonic acid (○) or 19:4n-5 acid (●) for 4 hr. Each point represents the mean ± SE of 6 rats. [a,b] Means not sharing a common superscript letter are significantly different at P<0.05.

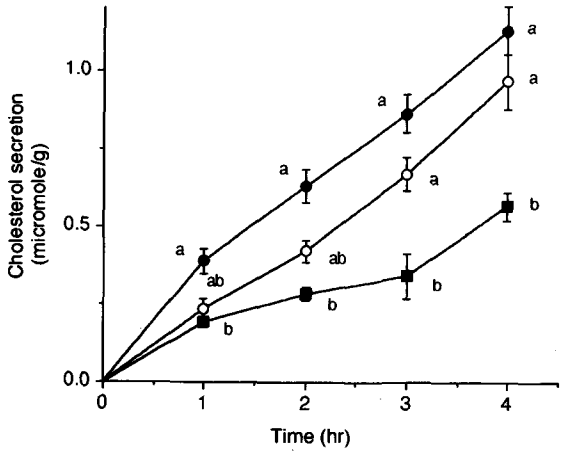


FIG. 3. Cholesterol secretion in perfused rat liver. Rat liver was perfused in the presence of oleic acid (■), arachidonic acid (○) or 19:4n-5 acid (●) for 4 hr. Each point represents the mean ± SE of 6 rats. [a,b] Means not sharing a common superscript letter are significantly different at P<0.05.

TABLE 1

Lipid Contents in Post-Perfused Rat Liver*

Fatty acids infused	Cholesterol	Triglyceride
	(μmol/g)	
Oleic acid	6.85 ± 0.30[a]	17.7 ± 1.5[a]
19:4n-5 acid	8.30 ± 0.40[b]	20.6 ± 1.4[a]
Arachidonic acid	6.40 ± 0.24[a]	13.3 ± 1.3[b]

*Each value represents the mean ± SE of 6 rats.

[a,b]Values in a column not sharing a common superscript letter are significantly different at P<0.05.

TABLE 2

Fatty Acid Composition (%) of Triglyceride in Perfusate at the End of Perfusion (4 hr)*

	Fatty acid infused		
	Oleic acid	19:4n-5	Arachidonic acid
16:0	15.4 ± 0.7	14.0 ± 0.8	13.1 ± 0.6
16:1	2.3 ± 0.2	1.9 ± 0.2	2.0 ± 0.2
18:0	1.8 ± 0.1[a]	2.4 ± 0.1[b]	2.2 ± 0.2[a,b]
18:1	55.8 ± 1.2[a]	13.5 ± 1.0[b]	14.5 ± 0.9[b]
18:2n-6	14.3 ± 0.5[a]	16.3 ± 0.4[b]	14.5 ± 0.7[a,b]
19:4n-5[d]	—	31.8 ± 2.0	—
20:3n-6	2.2 ± 0.0[a]	—	0.6 ± 0.1[b]
20:4n-6	2.1 ± 0.3[a]	7.3 ± 0.4[b]	43.6 ± 1.9[c]
20:5n-3	0.6 ± 0.0	—	0.4 ± 0.1
Unknown[e]	—	6.9 ± 1.5	—
22:4n-6	0.6 ± 0.1[a]	0.4 ± 0.1[a]	4.7 ± 0.5[b]
22:5n-3	1.2 ± 0.1[a]	1.1 ± 0.1[a]	0.7 ± 0.1[b]
22:6n-3	3.7 ± 0.2[a]	2.6 ± 0.2[b]	2.2 ± 0.1[b]

*Each value represents the mean ± SE of 6 rats.

[a,b,c]Values in the same row not sharing a common superscript letter are significantly different at $p<0.05$.

[d]Includes 20:3n-6.

[e]Tentatively identified as 21:4n-5 which includes 20:5n-3.

TABLE 3

Fatty Acid Composition (%) of Triglyceride in Post-Perfused Rat Liver*

	Fatty acid infused		
	Oleic acid	19:4n-5	Arachidonic acid
16:0	22.3 ± 0.9[a]	22.2 ± 0.6[a]	19.2 ± 0.8[b]
16:1	2.6 ± 0.4	3.2 ± 0.2	2.7 ± 0.2
18:0	2.8 ± 0.2[a,b]	3.1 ± 0.2[a]	2.3 ± 0.1[b]
18:1	34.9 ± 2.2[a]	24.5 ± 0.8[b]	20.2 ± 1.2[b]
18:2n-6	25.7 ± 1.9	24.2 ± 0.6	21.7 ± 1.2
19:4n-5[c]	—	9.9 ± 0.9	—
20:3n-6	0.4 ± 0.1	—	0.4 ± 0.0
20:4n-6	2.5 ± 0.4[a]	3.8 ± 0.3[a]	24.0 ± 2.5[b]
20:5n-3	0.5 ± 0.1	—	0.3 ± 0.1
Unknown[d]	—	2.1 ± 0.3	—
22:4n-6	0.9 ± 0.2[a]	0.6 ± 0.1[a]	2.7 ± 0.2[b]
22:5n-3	1.3 ± 0.2	1.2 ± 0.1	1.2 ± 0.2
22:6n-3	2.9 ± 0.4	2.3 ± 0.2	2.5 ± 0.3

*Each value represents the mean ± SE of 6 rats.

[a,b]Values in the same row not sharing a common superscript letter are significantly different at $p<0.05$.

[c]Includes 20:3n-6.

[d]Tentatively identified as 21:4n-5 which includes 20:5n-3.

DISCUSSION

The present results indicate that arachidonic acid when infused into liver influences lipid metabolism differently than does oleic acid as evidenced by the decrease in triglyceride secretion and the production of ketone bodies by the liver as well as by the decrease in hepatic triglyceride concentration. Consistent with our observations, Wong *et al.* (15) observed a depression of production and secretion of triglyceride in rat hepatocytes incubated with arachidonic acid as compared to oleic acid.

It is known that fatty acids infused into liver stimulate synthesis and secretion of VLDL (16–21). Kohout *et al.* (21) showed that the output of triglyceride from perfused liver was proportional to the number of carbon atoms when infusion of caproic, caprylic, lauric, palmitic and stearic acids was compared. When fatty acids containing 18 carbon atoms were infused, the output of triglyceride decreased as the number of double bonds in the fatty acids increased. However, only a small difference was observed in the rate of ketone body production among the various fatty acids infused. Wilcox *et al.* (18) also observed that unsaturated fatty acids (palmitoleic, oleic and linoleic acids) stimulated the secretion by perfused liver of VLDL-triglyceride more than did saturated fatty acids (myristic, palmitic and stearic acids), whereas the effects of the unsaturated fatty acids on VLDL secretion was comparable. On the other hand, when hepatocytes were incubated with oleic, linolenic, arachidonic, eicosapentaenoic or docosahexaenoic acid, synthesis and secretion of triglycerides progressively decreased with increasing fatty acid chain length and unsaturation (15). Therefore, triglyceride synthesis and secretion by the liver appear to decrease with infusion of saturated or highly polyunsaturated fatty acids (13,16,19). However, with 19:4n-5 acid the rate of triglyceride secretion did not decrease when compared to oleic acid infusion.

Salem *et al.* (20) showed that increased secretion of VLDL-triglyceride stimulated by infusion of oleic acid resulted in a simultaneous increase in cholesterol secretion and in the activity of hepatic cholesterol synthesizing enzymes. Thus, requirement of cholesterol as a component of VLDL may be increased when VLDL secretion is stimulated (20). This does not fit the observation we made with arachidonic acid in this study. Arachidonic acid, compared to oleic acid, stimulated cholesterol secretion, whereas it decreased triglyceride secretion. The results showed that the lipid composition of the VLDL fraction, a major lipoprotein in the perfusate, may be changed by infusion of arachidonic acid as compared to oleic acid. Although the results do not necessarily explain the cholesterol-lowering activity of n-6 PUFAs, there is a possibility that the change in the lipid composition of VLDL secreted from the liver may influence subsequent metabolism and thus plasma cholesterol levels (9). It is of interest in this context that Wilcox *et al.* (18) reported that VLDL secreted by the liver after infusion of unsaturated fatty acids (palmitoleic, oleic and linoleic acids) is composed of large particles with less phospholipid and cholesterol relative to triglyceride than VLDL produced after infusion of saturated fatty acids (myristic, palmitic and stearic acids).

In contrast to arachidonic acid, the effects of 19:4n-5 acid on ketone body production and triglyceride secretion as well as hepatic triglyceride concentration were similar to those observed with oleic acid. However, 19:4n-5 acid stimulated cholesterol secretion to the same extent as arachidonic acid. In addition, 19:4n-5 acid caused an increase in cholesterol concentration in post-perfused liver which was larger than that caused by arachidonic acid or oleic acid. The results suggest that 19:4n-5 acid, in comparison with oleic acid and arachidonic acid, stimulates cholesterol synthesis and/or inhibits bile acid synthesis in the liver. The effect of 19:4n-5 acid on liver lipid metabolism appears to be quite different from that of

arachidonic acid. This odd-numbered PUFA, in contrast to arachidonic acid which mainly altered triglyceride metabolism, influenced mainly cholesterol metabolism.

The triglyceride concentration in post-perfused liver was higher upon oleic and 19:4n-5 acid infusion than upon arachidonic acid infusion, although all stimulated triglyceride secretion from the liver. On the other hand, the cholesterol concentration in post-perfused liver was not correlated to the secretion of cholesterol from the liver. Interpretation of these reciprocal changes of the lipid concentration and secretion in liver is difficult at present. It was reported that there was no correlation between liver triglyceride output and liver triglyceride concentration when various fatty acids were infused (21).

When 19:4n-5 acid was perfused, the proportion of fatty acid which was tentatively identified as arachidonic acid on a SILAR 10C column increased in triglyceride of the perfusate and post-perfused liver. Further analysis of the fatty acid composition by CP-Sil 88 WCOT fused silica capillary column (50m × 0.25mm) (Chrompack International, Middelburg, Holland) also supported the view that a portion of 19:4n-5 acid might be converted to arachidonic acid. However, since it is difficult to explain the metabolic pathway leading 19:4n-5 acid to arachidonic acid in mammals, more detailed analyses will be required before definite conclusions are drawn. Jakschik *et al.* (22) reported that 19:4n-5 acid was converted to compounds that comigrated with 5-hydroxyeicosatetraenoic (5-HETE) and 5,12-dihydroxyeicosatetraenoic acids (5,12-HETE) in rat basophilic leukemia cells. Thus, 19:4n-5 acid might be elongated to arachidonic acid. Alternatively, it would be possible that 19:4n-5 acid replaces arachidonic acid in membrane lipids with the liberated arachidonic acid being subsequently incorporated into triglycerides. As the levels of 22:4n-6 in the perfusate and post-perfused liver triglyceride tended to decrease after perfusion with 19:4n-5 acid, it would also be possible that increased arachidonic acid levels might result from 22:4n-6 by retroconversion.

The present study shows characteristic differences in the effects of oleic, arachidonic and 19:4n-5 acids on lipid metabolism in isolated perfused rat liver. The possibility exists that these PUFAs also influence the concentration of plasma lipids differently when they are given *per os.*

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Cholesterol Ester Hydrolase Activity in Mammary Tissue of the Lactating Rat

D.W. West* and **J.H. Shand**
Hannah Research Institute, Ayr, Scotland, KA6 5HL

Cholesterol ester hydrolase activity has been studied in mammary glands of rats. Subcellular fractionation of the glands obtained in mid-lactation indicated that around 80% of the recovered activity was associated with particulate fractions. Two distinct cholesterol ester hydrolase activities were identified, one with an optimum pH of 7.5–9.0 and the second (approximately 5% of the total activity) with a more acidic pH optimum. Although the neutral cholesterol ester hydrolase had some properties in common with the lipoprotein lipase in mammary tissue, it was shown to be a separate entity by several criteria. Its activity could be increased following treatment with Mg-ATP and cAMP-dependent protein kinase, suggesting identity with the hormone sensitive lipase of adipose tissue. The cholesterol ester hydrolase activity in mammary glands just after parturition was greater than in glands obtained either from late-pregnant or mid-lactating animals. The subcellular distribution of the neutral cholesterol ester hydrolase suggested that it may have a different function to the neutral cholesterol ester hydrolase of adrenals and other tissues. Nevertheless the fact that the activity of the enzyme can be modulated by cAMP-dependent protein kinase suggests the possibility that hormonal control of this enzyme may be involved in the regulation of cholesterol metabolism in the mammary gland.
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A number of hormone-sensitive tissues contain cholesterol ester hydrolase (CEH) activity with neutral pH optima (1,2). Such enzymes have been reported to be localized in both soluble and particulate fractions of cell homogenates (3–5), although those occurring in the soluble fraction have been investigated most (6–8). One of the best characterized of these is the CEH of the bovine adrenal cortex (9). This enzyme catalyzes an important rate-limiting step in steroidogenesis (10) and its activity is regulated by the action of adrenocorticotrophic hormone acting *via* an increase in cAMP levels and consequent activation of protein kinase A (11). The increase in activity on phosphorylation by cAMP-dependent protein kinase resembles the activation seen with hormone-sensitive lipase from adipose tissue (12,13), and the two enzymes have recently been shown to be the same entity by immunological techniques (8). The mammary gland is also a hormone-sensitive tissue and during lactation requires a considerable throughput of cholesterol to support milk production (14). In the rat it has been calculated that between 60% and 80% of the milk cholesterol is derived in this way (15,16). Labelled cholesterol was rapidly taken up by the gland when it was infused into plasma but its subsequent appearance in the milk was surprisingly slow and the radiolabel required about 20 hr to attain its maximum activity in the milk (17,18). It was suggested that this time lag was due to the incorporation of the labelled cholesterol into tissue membranes from which it was only slowly released (17). The mammary gland has been reported to contain acyl-CoA: cholesterol acyltransferase activity (19) and these two results considered together would suggest that esterification of cholesterol, prior to storage in a cholesterol ester pool in the gland, had occurred. Evidence for the presence of cholesterol esters in the mammary gland has been presented (20). In spite of this, very little esterified cholesterol is secreted into milk (21). This suggests that active metabolism of this cholesterol ester pool occurs and there is some evidence in the literature supporting a rapid turnover of cholesterol esters in the mammary gland (22,23). Active turnover of cholesterol esters implies the presence of CEH activity and although the LPL activity of the mammary gland has been studied (24), no attempt appears to have been made to investigate or characterize its CEH activity.

In the study reported here, the lactating mammary gland is shown to contain both acid and neutral CEH activities which are mainly associated with the particulate cell fractions. The activity of the neutral CEH can be increased by cAMP-dependent protein kinase, suggesting that hormonal modulation of the activity of this enzyme may be involved in the control of cholesterol metabolism in the gland.

MATERIALS AND METHODS

Cholesteryl [1-^{14}C]oleate (52 mCi/mmol), [9,10(n)-^{3}H]oleic acid (5 Ci/mmol), glycerol tri-[9,10(n)-^{3}H]oleate (1 Ci/mmol), and uridine diphospho-D-[U-^{14}C]galactose (231 mCi/mmol) were purchased from Amersham International (Amersham, Bucks, U.K.). All biochemicals were purchased from Sigma Chemical Co. (Poole, Dorset, U.K.) or from BCL (Lewes, Sussex, U.K.).

Bovine serum was prepared as described by Clegg (24), but was inactivated by heating at 56°C for 10 min before final centrifugation and storage at −20°C. Lipids were removed from the heat-inactivated serum by preparation of an acetone/ether powder (25). The resultant preparation was redissolved to give the same protein concentration as the original serum.

Preparation of mammary microsomes and acini. Female Wistar rats were kept on a constant light cycle (lights on 08.00–20.00) and were used at 14.30. They were anaesthetized (pentobarbital; 80 mg/Kg), and the abdominal mammary glands dissected free from the skin and abdominal wall before being rapidly freeze-clamped with tongs pre-cooled in liquid nitrogen. Approximately 2 g of gland was homogenized (Polytron; PMA20N probe, 17000 rpm, 30 seconds) in 5 volumes of an ice-cold medium consisting of 250 mM sucrose, 100 mM KF, 1 mM EDTA, and 50 mM MES-50 mM Tris/HCl buffer pH 7.1. The homogenate was centrifuged (10,000 × *g*,

*To whom correspondence should be addressed.

Abbreviations: CEH, cholesterol ester hydrolase; HSL, hormone sensitive lipase; IBMX, isobutylmethyl xanthine; LPL, lipoprotein lipase; MES, 2-(*N*-morpholino)ethanesulfonic acid.

10 min), the pellet discarded and the supernatant recentrifuged for 30 min at 100,000 × *g*. The resultant pellet was resuspended in 50 mM MES-50 mM Tris/HCl buffer pH 7.1 containing 100 mM KCl and again centrifuged at 100,000 × *g* for 30 min. The pellet was then resuspended in the same medium at a concentration of 7 mg/mL, and stored at −80°C before use. Microsomes were similarly prepared in homogenization medium to which 0.5 M KCl had been added (salt-washed microsomes). Acini were prepared from lactating mammary tissue by collagenase digestion (26).

Subcellular fractionation of mammary tissue and of mammary acini. Subcellular fractionation was carried out after homogenization in a "high" ionic strength buffer containing 250 mM sucrose, 1 mM EDTA, 100 mM KCl, and 10 mM Tris/HCl pH 7.4 as described by Clegg (24). Four fractions were obtained, P1, nuclear/debris pellet (1000 × *g*); P2, mitochondrial pellet (3000 × *g*); P3, microsomal pellet (105,000 × *g*); and S3, the post-microsomal supernatant. Assays for marker enzyme activities were as follows: 5′-Nucleotidase (EC 3.1.3.5) was according to Michell and Hawthorn (27). Phosphodiesterase-1 (EC 3.1.4.1), NADPH-cytochrome C oxidoreductase (EC 1.6.2.4), acid phosphatase (EC 3.1.3.2), and cytochrome C oxidase (EC 1.9.3.1) were assayed as described by Brown *et al.* (28). Galactosyl transferase (EC 2.4.1.22) was assayed using UDP-D[U-^{14}C]galactose by a modification of the method of Morré *et al.* (29). LPL was determined according to Nilsson-Ehle and Schotz (30), as modified by Clegg (24), using bovine serum prepared as above to activate the substrate. Lactate dehydrogenase (EC 1.1.1.27) was measured using a standard photometric assay. A slightly cruder subcellular fractionation was also carried out after homogenization of the mammary gland in a "low" ionic strength buffer containing 250 mM sucrose, 1 mM EDTA, and 10 mM Tris/HCl pH 7.4. Three fractions were obtained P1+P2, a combined nuclear/mitochondrial pellet (3000 × *g*), P3, a microsomal pellet (105,000 × *g*) and S3, the post-microsomal supernatant, all of which were assayed for CEH activity. Acini were homogenized in the KCl-containing buffer and fractionated by differential centrifugation giving two pellet fractions (10,000 × *g* and 105,000 × *g*) and a post-microsomal supernatant fraction which were assayed for CEH activity.

Protein and DNA measurements. The protein concentrations were determined by the dye-binding technique of Bradford (31), and the DNA contents of mammary tissue and acini were assayed as described by Labarca and Paigen (32).

Treatment with heparin. Microsomes (0.4 mg) were mixed with heparin (50, 100 or 200 μg/mL) in 50 mM MES-50 mM Tris/HCl pH 7.1 containing 100 mM KCl. After standing for 30 min in ice, the mixture was centrifuged at 15,000 × *g* for 30 min at 4°C. The supernatant was collected and the pellet resuspended in the same volume of fresh buffer. CEH activity was measured in both.

Treatment with Triton X-114. Microsomes (1.8 mg) were mixed with Triton X-114 (final conc. 0.16% w/v) in 50 mM MES-50 mM Tris/HCl pH 7.1 containing 100 mM KCl. After standing for 60 min in ice, the mixture was incubated for 10 min at 30°C, to precipitate Triton X-114, before being centrifuged (15,000 × *g*, 5 min, 30°C). CEH activity was measured in the detergent-free supernatant.

Activation with Mg-ATP. Microsomes (0.04–0.1 mg) were preincubated for 10 min at 37°C with $MgCl_2$ (3 mM), ATP (5 mM), cAMP (0.01 mM), and IBMX (2.5 mM) in 25 mM MES-25 mM Tris/HCL buffer pH 7.1 with and without protein kinase A (0.26 mg/mL) before assaying for neutral CEH activity. Where present, protein kinase inhibitory peptide was at a concentration of 0.01 mg/mL. Enzyme protein, released into the detergent-free supernatant following treatment with Triton-X-114, was also incubated under the same conditions.

Assay of CEH. Activity was measured in terms of the release of [1-^{14}C]oleic acid from cholesteryl [1-^{14}C]oleate (33). The microsomal fraction (40 μg) and an ethanolic solution of the substrate (20 nmol, 10 μL, 100,000 dpm) were incubated at 37°C for 40 min in 50 mM MES-50 mM Tris-HCl pH 7.1 containing 0.7 mM $MgCl_2$, 25 mM KCl, and bovine serum albumin (1%, w/v), in both the presence and absence of 1M NaCl (total volume of 200 μL). In the presence of 1M NaCl, a three-fold higher specific activity substrate was used. The reaction was initiated by the addition of substrate and terminated by the addition of a chloroform/heptane/methanol (1.25:1:1.41, v/v/v) mixture followed by 0.45 mL of K_2CO_3/boric acid buffer pH 10.5. In each assay, appropriate controls without microsomes were incubated concurrently in order to correct for background. After the addition of [9,10(n)-^{3}H]oleic acid (2.1 μmole, 6000 dpm) and agitation on a vortex mixer, the mixture was separated by centrifugation and an aliquot of the upper aqueous layer was counted to determine the amount of fatty acid released. Corrections for recovery were applied by reference to the added oleic acid. The presence of 1 M NaCl invariably led to a lower recovery of the released fatty acid.

RESULTS

Optimization of assay conditions. The rate of hydrolysis of cholesteryl oleate (typically 300–700 pmol per min/mg of protein) was highest when the substrate was presented as an ethanolic solution. Activity was optimal at 100 μM cholesteryl oleate and was a linear function of time (to 40 min) and microsomal protein concentration (10 μg to 200 μg). In the absence of bovine serum albumin, a marked reduction in the hydrolytic rate was observed. The standard assay conditions were incubation at 37°C for 40 min in 50 mM MES-50 mM Tris/HCl pH 7.1 buffer containing bovine serum albumin (1%, w/v), 40 μg microsomal protein and 100 μM cholesteryl oleate (total volume 200 μL). The assay was carried out in the presence and absence of 1 M NaCl.

Subcellular fractionation. Regardless of the ionic strength of the buffer used in the homogenization, an average of just over 70% of the CEH activity of the homogenate was recovered during the subcellular fractionation, which compared favorably with the general recovery of the marker enzyme activities. The subcellular distribution of CEH activity in the mammary gland homogenized in the "high" ionic strength buffer is presented in the form of DeDuve plots (34) (Fig. 1) and is compared with that determined for various marker enzyme activities in lactating rat mammary tissue during a single representative experiment. Approximataely 21% of the recovered CEH activity was found in the S3 faction, but the bulk of the activity was present in membrane

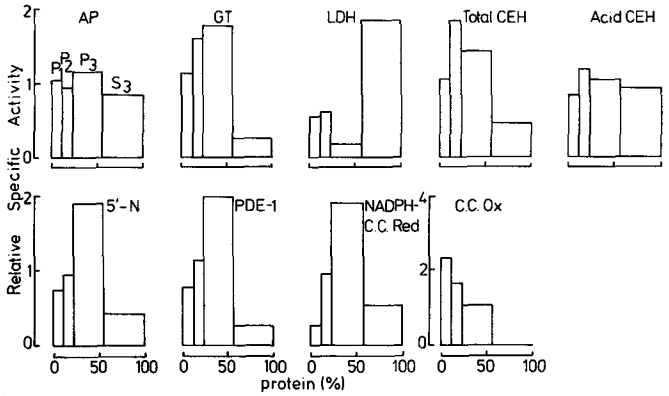


FIG. 1. Subcellular distribution of some enzyme activities in lactating mammary tissue. The tissue was homogenized in "high-ionic" strength buffer (see text). The results are presented in the form of DeDuve plots (34), where the width of any one histogram bar is proportional to the percentage of the total recovered protein in the fraction to which that bar relates. The relative specific activity is calculated by dividing the percentage of the total recovered activity in the fraction by the percentage of the total protein in that fraction. AP, acid phosphatase (lysosomes); GT, galactosyl transferase (Golgi); LDH, lactate dehydrogenase (cytosol); total CEH, total cholesterol ester hydrolase; acid CEH, salt-resistant cholesterol ester hydrolase; 5′-N, 5′-nucleotidase (plasma membrane), PDE-1, phosphodiesterase-1 (plasma membrane); NADPH-C.C.Red, NADPH-dependent cytochrome C oxidoreductase (endoplasmic reticulum); C.C.Ox, cytochrome C oxidase (mitochondria).

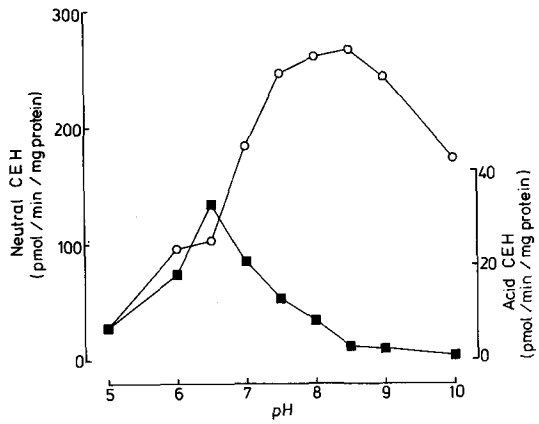


FIG. 2. Variation of CEH activity with pH. CEH activity was measured in the presence and absence of 1 M NaCl as described in the Materials and Methods section using 50 mM Tris-50 mM MES-50 mM glycylglycine adjusted to the correct pH with NaOH or HCl as required, as buffer throughout. The difference in the two readings at each pH value was plotted as the neutral esterase activity (○—○). The acid esterase activity was that measured in the presence of the high salt (■—■). Results are expressed as pmol cholesteryl oleate hydrolyzed per min/mg protein.

TABLE 1

The Distribution of Enzyme Activities Between the Particulate and the Soluble Fractions of Mammary Gland Homogenates[a]

Enzyme	Ionic strength of homogenizing buffer	Particulate (P1+P2+P3)	Soluble (S3)
NADPH-cyto C oxidoreductase	High	78.4	23.0
	Low	79.8	20.1
5′-Nucleotidase	High	81.3	18.7
	Low	67.9	32.1
Total cholesterol ester hydrolase	High	79.2	20.9
	Low	49.0	51.0

[a]The mammary glands were homogenized in both "low" and "high" ionic strength buffers (see text) and the amount of each enzyme activity present in each fraction expressed as percentage of the total recovered activity.

fractions. The distribution of specific CEH activity among these membrane fragments was broadly similar to those of NADPH-cytochrome C oxidoreductase (endoplasmic reticulum marker), 5′-nucleotidase and phosphodiesterase-1 (plasma membrane markers) and galactosyl transferase (Golgi marker). Homogenization in the "low" ionic strength buffer altered the distribution of the enzyme activities with a greater proportion of the CEH being recovered in the cytosolic fraction (Table 1). This was nevertheless mirrored by the plasma membrane marker 5′-nucleotidase, which exhibited a similar redistribution into the soluble fraction. In contrast, the endoplasmic reticulum marker NADPH-cytochrome C oxidoreductase showed no such change in its distribution between these subcellular fractions.

The CEH activity was strongly inhibited by 1 M NaCl with its activity being reduced to around 5% of the original. The salt-resistant hydrolase activity was a small but consistent portion of the total CEH activity associated with each of the subcellular fractions, but its distribution was slightly different to that observed for the total activity (Fig. 1) and resembled the pattern shown by acid phosphatase, a lysosomal marker. The salt-resistant ester hydrolase had an optimum activity at pH 6.5 (Fig. 2) rather higher than that normally associated (35,36) with the lysosomal enzyme. In contrast, the salt-inhibited hydrolase activity (total minus the salt-resistant activity) had a broad optimum over the range pH 7.5–9.0, similar to that shown by LPL and the neutral CEH activity reported in a number of other tissues (1–7). The microsomal pellet P3 was subjected to further fractionation on a discontinuous sucrose gradient similar to that used by Clegg (24) and the distribution of both CEH activities and various marker enzymes again compared (Table 2). The neutral CEH was relatively more concentrated at the 0.9/32% interface when compared with the other fractions. This distribution resembled that of the two plasma membrane markers 5′-nucleotidase and phosphodiesterase-1 and was unchanged by homogenization and fractionation in "low" ionic strength buffer.

The distribution was unlike that shown by NADPH-cytochrome C oxidoreductase and galactosyl transferase suggesting that the neutral CEH was preferentially associated with plasma membrane fragments rather than with endoplasmic reticulum or Golgi membranes. At "high" ionic strength the partition of the acid CEH between the gradient fractions resembled that of the neutral hydrolase, but at "low" ionic strength a significant proportion was shifted into the heavier fractions and in this characteristic it resembled the redistribution shown by acid phosphatase.

When acini were similarly homogenized and fractionated, 73% of the original CEH was recovered in the fractions obtained and again 75% of the recovered activity

TABLE 2

Density Gradient Purification of Rat Mammary Microsomal (P3) Fraction[a]

Enzyme	Ionic strength of homogenizing buffer	% of total activity recovered in fraction			
		F1	F2	F3	F4
NADPH-cytochrome C oxidoreductase	High	29.2	25.0	30.4	15.4
	Low	18.1	31.4	36.1	14.4
Galactosyl transferase	High	26.3	60.6	6.5	6.6
5′-Nucleotidase	High	50.9	25.7	18.5	4.9
	Low	56.2	32.1	9.5	2.2
Phosphodiesterase-1	High	53.4	19.3	17.0	10.3
	Low	47.0	31.2	16.5	5.3
Salt-inhibited (neutral) cholesterol ester hydrolase	High	41.6	21.2	24.0	13.2
	Low	39.5	17.8	22.5	20.2
Acid phosphatase	High	25.0	25.2	36.8	13.0
	Low	18.6	28.9	34.4	18.1
Salt-resistant (acid) cholesterol ester hydrolase	High	43.8	17.7	25.3	13.2
	Low	30.6	36.8	27.3	5.3
Lipoprotein lipase	Low	27.7	28.0	29.2	15.1

[a]Fraction P3 (47 mg protein) was layered onto a sucrose gradient consisting of 6 mL each 40%, 36%, and 32% (w/w) sucrose and carefully overlayered with 0.9% sucrose. All sucrose solutions were made up in 20 mM Tris-HCl pH 7.4. After centrifugation (105,000 × *g*, 90 min) membrane material was collected from the interfaces between the 0.9% and 32% layers (F1), the 32% and 36% layers (F2) and the 36% and 40% layers (F3). These three fractions, together with the pellet fraction (F4), were diluted with 20 mM Tris-HCl pH 7.4 and sedimentable material collected by centrifugation (105,000 × *g*, 45 min). The resultant pellets were suspended in the Tris buffer and assayed for enzyme activities as described in the Materials and Methods section. The salt-resistant hydrolase was taken as that activity measured in the presence of 1 M NaCl and having an acid pH optimum. The salt-inhibited hydrolase, showing a more neutral pH optimum, was the difference between the activities measured in the presence and absence of NaCl. The results are given as the percentage of the activity recovered in each interface fraction for the microsomal pellets obtained from the homogenization in both "high" and "low" ionic strength buffers.

TABLE 3

Solubilization of CEH Activity[a]

Condition	Neutral hydrolase	Acid hydrolase
Control	100	100
0.5 M KCl treated microsomes	21.5	28.4
Detergent-free supernatant	79.4 ± 3.6(7)	16.4
Resuspended Triton pellet	6.1	0
Heparin treatment		
50 μg/mL		
Supernatant	6.8 ± 2.9(2)	n.d.[b]
Pellet	93.3 ± 2.9(2)	n.d.
100 μg/mL		
Supernatant	7.5 ± 4.6(2)	n.d.
Pellet	92.5 ± 4.6(2)	n.d.
200 μg/mL		
Supernatant	17.4 ± 1.6(2)	n.d.
Pellet	82.6 ± 1.6(2)	n.d.

[a]Microsomes were either prepared in the presence of 0.5 M KCl or treated with Triton X-114 or heparin as detailed in the Materials and Methods section. Results are expressed as percentages of controls ± SEM with the number of animals in parenthesis. Control values were 523.0 and 11.0 pmol cholesteryl oleate hydrolyzed per min/mg of protein for the neutral and acid hydrolases, respectively.

[b]n.d. = not determined.

was associated with the particulate material (results not shown). Moreover, when the CEH activity was compared in homogenates of whole mammary glands and in acini prepared from the same glands, the hydrolase activity in the acini (5473 pmol per min/mg of DNA) averaged (for two preparations) 82.9% of the value determined for the whole tissue. Together these results, obtained on a cellular preparation free of adipose tissue, confirmed that the bulk of the CEH activity was definitely associated with the mammary epithelial cells.

The inhibition by high salt and the broad pH optimum indicated a similarity of the neutral CEH activity with the LPL associated with this tissue (24). However, only 11.6% of the neutral hydrolase and 19.8% of the acid hydrolase activity were retained following acetone/diethyl ether treatment (25) of the microsomal pellet; and the inclusion of heat-inactivated serum, at a concentration of serum protein reported to stimulate LPL activity (24), gave greater than 85% inhibition of the neutral hydrolase and a total loss of the acid hydrolase activity. Extraction of the serum with acetone/diethyl ether to remove lipids did not affect its inhibition of CEH. The results of these two tests taken together with the results obtained from the gradient fractionation (Table 2) indicate that the neutral CEH activity present in the microsomal fraction obtained from the mammary gland was distinct from the LPL activity previously identified to be in this fraction (24).

Solubilization of esterase activity. Following treatment of the microsomes with Triton X-114, 50.3 ± 3.9% (n=10) of the neutral hydrolase activity was released into the soluble fraction together with 70.3 ± 4.9% (n=10) of the protein. The specific activity of the enzyme was reduced relative to that in the microsomes (Table 3) but was reduced even further if the detergent was not removed from the solution (37). The acid hydrolase was inhibited by this detergent treatment. Around 80% of the activity was lost when the microsomes were washed with 0.5 M KCl, but heparin (200 μg/mL) released only 17% of the neutral hydrolase into the soluble fraction (Table 3).

Inhibition and activation of CEH. Taurocholate and cholate (1 mM) both significantly ($P<0.025$) enhanced the activity of the neutral CEH (Table 4). Increasing the cholate concentration to 20 mM caused inhibition. The acid hydrolase activity was also activated by 1 mM cholate and inhibited by the higher concentration of this bile salt. Potassium fluoride (10 mM) and sodium pyrophosphate (10 mM) were inhibitory and both CEH activities were essentially abolished by phenylmethyl sulfonyl fluoride. Phenyl boronic acid also greatly inhibited both hydrolases, but they were less susceptible to its butyl analogue. The inclusion of phosphate or vanadate had no significant effect, although performing

TABLE 4

Influence of Various Materials on the CEH Activities in Microsomes Prepared from Rat Lactating Mammary Gland[a]

Addition	Neutral hydrolase	Acid hydrolase
None (control)	100	100
Potassium fluoride (10 mM)	73.6 ± 4.8	64.6 ± 8.0
Potassium phosphate (25 mM)	91.7	129.3
Sodium vanadate (0.2 mM)	106.4 ± 2.7	113.5 ± 8.7
Sodium pyrophosphate (10 mM)	64.5 ± 3.0	48.2 ± 9.3
Sodium taurocholate (1.0 mM)	122.0 ± 2.94	n.d.[b]
Sodium cholate (1 mM)	120.0 ± 4.9	115.2 ± 6.0
Sodium cholate (20 mM)	0.9 ± 0.7	0.4 ± 0.4
Phenylmethylsulfonyl fluoride (1 mM)	0.0	15.6 ± 12.0
Phenyl boronic acid (0.4 mM)	3.6 ± 1.6	17.6 ± 14.31
Butyl boronic acid (1 mM)	52.0 ± 6.2	30.7 ± 23.2

[a]The assay for CEH activity was carried out as detailed in the Materials and Methods section with additions included at the final concentrations shown. The results are expressed as percentages of controls ± SEM for four animals. Control values wre 652.0 and 28.5 pmol cholesteryl oleate hydrolyzed per min/mg of protein for the neutral and acid hydrolases, respectively.

[b]n.d. = not determined.

TABLE 5

Activation of Neutral CEH[a]

Additions	Relative activity	
	Microsomes	Solubilized enzyme
None (control)	100.00	100.00
ATP	102.9 ± 0.4(3)	134.6 ± 3.9(3)
ATP + protein kinase A	105.8 ± 7.1(7)	156.5 ± 8.5(6)
ATP + protein kinase A + inhibitory peptide	n.d.[b]	108.3 ± 2.5(3)

[a]Microsomes or Triton-X114 solubilized enzyme protein (see Materials and Methods) (40–100 μg) were assayed for CEH activity in the presence of $MgCl_2$ (3 mM), cAMP (0.01 mM), and IBMX (2.5 mM), with and without ATP (5 mM). Where indicated, protein kinase A was present at a concentration of 0.25 mg/mL and the inhibitory peptide at 10 μg/mL. The substrate concentration for the CEH assay was reduced from 100 μM to 30 μM. Results are expressed as percentages of the controls ± SEM with the number of animals in parenthesis. The control values wre 276.5 and 208.0 pmol cholesteryl oleate hydrolyzed per min/mg of protein for the microsomes and solubilized enzyme, respectively.

[b]n.d. = not determined.

TABLE 6

CEH Activities in Microsomes Isolated from the Mammary Glands of Pregnant, Early- and Mid-Lactating Rats[a]

Animals	Neutral hydrolase	Acid hydrolase
Pregnant	119.7 ± 12.7 (6)	20.2 ± 2.2 (7)
Early-lactating	239.9 ± 46.5* (3)	10.4 ± 1.6** (4)
Mid-lactating	114.0 ± 40.6 (4)	6.3 ± 2.1***(4)

[a]The microsomes were prepared in the standard manner from the mammary glands of rats on day 18–19 of pregnancy and 2 days and 10–12 days after parturition and assayed for CEH activity as described in Materials and Methods. Results are expressed as pmol cholesteryl oleate hydrolyzed per min/mg of DNA ± SEM with the number of animals in parenthesis. Statistically significant differences between lactating and pregnant glands are indicated by *P<0.05, **P<0.01, and ***P<0.005.

the assay in phosphate buffer rather than MES-Tris/HCl led to a lowering of activity (from 254 to 92 pmol cholesteryl oleate hydrolyzed per min/mg of protein).

Activation by Mg-ATP. Incubation of microsomes with Mg-ATP, cAMP, cAMP-dependent protein kinase and IBMX caused only a small and very variable activation of the neutral hydrolase activity (Table 5). If the enzyme was first solubilized with Triton X-114 and the detergent subsequently removed, greater stimulation of enzyme activity was observed following incubation with this mixture. Inclusion of the cAMP-dependent protein kinase made little difference to the extent of activation of the solubilized enzyme, but the presence of the peptide inhibitor of the cAMP-dependent kinase (38) markedly inhibited the activation caused by Mg-ATP.

Influence of pregnancy and lactation. Microsomes obtained from the glands of animals early in lactation had a higher activity of the neutral CEH than glands from either the late pregnant or mid-lactating animals (Table 6), whereas the microsomes obtained from the pregnant glands had a significantly higher level of the acid CEH than either of the lactating groups.

DISCUSSION

Our data demonstrate that both a neutral and an acid CEH exist in the microsomal fraction of the lactating mammary gland. The resemblance of these activities in the mammary gland to those existing in other tissues is an obviously important point to consider. A number of tissues contain CEH activities, apparently all identical, that are activated by high concentrations of bile salts (39). Although low levels of such materials activated the neutral CEH present, in mammary tissue higher levels were markedly inhibitory, making it unlikely that the mammary enzyme was the same entity. The neutral CEH was inhibited by high salt concentrations, a property that it shares with LPL. Nevertheless, it was distinct from the LPL previously demonstrated to be present in this fraction since its partition on the sucrose gradient was different and it was destroyed by preparation of an acetone/diethyl ether powder, a procedure generally used to prepare LPL, and was inhibited by delipidized serum. In this, and in a number of other properties, it resembled the HSL and neutral CEH present in adipose tissue and in the adrenal cortex, respectively. The neutral CEH activities of adipose tissue, adrenal, and liver can all be activated by incubation with cAMP-dependent protein kinase and Mg-ATP suggesting that activation resulted

from phosphorylation of the enzyme. Slight activation of the neutral CEH in the microsomes from the mammary gland by Mg-ATP could also be demonstrated but was more readily observed when it had been first solubilized by detergent treatment. The extent of activation of between 30–84% was similar to that reported for HSL from rat adipose tissue (40), but was considerably less than that obtainable with the enzyme from chicken adipose tissue. Stimulation of enzyme activity by ATP could be almost completely inhibited by the presence of the inhibitor of protein kinase A. Thus, like HSL, the modulation of activity was most probably due to phosphorylation by the cAMP-dependent protein kinase indicating that the activity of the enzyme would be altered by changes in the cAMP content of the epithelial cell in response to hormonal signals. The inability to demonstrate reasonable activation of the unsolubilized enzyme by Mg-ATP could be due to a number of factors including limited access of the enzyme to the kinase or the presence of an active phosphatase which we have previously shown to exist in this microsomal fraction (41). Attempts to remove this phosphatase by a high salt wash of the microsomes, prior to treatment with Mg-ATP, led to a loss of activity in the preparation. Similarly, the presence of the normally used phosphatase inhibitors KF and pyrophosphate led to inhibition of the ester hydrolase activity, so it proved difficult to nullify easily this putative phosphatase activity.

Although the neutral CEH had many similarities to HSL, the distribution of the mammary enzyme indicated that it had a predominantly membrane location rather than the cytosolic location of the adrenal and adipose tissue enzymes, suggesting that it may function in a different manner to the latter enzymes. Nevertheless, the fact that the mammary enzyme can be regulated by a mechanism involving phosphorylation-dephosphorylation by cAMP-dependent protein kinase suggests the possibility that hormonal control of the activity of this enzyme has a role in manipulating the cholesterol metabolism of the mammary gland and in the secretion of cholesterol into milk.

The cholesterol ester levels in samples of prepartum milk have been shown to be higher than that of milk obtained after parturition (42,43) suggesting that the glands of pregnant animals contain a higher amount of cholesterol ester. Recent work in our laboratory has shown that the cholesterol ester content of microsomes from pregnant glands is about twice that found in early lactators. The doubling of the neutral CEH activiaties between late pregnancy and day two of lactation is therefore consistent with an inverse correlation between the hydrolase and the cholesterol ester content of the gland at these two stages of development.

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The Role of n-3 Essential Fatty Acids in Brain and Behavioral Development: A Cross-Fostering Study in the Mouse

P.E. Wainwright*[a], Y.S. Huang[a,b], B. Bulman-Fleming[a], D.E. Mills[a], P. Redden[b] and D. McCutcheon[a]

[a]Department of Health Studies, University of Waterloo, Waterloo, Ontario, N2L 3G1 and [b]Efamol Research Institute, Kentville, Nova Scotia, B4N 4H8 Canada

A cross-fostering design was used to examine the effects on brain and behavioral development in mice of pre- and/or postnatal dietary supplementation with n-3 fatty acids. Pregnant mice were fed either of two liquid diets, control (con) or experimental (exp). Each diet provided 3% of the calories in the form of n-6 fatty acids; the experimental diet was supplemented with an additional 1.5% from long chain n-3 fatty acids derived from fish oil. There were four treatment groups, with all pups fostered at birth. These groups were (prenatal diet/postnatal diet): Group 1, exp/exp; Group 2, exp/con; Group 3, con/exp; Group 4, con/con; a fifth control group (unfostered) was fed lab chow (LC) throughout the study. Animals from the exp/exp and con/con groups were weaned onto lab chow for later behavioral assessment. Prenatal n-3 supplementation resulted in a small acceleration of behavioral development. The adult animals did not differ on visual discrimination learning nor did they differ in visual acuity. During development the fatty acid composition of the brain membrane phospholipids reflected closely that of the pre- and postnatal dietary conditions. Levels of 22:5n-3 and 22:6n-3 increased in the n-3 supplemented groups, accompanied by a decrease in levels of 22:4n-6 and 22:5n-6; the net effect of these changes was to increase the total levels of C_{22} fatty acids. While these results support considerable plasticity of the fatty acid composition of the developing brain with respect to the immediate dietary availability of n-3 compounds, they do not support long term effects on learning capacity of n-3 supplementation during the developmental period.

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This paper addresses the putative functional role played by polyunsaturated fatty acids (PUFA) of the n-3 series in mammalian brain and behavioral development. The structural lipids of the grey matter of the mammalian nervous system are unique from others of the body in terms of their high proportion of PUFA, particularly arachidonic acid (AA, 20:4n-6) and docosahexaenoic acid (DHA, 22:6n-3) (1). In the brain, concentrations of DHA differ between subcellular fractions, with high levels in the microsomes and synaptosomes, particularly in the *sn*-2 position of phosphatidylethanolamine and phosphatidylserine (2,3). Similarly high concentrations have been reported in the retina (4). The fatty acid precursors of both the n-6 and n-3 series (LA, linoleic acid, 18:2n-6, and LN, α-linolenic acid, 18:3n-3) cannot be synthesized *de novo* by mammals, and are therefore considered essential dietary nutrients. LA and LN are desaturated and elongated by the same enzymes to form the longer carbon chain derivatives (5). Changes in membrane fatty acid composition have been associated with alterations in the activity of various membrane-bound enzymes (6,7). It has been suggested that such functional changes may underlie reported associations between dietary lipid composition and behavior in mature animals (8).

Because these long chain PUFA accrue rapidly in the brain during the prenatal and suckling periods (9-11), the question arises as to whether specific dietary deficiencies during gestation and lactation may have enduring consequences on brain lipid composition and function. Of the studies on the effects of essential fatty acid (EFA) deficiency on brain phospholipid composition, some have shown decreases in levels of 20:4n-6 and 22:6n-3, with most showing increases in 20:3n-9, 22:3n-9, and in 22:5n-6 (12-18). Studies which specifically address n-3 deficiency, while showing minimal effects on levels of 20:4n-6, show a consistent decrease in levels of 22:6n-3, accompanied by a corresponding increase in levels of 22:5n-6 (19-23). Nutritional rehabilitation of such animals results in a decrease in levels of 22:5n-6 with an increase in levels of 22:6n-3 (24-26). Together with these biochemical differences, studies in the rhesus monkey have reported effects of n-3 on the physiological responsiveness of the retina, as well as on behavioral measures of visual acuity—the retinal changes appear to be independent of later nutritional rehabilitation (23,27). Changes in retinal function associated with decreased levels of 22:6n-3 have also been reported in the rat (28), although another study finds no such effects in either the rat or the guinea pig (29). Studies conducted on rodents have compared the effects of dietary n-3 *vs* n-6 fatty acids on learning ability, and report improved associative capacities as a result of the provision of n-3 during development (30-34). However, much of this work does not dissociate possible effects on sensory thresholds from those on cognitive function. Moreover, in these studies, the animals were maintained on the dietary treatment until they were tested as adults, and they therefore do not provide information on the importance of specific developmental periods. These issues were addressed in the present study. Recent work from our laboratory (35,36), in which pregnant mice consuming ethanol received n-3 dietary supplementation, while showing no effect of n-3 on spatial reversal learning in a water maze, did show a modest effect in accelerating indices of sensory development in the pups, particularly eye-opening. Therefore, in this study, a cross-fostering design was used to ascertain whether there was a critical period, either pre- or postnatal, during which the provision of n-3 fatty acids in the maternal diet would affect behavioral development in the offspring. The study also examined whether supplementation of n-3 fatty acids throughout the prenatal and preweaning developmental period would have an effect on

*To whom correspondence should be addressed.

Abbreviations: AA, arachidonic acid; ANOVA, analysis of variance; BHT, butylated hydroxytoluene; DHA, docosahexaenoic acid; EFA, essential fatty acids; GLC, gas-liquid chromatography; GLM, general linear model; LA, linoleic acid; LC, lab chow; LN, α-linolenic acid; PC, phosphatidylcholine; PE, phosphatidylethanolamine; PUFA, polyunsaturated fatty acids; SAS, Statistical Analysis Systems; TLC, thin-layer chromatography.

later learning ability. This was accomplished by assessing the learning capacity of adult animals which had been fed either n-6 supplemented with n-3 or n-6 fatty acids only throughout gestation and lactation and then weaned onto a chow diet containing both n-3 and n-6 fatty acids. A visual discrimination learning task was used and the procedure incorporated a behavioral measure of visual acuity in order to separate effects on learning ability from those on sensory capacity. The fatty acid composition of the brain glycerophospholipids was measured during development and in the adult animals.

METHODS

Subjects. Parents were 10–12-week-old B6D2 F_1 hybrid mice purchased from Charles River Breeding Laboratories (St. Constant, P.Q., Canada). They were maintained under a reversed 12 hr light:12 hr dark schedule at 22 ± 1°C, with tap water and lab chow (Purina # 5001) available *ad libitum.* The animals were group-housed in standard plastic cages containing Beta-Chip hardwood bedding and toilet tissue for nesting material. Breeding commenced at about 20 weeks of age.

Experimental design and materials. Maternal animals were fed one of two liquid diets, each of which provided 20% of the calories from oil. The control diet (con) provided 3% of the calories as n-6 fatty acids (FA). The experimental diet (exp) similarly contained 3% n-6 FA and was supplemented with an additional 1.5% of long-chain n-3 FA derived from fish oil. The liquid diets were the sole source of food and fluid. They were the same as those used previously (35), formulated by BioServ (Frenchtown, NJ); 20% of the calories were provided as protein (fat-free casein, < 0.5% fat) and 60% as carbohydrate (maltose-dextrin), together with minerals and vitamins; a typical analysis indicates 56 i.u. of α-tocopherol per liter. In the control diet the oil was constituted from a mixture of safflower and olive oil, with the proportions of each based on prior analysis and calculated to yield 3.0% of the dietary calories as n-6 fatty acids. Similarly, in the experimental diet, the oil contained a mixture of safflower oil, olive oil, and Polepa (a fish oil concentrate, provided by Efamol Research Institute, Kentville, Nova Scotia, Canada), such that the contribution to the dietary calories was 3.0% n-6 and 1.5% n-3. The n-3 content of the Polepa was: 18:3, 2.1%; 20:5, 12.9%; 22:5, 1.7%; 22:6, 7.5%. The oil mixtures were also analyzed and a representative analysis of the EFA composition of each is shown in Table 1. There were four treatment groups in the study, which were constituted by cross-fostering all pups at birth, either to a dam receiving the same diet as their prenatal dam, or to one receiving the diet opposite to that of their prenatal dam. Thus the groups were (prenatal diet/postnatal diet): Group 1, exp/exp; Group 2, exp/con; Group 3, con/exp; and Group 4, con/con. A fifth untreated group was included [Group 5, lab chow (LC)], in which pregnant and lactating mice were allowed free access to lab chow (containing 22.3% 18:2n-6, 2.3% 18:3n-3, 1.3% 20:5n-3 and 1.1% 22:6n-3) and tap water throughout the course of the experiment. There is one point which should be addressed here with respect to the design of this study. The diets were formulated to provide *equivalent* amounts of n-6 fatty acids, with *additional* n-3 being added to constitute the n-3 group. Although the design might also be described as one which addresses n-3 *deficiency,* with the control group being the deficient group, the experimental dietary manipulation is better described as providing n-3 *supplementation.* The reason for this is that, regardless of levels of essential fatty acids in the maternal diet, these compounds may nevertheless remain available to the developing offspring through the presence of maternal stores. In order to obtain a true deficiency in the supply of n-3 fatty acids to the offspring, it may be necessary to deplete the maternal diet for successive generations (18) or gestations (37).

TABLE 1

Selected Fatty Acid Composition of Dietary Oils[a]

	Experimental[b]	Control[c]
18:2n-6	14.2	13.3
18:3n-3	0.8	<0.2
20:4n-6	0.3	—
20:5n-3	3.3	—
22:5n-3	0.4	—
22:6n-3	1.9	—

[a] Representative GLC analysis of oil mixtures, where value represents % of total fatty acids. Note that levels of n-6 fatty acids are similar in each group, with the experimental oil supplemented with n-3 fatty acids derived from fish oil concentrate.

[b] Experimental = olive/safflower/polepa, 100 g:27.5 g:72.5 g.

[c] Control = olive/safflower, 9 g:1 g.

Procedure. Animals were mated daily, starting at the beginning of the dark phase, and were checked for the presence of copulatory plugs about 7 hr later. The day on which a vaginal plug was detected was considered day 0 of gestation. All pregnant dams were weighed and then randomly assigned to the exp, con or lab chow condition in the ratio 2:2:1, respectively. Dams in the liquid dietary groups were fed 0.75 mL × body wt. (g) daily until day 20. The animals were weighed on days 1, 5, and every two days thereafter, and the amount of food adjusted accordingly. Birth occurred on day 19 or 20 and all dams and litters were weighed on day 20. At this time, litters were culled to six (three of each sex), and these pups were placed with their appropriate foster mother (groups 1–4 —dietary treatments) or returned to their natural mother (group 5—lab chow). From day 20 until day 32 (post-conception), when the pups were tested on behavioral development, lactating mothers were fed the appropriate liquid diet *ad libitum* at 1.5 × strength. Daily food intake was monitored throughout the feeding period.

Day 32 behavioral development. On day 32 post-conception litters were coded by an independent observer so that the experimenter was blind to their experimental condition. Then all pups were weighed, and two pups of each sex were selected randomly from each litter, and subjected to a battery of 12 tests to measure behavioral development. These tests are identified as follows: i) Righting reflex; ii) cliff aversion; iii) forelimb and hindlimb grasp reflex iv) vibrissae-placing reflex; v) level screen test; vi) vertical screen test; vii) screen climbing test; viii) pole grasp; ix) forelimb and hindlimb stick grasp; x) eye opening; xi) visual placing reflex; and xii) auditory startle response. The pup was assigned a score on each test, using standardized criteria, and these scores ranged from 1 to 10. The scores were then averaged over all the

tests to yield a mean behavioral test score. By substituting the mean score of each pup into a previously derived quadratic equation predicting chronological age from behavioral test score, it was possible to obtain a measure of "B6D2F$_2$-equivalent behavioral age" for each mouse. (See references 38 and 39 for a full description of these procedures.) Previous studies have employed this technique to investigate the effects of nutritional availability (40) and interactions between dietary fatty acids and prenatal ethanol exposure (35,41,42) on behavioral development in the mouse. Following testing on day 32, one animal of each sex was selected randomly, anaesthetized with halothane and then decapitated. The brains of these animals were extracted and trimmed by removal of the olfactory bulbs anteriorly and 2 mm below the medulla posteriorly. Within two min the brains were then weighed to the nearest 0.1 mg, and frozen at −80°C to await biochemical analysis. All remaining pups were discarded, except those in Groups 1 (exp/exp) and 4 (con/con), which were left with the dam until weaning at day 46 post-conception. Dams were fed the appropriate liquid diet up to day 35. Thereafter feeding reverted to lab chow and tap water *ad libitum*.

Water maze testing. Twenty animals (10 males and 10 females) from each of the exp/exp and con/con groups were tested in a water maze at 90 ± 12 days of age. The apparatus consisted of a stainless steel "T"-shaped container; the dimensions have been described in detail previously (35). The container was filled to a depth of 15 cm with 22°C tap water to which was added enough milk powder to ensure opacity. An escape platform was available in either the right or the left arm; the presence of this platform was indicated by striped stimulus cards which were placed inside plexiglass holders which lined the walls of the appropriate arm. These striped cards were generated by computer and had alternating vertical black and white stripes of width 0.6 cm, such that 50% of the area was black and 50% white. The arm of the maze which did not contain the platform also had computer-generated cards, with the same percentage of black and white as the striped card, but comprised of black dots on a white background, such that they appeared a uniform grey. It is customary in vision research to express the intensity of a striped stimulus grating in terms of "cycles per degree," which describes the relationship, at a constant distance, between the spatial frequency of the grating and the visual angle subtended by the animal's eye (43). The closer together the cycles, the smaller the visual angle between cycles, therefore the more cycles per degree of visual angle. Assuming that the behavioral choice occurred when the animal had reached the border of the T junction, and was facing the stripes on the back wall, the intensity of the striped stimulus can be expressed as 0.095 cycles per degree. Following two days of acclimation, which was comprised of a two min exposure to the maze with the striped stimulus cards and escape platform in each arm, each animal was tested on a block of six trials twice daily for five consecutive days. During testing the sequence of sides on which the platform was placed was randomized, with the constraint that there always be three presentations on each side in any one block. Following placement of the animal in the stem of the T, the number of seconds for it to reach the platform was recorded, as was the number of wrong turns it made. A wrong turn was defined as the animal's whole body (tip of nose to base of tail) pointing down an arm of the maze which did not contain the platform. The animal was allowed to rest on the platform for 20 seconds before being placed in a warmed, paper towel-lined cage, on top of a domestic heating pad, for a 30 second inter-trial interval. An animal was considered to have learned the task when only two wrong turns were made during two consecutive blocks (10/12 correct). When an animal reached this criterion, its next block of trials consisted of five trials, in which there was one presentation of the original stripe width, and one of each of four decreasing stripe widths, 0.6 cm, 0.3 cm, 0.15 cm, 0.075 cm and 0.0375 cm (*i.e.*, increasing cycles per degree, 0.095, 0.19, 0.38, 0.75 and 1.5). The order of presentation was randomized, as was the side of the "T" on which the stimulus cards were placed (left or right, relative to the grey cards). The objective of this last block of trials was to assess the limits of the animal's visual acuity, the rationale being that as the stripe width decreased, the animal would eventually be confronted by a stimulus which it would be unable to distinguish from grey, and would therefore have difficulty locating the platform.

Brain lipids. All tissue was coded such that the biochemical analysis was conducted independently of prior knowledge of the treatment conditions. The homogenized brains were extracted using a modified Bligh and Dyer method (44), utilizing chloroform/methanol (1:1, v/v), with 0.02% BHT (w/v). After separation and drying under nitrogen, the total lipids were fractionated by one-dimensional thin-layer chromatography (TLC), using silica gel plates (Analtech GF, Newark, DE) and a chloroform/methanol/acetic acid/water (50:30:4:2, by vol) solvent system. The fatty acids in the resulting phospholipid fractions were transesterified with 14% boron trifluoride in methanol, and analyzed on a gas chromatograph (Perkin-Elmer 8420, Norwalk, CT) equipped with a flame ionization detector and a 15 m × 0.32 m (i.d.) capillary column (Supelco Wax 10, Bellefonte, PA). The temperature program for the GLC run consisted of 2 min at 160°C, followed by a 2°/min increase to 190°C. After 10 min at 190°C the temperature was increased 5°/min to 220°C, followed by 2 min at 220°C.

Statistical analyses. The data were analyzed using the general linear model (GLM) provided by the Statistical Analysis System (SAS) to do analysis of variance (ANOVA), with the α level set at 0.05. The litter mean score, collapsed across sex, was used as the unit of analysis for the pup data, except for those measures obtained in the water maze, where one pup from each litter was tested. As the fatty acid determinations were conducted on the pooled brains from a litter, the litter also represents the unit of analysis for these measures. The sample sizes are shown in the respective tables, with an average of 15 litters per group. The few instances of missing data are due to experimenter oversight or technical problems. The experimental hypotheses were addressed by the pre-planned linear comparisons outlined here: i) Prenatal n-3 supplementation: exp/exp + exp/con *vs* con/exp + con/con; ii) postnatal n-3 supplementation: exp/exp + con/exp *vs* exp/con + con/con; iii) prenatal × postnatal interaction: exp/exp + con/con *vs* exp/con + con/exp; and iv) nutritional effects: exp/exp *vs* LC, con/con *vs* LC.

TABLE 2

Maternal and Pup Variables as a Function of Pre- and Postnatal Dietary Supplementation with n-3 Fatty Acids[a]

Prenatal diet	Exp	Con	LC	Effects[b]
Maternal wt. day 0 (g)	24.0 ± 0.2 (32)	24.2 ± 0.2 (36)	24.1 ± 0.3 (17)	
Maternal wt. gain to day 17 (g)	15.3 ± 0.7 (15)	15.6 ± 0.7 (15)	16.5 ± 0.6 (17)	
Pup birth wt. (g)	1.42 ± 0.02 (32)	1.42 ± 0.02 (35)	1.49 ± 0.02 (17)	nut[c]

Pre/post	exp/exp	exp/con	con/exp	con/con	LC	Effects[b]
Pup body wt. day 32 (g)	8.5 ± 0.09 (17)	8.8 ± 0.15 (15)	8.4 ± 0.17 (15)	8.6 ± 0.17 (20)	8.0 ± 0.16 (17)	nutexp[c] nutcon[d]
Pup brain wt. day 32 (mg)	364.2 ± 2.6 (17)	362.2 ± 3.0 (15)	362.2 ± 2.7 (15)	367.2 ± 2.8 (20)	370.7 ± 2.1 (17)	

[a]Values represent group means ± SEM with sample size = (n) litters.

[b]Abbreviations for experimental effects: exp, experimental diet (n-3 supplemented); con, control diet; pre, main effect of prenatal n-3 supplementation; post, main effect of postnatal n-3 supplementation; int, prenatal/postnatal interaction; nutexp, exp/exp *vs* lab chow; nutcon, con/con *vs* lab chow; and nut, lab chow *vs* dietary groups combined.

[c]$p < 0.05$.

[d]$p < 0.01$.

RESULTS

Maternal and pup weight. These data are presented in Table 2. Our previous work had shown no differences between groups in maternal diet consumption, and this was confirmed in the present study by analysis of the data for only half the dams (randomly chosen). There were no differences among groups in the average weight of the mothers at the beginning of the experiment, nor in the amount of weight gained during pregnancy. At birth, the exp and con pups were similar in weight, but weighed less than the LC pups, $F(1,81) = 9.85$, $p < 0.01$). There were no differences in litter size (data not shown). On day 32 postconception the pups of the dams fed liquid diets did not differ in weight, but were heavier than those of mothers fed lab chow, $F(4,79) = 4.20$, $p < 0.01$. There were no significant differences among the groups on day 32 for brain weight.

Behavioral development. As is seen in Figure 1, the overall development of the pups of dams which received the n-3 supplemented diet prenatally was advanced in comparison with those which received the control diet prenatally, $F(1,79) = 5.22$, $p < 0.025$. There was also a nutritional effect, in which the pups in the LC group were developmentally advanced when compared both with those in the exp/exp group and in the con/con group, $F(1,79) = 4.97$, $p < 0.05$ and $F(1,79) = 11.46$, $p < 0.01$, respectively. Table 3 shows the scores obtained on each of the individual tests, together with the outcome of the respective statistical analyses. It can be seen from the general pattern of these results that the major effect (other than the nutritional effects) was due to prenatal n-3 supplementation. In the single case for which there was a postnatal n-3 effect, the level screen test, the performance of the con/exp group was much worse than those of the other groups.

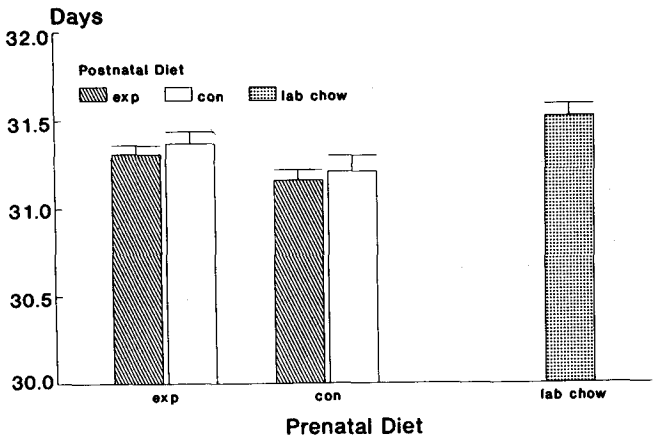


FIG. 1. Effect of pre- and postnatal dietary supplementation with n-3 fatty acids on behavioral development, expressed as "days postconception" in B6D2F$_2$ mice tested on day 32 postconception (exp = n-3 supplemented diet; con = control diet).

Water maze learning. There was no difference between the exp/exp and con/con groups with respect to the number of trials to reach criterion (exp Mean = 51.6 ± 3.0 SEM; con Mean = 51.9 ± 2.8 SEM). As shown in Figure 2 and supported by a repeated measures analysis, there was a clear effect of decreasing stripe width in increasing the time taken to escape to the platform, $F(4,25) = 7.87$, $p < 0.001$. This reflected the significant increase in the number of errors made by the animals as the stripe width decreased (data not shown). Neither escape time nor

TABLE 3

Effects of Pre- and Postnatal Dietary Supplementation with n-3 Fatty Acids on Behavioral Development on Day 32 Postconception[a]

Pre/post	exp/exp (n=17)	exp/con (n=15)	con/exp (n=15)	con/con (n=20)	LC (n=17)	Effects[b]
Righting reflex	8.8 ± 0.2	8.8 ± 0.2	8.4 ± 0.2	8.7 ± 0.3	8.9 ± 0.2	
Cliff aversion	8.2 ± 0.2	8.8 ± 0.2	8.4 ± 0.2	8.5 ± 0.2	8.7 ± 0.2	
Limb grasp	5.9 ± 0.1	5.9 ± 0.1	5.8 ± 0.2	5.8 ± 0.1	6.0 ± 0.1	
Vibrissae place	4.0 ± 0.1	4.1 ± 0.2	3.9 ± 0.1	4.2 ± 0.1	4.3 ± 0.2	
Level screen	9.4 ± 0.1	9.6 ± 0.1	9.0 ± 0.2	9.5 ± 0.1	9.6 ± 0.1	post[c]
Vertical screen	10.0 ± 0.0	10.0 ± 0.0	10.0 ± 0.0	10.0 ± 0.0	10.0 ± 0.0	
Screen climb	8.9 ± 0.2	9.0 ± 0.1	8.6 ± 0.2	8.6 ± 0.2	8.8 ± 0.2	pre[c]
Pole grasp	6.9 ± 0.2	6.6 ± 0.2	6.6 ± 0.1	6.8 ± 0.2	7.8 ± 0.2	nutexp[d]; nutcon[e]
Stick grasp	7.0 ± 0.1	6.9 ± 0.2	6.7 ± 0.3	6.6 ± 0.2	7.4 ± 0.2	nutcon[d]
Eye opening	6.2 ± 0.1	6.4 ± 0.1	6.0 ± 0.2	6.0 ± 0.2	6.7 ± 0.2	nutcon[d]; nutexp[c]; pre[c]
Visual placing	1.2 ± 0.4	1.0 ± 0.4	1.4 ± 0.5	1.3 ± 0.5	0.8 ± 0.3	
Auditory startle	2.2 ± 0.2	2.6 ± 0.1	2.2 ± 0.2	2.4 ± 0.1	2.4 ± 0.1	

[a]Values represent group means ± SEM with sample size = (n) litters. The tests are identified in the methods section of the text. [b]See Table 2 for abbreviations. [c]$p < 0.05$. [d]$p < 0.01$. [e]$p < 0.001$. [f]$p < 0.0001$.

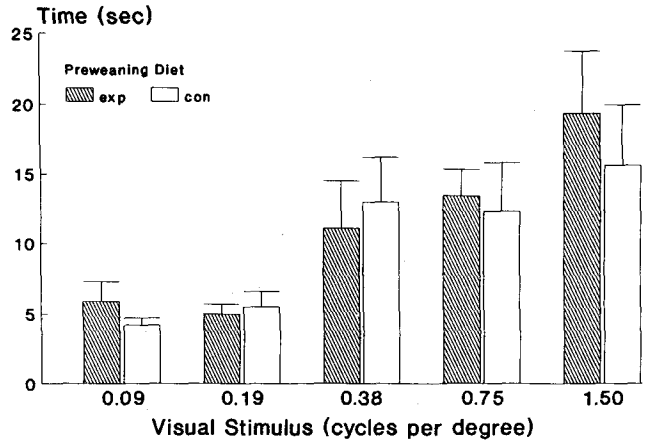


FIG. 2. Effect of stimulus intensity (*i.e.*, decreasing stripe width, expressed as increasing "cycles per degree") on visual discrimination in adult B6D2F$_2$ mice (exp = n-3 supplemented diet; con = control diet; preweaning = includes prenatal period and postnatal period prior to weaning).

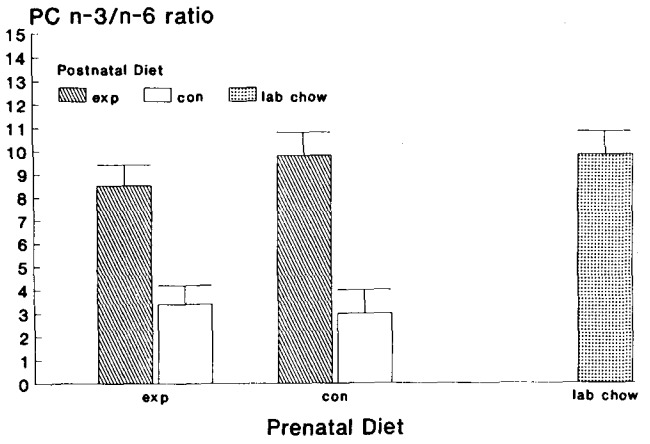


FIG. 3. Effect of pre- and postnatal dietary supplementation with n-3 fatty acids on long chain n-3:n-6 Ratio [(22:5n-3 + 22:6n-3)/(22:4n-6 + 22:5n-6)] of brain phosphatidylcholines in B6D2F$_2$ mouse pups on day 32 postconception (exp = n-3 supplemented diet; con = control diet).

number of errors showed a significant difference between the two dietary groups.

Brain phospholipid analysis. The fatty acid compositions of the brain phosphatidylcholine (PC) and phosphatidylethanolamine (PE) fractions from day 32 animals are shown in Tables 4 and 5; these include a detailed description of the significant results. At both ages, in both the PC and PE fractions, 20:4n-6 (AA) and 22:6n-3 (DHA) were the predominant long chain n-6 and n-3 fatty acids, respectively. At day 32, the PUFA composition of the brain reflected that of the pre- and postnatal dietary n-3 conditions. Dietary supplementation with n-3 compounds increased levels of n-3 fatty acids, particularly 22:5n-3 and 22:6n-3, with a concomitant decrease in levels of 22:4n-6 and 22:5n-6 in the groups which received the n-3 diet postnatally. This can be summarized by the long chain n-3/n-6 ratio, [(22:5n-3 + 22:6n-3)/(22:4n-6 + 22:5n-6)], which is shown in Figures 3 and 4. In the PC fraction this was increased by postnatal n-3, $F(1,73) = 46.98$, $p <$

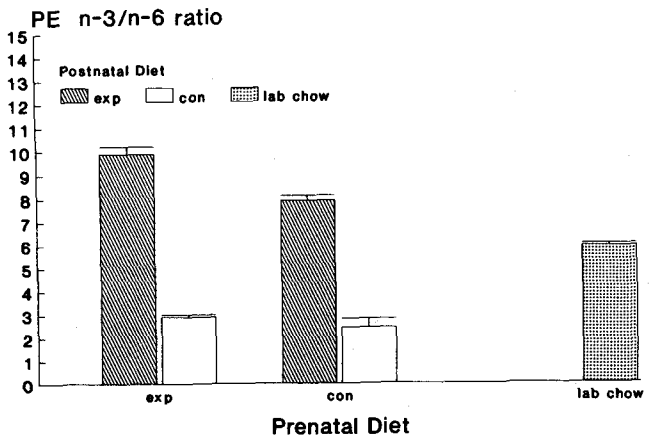


FIG. 4. Effect of pre- and postnatal dietary supplementation with n-3 fatty acids on long chain n-3:n-6 ratio [(22:5n-3 + 22:6n-3)/(22:4n-6 + 22:5n-6)] of brain phosphatidylethanolamines in B6D2F$_2$ mouse pups on day 32 postconception (exp = n-3 supplemented diet; con = control diet).

TABLE 4

Effect of Pre- and Postnatal Dietary Supplementation with n-3 Fatty Acids on Selected Fatty Acid Composition of Brain Phosphatidylcholines in B6D2F$_2$ Mouse Pups on Day 32 Postconception[a]

Pre/post	exp/exp (n=17)	exp/con (n=15)	con/exp (n=14)	con/con (n=17)	LC (n=17)	Effects[b]
16:0	46.7 ± 0.5	46.4 ± 0.6	48.1 ± 0.7	48.6 ± 0.7	47.9 ± 0.3	pre[d]
16:1n-7	5.0 ± 0.1	4.9 ± 0.1	4.6 ± 0.1	4.6 ± 0.3	4.7 ± 0.1	
18:0	4.7 ± 0.2	4.9 ± 0.2	6.4 ± 0.3	6.1 ± 0.3	6.8 ± 0.3	pre[f]; nutexp[f]
18:1n-9	20.3 ± 0.2	20.7 ± 0.3	22.4 ± 0.2	22.1 ± 0.5	21.0 ± 0.2	pre[f]; nutcon[c]
18:2n-6	1.6 ± 0.04	1.4 ± 0.07	1.7 ± 0.05	1.4 ± 0.07	1.5 ± 0.04	post[e]
18:3n-6	0.1 ± 0.01	trace	trace	0.1 ± 0.03	0.2 ± 0.2	
18:3n-3	0.4 ± 0.03	0.4 ± 0.05	0.4 ± 0.08	0.4 ± 0.06	0.6 ± 0.05	nutcon[d]; nutexp[d]
20:3n-6	0.8 ± 0.04	0.6 ± 0.04	0.7 ± 0.04	0.4 ± 0.04	0.8 ± 0.04	pre[f]; post[f]; nutcon[f]
20:4n-6	7.5 ± 0.5	7.8 ± 0.8	6.8 ± 0.4	7.2 ± 0.5	7.8 ± 0.1	
22:4n-6	0.5 ± 0.04	1.1 ± 0.2	0.4 ± 0.05	0.7 ± 0.05	0.5 ± 0.03	pre[d]; post[f]
22:5n-6	0.2 ± 0.05	0.6 ± 0.09	0.2 ± 0.09	0.9 ± 0.09	0.1 ± 0.05	post[f]; nutcon[f]; int[c]
22:5n-3	0.2 ± 0.06	0.2 ± 0.1	0.1 ± 0.02	trace	trace	pre[d]; nutexp[c]
22:6n-3	5.4 ± 0.2	4.0 ± 0.2	4.7 ± 0.2	3.3 ± 0.3	4.3 ± 0.1	pre[e]; post[f]; nutcon[e]; nutexp[e]

[a]Values represent group means ± SEM with sample size = (n) litters. [b]See Table 2 for abbreviations. [c,d,e,f]See Table 3 for values.

TABLE 5

Effect of Pre- and Postnatal Dietary Supplementation with n-3 Fatty Acids on Selected Fatty Acid Composition of Brain Phosphatidylethanolamines in B6D2F$_2$ Mouse Pups on Day 32 Postconception[a]

Pre/post	exp/exp (n=16)	exp/con (n=15)	con/exp (n=15)	con/con (n=16)	LC (n=17)	Effects[b]
16:0	11.0 ± 0.3	11.4 ± 0.3	11.8 ± 0.5	13.8 ± 0.7	11.2 ± 0.3	pre[e]; post[d]; nutcon[f]
16:1n-7	0.6 ± 0.04	0.6 ± 0.04	0.5 ± 0.07	0.7 ± 0.06	0.6 ± 0.03	
18:0	6.2 ± 0.5	5.7 ± 0.6	9.8 ± 0.8	6.3 ± 0.6	5.2 ± 0.9	
18:1n-9	9.5 ± 0.1	9.7 ± 0.1	0.8 ± 0.4	10.8 ± 0.2	9.5 ± 0.1	pre[e]; post[d]; int[c]; nutcon[e]
18:2n-6	1.0 ± 0.1	0.6 ± 0.03	0.7 ± 0.04	0.7 ± 0.09	0.7 ± 0.02	post[c]; int[c]; nutexp[c]
18:3n-6	0.3 ± 0.06	0.2 ± 0.02	0.1 ± 0.03	0.1 ± 0.03	0.3 ± 0.01	pre[c]; post[c]; int[c]; nutcon[d]
18:3n-3	0.5 ± 0.04	0.4 ± 0.03	0.4 ± 0.05	0.4 ± 0.05	0.6 ± 0.02	nutcon[e]
20:3n-6	0.9 ± 0.03	0.6 ± 0.03	0.8 ± 0.04	0.4 ± 0.05	0.8 ± 0.02	pre[f]; post[f]; nutcon[f]; nutexp[c]
20:4n-6	17.1 ± 0.4	22.1 ± 0.5	17.1 ± 0.4	22.3 ± 0.5	19.9 ± 0.4	post[f]; nutcon[e]; nutexp[f]
22:4n-6	3.1 ± 0.08	5.1 ± 0.1	3.3 ± 0.1	4.7 ± 0.2	4.3 ± 0.1	post[f]; int[c]; nutcon[c]; nutexp[f]
22:5n-6	0.5 ± 0.05	4.0 ± 0.1	0.9 ± 0.03	5.2 ± 0.4	0.7 ± 0.04	pre[f]; post[f]; int[c]; nutcon[f]
22:5n-3	1.0 ± 0.02	0.3 ± 0.02	0.9 ± 0.02	0.2 ± 0.06	0.5 ± 0.01	pre[d]; post[f]; nutcon[f]; nutexp[f]
22:6n-3	33.9 ± 0.5	26.4 ± 0.4	32.5 ± 1.3	21.4 ± 1.0	28.5 ± 0.3	pre[f]; post[f]; int[c]; nutcon[f]; nutexp[f]

[a]Values represent group means ± SEM with sample size = (n) litters. [b]See Table 2 for abbreviations. [c,d,e,f]See Table 3 for values.

0.0001. The PE fraction showed significant effects of both pre-, F (1,72) = 25.91, $p < 0.0001$, and postnatal supplementation, F (1,72) = 616.63, $p < 0.0001$, with an interaction between the pre- and postnatal treatments, F (1,72) = 8.46, $p < 0.01$, due to a slightly larger postnatal effect in the exp/exp group. When compared with the group fed lab chow, in the PE fraction the ratio in the exp/exp group was greater, and in both the PE and PC fractions this value was lower in the con/con group. The total percentages of the C_{22} fatty acids are shown in Figures 5 and 6. In the PC fraction there was a significant prenatal effect, F (1,75) = 19.40, $p < 0.0001$, and a marginal postnatal effect, F (1,75) = 3.04, $0.1 > p > 0.05$, with n-3 supplementation increasing the amount of these long chain compounds. The PE fraction, while showing pre-, F (1,72) = 10.67, $p < 0.01$, and large postnatal effects, F (1,72) = 31.22, $p < 0.0001$, also showed a significant interaction, F (1,72) = 4.57, $p < 0.05$, due to a large increase due to postnatal supplementation in the con/exp group. In both fractions, the total C_{22} fatty acids in the exp/exp group exceeded that of the lab chow control. With respect to the individual fatty acids, it is worth noting that in the PE fraction levels of 22:6n-3 in the con/exp group were equivalent to those found in the exp/exp group, Interaction F (1,72) = 5.3, $p < 0.05$. There were also decreases in levels of 20:4n-6 due to postnatal n-3 supplementation, F (1,72) = 127.11, $p < 0.0001$. In both the PC and PE fractions the levels of 20:3n-6 were increased by prenatal [PC: F (1,75) = 17.15, $p < 0.0001$; PE: F (1,72) = 16.27. $p < 0.001$] and postnatal n-3 supplementation, [PC: F (1,75) = 69.51, $p < 0.0001$; PE: F (1,72) = 91.24, $p < 0.0001$]. This reflects the inhibition of the activity of Δ5 desaturase by long chain n-3 compounds, and is consistent with our previous findings (36,42). The analysis of the adult brains from the exp/exp and con/con dietary groups (which were subsequently weaned onto lab chow)

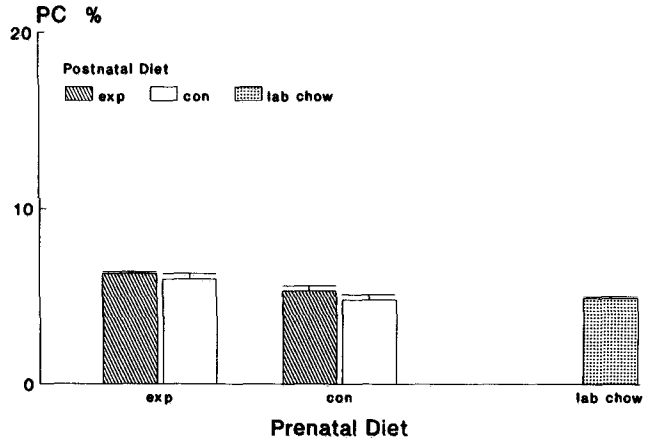


FIG. 5. Effect of pre- and postnatal dietary supplementation with n-3 fatty acids on total percentage of C_{22} polyunsaturated fatty acids [(22:5n-3 + 22:6n-3 + 22:4n-6 + 22:5n-6)] of brain phosphatidylcholines in B6D2F$_2$ mouse pups on day 32 postconception (exp = n-3 supplemented diet; con = control diet).

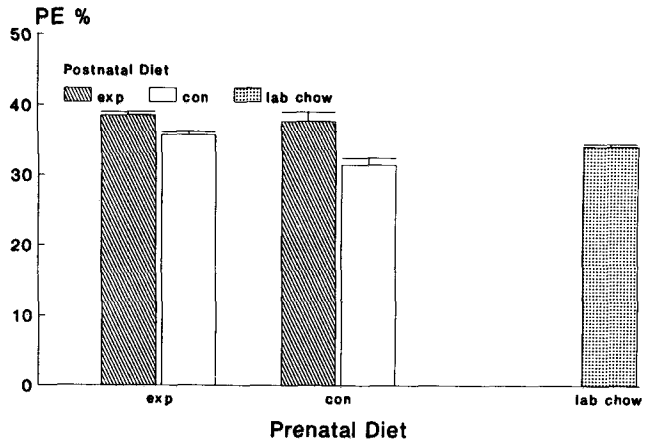


FIG. 6. Effect of pre- and postnatal dietary supplementatiuon with n-3 fatty acids on total percentage of C_{22} polyunsaturated fatty acids [(22:5n-3 + 22:6n-3 + 22:4n-6 + 22:5n-6)] of brain phosphatidylethanolamines in B6D2F$_2$ mouse pups on day 32 postconception (exp = n-3 supplemented diet; con = control diet).

are shown in Table 6. The only notable differences were in the PE fraction and included increases in 18:2n-6, 18:3n-6 and 22:5n-6 in the con/con group.

Dependent variable correlations. Pearson product-moment correlation coefficients were calculated within groups for the litter-mean scores obtained on the overall developmental score, eye opening, and PC and PE levels of 22:6n-3. As expected, there were consistent positive correlations between the overall score and eye-opening, but neither of these scores correlated with the DHA levels (data not shown).

DISCUSSION

The hypothesis of this study was that supplementation of the maternal diet with n-3 fatty acids during the pre- and/or postnatal periods would accelerate indices of sensorimotor development in the pups, and would also contribute to differences in discrimination learning ability

TABLE 6

Effect of Pre- and Postnatal Dietary Supplementation with n-3 Fatty Acids on Selected Fatty Acid Composition of Brain Phospholipids in Adult B6D2F$_2$ Mice[a]

	Phosphatidylcholine		Phosphatidylethanolamine	
Pre/post	exp/exp	con/con	exp/exp	con/con
16:0	43.3 ± 0.4	42.1 ± 0.6	7.9 ± 0.2	7.8 ± 0.3
16:1n-7	1.1 ± 0.1	1.1 ± 0.1	0.3 ± 0.04	0.3 ± 0.05
18:0	3.9 ± 0.8	3.5 ± 0.8	7.2 ± 0.5	5.7 ± 0.6
18:1n-9	27.5 ± 0.2	28.4 ± 0.2[d]	18.0 ± 0.3	17.7 ± 0.4
18:2n-6	0.9 ± 0.1	0.9 ± 0.1	0.5 ± 0.1	2.2 ± 0.6[c]
18:3n-6	0.2 ± 0.02	0.1 ± 0.02	trace	0.6 ± 0.3[c]
18:3n-3	1.3 ± 0.1	1.2 ± 0.1	0.2 ± 0.02	0.3 ± 0.04
20:3n-6	0.4 ± 0.03	0.3 ± 0.02	0.5 ± 0.03	0.5 ± 0.02
20:4n-6	5.8 ± 0.1	5.9 ± 0.1	13.6 ± 0.3	13.3 ± 0.4
22:4n-6	0.6 ±0.1	0.5 ± 0.03	4.2 ± 0.1	4.1 ± 0.1
22:5n-6	0.2 ± 0.06	0.1 ± 0.04	0.2 ± 0.04	0.5 ± 0.04[f]
22:5n-3	trace	trace	0.4 ± 0.03	0.5 ± 0.02
22:6n-3	6.5 ± 0.2	6.8 ± 0.2	31.3 ± 0.7	30.9 ± 1.2

[a]Values represent means ± SEM with sample size = 20 animals/group.

[c,d,f]See Table 3 for values.

and visual acuity in these animals as adults. The design of the study incorporated methodological controls which are important in interpreting effects of n-3 supplementation during development. First, cross-fostering procedures are necessary to separate pre- from postnatal effects on development. Similarly, in order to establish that the provision of n-3 compounds during prenatal and preweaning development is indeed critical with respect to adult behavior, all pups which are tested as adults should be weaned onto a control diet. This is because considerable plasticity has been demonstrated in the brains of postweaning and adult animals with respect to dietary fatty acid content, particularly the n-3 compounds, with the observed changes occurring within days to a few weeks (17-19,45-48). Second, before differences on a learning task can be attributed unequivocally to differences in ability to make the required association, it is necessary to exclude possible effects on sensory thresholds. Finally, in conducting developmental studies in a species where multiple births are the norm, the litter, and not the individual pup, should be used as the independent unit of analysis; failure to do this may inflate artificially the power of the design (49).

The results on behavioral development support our previous work in showing an effect of n-3 supplementation, and identify the prenatal period as critical. In the present study both sensory and motor categories were affected and again there was a strong effect on the eye-opening score. This replication of the n-3 effects occurred despite the fact that in this study the differences in maternal weight gain observed previously were not there (35,42), and by day 32 postconception the pups in both liquid diet groups were slightly heavier than the lab chow controls. This confirms that the effects on behavioral development were not related to differences in caloric availability or utilization. However, these effects were very modest in size, with the statistical significance reflecting the power provided by the large number of litters tested. There were no significant differences between

adult animals from the exp/exp and con/con groups when they were tested on visual discrimination learning ability. Neither did they appear to differ in visual acuity, with a similar increase in the number of errors with decreasing stimulus width. These results are at variance with those cited previously (31-34). However, this may be explained by differences among the studies related to the methodological issues discussed above, particularly with respect to the learning behavior, where in the previous work the animals were tested while still undergoing dietary treatment.

The analyses of the fatty acid content of the PC and PE fractions from the pup brains on day 32 postconception show that the long chain PUFA composition reflects both the pre- and postnatal dietary treatments, with an increase in 22:5n-3 and 22:6n-3 in the n-3 supplemented group, with a concomitant decrease in 22:5n-6 and 22:4n-6. Previous work has shown that the fatty acid composition of the newborn brain reflects that of the maternal diet during gestation (20,35,42), and the present results indicate considerable plasticity of these measures with respect to the composition of the maternal diet during lactation as well. Noteworthy in this respect is the large effect of the postnatal supplementation in the PE fraction, where the total amount of 22:6n-3 was as high in the con/exp group, which received the n-3 supplementation only during the 12 day postnatal period, as in the exp/exp group which was supplemented during gestation and lactation. Thus, these results show that the n-3/n-6 ratio of the long chain PUFA in the brain of the developing offspring can be altered within days through manipulation of the n-3 content of the maternal diet during lactation. Another potentially important finding of this work is that of the increase in the C_{22} fatty acids by the long chain n-3 supplementation. However, it should be noted that while our analysis shows a proportional increase, in the absence of absolute values it is not possible to determine whether there were changes in the actual lipid pool sizes. Nevertheless, these data are consistent with reports of the preferential uptake by the developing brain of preformed, longer chain, more unsaturated fatty acids (50,51). It is possible that, as has been shown in adult animals, the developing brain is not protected against an excess of very long chain PUFA (48). Previous work has shown that rats supplemented with n-3 during development maintain higher levels of 22:6n-3, even following a further two months on a n-3 deficient diet (52). Because of the possible functional consequences of such changes, and the increasing commercial availability of highly purified fatty acids, future work should address this question in the context of a dose-response relationship. The fact that the adult animals in the con/con group had similar n-3 levels to those of the exp/exp group shows an ability to regain normal composition by incorporating dietary n-3 after weaning. The lingering increase in 22:5n-6 seen in the adult animals of the con/con group is consistent with some previous work on EFA deficiency during early development (53).

In summary, these results show that the long chain n-3 fatty acid content of developing mouse brain, when measured in the preweaning period, very readily reflects the n-3 content of the pre- and postnatal maternal diet; most of these effects are reversible by the postweaning diet. Despite a small acceleration of indices of sensorimotor development, particularly eye-opening, by prenatal n-3 dietary supplementation, there is no enduring effect of n-3 supplementation during both the prenatal and preweaning periods on functional abilities related to visual learning capacity.

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Effect of *trans* Fatty Acids on Plasma Lipids, Platelet Function and Systolic Blood Pressure in Stroke-Prone Spontaneously Hypertensive Rats

M.T. Chiang[a,*], M.I. Otomo[a], H. Itoh[a], Y. Furukawa[a], S. Kimura[a] and H. Fujimoto[b]

[a]Laboratory of Nutrition Chemistry, Department of Food Chemistry, Faculty of Agriculture, Tohoku University, Sendai 981 and [b]Department of Clinical and Laboratory Medicine, Tohoku University School of Medicine, Sendai 981, Japan

To investigate the effect of *trans* fatty acids on plasma lipid levels and systolic blood pressure, hydrogenated corn oil was fed to SHRSP (stroke-prone spontaneously hypertensive rats) and WKY (Wistar-Kyoto) rats for 30 days. Significantly lower systolic blood pressure and plasma total cholesterol were observed in SHRSP rats fed *trans* fatty acids when compared with rats fed *cis* fatty acids from olive oil. In addition, higher HDL cholesterol and lower VLDL plus chylomicron cholesterol levels were found in SHRSP rats fed *trans* fatty acids. Although no significant changes of systolic blood pressure and plasma total cholesterol levels were observed in WKY rats after *trans* fatty acids treatment, WKY rats fed *trans* fatty acids had lower plasma LDL cholesterol and higher HDL cholesterol levels. In addition, platelet aggregation induced by collagen was decreased in WKY rats fed *trans* fatty acids. It is interesting that *trans* fatty acids increased the activity of plasma lecithin:cholesterol acyltransferase (LCAT) in both SHRSP and WKY rats. The observed influence of *trans* fatty acids on plasma lipid levels, systolic blood pressure and platelet aggregation suggests that *trans* fatty acids might prevent thrombotic disorders in SHRSP rats.

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The unsaturated fatty acids of vegetable and animal fats possess the *cis* configuration almost exclusively. In the 18:1 and 18:2 fatty acids, the double bonds are typically located at the ω9 and ω6 positions. Hydrogenated vegetable oil used as cooking oils, shortenings and margarines contains *trans* unsaturated fatty acids, which can amount to more than one-third of the total fatty acids present (1–4).

Dietary *trans* fatty acids have been shown to be incorporated into various tissues of rat (5–7). Autopsy and biopsy studies have revealed that they are also found in human tissues, with highest concentrations in liver and adipose tissues (8). In addition, *trans* fatty acids have also been identified in human milk (9). Although the amount of *trans* fatty acids that can be deposited in tissues has been shown to be related to the amount of *trans* fatty acids in the diet (10), Adlof and Emken (8) demonstrated that no *trans* 18:3 isomers were detected in human tissues and that *trans* 18:2 isomers would not be expected to have a significant physiological impact. The studies on the biological effects of *trans* fatty acids were typically focused on elaidic acid (*trans*-9-octadecenoate), because *trans* monoenoic acids (*t*-18:1) are quantitatively important components of our diet (11).

Although the effects of *trans* fatty acids on serum lipid levels and on the development of atherosclerosis have been studied in both animals and human (12–15), results have been inconclusive. Sugano *et al.* (16) reported that *trans* fatty acids are hypercholesterolemic in some cases and normocholesterolemic in others. A major reason for this discrepancy seems to be the difference not only in the types and amounts of *trans* fatty acids ingested, but also in the amount of dietary linoleic acid present in the experiments. It is, therefore, of considerable importance to examine the metabolism and biological properties of *trans* 18:1 fatty acids.

In the present study, the effect of *trans* fatty acids on plasma lipids and platelet function was studied using SHRSP (stroke-prone spontaneously hypertensive rats) and WKY (Wistar-Kyoto) rats given 1% NaCl water for four weeks.

MATERIALS AND METHODS

Eight-week-old SHRSP rats were used as experimental models and WKY rats of the same age were chosen as controls. Each of the two groups was divided into two subgroups (*cis*-FA and *trans*-FA) of equal body weight and systolic blood pressure. They were housed in individual stainless steel cages in a room kept at 23 ± 1°C. Fresh diets and 1% NaCl drinking water were available *ad libitum* for 30 days. The composition of the basal diet is shown in Table 1 and the fat composition of each diet is shown in Table 2 (17).

TABLE 1

Composition of Experimental Diet

Casein	20%
Mineral mixture[a]	4
Vitamin mixture (water souble)	1
Vitamin mixture (fat souble)[b]	1
Cholesterol	0.5
Cholic acid	0.3
cis or *trans* Fat[c]	10
Corn starch	up to 100

[a]Harper's salt mixture.

[b]100 Grams of vitamin mixture contain: Vitamin A, 150,000 IU; vitamin E, 2 gram; *p*-aminobenzoic acid, 1 gram; and choline chloride, 15 gram.

[c]*cis* Fat: 86% olive oil + 14% tristearin; *trans* fat: 94% + hydrogenated corn oil + 6% safflower oil; 500 gram of fats contain 10,000 IU vitamin D.

*To whom correspondence should be addressed at the Department of Marine Food Science, National Taiwan Ocean University, 2 Pei-Ning Road, Keelung 20224, Taiwan, R.O.C.

Abbreviations: ADAM, 9-anthryldiazomethane; FA, fatty acids; HDL, high density lipoprotein; LCAT, lecithin:cholesterol acyltransferase; LDL, low density lipoprotein; PG, prostaglandin; PPP, platelet poor plasma; PRP, platelet rich plasma; SHRSP, stroke-prone spontaneously hypertensive rats; TBA, thiobarbituric acid; TX, thromboxane; VLDL, very low density lipoprotein; WKY, Wistar-Kyoto.

TABLE 2

Fatty Acid Composition of Dietary Fat[a]

Type of fatty acid[b]	*cis*-FA (%)	*trans*-FA (%)
12:0	0.2	0.2
14:0	0.4	0.2
16:0	12.7	13.4
18:0	12.0	12.2
18:1(*t*)	—	41.2
18:1(n-9)(*c*)	68.5	25.1
18:1(n-7)	tr	0.8
18:1(n-5)	—	0.3
18:2(*tt*)	—	0.3
18:2(*ct*+*tc*)	—	0.5
18:2(*cc*)	5.2	5.0
20:0	0.4	0.2
22:0	0.1	0.6

[a] Fatty acid compositions were determined by gas liquid chromatography (17).

[b] C: *cis*-isomers; T: *trans*-isomers.

Determination of blood pressure. The arterial blood pressure of unaesthetized rats was measured by the indirect method using the caudal artery (18).

Fatty acid analysis. Analysis of fatty acyl groups of total lipids was done by gas-liquid chromatography of the corresponding methyl esters (19). Fatty acid esters were analyzed using a quadrex fused silica capillary column (20) (Silar 10C, 0.25 mm I.D. × 25 m × 0.25 μ film; oven temperature, 160°C to 170°C at 0.5°C/min) and a 15% DEGS column. Methyl esters of 18:2 (*tc* + *ct*) used as standards were generously supplied by the Japan Institute of Oils & Fats, Other Foods Inspection Foundation (Tokyo, Japan) (17); other fatty acid ester standards were purchased from Funakoshi Pharmaceutical Co. (Tokyo, Japan).

Determination of plasma lipids and lipid peroxides. Total cholesterol, phospholipids, triacylglycerols and free cholesterol in plasma were determined by enzymatic methods (20) using kits purchased from Wako Pure Chemical Company (Osaka, Japan). High density lipoprotein (HDL), low density lipoprotein (LDL), and very low density lipoprotein (VLDL) plus chylomicrons in plasma were determined by ultracentrifugation (21). The thiobarbituric acid (TBA) value of rat plasma was determined by the method of Yagi *et al.* (22).

Platelet aggregation induced by collagen. The platelet count of platelet rich plasma (PRP) was adjusted to a concentration of 6 × 10^6 cells/μL with autologous platelet poor plasma (PPP) (23). PRP was incubated with 60 μg/mL of collagen (Hormon-Chemie, Munich, Germany) in a Lumi-Aggregometer (Chrono-Log Corporation, Broomall, PA). The change in light transmission after the addition of aggregants was recorded.

Determination of prostaglandins (PG) and thromboxanes (TX). Rat kidney and platelet PG and TX were determined by the method of Kiyomiya *et al.* (24). The arachidonate metabolites were extracted by the method of Powell (25) using a Sep-pak C18 cartridge (26). The resultant fraction containing PG and TX was allowed to react with 9-anthryldiazomethane (ADAM). After removing contaminants and degraded reagent by silica gel Sep-pak, samples were applied to reverse phase high-performance liquid chromatography on octadecylsilyl silica gel (Cosmosil 5 C18, 4.6 mm I.D. × 150 mm; Nacalai; Tesque Inc., Kyoto, Japan) and monitored by a fluorescent detector. PGE_2, $PGF_{2\alpha}$, 6-keto-$PGF_{2\alpha}$ and TXB_2 were generously supplied by Ono Pharmaceutical Co. (Osaka, Japan). ADAM was purchased from Funakoshi Pharmaceutical Co. Silica gel Sep-pak and C18 Sep-pak were purchased from Waters Associates (Milford, MA).

Measurement of urine volume. Rats were moved into metabolic cages on day 28. The volume of urine for a 24-hr period was recorded at 3:00 p.m. The room temperature was kept at 23 ± 1°C.

Determination of LCAT activity. LCAT activity was measured by the method of Strokke and Norum (27) and was expressed as percentage of labeled cholesterol acylated per hour.

RESULTS

No significant difference in body weights was observed between the *cis* fat group and the *trans* fat group of SHRSP rats (range, 210–230 g) or WKY rats (range, 255–270 g) at the end of the fourth week.

Systolic blood pressure. The systolic blood pressure of the rats fed the experimental diets is shown in Figure 1. SHRSP rats fed *trans* fat showed a significant reduction in systolic blood pressure at the end of the experimental period. However, no significant influence on systolic blood pressure was observed in WKY rats fed the experimental diets.

Plasma lipids. Statistical analysis of plasma total cholesterol and lipoprotein cholesterol levels revealed a significant difference between animals fed *trans* fat and those fed *cis* fat. SHRSP rats fed hydrogenated corn oil showed significantly increased HDL cholesterol and decreased total cholesterol levels (Table 3). In addition, a significant decrease in the plasma lipoprotein cholesterol levels associated with LDL and VLDL plus chylomicrons was also observed in SHRSP rats fed *trans*

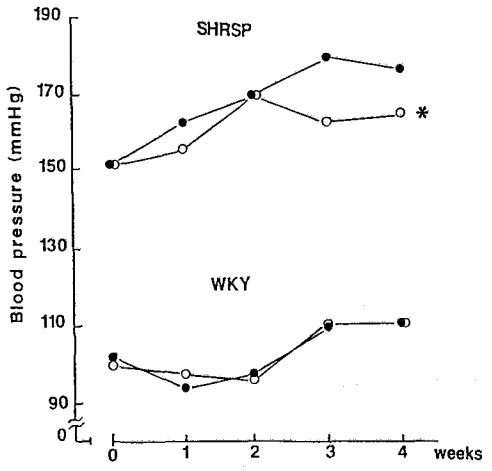


FIG. 1. Effect of *trans* fatty acids on systolic blood pressure in rats. Each point represents the average value from eight rats. *cis* Fat group, —●—; *trans* fat group, —○—. *Significant difference from the *cis* fat group at $p < 0.05$.

TABLE 3

Effect of Dietary *trans* Fatty Acids on Plasma Total Cholesterol and Cholesterol Associated with HDL, LDL, and VLDL plus Chylomicrons[a]

Exp. group	Diet	Cholesterol (mg/100 mL)							
				HDL		LDL		VLDL + Chylomicrons	
		Total	Free	Total	Free	Total	Free	Total	Free
SHRSP	*cis*-FA	83 ± 13[a]	20 ± 3[b]	24 ± 2[c]	6 ± 2	15 ± 3[d]	4 ± 1	32 ± 5[e]	9 ± 3
	trans-FA	50 ± 10[a]	10 ± 2[b]	31 ± 1[c]	5 ± 2	8 ± 3[d]	2 ± 1	16 ± 4[e]	5 ± 2
WKY	*cis*-FA	68 ± 12	16 ± 4	24 ± 3[f]	5 ± 2	21 ± 3[g]	6 ± 1	19 ± 2	7 ± 1
	trans-FA	58 ± 7	12 ± 3	38 ± 4[f]	7 ± 1	12 ± 2[g]	4 ± 2	14 ± 2	4 ± 1

[a]Results are expressed as mean ± SD for 8 rats per each dietary group. Values in the same column of each experiment with the same superscript letter are significantly different ($p < 0.05$).

TABLE 4

Effect of Dietary *trans* Fatty Acids on Plasma Triacylglycerols and Phospholipids[a]

Exp. group	Diet	Triacylglycerols (mg/100 mL)	Phospholipids (mg/100 mL)
SHRSP	*cis*-FA	91 ± 8	112 ± 3[a]
	trans-FA	100 ± 12	140 ± 16[a]
WKY	*cis*-FA	62 ± 15	98 ± 6
	trans-FA	70 ± 11	110 ± 7

[a]Results are expressed as mean ± SD for eight rats per each dietary group. Values in the same column of each experiment with the same superscript letter are significantly different ($p < 0.05$).

fat. Although there was no significant change in plasma total cholesterol in WKY rats fed the experimental diets, higher HDL cholesterol was found in the rats fed *trans* fat. The results for plasma phospholipids and triacylglycerols are given in Table 4. A significant increase in plasma phospholipid levels was observed in SHRSP rats fed *trans* fat, but no significant change was found in WKY rats fed the experimental diets. No significant difference in triacylglycerol values was observed between rats on the *cis* fat diet and on the *trans* fat diet. It is interesting to note that the ratio of plasma total cholesterol to phospholipids in rats fed *trans* fat were significantly ($p < 0.05$) decreased not only in SHRSP rats (mean ± SD; 0.7 ± 0.0 for *cis* fat group and 0.4 ± 0.1 for *trans* fat group), but also in WKY rats (mean ± SD; 0.6 ± 0.0 for *cis* fat group and 0.5 ± 0.0 for *trans* fat group).

Platelet aggregation. As shown in Figure 2, platelet aggregation induced by 60 μg/mL collagen was markedly reduced in WKY rats fed *trans* fat. Because of the hypoaggregability in SHRSP rats (28), there was no significant difference between the SHRSP groups.

Prostaglandins production of kidney. Figures 3 and 4 show the levels of PGE_2 and $PGF_{2\alpha}$ in kidney from rats fed experimental diets for four weeks. Although *trans* fat significantly decreased the level of $PGF_{2\alpha}$ production in kidney of SHRSP rats, the amount of PGE_2 formed in rats fed *trans* fat was only slightly decreased, which resulted in an increase of the ratio of $PGF_2/PGF_{2\alpha}$ in kidney of SHRSP rats. Both the levels of PGE_2 and $PGF_{2\alpha}$ in kidney from WKY rats fed the *trans* fat were significantly decreased, but no significant difference was found in $PGE_2/PGF_{2\alpha}$ ratios of the two dietary groups (Fig. 5).

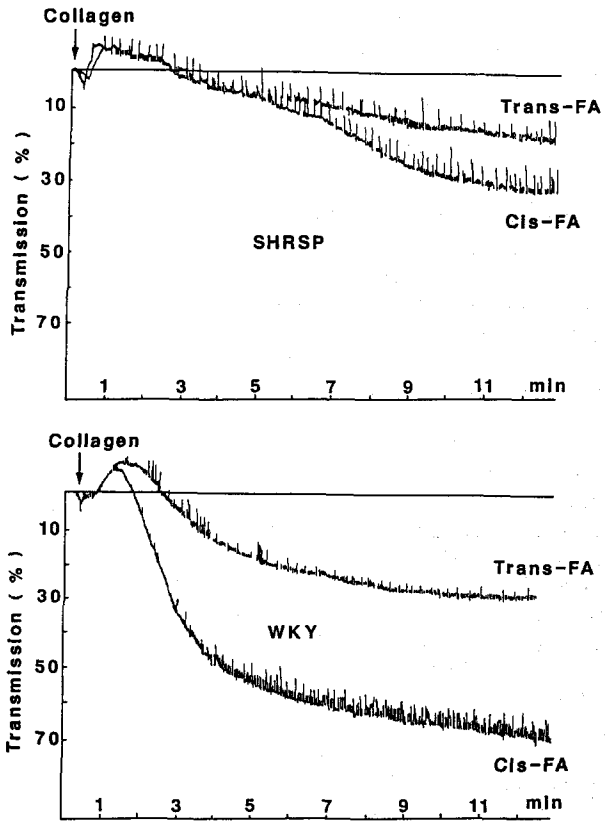


FIG. 2. Effect of *trans* fatty acids on platelet aggregation in SHRSP and WKY rats. Citrate PRP was preincubated for 2 min at 37°C, then the platelet response to collagen (60 μg/mL) was tested.

Urine excretion. The volume of urine excreted is shown in Figure 6. SHRSP rats fed *trans* fat showed a decrease in urine excretion, which reflected the diuretic effect of *trans* fatty acids induced by the higher ratio of $PGF_2/PGF_{2\alpha}$ in kidney when compared to that of rats fed *cis* fat. In WKY rats fed *cis* fat and *trans* fat, there was no significant difference in the ratio of $PGE_2/PGF_{2\alpha}$ in kidney, and there was no significant change in urine excretion volume.

Plasma lipids peroxide. The plasma thiobarbituric acid (TBA) values for SHRSP rats (4.0 ± 0.5 nmole MDA/mL)

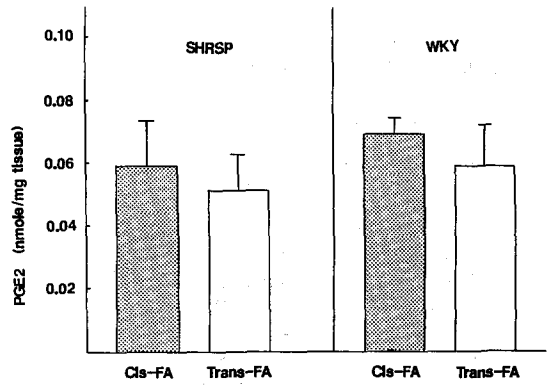


FIG. 3. Effect of dietary *trans* fatty acids on PGE_2 level in kidney of rats. Results are expressed as mean ± SD for eight rats in each dietary group.

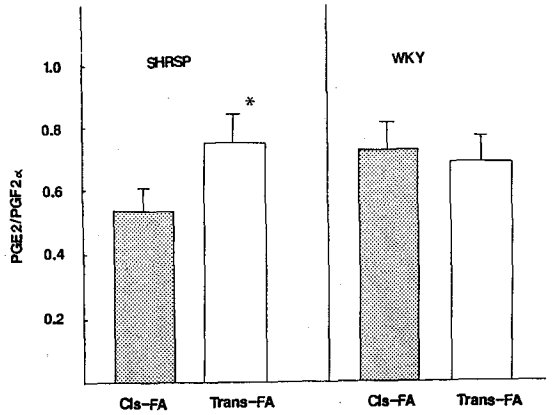


FIG. 5. Effect of dietary *trans* fatty acids on $PGE_2/PGF_{2\alpha}$ in kidney of rats. Results are expressed as mean ± SD for eight rats in each dietary group. *Significant difference from the *cis* fat group at $p < 0.05$.

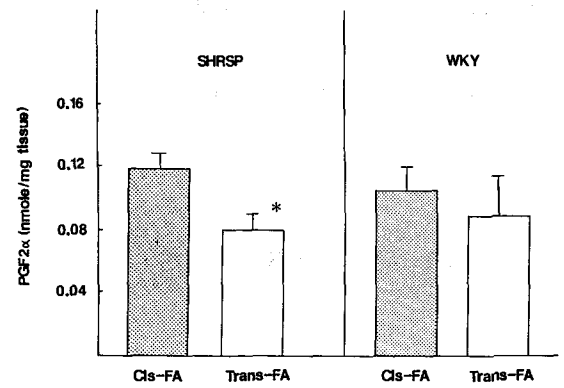


FIG. 4. Effect of dietary *trans* fatty acids on $PGF_{2\alpha}$ level in kidney of rats. Results are expressed as mean ± SD for eight rats in each dietary group. *Significant difference from the *cis* fat group at $p < 0.05$.

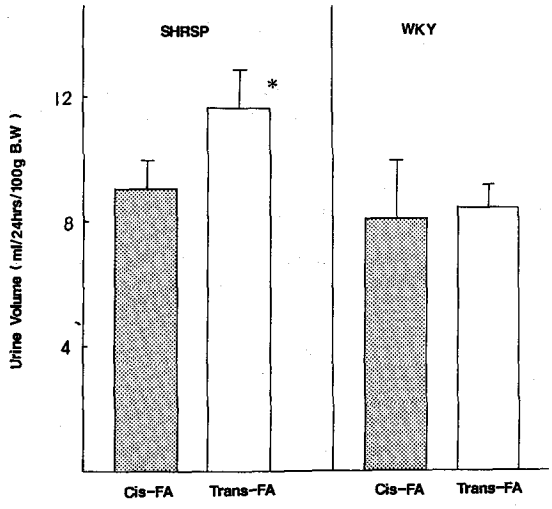


FIG. 6. Effect of dietary *trans* fatty acids on urine excretion for 24 hr in rats. Results are expressed as mean ± SD for eight rats in each dietary group. *Significant difference from the *cis* fat group at $p < 0.05$.

and WKY rats (5.3 ± 0.5 nmole MDA/mL) on the *trans* fat diet were significantly decreased ($p < 0.05$) when compared to animals that received the *cis* fat diet (2.8 ± 0.2 nmole MDA/mL in SHRSP rats, and 4.0 ± 0.5 nmole MDA/mL in WKY rats) for 30 days.

Fatty acid composition of the tissues. In platelets, *trans* 18:1 fatty acid was found in total lipids in both SHRSP and WKY rats. Although there was no significant change of the level of 20:4n-6 in total lipids of platelets from rats fed the experimental diets, the ratio of 20:4/18:2 decreased with dietary *trans* fatty acids. In addition, linoleic acid (18:2) levels in platelets of animals fed the *trans* fat were higher than in platelets of animals fed the *cis* fat, as shown in Table 5. On the other hand, the changes of fatty acid composition in kidney appeared to be similar to those of platelets in animals fed the experimental diets (Table 6).

LCAT activity. Significant ($p < 0.05$) increases in plasma LCAT activity were found in rats fed the *trans* fat for 30 days. The LCAT activities in plasma of animals of the *cis* fat group and the *trans* fat group were 5.8 ± 2.1 and 10.9 ± 1.5% cholesterol esterified/hr in SHRSP rats and 7.8 ± 0.9 and 10.3 ± 1.2% cholesterol esterified/hr in WKY rats.

DISCUSSION

Various studies have indicated a relationship between dietary intake, particularly of dietary fat, and cardiovascular disease in different populations (29). It has been shown that the degree of atherosclerosis can depend on the type of dietary fat and that it closely correlates with increased blood lipid concentrations (15,30-32).

Hydrogenation of vegetable oil causes positional and geometrical isomerization of the natural *cis* double bonds in fatty acids. These isomeric fats, which can constitute 5-8% of the fatty acids in human diets, are readily absorbed and deposited in tissues. Deposition of *trans* fatty acids in tissues appears to be selective. *trans* Octadecenoate is primarily incorporated into phospholipids and triacylglycerols of plasma, liver, heart, adipose, kidney, and red blood cells (33-35). Adipose tissue and liver generally contain higher levels than do other tissues (8). Minimum deposition of *trans* fatty acids occurs in the brain (36). Higher levels of 16:1 and 18:2 in platelets and

TABLE 5

Changes in Fatty Acid Composition of Platelet Total Lipids in SHRSP and WKY Rats Fed Experimental Diets[a]

Type of fatty acids[b]	SHRSP		WKY	
	cis-FA (%)	*trans*-FA (%)	*cis*-FA (%)	*trans*-FA (%)
14:0	4.3 ± 2.4	2.8 ± 1.2	0.7 ± 0.0	0.7 ± 0.1
16:0	31.1 ± 7.2	27.9 ± 4.2	33.0 ± 0.5	35.0 ± 1.4
16:1(*t*)	tr	0.8 ± 0.3	tr	0.8 ± 0.2
16:1(*c*)	2.0 ± 0.4[c]	3.6 ± 1.2[c]	1.7 ± 0.2	1.3 ± 0.4
18:0	14.1 ± 0.5	11.4 ± 1.3	14.6 ± 0.8	11.8 ± 0.7
18:1(*t*)	tr	5.0 ± 1.3	tr	4.0 ± 0.2
18:1(*c*)	17.0 ± 3.3	15.8 ± 1.4	18.9 ± 0.7	15.1 ± 0.9
18:2(*ct*+*c*)	tr	0.3 ± 0.1	tr	0.1 ± 0.0
18:2(*cc*)	4.2 ± 2.1[d]	6.2 ± 2.8[d]	3.9 ± 0.3[e]	4.7 ± 0.2[e]
20:4	27.0 ±6.4	25.2 ± 6.9	27.2 ± 1.4	26.1 ± 1.3
20:4/18:2 ratio	7.1 ± 1.5	5.9 ± 2.2	7.1 ± 0.9	5.6 ± 0.4

[a]Results are expressed as mean ± SD for eight rats in each group. Fatty acid compositions were determined by GLC using DEGS or silica capillary columns as described in Materials and Methods.

[b]C: *cis*-isomers; T: *trans*-isomers.

[c,d,e]Values in the same column of each experiment with the same superscript letter are significantly different ($p < 0.05$).

TABLE 6

Changes in Fatty Acid Composition of Kidney Total Lipids in SHRSP and WKY Rats Fed Experimental Diets[a]

Type of fatty acid[b]	SHRSP		WKY	
	cis-FA (%)	*trans*-FA (%)	*cis*-FA (%)	*trans*-FA (%)
14:0	0.7 ± 0.1	0.8 ± 0.1	0.5 ± 0.1	0.7 ± 0.1
16:0	21.0 ± 0.8	20.7 ± 0.9	20.6 ± 0.9	21.2 ± 1.3
16:1(*t*)	tr	1.2 ± 0.2	tr	0.5 ± 0.2
16:1(*c*)	3.3 ± 0.5	2.8 ± 0.4	3.3 ± 0.6	3.4 ± 0.5
17:0	0.2 ± 0.1	0.1 ± 0.1	0.1 ± 0.0	0.1 ± 0.0
17:1	0.4 ± 0.0	0.5 ± 0.2	0.1 ± 0.1	0.0 ± 0.0
18:0	13.1 ± 1.5	11.6 ± 0.6	14.3 ± 1.1	12.4 ± 1.3
18:1(*t*)	tr	5.4 ± 1.9	tr	4.0 ± 0.3
18:1(*c*)	34.3 ± 3.4	26.5 ± 1.4	25.4 ± 3.0	23.0 ± 2.6
18:2(*ct*+*tc*)	tr	0.1 ± 0.0	tr	0.3 ± 0.1
18:2(*cc*)	6.8 ± 0.2[c]	8.6 ± 1.2[c]	8.1 ± 1.0	9.5 ± 1.1
20:3	0.5 ± 0.2	0.4 ± 0.2	0.9 ± 0.1	0.7 ± 0.1
20:4	20.0 ± 1.7	19.2 ± 1.6	25.2 ± 2.9	23.1 ± 2.3
20:4/20:3 ratio	34.1 ± 6.0	48.8 ± 12.7	27.3 ± 2.5	32.9 ± 4.2
20:4/18:2 ratio	2.8 ± 0.1	2.3 ± 0.3	3.3 ± 0.2	2.5 ± 0.3

[a]Results are expressed as mean ± SD for eight rats per each group. Fatty acid compositions were determined by GLC using DEGS or silica capillary columns as described in Materials and Methods.

[b]C: *cis*-isomers; T: *trans*-isomers.

[c]Values in the same column of each experiment with the same superscript letter are significantly different ($p < 0.05$).

kidney of rats fed *trans* fat were observed when compared with rats fed *cis* fat diet. This could indicate that elaidic acid (*t*-18:1n-9) may inhibit the conversion of 18:2n-6 to 20:4n-6 by acting as a competitive inhibitor of desaturases (*e.g.*, Δ6 desaturase) (37). Other studies have shown that *trans* fatty acids have no adverse effects if rats are also fed adequate amounts of essential fatty acids (38,39); this is similar to what we found in the present study.

Many investigators have focused on the effect of *trans* fatty acids on eicosanoid metabolism, because there is evidence that dietary *trans* fatty acids may regulate prostaglandin (PG) synthesis by affecting the enzymes of PG metabolism (37). Zevenbergen and Haddeman (11) found that high amounts of *trans* fatty acids in hydrogenated vegetable oil do not interfere with the cyclooxygenase or lipooxygenase pathways. Our results, however, indicate

that *trans* fatty acids may possibly influence eicosanoid metabolism by affecting tissues phospholipase A_2 activities in both SHRSP and WKY rats.

Douglas *et al.* (40) reported that vitamin E inhibited phospholipase A_2 *in vivo* and *in vitro* in a dose-dependent manner. It is also well known that higher TBA values typically correlate with lower vitamin E concentrations in tissues. Results of the present study indicate that *trans* fatty acids may reduce the consumption of vitamin E in plasma, which may result in lower phospholipase A_2 activity and in a reduction in platelet aggregability.

On the other hand, studies on the relationship between cholesterol levels and platelet aggregation by Shattil *et al.* (41) showed that cholesterol acquired by platelets may exert its effect by modifying the platelet membrane. Wörner and Patscheke (42) reported that increases in the cholesterol/phospholipid ratios of platelets resulted in increased aggregation and liberation of arachidonate. In addition, more recent studies demonstrated that in cholesterol-enriched platelets (pre-labelled with [^{14}C]arachidonic acid), thrombin stimulation led to the release of more arachidonic acid from pre-labelled platelet phospholipids (43). It is possible that cholesterol induces an increase in platelet membrane microviscosity, thereby affecting the activity of phospholipase A_2, which can be responsible for the release of arachidonic acid. Therefore, lower platelet aggregability is possibly related to lower cholesterol-to-phospholipid ratios in blood.

Furthermore, studies on the acute effects of lipoproteins on platelet function showed that VLDL, as well as LDL, increased platelet aggregation (28). Brook *et al.* (44) found that the platelet [^{14}C]serotonin release and platelet aggregation paralleled changes in LDL levels. It is thus suggested that lower platelet aggregability in rats fed *trans* fatty acids might be due to the lower cholesterol-to-phospholipid ratios and the lower levels of LDL and VLDL cholesterol.

SHRSP rats fed *trans* fatty acids having a decreased systolic blood pressure might show a lower risk of thrombosis, since the incidence of stroke in SHRSP rats is dramatically diminished if the blood pressure in controlled (45). The hypotensive effect of *trans* fatty acids in SHRSP rats may be due to the high ratio of PGE_2-to-$PGF_{2\alpha}$ in kidney.

The two principal prostaglandins, PGE_2 and $PGF_{2\alpha}$, have opposing effects—PGE_2 is a vasodilator which increases blood flow and participates in the regulation of water and salt excretion; $PGF_{2\alpha}$ is a vasoconstrictor. Although lower phospholipase A_2 (PLA_2) activity in rats fed *trans* fatty acids could decrease the synthesis of PGE_2 and $PGF_{2\alpha}$, SHRSP rats require adequate amounts of PGE_2 to facilitate excretion of excess sodium, since SHR rats have a high preference for NaCl (46). Results of the present study showed that slightly decreased PGE_2 levels and significantly decreased $PGF_{2\alpha}$ levels caused the higher ratios of PGE_2-to-$PGF_{2\alpha}$ that occurred in SHRSP rats fed the *trans* fatty diet. Higher $PGE_2/PGF_{2\alpha}$ ratios in the kidney might thus play an important role in protecting against the development of hypertension and be beneficial in the prevention of thrombosis in SHRSP rats. We therefore suggest that dietary *trans* fatty acid may influence the type of prostaglandins produced in the kidney that exert the hypotensive effect in SHRSP rats.

LCAT is thought to prevent the accumulation in plasma of unesterified cholesterol derived from the surface of chylomicrons and VLDL. In addition, LCAT may play a role in the transport of unesterified cholesterol from peripheral tissues to the liver (47). Because some cholesteryl ester could arise from LCAT activity in that the cholesteryl esters of the HDL exchange with those of LDL and VLDL plus chylomicrons (48), the appearance of the ester in serum LDL and VLDL might serve as an indicator of the extent of the exchange reaction (49), as no cholesteryl eicosatrienoate appears to be produced in liver. It is thus suggested that the lower level of plasma VLDL plus chylomicron cholesterol in rats fed *trans* fat possibly resulted from the higher plasma LCAT activity.

Moore *et al.* (20) has reported that plasma LCAT activity generally decreased when *trans* fatty acids were fed to rats, and the decreased esterification of cholesterol might be related to a reduced amount of free cholesterol in serum. However, other investigators showed that there was no or an inverse correlation between LCAT activity and serum cholesterol levels (50,51). Takatori *et al.* (49) demonstrated that an initial increase in LCAT activity was observed in rats fed a concentration of elaidic and linoelaidic acids. In the present study, higher HDL cholesterol and lower total cholesterol levels were found in rats fed *trans* fat.

Several earlier studies had shown that *trans* fatty acids have a plasma cholesterol-elevating effect (52–54). Other investigators found that there was no significant increase of plasma cholesterol levels due to *trans* fatty acids (13,20). Although data are available on the effect of dietary *trans* fatty acids, they are often inconclusive because of poor experimental design, lack of appropriate controls, and because of poorly defined diets. Recent studies showed that *trans* monoenoic acids have either no effect or a lowering effect on plasma cholesterol levels (16,17). Several hypotheses have been advanced to explain why blood cholesterol levels are lowered by these fatty acids, including a possible reduction of cholesterol absorption together with a lower rate of fat absorption and modulation of intestinal cells to process cholesterol (16). According to the results of the present study, it is possible that the low level of the total plasma cholesterol may be explained on the basis of impaired secretion of cholesterol and cholesteryl ester of *trans* fatty acids from liver, together with an increase in plasma LCAT activity.

Thus, it appears that selectively hydrogenated vegetable oils, as they are used in margarine manufacture, and which contain positional and geometrical isomers of the unsaturated fatty acids, not only are digestible as determined by long-term studies (38), but also appear to offer a prophylactic approach to the prevention of thrombotic disorders as shown in SHRSP rats.

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Possible Essentiality of Docosahexaenoic Acid in Japanese Monkey Neonates: Occurrence in Colostrum and Low Biosynthetic Capacity in Neonate Brains

Ayako Kanazawa[a], Teruo Miyazawa[a], Haruko Hirono[b], Motoharu Hayashi[c] and Kenshiro Fujimoto[a,*]
[a]Department of Food Chemistry, Faculty of Agriculture, [b]College of Medical Sciences, Tohoku University, Sendai and [c]The Primate Research Institute, Kyoto University, Inuyama, Japan

The importance of mother's milk as a source of docosahexaenoic acid (DHA) in Japanese monkey neonates was investigated. The DHA content in monkey colostrum total lipids was 2.2%, similar to or slightly higher than in humans. A comparison of the biosynthetic capacity of brain microsomes from monkeys of different age (up to 10 years) showed that chain elongation/desaturation of linolenic acid and eicosapentaenoic acid in neonates was significantly less pronounced than in adults. In particular, the formation of DHA, which is the product of Δ4 desaturase, was negligible. These results suggest that milk is an important source of DHA in Japanese monkey neonates.
Lipids 26, 53–57 (1991).

Long-chain polyunsaturated fatty acids (PUFA), which are major fatty acid components at the 2-position of glycerophospholipids in membranes, are thought to be essential for maintaining fluidity and physiological activity. Docosahexaenoic acid (DHA, 22:6n-3) is particularly abundant in phosphatidylethanolamine of brain grey matter (1) and retina (2). In these tissues, most of the n-3 PUFA is DHA, not eicosapentaenoic acid or linolenic acid. DHA is known to be selectively incorporated into the developing brain (3,4). Holman *et al.* (5) reported a neurological disturbance in a 6-year-old girl who was maintained on parenteral nutrition that lacked n-3 PUFA. Neuringer *et al.* (6,7) found that dietary n-3 fatty acid deprivation caused subnormal visual acuity in rhesus monkeys. Feeding of n-3 PUFA-deficient oils to rats was shown to decrease DHA level in brain phospholipids (8–10) and be associated with inferior learning capacity (8,9), whereas supplementation with DHA partially restored learning ability (11). These findings suggest that DHA might be essential in maintaining nervous system function.

In animals, long-chain PUFA such as 20:4n-6, 22:4n-6 and 22:6n-3 are synthesized from dietary C_{18} precursors, 18:2n-6 and 18:3n-3, which are synthesized *de novo* in plants. Animal studies have suggested that the capacity for chain elongation and desaturation is species and age dependent. Indirect evidence indicated that newborn human infants cannot produce sufficient quantities of long-chain PUFA from C_{18} precursors and, hence, depend on maternal supply (12,13). This would explain why DHA is present in human milk, although its content is variable and partially dependent on dietary fat intake (14). However, even the milk from vegetarians contains a considerable amount of DHA (15). The milk from various animals (16), including rats (17–21), rabbits (22), guinea pigs (22) and pigs (23), contains only trace or negligible amounts of DHA, whereas fish-eating marine animals have high DHA levels (16). Human milk provides DHA for neonates, whose DHA biosynthetic activity may be low at the time the human brain develops (12,13).

In the present study, Japanese monkeys were used as experimental models to investigate the effects of age on the biosynthetic capacity of long-chain PUFA in brain. First, the fatty acid composition of colostrum lipids was analyzed for comparison with that of humans. Then, the ability of brain microsomes to produce long-chain PUFA from their precursors was determined *in vitro* for animals of different age.

To test whether there is any relationship between the local PUFA content and the local synthetic activity of PUFA in cerebral cortex, three major regions of cerebral cortex were compared in respect to both long-chain PUFA content and synthetic capacity to produce long-chain PUFA.

MATERIALS AND METHODS

Materials. [1-^{14}C]Linolenic acid (2.07 GBq/mmole) and [1-^{14}C]eicosapentaenoic acid (2.17 GBq/mmole) were purchased from Amersham Japan (Tokyo, Japan). Radioactive purities were checked by high performance liquid chromatography (HPLC) and liquid scintillation counting as described below. Non-radioactive linolenic and eicosapentaenoic acids were obtained from Sigma Chemical Co. (St. Louis, MO). Octadecatetraenoic (n-3), eicosatetraenoic (n-3) and docosapentaenoic (n-3) acids used as standards were provided by Nippon Suisan Kaisha (Tokyo, Japan). Arachidonic and octadecatetraenoic acids (n-3) were provided by Idemitsu Petrochemical Co. (Tokyo, Japan). Coenzymes were purchased from Oriental Yeast Co. (Tokyo, Japan).

Animals. Cerebral cortices of Japanese monkeys [*Macaca fuscata fuscata;* two newborns, (a male and a female), a male 2 years of age, a male 6.5 years of age, and a male 10 years of age] were obtained from the Primate Research Institute, Kyoto University, Inuyama, Japan. Animals were fasted for 24 hr prior to killing. Tissues were frozen and kept at −80°C until use. The frontal lobe from a 6.5-year-old animal and the occipital lobes from a 2-, a 6.5- and a 10-year-old animal were used for the preparation of microsomes. The specific origin of the samples from the two neonatal brains was unknown.

The colostrum was obtained from a 10-year-old female animal four days after delivery. All animals had been fed a commercial monkey diet, Oriental APF (Oriental Yeast Co.).

*To whom correspondence should be addressed at Department of Food Chemistry, Faculty of Agriculture, Tohoku University, 1-1 Tsutsumidori-Amamiyamachi, Aoba-ku, Sendai 981, Japan.

Abbreviations: DHA, docosahexaenoic acid; GLC, gas-liquid chromatography; GSH, glutathione (reduced form); HPLC, high performance liquid chromatography; PUFA, polyunsaturated fatty acids.

Isolation of microsomes. The cerebral cortex was homogenized using a Potter-Elvehem homogenizer in 5 vol of a solution containing 0.25 M sucrose, 0.15 M KCl, 5 mM $MgCl_2$, 1.5 mM glutathione (reduced form) (GSH) and 50 mM K_2HPO_6 at pH 7.0. The homogenate was centrifuged at 800 × *g* for 10 min to remove cell debris, and then the supernatant was centrifuged at 10,000 × *g* for 20 min. The supernatant at 10,000 × *g* was further centrifuged at 100,000 × *g* for 1 hr to obtain the microsomal fraction. The microsomal pellet was suspended in a buffer containing 0.15 M KCl, 5 mM $MgCl_2$, 0.15 mM GSH and 50 mM K_2HPO_4 at pH 7.0. The entire isolation procedure was carried out at 0–4°C. The protein content of the microsomal solution was measured using the method of Lowry *et al.* (24).

Assay of PUFA chain elongation-desaturation. The assay conditions were basically those of the procedure described by Purvis *et al.* (25). Typically, reaction mixtures contained 100 mM fatty acids, 150 mM $MgCl_2$, 1.5 mM GSH, 5 mM ATP, 1.25 mM NADH, 1.25 mM NADPH, 0.3 mM CoA, 0.3 mM malonyl CoA and 50 mM K_2HPO_6 at pH 7.0 in a final volume of 0.5 mL. The fatty acids were a mixture of labeled and unlabeled 18:3 or 20:5 and were used in experiments after conversion to ammonium salt and suspension in 1% aqueous solution of Triton WR1339 containing fatty acid-free bovine serum albumin (Sigma Chemical Co., St. Louis, MO) at a molar ratio of 4:1. The radioactivities in reaction mixtures were in the range of 5.0 to 8.3 × 10^3 Bq. Incubation was initiated by adding 0.5 to 1.0 mg of microsomal protein to the reaction mixture and carried out at 37°C for 20 min. Assays were terminated by addition of 0.5 mL of 20% KOH containing 1 mg BHT. The mixtures were saponified at 80°C for 30 min, acidified with 0.5 mL of 5 M HCl, and the fatty acids were extracted with chloroform/methanol (2:1, v/v). After evaporation to dryness, the mixture was methylated with 13% (w/v) BF_3/methanol at 80°C for 5 min (26). Reaction mixtures without microsomes were used for control experiments. Recovery of radioactivity in the control experiment was 72.6%.

Separation of fatty acids and liquid scintillation counting. Fatty acid methyl esters were separated by HPLC essentially as described by Hirata *et al.* (27) using a Hitachi chromatograph model 635A (Hitachi, Tokyo) fitted with two Lichrosorb RP-18 columns (5 μm particle size, 4 × 250 mm connected with 7 μm particle size, 4 × 250 mm; Kanto Chemical Co., Tokyo) in series. The methyl esters were eluted stepwise with aqueous methanol (14%, 10% and 6%) at a flow rate of 0.6 mL/min. Fractions were detected with a UV monitor set at 210 nm and identified by comparison of their retention times with those of standards.

The radioactivities of the methyl esters were determined by counting fractions collected at 1-min intervals in scintillation vials containing 6 mL of NT scintillation fluid (28) using an Aloka liquid scintillation system, LSC-903 (Aloka, Tokyo).

Lipid analysis. Colostrum lipids were extracted by the Bligh-Dyer method (29). The lipid classes were separated by silicic acid and Florisil column chromatography (30). The lipids of the diet and tissues were extracted by the Folch method (31). Tissue lipids were fractionated into lipid classes on a Sep-Pak Cartridge Silica (Waters Associates, Milford, MA) (32). Lipids were transmethylated with sodium methylate. Microsomal lipids were saponified, extracted with chloroform/methanol (2:1, v/v), and then methylated with 13% BF_3/methanol as described above. Fatty acid methyl esters were analyzed by gas-liquid chromatography (GLC) on a JEOL JGC-20K apparatus (JEOL, Tokyo) equipped with a glass column (2.0 mm × 2 m) containing 10% Silar 10C on Chromosorb W (60/80 mesh). Column temperature was programmed from 170–220°C at 1°C/min. Duplicate determinations were carried out. Fatty acid methyl esters were identified by comparison of their retention times with those of standards.

RESULTS

Fatty acid compositions of diet and colostrum. The diet for monkeys contained 10.3% total lipids (w/w), of which the major fatty acids were as follows: 37.3% linoleic acid, 36.4% oleic acid, 13.8% palmitic acid, 2.7% linolenic acid and 2.6% stearic acid. Percentages of n-6 and n-3 PUFA in the total lipids were 41.7% and 3.6%, respectively.

Fatty acid compositions of the colostrum from *Macaca fuscata fuscata*, together with the cited data for human samples, are presented in Figure 1. The fatty acid profile in the monkey is similar to that in humans, although the linoleate content is a little higher in the monkey while palmitate is lower than in human colostrum. The total n-3 fatty acid levels in the monkey were similar to those in humans (33). The n-6/n-3 ratio in monkey was 5.9 and slightly higher than in humans. The DHA content in monkey was 2.2% and essentially identical to that in humans (33,34).

Synthesis of chain elongation-desaturation products by brain microsomes. Synthesis of chain elongation-desaturation products from linolenate was substantially influenced by age in the monkeys tested (Fig. 2). Judging from the total amount of metabolites produced, the activity in newborns was lower than in older monkeys. The synthesis of 22:6 was highly variable, *i.e.*, the greatest activity was found in a 6.5-year-old, while the activity in newborns was negligible. Similar results were obtained

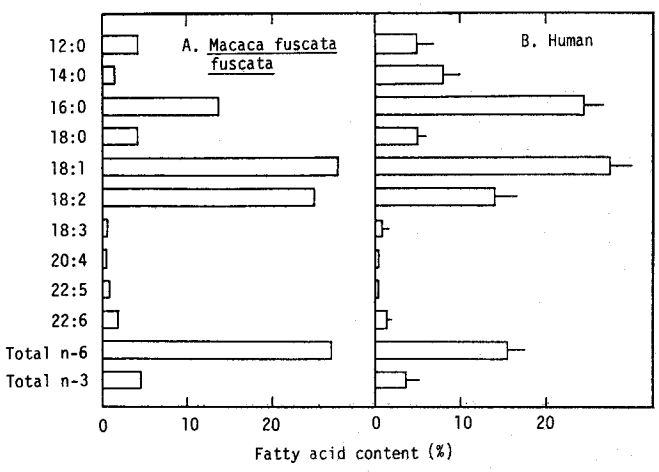


FIG. 1. Fatty acid composition of breast milk. Comparison between colostrum of *Macaca fuscata fuscata* and human (29). The monkey colostrum was collected from a 10-year-old female four days after delivery.

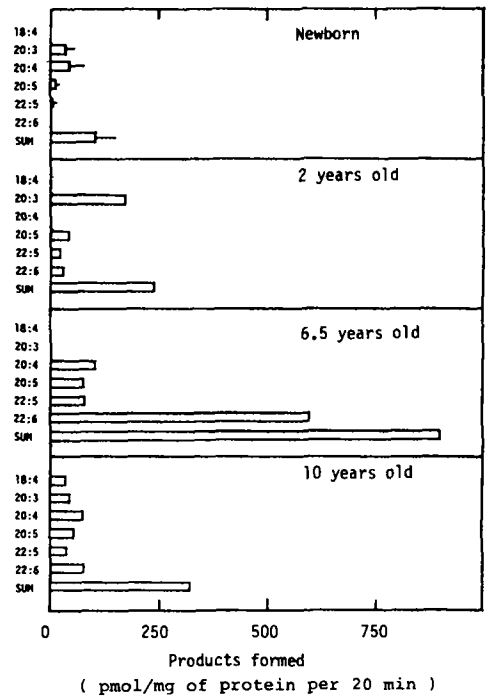


FIG. 2. Metabolites from 18:3n-3 by brain microsomes from *Macaca fuscata fuscata* of different ages. Sizes of brains are given in parentheses (longitudinal axis × bilateral axis, cm). A female and a male newborn (6.2 × 5.1), a 2-year-old male (6.8 × 5.5), a 6.5-year-old male (7.5 × 6.1) and a 10-year-old male (7.0 × 6.1) were tested.

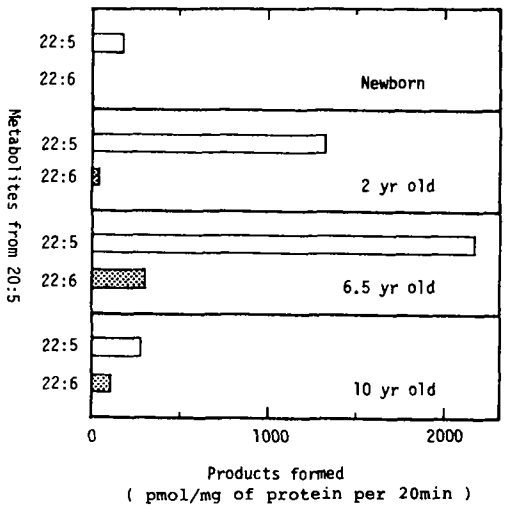


FIG. 3. Metabolites from 20:5n-3 by brain microsomes from *Macaca fuscata fuscata* of different ages. A female newborn, a 2-year-old male, a 6.5-year-old male, and a 10-year-old male were tested.

for the metabolism of 20:5 which was elongated and desaturated most extensively in a 6.5-year-old monkey, while the formation of 22:6 in the newborn again was negligible (Fig. 3).

Regional fatty acid composition and synthesis of long-chain PUFA in cerebral cortex. The DHA content of total lipids varied considerably between cerebral regions. DHA was found in decreasing abundance in occipital, in temporal and in frontal lobes (Table 1). The reverse was observed for n-6 PUFA, such as 20:4 and 22:4. A similar pattern of PUFA distribution was found in microsomal lipids (Table 2) with DHA being most abundant in the occipital lobe. On the other hand, brain microsomes prepared from frontal and occipital lobes showed almost the same activity for producing DHA from linolenic acid (Fig. 4). Thus, no direct relationship was apparent between long-chain PUFA synthetic activity and PUFA content of the cerebral cortex.

TABLE 1

Regional Variation in Fatty Acid Composition of Grey Matter Lipids of a 10-Year-Old Male *Macaca fuscata fuscata*

	Frontal	Temporal	Occipital
16:0	19.4%	20.7%	21.9%
18:0	22.8	21.7	20.3
18:1n-9	19.9	21.6	19.1
18:2n-6	2.7	2.8	3.7
18:3n-3	tr	tr	tr
20:4n-6	6.8	5.8	4.1
20:5n-3	tr	tr	tr
22:4n-6	6.7	5.6	3.5
22:5n-3	1.2	1.0	1.1
22:6n-3	11.0	13.9	16.6

TABLE 2

Regional Variation in Fatty Acid Composition of Brain Microsomal Lipids of a 10-Year-Old Male *Macaca fuscata fuscata*

	Frontal	Temporal	Occipital
16:0	21.4%	15.8%	17.3%
18:0	22.0	18.6	19.2
18:1n-9	18.3	15.6	16.7
18:2n-6	1.0	1.5	0.8
18:3n-3	tr	tr	tr
20:4n-6	8.5	10.3	7.2
20:5n-3	tr	tr	tr
22:4n-6	1.2	1.0	1.1
22:6n-3	12.1	14.6	15.8

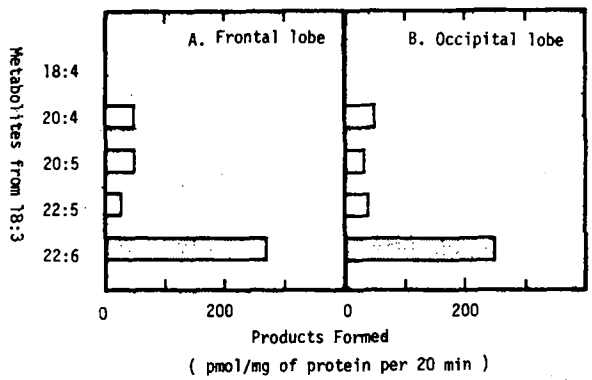


FIG. 4. Comparison of capacity for chain elongation/desaturation between frontal and occipital lobes from *Macaca fuscata fuscata*. A 6.5-year-old male was tested.

DISCUSSION

Although the fatty acid composition of milk of a wide variety of animals has been reported (16–23), the occurrence of DHA in milk was only noted in marine animals which live on a fish diet rich in DHA (16). This does not necessarily rule out the occurrence of trace amounts of DHA in animal milk, because in many of the studies no special attention appears to have been paid to the possible occurrence of small amounts of DHA. In human milk, DHA is always present, although its content varies with dietary habits. It also must be emphasized here that the DHA content in colostrum is highest and that it decreases somewhat in mature milk (33,35,36). This has been used as an argument for the maternal origin of long-chain PUFA needed by human newborns. Because the composition of lipids and fatty acids in the Japanese monkey's colostrum is similar to that in humans, it appears that *Macaca fuscata fuscata* would be a superior model to elucidate PUFA metabolism in neonates relevant to humans.

In the present study, the biosynthetic activity of n-3 long-chain PUFA in monkey brain was assessed using microsomes *in vitro*. Kinetic studies on the metabolic conversion of PUFA have shown that the rate of activation of a fatty acid to its CoA thioester in rat liver microsomes is greater than either elongation or desaturation of the acyl CoA ester (37–39). The incorporation of the metabolic products into lipids is also not rate limiting—the rate of acylation has been found to be of the same order or greater than the rate of activation in rat liver (37). Therefore, the ease of conversion of short-chain PUFA is commonly examined. Our data suggest that, in monkey brain, desaturation steps and not chain-elongation steps are rate-limiting in converting 18:3n-3 to DHA, because among the intermediates of this reaction (Fig. 2), the substrates for the desaturation (20:3, 20:4 and 22:5) were more abundant than the substrates for the chain-elongation (18:4 and 20:5). The ratios of the two were 6.6 for newborns, 4.6 for 2-year-olds, 2.5 for 6.5-year-olds and 2.3 for ten-year-olds.

In the case of rats, Strouvé-Vallet and Pascaud (40) reported that the brain microsomal activity for elongation and desaturation of linoleic acid was high in the first four days after birth, but decreased rapidly thereafter. Studies by Cook (41,42) also confirmed that the highest Δ6 desaturase activity is associated with neonatal rat brain. In liver, desaturase activity is stable up to 30 days (40) or increases slightly after weaning (41,42).

In the case of perinatal piglets, the brain enzyme activity was also greatest in the neonatal period, while the liver enzyme activity was higher in the postnatal period. Δ5 Desaturase activity, in particular, was very low during the neonatal period (25,43).

The importance of the liver in supplying DHA for the nervous system has recently been reported in rat (44). In our study, however, we could not assess the metabolizing activity of liver, because monkeys had been fasted for 24 hr. Under this condition, Δ6 desaturase activity is known to be very low (45,46). In contrast, the activity in brain is quite independent of diet (data not shown).

In the present study, the capacity of the brain for elongation/desaturation was found to be lower in newborn monkeys than in older animals. In particular, Δ4 desaturation and formation of DHA in newborns was negligible (Figs. 3 and 4). The lower chain elongation/desaturation activity in monkey neonates observed in the present study is quite different from the results previously reported for rats (see above). The cause for this is unknown, but differences in the developmental stage of brain neural cells at parturition may be responsible for that, as primates are born at the end of the cell-stretching stage, while rat neonates are at the end of the cell-multiplication stage (47). Therefore, from the point of brain neural cell development, monkey neonates correspond to the stage of rats 10 days after birth, when Δ6 desaturase activity is decreased to very low levels (40). It appears reasonable to assume that the occurrence of DHA in primate colostrum compensates for the low biosynthetic activity in the neonate brain.

Besides the low desaturase activity, another factor responsible for the low capacity of long-chain PUFA biosynthesis may be the competitive compartmentalization of linolenate and its intermediate metabolites between acylation to form glycerophospholipids and desaturation. In Macaque monkey, all neurons have completed cell division during the 120 days of gestation, and after birth only the multiplication of glia can be observed (48). Furthermore, the size of the brain and the number of synapses reach almost steady levels after three years (49). The brain of a 6.5-year-old monkey used in the present study is thought to be mature. In neonatal monkeys, a marked increase of glia cells would occur. Therefore, the accelerated incorporation of PUFA into microsomal membranes of glia cells might result in a low rate of desaturation. However, the lipid analyses of astrocytes and oligodendrocytes in rats showed a very low content of n-3 PUFA other than DHA, similar to neurons (50). This observation again supports the idea of a specific affinity of DHA to neural tissues. Judging from these results, the compartmentalization of 18:3, or intermediate n-3 PUFA, in membrane phospholipids may not be the principal cause for the poor desaturation activity in monkey neonates.

Lowered capacity for long-chain PUFA synthesis in rhesus monkey neonates was also suggested by the observation that the plasma DHA level drastically decreased after birth (6,51). It has been suggested that Δ4-desaturase in humans (52–54) and monkeys (55) is much less predominant than Δ5 and Δ6 desaturase activities, because administration of linolenic acid or 20:5 resulted in accumulation of 22:5n-3 rather than DHA. Although the number of monkeys used in the present studies was quite limited, the data suggest the possibility that Δ4 desaturase activity in primate newborns is too low to produce sufficient DHA for brain development and that a large portion of the long-chain polyunsaturated fatty acids required may depend on maternal supply and/or the metabolites of the liver as source.

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Vitamin E and the Susceptibility of Erythrocytes and Reconstituted Liposomes to Oxidative Stress in Aged Diabetics

Shiro Urano*[a], Midori Hoshi-Hashizume[a], Noriko Tochigi[a], Mitsuyoshi Matsuo[a], Masataka Shiraki[b] and Hideki Ito[b]

[a]Tokyo Metropolitan Institute of Gerontology and [b]Tokyo Metropolitan Geriatric Hospital, Itabashi-ku, Tokyo 173, Japan

A remarkable increase in the permeability of erythrocyte ghosts and liposomal membranes composed of erythrocyte lipids from aged diabetics was revealed by measuring [^{14}C]glucose leakage. There were no significant differences in the contents of free cholesterol or phospholipids, or in the cholesterol/phospholipid ratio between diabetic and normal erythrocyte membranes, but significantly higher amounts of unsaturated fatty acids, arachidonic acid and docosahexaenoic acid were observed in the erythrocyte membranes of diabetics. Reconstituted liposomes prepared from aged diabetic erythrocyte lipids were highly susceptible to superoxide-induced oxidative stress. Vitamin E was highly effective in suppressing the peroxidative lysis of liposomes composed of diabetic erythrocyte lipids. The effect of superoxide dismutase (SOD) on the inhibition of peroxidation of unsaturated lipids within liposomal membranes was less than that of vitamin E.

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It is well recognized that one of the serious problems facing aged diabetic patients is the high prevalence of thrombotic disorders (1). However, the pathogenesis of these disorders is not well understood. One possible factor contributing to this morbid state is thought to be due to hemorheologic changes in diabetics, since abnormalities in the physical properties of erythrocytes from diabetic patients have been reported. These abnormalities include increased whole-blood and plasma viscosity, reduced erythrocyte deformability, and an increased adhesion of erythrocytes to endothelial cells (2-6).

In general, membrane phospholipids appear to play an important role in such physicochemical properties of erythrocyte membranes (7). In fact, there is substantial evidence to suggest that dietary supplementation with polyunsaturated fatty acids induces changes in membrane-associated cell functions and physical properties such as fluidity (8-11). However, the direct relationship between the physicochemical properties and membrane fatty acid composition of erythrocytes from diabetic patients has not yet been examined.

Polyunsaturated lipids, which influence membrane properties, are abundant in biological membranes, and their peroxidation is thought to constitute a serious threat to membrane integrity. Once peroxides are produced, the physicochemical properties of membranes are believed to be affected, and hence, membrane function could be altered (12). In order to clarify the question whether the abnormalities in erythrocytes from aged diabetics arise from differences in their polyunsaturated lipid content, and whether vitamin E protects erythrocyte membranes against peroxidation, the permeability of erythrocyte ghosts and reconstituted liposomes prepared from erythrocyte membrane lipids was investigated. Also the susceptibility of erythrocyte lipids from aged diabetics to superoxide and the inhibitory effect of vitamin E on lipid peroxidation in erythrocytes from aged diabetics were examined.

*To whom correspondence should be addressed at the Tokyo Metropolitan Institute of Gerontology, 35-2 Sakae-cho, Itabashi-ku, Tokyo 173, Japan. (TMIG-I No. 158.)

Abbreviations: MDA, malondialdehyde; PBS, phosphate buffered saline; SOD, superoxide dismutase; TBARS, thiobarbituric acid reactive substance.

MATERIALS AND METHODS

Materials. Erythrocytes were obtained from 70- to 80-year-old diabetic patients and normal age-matched subjects. Patients with diabetes were receiving insulin therapy. All subjects took an almost identical restricted diet (amount of dietary lipids, 40 g/day). The studies were performed after informed consent was obtained from all subjects. Fresh, heparinized blood was centrifuged at 1,500 × *g* for 10 min, and the erythrocytes were washed three times with phosphate buffered saline (PBS, pH 7.4). Erythrocyte ghosts were obtained by the method of Steck (13). Xanthine, xanthine oxidase, superoxide dismutase (SOD), and cytochrome c were purchased from Sigma Chemical Co. (St. Louis, MO). D-[U-^{14}C]glucose was obtained from Amersham International PLC (Buckinghamshire, UK); α-tocopherol (vitamin E) was from E. Merck (Darmstadt, FRG). All other chemicals were of the highest grade available.

Preparation of liposomes. The erythrocyte ghosts were extracted with a mixture of chloroform and methanol (2:1, v/v) following a previously reported procedure (14). Briefly, the extracted lipids (3.12 mg), dicetyl phosphate (0.24 mg) and α-tocopherol (if necessary, 0.3 molar ratio to lecithin) were dissolved in 1 mL of a mixture of chloroform and methanol (2:1, v/v). After rotary evaporation under N_2, 0.2 mL of [^{14}C]glucose solution in PBS (pH 7.4) was added to the dried lipids (under N_2). The mixture was shaken vigorously on a vortex mixer to obtain a suspension. The suspension was allowed to swell for 2 hr at room temperature and was then applied to a 20-cm column of Sephadex G-200. The column was eluted with PBS to yield a liposome suspension.

Measurement of the permeability of erythrocyte ghosts and liposomes to [^{14}C]glucose. Erythrocyte ghosts (1 mL of suspension) were mixed with [^{14}C]glucose and 0.1 mM $MgSO_4$ in 0.5 mM sodium phosphate and incubated at 4°C for 15 hr. The radioactive marker was sealed inside by the shear stress through a No. 27 gauge hypodermic needle as previously reported (15). The vesicles were then washed twice, suspended in cold 50 mM sodium phosphate (pH 7.8), and incubated at 25°C. The vesicle suspensions were centrifuged at 15,000 × *g* for 20 min at various intervals up to 7 hr. The radioactivity of each supernatant was measured using a scintillation counter.

Triplicate 0.2-ml aliquots of liposome suspension were placed into dialysis bags (0.8 cm diameter) containing appropriate reagents (xanthine and xanthine oxidase, and

if necessary, SOD) in 1 mL of PBS and were dialyzed at 22°C against 4 mL of PBS with constant mixing by rotation. At various intervals up to 24 hr, the dialysis bags were removed and placed in 4 mL of fresh PBS at each time point. [^{14}C]Glucose leakage was determined by measuring the radioactivity of the dialysate using a scintillation counter. Results were expressed as percent of total glucose released from erythrocyte ghosts or liposomes in suspensions to which the detergent, Triton X-100 (1% v/v), had been added.

Superoxide generating system. The complete system consisted of xanthine (2.5 μmol) and xanthine oxidase (0.2 U). The generation of superoxide (O_2^-) by this system was measured as previously reported (16). The inhibitory effect of α-tocopherol (0.3 molar ratio relative to lecithin) or SOD (30 μg) on the peroxidation of membranes was assessed by the inhibition of the O_2^--induced increase in membrane permeability to [^{14}C]glucose.

Analysis of erythrocyte membrane lipids. Erythrocyte membrane lipids were extracted with a mixture of chloroform and methanol (2:1, v/v) as described previously (13). Aliquots were applied to a silica gel plate and developed with chloroform/methanol/acetic acid/formic acid/water (37.5:12.5:6:3:1, by vol) as the first solvent, and hexane/isopropyl ether/acetic acid (3:95:2, v/v/v) as the second solvent. The fractions were analyzed using a density chromatoscanner (Shimadzu, CS-910). In order to analyze the fatty acid composition of the extracted lipids, portions of the lipids were mixed with butylated hydroxytoluene and heated at 100°C for 2 hr with 5% HCl solution in methanol. The reaction mixture was extracted with hexane and washed with water. The hexane solution was evaporated under nitrogen gas and lipids were taken up to 200 μL of hexane. The esterified fatty acids were analyzed by gas chromatography on an EGSS-X column. For analysis of vitamin E content in erythrocyte membranes, portions of the lipids extracted were re-extracted with ethyl acetate, and the ethyl acetate extract was injected on the guard column of the HPLC (Wakogel LC-5H, Wako Pure Chemical Co., Osaka, Japan). The experimental conditions of HPLC used for analysis were identical to those described earlier (17).

RESULTS AND DISCUSSIONS

Erythrocyte membranes from aged diabetics were significantly more permeable to [^{14}C]glucose than those from normal subjects as shown in Figure 1 ($p<0.05$). One of the contributing factors to changes in the permeability of membranes is thought to be due to variation in the proportion of unsaturated lipids. It seems that the erythrocyte membrane lipids of aged diabetics are less saturated than those of normal aged subjects. We therefore analyzed the phospholipid, cholesterol, vitamin E and fatty acid contents of various erythrocyte membranes. Table 1 shows a comparison of the content of vitamin E and the compositions of erythrocyte ghost lipids between aged diabetics and normal subjects. There were no significant differences in the content of vitamin E, free cholesterol or phospholipids, or in the cholesterol/phospholipid ratios between diabetic and normal erythrocyte membranes. On the other hand, as shown in Table 2, we found that diabetic erythrocyte membranes contain a significantly higher proportion of arachidonic acid (20:4, 16.5%) and

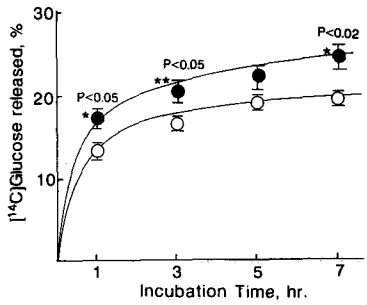


FIG. 1. Comparison of the permeability of erythrocytes to [^{14}C]-glucose. The PBS suspensions of diabetic (●) and normal (○) erythrocyte ghosts resealed with radioactive glucose were incubated at 25°C. Released glucose was measured by scintillation counting as described under Materials and Methods. Percent release of glucose at each time point is expressed in accumulated counts of radioactivity released.

TABLE 1

Phospholipid Composition, Free Cholesterol and Vitamin E Content of Erythrocyte[a]

	Normal (n=12)	Diabetic (n=12)
Phosphatidylethanolamine (%)	34.3 ± 0.9	33.7 ± 1.0
Phosphatidylserine (%)	14.5 ± 0.7	14.2 ± 1.0
Phosphatidylcholine + phosphatidylinositol (%)	21.9 ± 0.6	22.1 ± 0.4
Sphingomyelin (%)	29.3 ± 1.3	30.0 ± 0.4
Free cholesterol (μg/mg protein)	207.5 ± 13.8	214.6 ± 12.9
Cholesterol/phospholipid ratio	1.0 ± 0.1	1.2 ± 0.3
Vitamin E (μg/mL packed cell)	2.3 ± 0.1	2.1 ± 0.1
(μg/mL plasma)	9.0 ± 0.2	8.5 ± 0.3

[a]Values are means ± SE.

TABLE 2

Fatty Acid Composition of Erythrocyte Membranes[a]

Fatty acid (%)	Normal (n=14)	Diabetic (n=21)
16:0	31.3 ± 1.1	29.7 ± 1.1
18:0	19.3 ± 0.6	17.9 ± 0.8
18:1	16.4 ± 0.4	14.7 ± 0.3[b]
18:2	10.2 ± 0.4	10.0 ± 0.4
20:4	14.2 ± 0.6	16.5 ± 0.5[c]
22:6	9.3 ± 0.7	11.1 ± 0.3[d]

[a]Values are means ± SE.
[b]Less *vs* normal $P<0.01$.
[c]Greater *vs* normal $P<0.01$.
[d]Greater *vs* normal $P<0.05$.

docosahexaenoic acid (22:6, 11.1%) as compared to normal erythrocyte membranes. Considering high arachidonic acid contents in either erythrocyte membrane in young diabetic women or in platelets of aged diabetics (18,19), the results suggest that there exists a difference in the lipid content of erythrocyte membranes between aged diabetics and normal subjects that can largely be ascribed to differences in the levels of unsaturated fatty acids. In the present study, both groups had essentially

the same dietary lipid intake. It is therefore suggested that the abnormal lipid content of erythrocyte membranes in aged diabetics could be due to disease-induced changes rather than diet-induced ones, although this mechanism can presently not be verified.

In order to investigate whether the increase in the level of unsaturated fatty acids in erythrocyte membranes of aged diabetics induces the increased permeability of the membranes to [^{14}C]glucose, the permeability of reconstituted liposomes prepared from lipids extracted from diabetic erythrocyte ghosts was investigated.

Although the permeability of liposomes composed of diabetic erythrocyte lipids to [^{14}C]glucose was greater than that of liposomes composed of normal erythrocyte lipids, the difference was not statistically significant in the early stage of incubation (Fig. 2). After 18 hr, however, the rate of glucose leakage from liposomes composed of diabetic erythrocyte membrane lipids was significantly higher. As shown in Figure 2, the peroxidation of the diabetic and normal lipids (thiobarbituric acid reactive substances—TBARS value) was progressive after 18 hr. These results suggest that as the degree of unsaturation of the lipids in erythrocyte membranes becomes higher, the leakage of glucose increases, and that the large difference in glucose leakage between diabetic and normal erythrocytes arises from differences in their susceptibility to peroxidation. Previously it has been shown that the oxidation of xanthine by xanthine oxidase caused lysis of erythrocyte membranes and liposomes, and that their permeability to glucose or chromate ions was significantly increased (15,16). Consequently, in order to clarify the susceptibility of liposomes to oxidative stress, liposomes were exposed to superoxide (O_2^-) generated by the xanthine-xanthine oxidase system (16). Figure 3 shows the kinetics of liposome damage caused by exposure to O_2^-. In this experiment, the oxidation of lipids in both types of liposomes progressed with incubation time. Although glucose leakage from liposomes composed of either diabetic or normal erythrocyte lipids was enhanced by the addition of O_2^-, the leakage from the diabetic liposomes increased more markedly than that from normal liposomes. From these results, it would seem that diabetic erythrocyte membranes are more readily oxidizable probably due to their greater unsaturated fatty acid, especially arachidonic acid and docosahexaenoic acid, contents.

It is recognized that aerobic organs and cells are protected from oxygen toxicity by an array of defense systems, including preventive antioxidants, such as vitamin E, and antioxidative enzymes, such as superoxide dismutase (SOD) (20-22). In order to verify the inhibitory effects of vitamin E and SOD on the oxidative damage of diabetic erythrocyte membranes by O_2^-, we examined the protection of liposomes against oxidation by vitamin E inside the membranes or SOD outside the membranes. As shown in Figure 4B, the inhibitory effect of vitamin E on the oxidative damage of normal liposomes was similar to the effect of SOD. In contrast, Figure 4A shows that vitamin E was much more effective than SOD in suppressing the oxidative damage to diabetic liposomes. In this experiment, no significant inhibitory effect of vitamin E against lipid peroxidation was observed by the use of physiological vitamin E content in liposomes, probably because of the nonphysiologically high

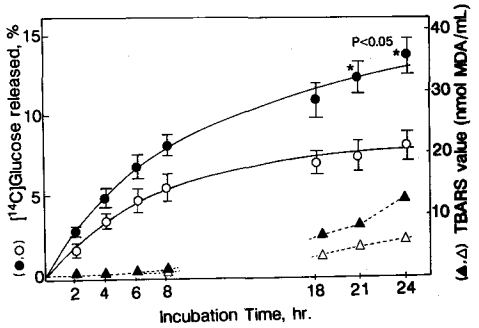


FIG. 2. Kinetics of [^{14}C]glucose leakage from reconstituted liposomes upon lipid peroxidation. Suspensions of liposomes, sealed with radioactive glucose, composed of lipids from diabetic erythrocyte membranes (●) or from normal erythrocyte membranes (○) were incubated at 22°C for 24 hr. At various intervals up to 24 hr, glucose leakage into the outside PBS solution of the dialysis bag was measured by scintillation counting. Lipid peroxides in diabetic (▲) and normal (△) liposomes were measured by the thiobarbituric acid method (27). Results are expressed as quantities of malondialdehyde (MDA) formed.

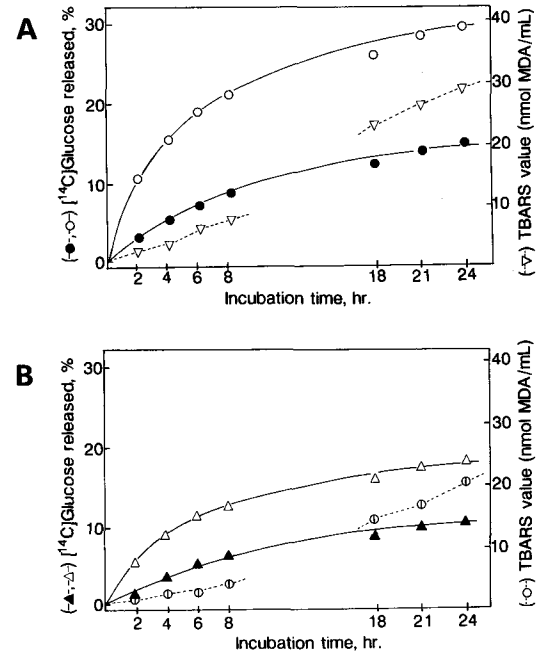


FIG. 3. Comparison of liposome damage caused by exposure to superoxide (O_2^-) and O_2^--induced lipid peroxidation. (A) ●, diabetic liposomes, no O_2^- or ○, diabetic liposomes with O_2^- exposure; (B) ▲, normal liposomes, no O_2^- or △, normal liposomes with O_2^- exposure was incubated at 25°C for 24 hr. The incubation system consisted of 2.5μM xanthine and 0.2 unit xanthine oxidase in 1 mL of PBS (pH 7.4). Vitamin E contents in diabetic and normal liposomes were 0.3 μg and 0.4 μg/mg lipids. TBARS values in diabetic (▽) and normal (⊖) liposomes upon O_2^- exposure were measured at each time point as previously described (27).

amount of O^-_2 used. Consequently, we also used a relatively high amount of vitamin E (0.3 molar ratio to lecithin). Although the physical properties of the membranes could be altered under these conditions, it is reasonable to assume that the membranes are stabilized by the addition of vitamin E (23,24), and that the inhibitory effect of vitamin E in both types of liposomes,

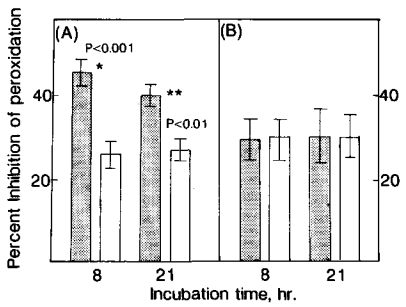


FIG. 4. Inhibitory effects of vitamin E and SOD on oxidative damage to liposomes. Liposomes prepared from diabetic (A) or normal (B) erythrocyte lipids were incubated with the xanthine-xanthine oxidase system in the presence of vitamin E (0.3 molar ratio to lipids, closed columns) or SOD (30 μg, open columns) at 22°C as described under Materials and Methods. Percent inhibition was expressed as the percentage decrease of glucose leakage from liposomes.

which include the same amount of vitamin E, can be compared under these conditions.

As it has been proven that O_2^- generated outside the membrane can freely diffuse across the membrane (25), it seems that O_2^- or other oxygen radicals derived from O_2^- (for example, $^{\bullet}O_2H$, H_2O_2 and $^{\bullet}OH$) induce oxidative chain reactions within membranes. In the diabetic erythrocyte membrane, this chain oxidation may propagate more readily, because diabetic erythrocyte lipids are more unsaturated than the lipids of normal erythrocytes. Vitamin E is known to act as a chain-breaking antioxidant rather than a deactivator of oxygen radical species in membranes. Consequently, the effect of vitamin E on oxidative damage to liposomes made from diabetic erythrocyte membrane lipids is greater than the effect on damage to normal erythrocyte lipid liposomes. On the other hand, SOD directly scavenges only O_2^- outside the membrane, while other active oxygen species derived from O_2^- may not be scavenged there and may diffuse across the membrane thus inducing oxidative chain reactions. Consequently, the inhibitory effect of SOD in diabetic liposomes would be less than that of vitamin E (Fig. 4).

In conclusion, erythrocyte membranes from aged diabetics are significantly more permeable to glucose than erythrocyte membranes from normal aged subjects. The content of unsaturated fatty acids is higher in diabetic erythrocyte membranes, making them more susceptible than normal erythrocytes to oxidative stress. Furthermore, vitamin E is highly effective in suppressing peroxidation within diabetic erythrocyte membranes in accordance with its excellent chain-breaking activity. Although it is impossible at present to verify the mechanism of the increased leakage of glucose from diabetic erythrocyte membranes, it appears reasonable to suggest that the abnormal lipid environment in these membranes and their high susceptibility to oxidative stress would affect glucose transporter activity and thus glucose flux from erythrocytes (26).

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Phospholipid Synthesis by Chick Retinal Microsomes: Fatty Acid Preference and Effect of Fatty Acid Binding Protein

Peggy A. Sellner[a,b,*] and **Ariana R. Phillips**[a]
Departments of [a]Anatomy and Cell Biology and [b]Ophthalmology, University of Kansas Medical Center, Kansas City, Kansas 66103

The acylation of 1-palmitoyl-*sn*-glycerophosphocholine (1-16:0-GPC) or 1-palmitoyl-*sn*-glycerophosphoethanolamine (1-16:0-GPE) was measured using the microsomal fraction prepared from retinas of 14–15-day-old chick embryos. Rates of incorporation of exogenously supplied fatty acids into diacyl-GPC were generally 5–7 times greater than into diacyl-GPE. Substrate preferences for incorporation into diacyl-GPC and diacyl-GPE were, respectively, 18:2 > 18:3 = 20:5 > 20:4 > 18:1 > 22:6 = 18:0 and 18:2 > 22:6 ≥ 18:3 = 18:0 ≥ 20:4 = 18:1 > 20:5. The apparent selectivities were not consistent with the reported fatty acid compositions of these lipid classes. The addition of partially purified fatty acid binding protein (FABP) to the reaction had no effect either on overall rates of incorporation or on the substrate preference. When fatty acyl-CoA substrates were used, rates of incorporation of the 18:0 derivative were much higher than with the fatty acid, while rates with other fatty acyl-CoA were similar to those with the respective fatty acid. Substrate preferences for CoA derivatives incorporated into diacyl-GPC were: 18:0 > 20:4 > 18:2 ≥ 22:6, and into diacyl-GPE: 20:4 = 22:6 > 18:0 > 18:2. Polyunsaturated fatty acyl CoA (PUFA-CoA) were thus favored for incorporation into diacyl-GPE, and to a lesser extent into diacyl-GPC, a result that is consistent with composition data. When purified FABP was added to the reactions, there was an increase in the incorporation of 18:0-CoA and a decrease or no change in the incorporation of PUFA-CoA. The deacylation/reacylation cycle thus appears to play a role in the modification of phospholipid composition. The data are not consistent, however, with a role for FABP in directing PUFA toward membrane lipid synthesis.
Lipids 26, 62–67 (1991).

The fatty acid composition of retinas, like other neural tissue, is remarkably high in polyunsaturated fatty acids (PUFA), particularly of the n-3 family (1,2). Within the retina, the concentration of n-3 fatty acids is highest in the photoreceptor outer segments, where 22:6n-3 alone may constitute over 50% of fatty acids present in a given phospholipid class (1,3). Within the major phospholipid classes, PUFA are more prevalent among diacyl-GPE than among diacyl-GPC. For example, in adult chicken retina, PUFA constitute 48.5% of fatty acids in diacyl-GPE, but only 4.5% in diacyl-GPC (4).

The incorporation of PUFA into phospholipids first requires the activation of the fatty acid to its coenzyme A (CoA) derivative *via* the enzyme long-chain acyl-CoA synthetase. Two forms of the enzyme are generally recognized, one that is non-specific and one that is selective for arachidonic acid. The non-specific form is present in microsomes, mitochondria, and peroxisomes of liver (5,6). The cDNA for this protein has recently been shown to be expressed in liver and heart, and to a much lesser extent in brain (7). The arachidonic acid-specific form of acyl-CoA synthetase has been described in blood vessel endothelium (8), brain (9) and retina (10). In the latter two tissues, activation of docosahexaenoic acid was also demonstrated with a K_m similar to that for arachidonic acid (11,12). Thus, this 20:4-selective CoA synthetase may play an important role in the incorporation of these two PUFA into phospholipids.

The fatty acyl-CoA can then serve as a substrate for acyl-CoA:1-acyl-GP acyltransferase for *de novo* synthesis of phosphatidic acid, or for acyl-CoA:1-acyl-GPC (or -GPE) acyltransferase, involved in the tailoring of existing diacyl-GPC (or -GPE). The specificity of these acyltransferase enzymes for particular fatty acids could influence the composition of a particular membrane. For example, retinal microsomes can incorporate [^{3}H]22:6 into phosphatidic acid (13,14) suggesting the introduction of long-chain PUFA during *de novo* synthesis. In other tissues the deacylation/reacylation cycle is involved in the restructuring of *de novo* synthesized phospholipids (15). Lands *et al.* (16) have shown that the reacylation of 1-acyl-GPC in rat liver preferentially incorporates PUFA. That this latter pathway also exists in the photoreceptor is consistent with the detection of phospholipase A_2 activity in photoreceptor outer segments (17–19), thus providing a means of generating the lysophospholipids used in the acyltransferase reaction. Differences in turnover rates of individual molecular species of phospholipids in the retina (20) also imply active replacement of part or all of the membrane lipid molecules.

The above reactions involve the intracellular trafficking of fatty acids between cytoplasmic and/or membrane compartments; this movement may involve one or more binding proteins as intracellular carriers. Fatty acid binding protein (FABP) is a 12–14,000 dalton protein found in the cytosol of many tissues (for reviews, see refs. 21–24) including retina (25). This protein has been reported to have a binding preference for unsaturated fatty acids or their CoA esters (22), and has been suggested to play a role in directing these substrates to various metabolic pathways such as oxidation or esterification. For example, FABP stimulates the activities of acyl-CoA:glycerol-3-phosphate acyltransferase (26–28) and acetyl-CoA carboxylase (29). Peeters *et al.* (30) have demonstrated the ability of liver and heart FABP to mediate the accelerated transfer of fatty acids between mitochondria and lipid vesicles. While functional studies with retinal FABP have not been performed, this protein could be an important mechanism for channelling PUFA into the phospholipids of outer segment membranes.

We investigated the reacylation reaction in embryonic

*To whom correspondence should be addressed at the Department of Anatomy and Cell Biology, University of Kansas Medical Center, 39th and Rainbow Boulevard, Kansas City, KS 66103.

Abbreviations: ATP, adenosine triphosphate; CoA, Coenzyme A; DTT, dithiothreitol; FABP, fatty acid binding protein; GPC, glycerophosphocholine; GPE, glycerophosphoethanolamine; GP, glycerophosphate; PMSF, phenylmethylsulfonyl fluoride; PUFA, polyunsaturated fatty acids; ROS, rod outer segments; RPE, retinal pigment epithelium; fatty acids are listed as chain length:number of double bonds.

chick retinas using synthetic lysophospholipids and seven fatty acids as the substrates. 1-Acyl-GPC and 1-acyl-GPE acyltransferase activities were measured using the microsomal fraction prepared from these retinas. FABP was partially purified from the retina cytosol, and its effect on the reaction was measured. Under these conditions, rates of incorporation of fatty acid did not correlate well with composition data, and FABP did not significantly influence either the reaction rate or the inherent substrate preference of the reactions. However, when CoA esters of four fatty acids were prepared and tested, there was greater incorporation of PUFA into diacyl-GPE and, to a lesser extent, into diacyl-GPC. The addition of purified FABP to this reaction increased the incorporation of the saturated fatty acid and decreased the incorporation of PUFA.

MATERIALS AND METHODS

Fertilized chicken eggs were obtained from a local supplier and were incubated for 14–15 days. Embryos were sacrificed by decapitation. The anterior segment of the eye was cut away and the vitreous was lifted out. A few drops of 0.1 M Tris-HCl, pH 7.8 (containing the following protease inhibitors in μg/mL: phenylmethylsulfonyl fluoride (PMSF), 2; pepstatin, 3.4; aprotinin, 5; leupeptin, 2.4; and chymostatin, 5) were added to facilitate peeling away of the retina with fine forceps. At the region of the pecten, the retina was loosened by stroking with the tip of the forceps. At this stage of embryonic development, the retinal pigment epithelium does not adhere to the retina, which can thus be isolated cleanly.

Retinas were kept on ice in a small volume of Tris-HCl plus protease inhibitors and were homogenized in a glass Potter-Elvehjem homogenizer. The homogenate was centrifuged at 12,000 × *g* for 20 min to remove cell debris. The supernatant was then centrifuged at 100,000 × *g* to pellet the microsomal fraction, which was resuspended in 0.1 M Tris-HCl, pH 7.8 plus 5 mM dithiothreitol (DTT).

The high-speed supernatant was layered onto a Sephadex G-100 column and eluted with 0.1 M Tris-HCl buffer, pH 7.8, containing 0.2% sodium azide. The elution volume of FABP was determined in initial preparations by mixing an aliquot of supernatant with a trace amount of [1-^{14}C]oleic acid and counting 200 μL from each of the eluted fractions. In subsequent preparations, non-labelled fractions containing FABP were pooled and concentrated using an Amicon ultrafiltration cell. This concentrated "FABP fraction" was used in most of the enzyme assays. FABP was not purified further for these experiments; this allowed for the use of microsomes and FABP from the same preparation. All of the above procedures were carried out at 0–4°C.

In the experiments with fatty acyl-CoA derivatives, FABP was further purified (Sellner, P.A., and Phillips, A.R., manuscript in preparation). Briefly, the "FABP fraction" prepared as described above was separated by native polyacrylamide gel electrophoresis. After staining one of the lanes with Coomassie Blue to locate the FABP band, the corresponding regions from the remaining gel were cut. FABP was then eluted from the gel using the BioRad Electroeluter and concentrated by lyophilization.

In most experiments, all embryos (7 dozen) were sacrificed on the same day, and microsomes were kept refrigerated overnight to be used the following day after the FABP fraction had been prepared. Acyltransferase activity was lost or greatly compromised if the microsomes were stored at −20°C or if they were kept refrigerated for 2 days.

Preparation of fatty acyl-CoA. Derivatives of [1-^{14}C]-18:0, 18:2, 20:4, and 22:6 were prepared according to the method of Schmidt and Burns (31). We used 200 nmol of unlabelled fatty acid with 2 μCi labelled fatty acid in the synthesis to achieve a final concentration in our assays of 100 μM fatty acyl-CoA.

Acyltransferase assays. Each assay contained the following in a total volume of 0.1 mL: 0.1 mM 1-16:0-GPC or -GPE (pipetted in chloroform/methanol solution onto the bottom of the vial, solvent was evaporated with a stream of nitrogen), 0.1 M Tris-HCl, pH 7.8 plus 5 mM dithiothreitol (DTT), 1 mM adenosine triphosphate (ATP), 10 mM $MgCl_2$, 0.1 mM CoA, and 100 μM (0.1 μCi) [1-^{14}C]fatty acid (18:0, 18:1n-9, 18:2n-6, 18:3n-3, 20:4n-6, 20:5n-3, or 22:6n-3), added in 5 μL of propylene glycol. The fatty acyl-CoA (18:0, 18:2, 20:4, or 22:6) was added in 40 μL of aqueous suspension. The solution was vigorously vortexed after the addition of each constituent. Following a 5-min preincubation, 0.1–0.2 mg microsomal protein in 10 μL was added, and the reaction was allowed to proceed at 37°C for 6 min. For those assays containing FABP fraction, the protein was added (10–20 μg in 10 μL) after the preincubation. Another 5-min incubation was allowed prior to the addition of microsomes. The reaction was stopped by the addition of 0.9 mL of $CHCl_3$/MeOH (2:1, v/v). Lipids were extracted by the method of Folch *et al.* (32), and separated by thin-layer chromatography on 0.25 mm Silica Gel H plates (Analtech, Newark, DE) containing 7.5% magnesium acetate. The plate was developed twice in $CHCl_3$/MeOH/H_2O (60:30:5, v/v/v), each time one-third the way up; then in hexane/diethyl ether/formic acid (80:20:2, v/v/v), developed to within 1 cm from the top (33). Standard lanes were sprayed with 2′,7′-dichlorofluorescein and spots were visualized under ultraviolet light. The phospholipid and free fatty acid spots were scraped directly into scintillation vials and counted. Counts were converted to nmol after determining the specific activity from a 1 μL aliquot of the fatty acid-propylene glycol mixture. The data are expressed as nmol diacyl-GPC or -GPE formed/mg protein/6 min.

Protein was determined by the method of Lowry *et al.* (34). Non-radiolabelled fatty acids were obtained from Sigma Chemical Company (St. Louis, MO); radiolabelled fatty acids were from Dupont/New England Nuclear (Wilmington, DE). All other chemicals were of reagent grade.

RESULTS

The elution profile of retina cytosolic fraction (high-speed supernatant) mixed with [1-^{14}C]18:1 from a Sephadex G-100 column is shown in Figure 1. FABP elutes between 130–160 mL; the peak corresponds to a molecular weight of around 12,000 daltons. When an aliquot of this fraction with a trace amount of [1-^{14}C]18:1 methyl ester is electrophoresed on a native polyacrylamide gel, only one band (FABP) contains radioactivity, though several bands are present upon staining with Coomassie Blue (data not shown). Thus, we reasoned that any effect of

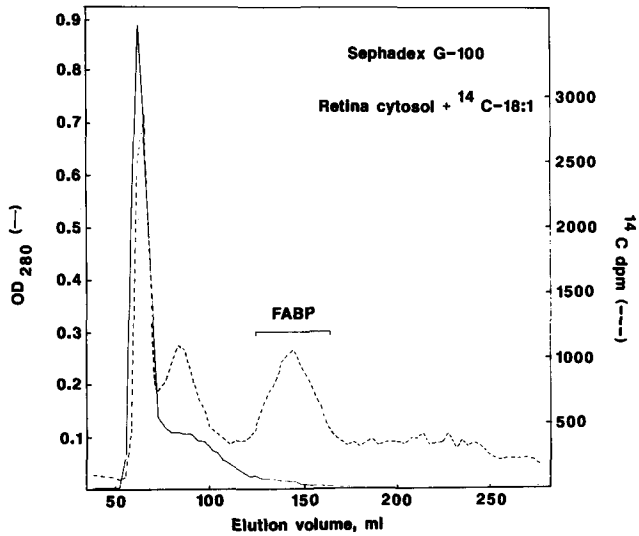


FIG. 1. Elution of FABP fraction in retina cytosol. An aliquot of retina cytosolic fraction was mixed with a trace amount (0.5 μCi) of [^{14}C]18:1. The labelled cytosol was eluted from a column of Sephadex G-100 as described in Materials and Methods. The solid line represents protein (OD_{280}); the dashed line represents fatty acid bound to protein (cpm). In subsequent runs without added fatty acid, the fractions marked "FABP" were pooled, concentrated, and used without further purification.

TABLE 1

Incorporation of Fatty Acids into Diacyl-GPC and the Effect of FABP[a]

Fatty acid	nmol GPC formed/mg protein/6 min	
	no FABP	+ FABP
18:0	0.44 ± 0.12 (4)	0.46 ± 0.25 (4)
18:1n-9	1.18 ± 0.58 (5)	1.11 ± 0.51 (5)
18:2n-6	3.24 ± 0.94 (5)	3.66 ± 1.20 (4)
18:3n-3	2.50 ± 0.84 (5)	1.92 ± 0.80 (5)
20:4n-6	1.58 ± 0.64 (4)	1.04 ± 0.72 (4)
20:5n-3	2.53 ± 0.69 (5)	2.69 ± 1.03 (4)
22:6n-3	0.37 ± 0.19 (5)	0.44 ± 0.32 (4)

[a]Acyltransferase activities were measured as described in Materials and Methods, using 1-palmitoyl-*sn*-GPC and the indicated [^{14}C]labelled fatty acid as substrates. Where indicated, FABP fraction (20 μg total protein) was added prior to addition of microsomes. Values represent means ± SEM for the number of experiments indicated in parentheses

the addition of this fraction on the reaction was attributable to FABP.

The incorporation of fatty acid into diacyl-GPC and the effect of FABP is shown in Table 1. While the absolute reaction rates varied considerably between experiments, within a given experiment, some general trends could be observed. For 1-16:0-GPC, the maximum rates of incorporation were with 18:2 as the substrate, followed by 18:3 = 20:5 > 20:4 > 18:1 > 22:6 = 18:0. The incorporation of 18:0 was low, as would be expected for acylation of the 2-position of the phospholipid, but rates for the incorporation of 20:4 and 22:6 were also lower than would be expected from the composition data (4).

TABLE 2

Incorporation of Fatty Acids into Diacyl-GPE and the Effect of FABP[a]

Fatty acid	nmol GPE formed/mg protein/6 min	
	no FABP	+ FABP
18:0	0.34 ± 0.18 (4)	0.28 ± 0.11 (4)
18:1n-9	0.24 ± 0.10 (5)	0.26 ± 0.07 (4)
18:2n-6	0.60 ± 0.23 (5)	0.68 ± 0.39 (3)
18:3n-3	0.36 ± 0.13 (3)	0.92 ± 0.56 (4)
20:4n-6	0.27 ± 0.16 (3)	0.26 ± 0.14 (4)
20:5n-3	0.17 ± 0.05 (5)	0.26 ± 0.04 (3)
22:6n-3	0.40 ± 0.15 (3)	0.20 ± 0.03 (2)

[a]Acyltransferase activities were measured as described in Materials and Methods, using 1-palmitoyl-*sn*-GPE and the indicated [^{14}C]-labelled fatty acids as substrates. Other information is as for Table 1.

The addition of 20 μg of FABP fraction caused a decrease (not statistically significant) in the incorporation of 18:1, 18:3, and 20:4, and a slight increase in rates with 18:0, 18:2, 20:5, and 22:6. In conjunction with these minor effects on absolute reaction rates, the effect of FABP on the overall substrate preference was minimal. Thus, for diacyl-GPC, 18:2 was again the substrate with maximal rates of incorporation, followed by 20:5 > 18:3 > 18:1 = 20:4 > 18:0 = 22:6. This represents a trend toward increasing the incorporation of 20:5 over 18:3 and decreasing the incorporation of 20:4 relative to 18:1 in the absence of FABP.

Rates of incorporation of fatty acids into diacyl-GPE and the effects of FABP are given in Table 2. Again, 18:2 was the substrate with the highest rates of incorporation, followed by 22:6 ⩾ 18:3 = 18:0 ⩾ 20:4 = 18:1 > 20:5.

In the presence of FABP, the incorporation of 18:3 was increased substantially; most of this increase is due to the result of one experiment in which an unusually large amount of this fatty acid was incorporated. Smaller increases were seen with 18:1, 18:2, and 20:5, while the reaction rates with 18:0, 20:4, and 22:6 declined. The substrate preference was changed to 18:3 > 18:2 > 18:0 = 18:1 = 20:5 = 20:4 ⩾ 22:6. There was a greater similarity of reaction rates among many of the fatty acids, and a trend, surprisingly, toward decreasing the incorporation of 22:6 into this phospholipid.

Incorporation rates with 1-16:0-GPE were generally 5-7-fold lower than for 1-16:0-GPC. Interesting exceptions were the rates of 18:0 and 22:6 incorporation, which were nearly equal in the two phospholipids, and 20:5, which had rates of incorporation into diacyl-GPC that were 15-fold greater than into diacyl-GPE. In neither phospholipid was the pattern of substrate preference consistent with the composition of PUFA in these membrane lipids.

To bypass the CoA activation step and thus eliminate any selectivity the acyl-CoA synthetase might have on the incorporation, we performed an experiment using synthesized fatty acyl-CoA derivatives (Table 3). Rates were generally similar to those obtained with the fatty acids, except for a 10-fold higher rate of incorporation of 18:0-CoA into diacyl-GPC. There were some differences in substrate preference: for diacyl-GPC, 18:0 showed the

TABLE 3

Incorporation of Fatty Acyl-CoA into Diacyl-GPC and Diacyl-GPE, and the Effect of FABP[a]

	nmol formed/mg protein/6 min			
	diacyl-GPC		diacyl-GPE	
Fatty acyl-CoA	no FABP	+ FABP	no FABP	+ FABP
18:0	5.39	6.04	1.65	3.21
18:2n-6	0.58	0.17	0.15	0.22
20:4n-6	1.87	1.46	2.32	1.04
22:6n-3	0.31	0.23	2.19	1.38

[a]CoA esters of [^{14}C]labelled fatty acids were prepared as described in Materials and Methods. Acyltransferase activities were measured as for Tables 1 and 2, except that 10 μg of purified FABP was added rather than FABP fraction. Values represent the means of duplicate determinations.

highest rates of incorporation, followed by 20:4, 18:2, and 22:6. For diacyl-GPE, 20:4 and 22:6 were equally effective, followed by 18:0 and 18:2. Interestingly, the effect of FABP was to increase the incorporation of 18:0, and to decrease or have no effect on the incorporation of the unsaturated fatty acids.

DISCUSSION

The purpose of the high n-3 fatty acid content of retina is unclear, but is not limited to providing a highly fluid environment for the visual pigment (35,36). The function of n-3 fatty acids may be related to other characteristics of the outer segment disk membrane, such as flexibility, permeability, or the high degree of membrane turnover in the photoreceptor outer segment. Clearly unique mechanisms exist within the photoreceptor or photoreceptor-retinal pigment epithelium (RPE) complex to create and maintain the unusual composition. One possibility is that the acyltransferase could prefer PUFA, as has been shown in other systems. Additionally, FABP could direct certain PUFA toward incorporation into lysophospholipids and away from oxidation or other routes, thus tailoring the composition of *de novo* synthesized molecular species.

In these studies, rates of incorporation of fatty acids into diacyl-GPC were much greater than into diacyl-GPE, despite the fact that these phospholipids are present in approximately the same molar concentrations in these membranes (4). Masuzawa *et al.* (37) also found that 1-acyl-GPC was the preferred acceptor over 1-acyl-GPE for the CoA- and ATP-dependent acylation. Similar results in retina were found by investigators using different experimental protocols. For example, when rat eyes were injected intravitreally with radiolabelled 20:5n-3, more label was recovered in the diacyl-GPC than diacyl-GPE fraction at all time points (38). Bovine retinas incubated *in vitro* with 20:4n-6, 20:5n-3, or 22:6n-3 labelled diacyl-GPC to a greater extent in all subcellular fractions than diacyl-GPE (39). This may suggest that *in vivo* the principal species synthesized is diacyl-GPC, and diacyl-GPE is prepared from this *via* base exchange or other conversion.

Louie *et al.* have found 10-fold greater specific activities among 22:6-containing species of diacyl-GPC than diacyl-GPE in frog rod outer segments (ROS) prepared from eyes injected with [^{3}H]glycerol. In addition, half-lives of all 22:6-containing species were longer in diacyl-GPE than in diacyl-GPC. Similarly, *in vitro* experiments with isolated bovine ROS incubated with [^{3}H]22:6 yielded specific activities of diacyl-GPC that were 10 times greater than those of diacyl-GPE or diacyl-glycerophosphoserine (40). Specific activities of 22:6-22:6 species were higher than any other 22:6-containing species for all phospholipids studied. Thus, while more diacyl-GPC is initially formed, it may also be rapidly turning over, a process which could not be estimated from our short incubations.

Low rates of incorporation of 18:0 would be expected for the 2-position of GPC and GPE, and our observed rates were therefore consistent with composition data. However, the rates of incorporation with 18:0-CoA were much higher. We have no explanation for this, even though disaturated species of GPC have been isolated (41). The greatest rates of incorporation of fatty acid in our studies were seen with 18:2, although when the CoA esters were used, 18:2 was the least utilized substrate. This suggests that in the assays with fatty acids, conditions were optimal for the activation and incorporation of 18:2 over other substrates. The acyltransferase, on the other hand, is not selective for this fatty acyl-CoA. Johnston and Hudson (4) report 0.1 mol% of 18:2 in whole retina diacyl-GPC and 7.8 mol% in diacyl-GPE. We have found very little (<1%) 18:2 in chick retina total phospholipids throughout development (Sellner, P.A., and Clough, J.A., submitted for publication). This difference from Johnston and Hudson's data (4) may reflect the influence of diet in the hen, an effect that can be significant (42), or the effect of developmental stage. Even if the 18:2 that we find in the retina phospholipid fraction is concentrated in the photoreceptors, our data would be consistent with a major role for the acyl-CoA synthetase in selectively activating this fatty acid for subsequent targeting, as the acyltransferase itself does not appear to select for this substrate (Table 3).

Johnston and Hudson (4) also report 11.4 mol% 20:4 and 19.3 mol% 22:6 in diacyl-GPE, figures that correspond with the rates of acyltransferase activity observed in this study with the fatty acyl-CoA derivatives, but not with the fatty acids. In general, the substrate preference using fatty acids was not consistent either with the final fatty acid composition or with the expected pattern of incorporating PUFA, particularly into diacyl-GPE. A similar conclusion was reached by Zimmerman and Keys (18) in their studies of acyltransferase in isolated bovine rod outer segments. In our studies using microsomes, however, the result apparently reflects the activity of the activation step rather than the acylation. The deacylation/reacylation cycle is contributing in part to the introduction of CoA derivatives of PUFA into these lipids. This path can work in conjunction with the incorporation of PUFA into phosphatidic acid *via* the Kennedy pathway, as Bazán and Bazán have shown (14).

Other mechanisms responsible for establishing a PUFA-rich composition, whether in retina or other tissues, have been suggested (35,43). In retina, there may be selective delivery of particular fatty acids to the photoreceptors *via* the blood and retinal pigment epithelium

(RPE) using special receptors and/or binding proteins. Evidence from Scott and Bazán (44) suggests that in the rat n-3 acids are delivered to the RPE in the form of long-chain PUFA (*e.g.*, 22:6) rather than as the precursor (18:3). In addition, rates of synthesis of long-chain PUFA may be greater for n-3 fatty acids than for other families. While there is no direct evidence for the capacity of the RPE or photoreceptors to synthesize long-chain PUFA from their 18-carbon precursors, synthesis of 22:6 from 22:5n-3 *in vitro* (45) or from 20:5n-3 following intravitreal injection (38,46) does occur, albeit at a low rate.

Restructuring of existing phospholipids may involve other subcellular fractions, as shown in bovine retina by Rotstein and Aveldaño (47). Their data indicate that the mitochondria are not only enriched in PUFA, but actively incorporate exogenous PUFA supplied *in vitro*. This is an especially intriguing possibility in light of the mitochondria-rich region of the photoreceptor adjacent to the outer segment. Other reactions such as CoA-dependent and CoA-independent transacylation may be important in modifying existing phospholipids. In brain microsomes, the latter reaction has been shown to be a significant path for the introduction of 22:6 and 20:4 (37). The transacylation of 16:0 in retina ROS was not significant (48), but similar measurements with PUFA have not been performed. Rates of incorporation can also be different depending on the presence of other fatty acids in the pool. This was pointed out by Lands *et al.* (16), who estimated the relative effectiveness of competing fatty acyl-CoA in the esterification of 20:4-CoA by rat liver microsomes. Such competition studies or determination of fatty acid or acyl-CoA pools would help clarify the mechanisms involved in phospholipid restructuring within a particular system.

The role of FABP has not been clearly established, though it is thought to play a role in intracellular trafficking of potentially toxic hydrophobic substances (22). FABP has been shown to affect several lipid-synthesizing enzymes, including glycerol-3-phosphate acyltransferase and acyl-CoA synthetase, though not always in a consistent manner. The lack of effect of FABP on the incorporation of fatty acid into diacyl-GPC and diacyl-GPE in this study suggests that FABP may be serving simply as a nondiscriminate carrier in the cell, or that its action is directed toward some other pathway of lipid metabolism, *e.g.*, oxidation. This conclusion is substantiated by the data from the experiments with acyl-CoA, in which incorporation of saturated fatty acid was increased and that of PUFA decreased or was unchanged. However, recent results of Bordewick *et al.* (49) using rat liver microsomes and FABP suggest that FABP greatly increases the incorporation of 16:0-CoA, 18:0-CoA, and 18:1-CoA into diacyl-GP and 1-acyl-GP. These authors did not use CoA derivatives of PUFA in their experiments, therefore the enhancement of PUFA incorporation into diacyl-GP during *de novo* phospholipid synthesis could not be assessed. Such a role for FABP in the retina could explain Bazán's result, *i.e.*, a high proportion of phosphatidic acid that contains 22:6, thus establishing a physiological function for this enigmatic protein.

Finally, both the substrate preference data and the effect of FABP may depend on the stage of development of the chick retina. At 14–15 days, the outer segments of the photoreceptors are not fully developed, thus there may not be the demand for long-chain PUFA as when disk membranes are being synthesized at a maximal rate. The data may reflect regulation of acyl-CoA pools *via* acyl-CoA synthetase or *via* the activities of PUFA synthesizing enzymes. Future studies will address this question at a later developmental stage, and will determine if there is a sudden appearance of PUFA when outer segments are being formed, that is, 18–19 days (50). We have found that the 22:6 content of retina phospholipids indeed increases from 4.5% on day 15 to 11.1% on day 21 (Sellner and Clough, submitted for publication). The fact that the chick retina is cone-dominant makes this an interesting and useful comparison with rod-dominant retinas, such as rat or bovine. The cone outer segment is not fully enclosed by a plasma membrane, as is the rod outer segment. Thus, while many lipid synthesizing activities have been described in the rod outer segment, the corresponding enzymes in the cone outer segment have not been studied and may not, in fact, be present. Hence, the mechanisms utilized by the immature retina for the synthesis of PUFA-containing phospholipids may persist in the adult retina.

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Triolein-Phosphatidylcholine-Cholesterol Emulsions as Substrates for Lipoprotein and Hepatic Lipases

Susanne Bennett Clark* and **Henry L. Laboda**[1]
Departments of Biophysics, Medicine and Physiology, Boston University School of Medicine, Housman Medical Research Center, Boston, MA 02118-2394

Lipolysis of emulsified glycerol tri[9,10-^{3}H]oleate by lipoprotein lipase purified from bovine milk (E.C. 3.1.1.34) and by hepatic lipase purified from rat liver perfusate was studied as a function of the phosphatidylcholine molecular species and the cholesterol content of the emulsions. Overall, the activities of the two enzymes were similar on a molar basis. Lipoprotein lipase initial lipolysis rates also were comparable for emulsions made with egg phosphatidylcholine or with saturated (dimyristoyl, dipalmitoyl and distearoyl) phosphatidylcholines when cholesterol was low. Increasing the cholesterol content of the emulsion from 2–3 mole percent to 7–14 mole percent reduced triolein lipolysis by lipoprotein lipase in emulsions made with saturated phosphatidylcholines. Rat hepatic lipase was more sensitive to increased cholesterol in emulsions made with saturated phosphatidylcholines than was lipoprotein lipase. The ability to maintain triolein lipolysis during longer incubations differed strikingly among the emulsions and for the two enzymes. Lymph chylomicrons were better substrates for both enzymes than any of the emulsions.
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Lipoprotein lipase(s) (LPL, E.C. 3.1.1.34) and hepatic lipase (HL) are important enzymes which contribute to the metabolism of lipoproteins (LP), particularly the triglyceride (TG)-rich LP, namely chylomicrons (CM), chylomicron remnants (CR) and very low density lipoproteins (VLDL). Both enzymes catalyze the hydrolysis of triacylglycerol (TG), releasing fatty acids (FA) which are ultimately metabolized. The enzymes differ in several respects. HL is confined to the liver whereas LPL, which may represent a group of proteins of differing molecular weights and specific activities (1), is found in many different tissues. LPL, but apparently not HL, requires a cofactor for maximal activity, namely apolipoprotein C-II (apoC-II) which is found in plasma associated mainly with CM, VLDL, and high density lipoproteins (HDL). LPL is also sensitive to high salt concentrations (1M NaCl), unlike HL.

Native substrates for HL have been reported to include HDL_1, HDL_2 (2), apoE containing intermediate density lipoprotein (IDL) (3) and chylomicron remnants (4). Native substrates for LPL are, mainly, VLDL and chylomicrons (5,6). Triglyceride-rich emulsions thus are reasonable models for many of the natural substrates of both enzymes.

Lipoprotein and emulsion surfaces are bounded by phospholipid monolayers, which contain up to 3 mol % TG (7,8) and whose compositions can vary greatly. Studies of LPL have shown that hydrolysis of TG incorporated into planar monolayers is critically dependent on how the substrate molecules are organized at the lipid-water interface; the enzyme activity varies with the surface pressure and the composition of the lipid film (see (5) for review). The lipolytic activity of HL on TG incorporated into monolayers is also influenced by the phospholipid (PL) composition, the unesterified cholesterol (UC) content and the physical state of the monolayer (9,10). Similarly, triolein emulsified in a variety of PL differs in reactivity to rat adipose tissue LPL (11). The present study examines the effect of phosphatidylcholine (PC) molecular species differences and UC content on the lipolysis, by LPL and HL, of a fluid TG (triolein) incorporated into well-characterized, chylomicron-sized emulsion particles of differing surface composition.

MATERIALS AND METHODS

LPL and HL were purified respectively from bovine milk and rat liver perfusate, essentially according to published methods (12,13). Both enzymes showed a single band on 10% SDS-PAGE with a mass of about 50 kD (14,15). Aliquots of a single, combined preparation of each enzyme were stored at −80°C in 50% aqueous glycerol and were used within minutes after thawing.

ApoC-II was isolated from human plasma VLDL (16–18), and its purity was established by IEF electrophoresis and amino acid analysis. Aliquots of an aqueous solution were stored at −80°C.

Emulsions were prepared by sonication above the respective PC chain melting temperatures (19). The final emulsions had surfaces that, at 37°C, were either very fluid, fluid but viscous, very viscous, just below the chain melting transition, or partly solid, depending on the PC composition and the cholesterol content (see Table 2). Aliquots of chloroform solutions of [9,10-^{3}H]trioleoylglycerol (40 mg; SA 0.5 μCi/μmol TG), cholesterol (0.75 or 4.0 mg), and either egg yolk lecithin (EYPC), dimyristoyl phosphatidylcholine (DMPC), dipalmitoyl phosphatidylcholine (DPPC) or distearoyl phosphatidylcholine (DSPC) (9.25 or 6.0 mg, respec-

*To whom correspondence should be addressed at the Department of Biophysics, BUSM, 80 E. Concord St., Boston, MA 02118-2394.
[1]Present address: Plough, Inc., Analytical R&D, 3030 Jackson Avenue, Memphis, TN 38151.

Abbreviations: apoC-II, apolipoprotein C-II; BSA, bovine serum albumin; CM, chylomicrons; CR, chylomicron remnants; DG, diglycerides; DMPC, dimyristoyl phosphatidylcholine; DPPC, dipalmitoyl phosphatidylcholine; DSPC, distearoyl phosphatidylcholine; EYPC, egg yolk phosphatidylcholine; FA, fatty acid; GLC, gas-liquid chromatography; HDL, high density lipoprotein; HL, hepatic lipase; IDL, intermediate density lipoprotein; KPB, potassium phosphate buffer; LP, lipoprotein; LPL, lipoprotein lipase; MG, monoglycerides; PC, phosphatidylcholine; PL, phospholipid; TG, triacylglycerol; TLC, thin-layer chromatography; TO, triolein; UC, unesterified cholesterol; VLDL, very low density lipoprotein.

TABLE 1

Lipid Compositions of Emulsions and Chylomicrons

	UC mol%	PC mol%	TG mol%	Diam[a] nm	Surface TG[a] molecules per particle
EYPC-LoC	2.2 (0.1)[b]	13.4 (2.3)	84.4 (2.3)	104 (14)	1340 (303)
EYPC-HiC	14.0 (1.0)	10.5 (0.7)	75.5 (1.5)	89 (5)	702 (87)
DMPC-LoC	1.7 (0.3)	12.9 (0.7)	85.5 (0.7)	105 (6)	1347 (150)
DMPC-HiC	7.4 (0.4)	12.1 (0.9)	80.4 (0.8)	91 (6)	834 (96)
DPPC-LoC	2.3 (0.1)	13.5 (0.9)	84.1 (0.8)	98 (7)	1166 (182)
DPPC-HiC	8.1 (0.2)	11.5 (0.3)	80.4 (0.4)	92 (3)	836 (45)
DSPC-LoC	2.6 (0.1)	15.1 (0.5)	82.2 (0.6)	85 (3)	851 (61)
DSPC-HiC	8.5 (0.3)	10.4 (0.3)	81.2 (0.4)	99 (3)	936 (52)
Chylomicrons[c]	3.0	6.7	87.9	150	1960

[a]Computed from the phase analysis program developed by Miller and Small (23,24) and Spooner and Small (25).
[b]Mean (SEM) of 5–7 separate preparations.
[c]Single batch. Chylomicrons also contained cholesteryl esters (CE) (2.4 mol% total lipid) and protein (2.3 wt% total chylomicron mass).

tively) were pipetted into 10-mL glass vials. Solvent was blown off with a stream of N_2, and the vials were placed into a vacuum desiccator at 4°C for at least 18 hr. Saline (80 mM NaCl, pH 7.2) was added (10 mL) and the vials were heated or cooled to the appropriate temperature (0°C for EYPC, 25–30°C for DMPC, 45–50°C for DPPC and 60–65°C for DSPC). The mixtures were sonicated continuously at the respective temperatures for 11 min at approximately 30% of maximum power output (Branson Sonifier Model 350, 1 cm probe tip). The sonicated mixtures were then transferred, at room temperature, to ultracentrifuge tubes (SW41), overlayered with 1.5–2.0 mL distilled water and centrifuged for 11 min at 23,000 rpm at 25°C. The floated emulsion particles, of a narrow size range, were harvested by tube slicing and were used in enzyme assays within 15 min. Negative stain electron microscopy of representative preparations revealed that all of the emulsion types displayed unimodal populations of particles with round profiles and no fragmented particles.

Chylomicrons were isolated from rat mesenteric lymph which was collected, on ice, for 10 hr after a gastric dose of 0.5 mL triolein containing 100 μCi [9,10-^{3}H]trioleoylglycerol, during continuous gastric infusion of saline at 2 mL/hr. CM were washed and concentrated by ultracentrifugation through an overlay of 6 mL distilled water per tube, in a swinging bucket rotor (SW41; 30,000 rpm for 30 min at 12°C). The final specific activity was approximately 300,000 dpm/μmol TG.

Analytical. Emulsions (5–7 preparations of each emulsion type) and chylomicrons (single preparation) were chemically characterized by standard methods, as described (20). The compositions of the emulsions are shown in Table 1.

Enzyme assays. Both enzyme activities were monitored simultaneously, with each individual emulsion. Assays were performed in duplicate for each preparation in round-bottom glass tubes. Blank tubes contained all ingredients except enzyme. Single pools of apoC-II, albumin and heparin aqueous solutions were used throughout the entire series of experiments.

Lipoprotein lipase. The components were added at room temperature in the order NaCl, pH 7.4 potassium phosphate buffer (KPB), heparin, emulsion, apoC-II, FA-free bovine serum albumin (BSA). The final concentrations in the reaction mixture were NaCl 150 mM, heparin 2.65 μg/mL, apoC-II 16.7 μg/mL, emulsion TG 3.33 μmol/mL, BSA 15 mg/mL, 10 mM pH 7.4 KPB. Tubes were warmed to 37°C for 5 min after apoC-II was added and before adding BSA. The reaction was then initiated by the addition of LPL, freshly diluted in 10 mM pH 7.4 KPB, to yield an enzyme/substrate ratio of 100 ng protein/μmol TG.[2] The LPL and apoC-II concentrations used provided about 1 and 300 molecules per emulsion particle, respectively, giving an apoC-II/LPL ratio of 300. A ratio of 50 has been shown to produce maximal lipolysis in PC monolayers (21). Incubations were routinely performed for both 10 and 20 min and, in certain instances, for up to 1 hr (see Results) with continuous shaking at 1 vibration/sec. The reaction was stopped by addition of a mixture of chloroform/methanol/benzene (0.5:1.2:1.0,v/v/v), containing 0.1 mM oleic acid, and the released FA was partitioned (93%) into an aqueous phase with 0.3M NaOH, as described (20). Aliquots of the aqueous phase were taken for liquid scintillation spectrometry in Liquiscint (National Diagnostics, Manville, NJ).

Hepatic lipase. The reaction mixtures contained NaCl 100 mM, BSA 15 mg/mL and emulsion TG 3.33 μmol/mL in 10 mM pH 7.4 KPB. The reaction was initiated by the addition of HL freshly diluted in 10 mM pH 7.4 KPB, in a ratio of 100 ng protein to 1 μmol TG. Lipolysis was followed at 37°C, routinely for 10 and 20 min but occasionally (see Results) for up to 1 hr, and was stopped as described above for LPL.

[2]In a single series of assays using triolein (TO) emulsified with a non-hydrolyzable PC ether (1-*O*-hexadecyl-2-*O*-oleyl-glycerophosphocholine), the lipolysis rate (FA released/hr/mol TO/mg LPL) was constant over an enzyme/substrate range of 12.5–150 ng LPL/μmol TO (Laboda, unpublished data).

Materials. Nonradioactive lipids were used without further purification. All were >99% pure, as stated by the suppliers and confirmed by thin-layer chromatography (TLC) for lipid class purity and by gas-liquid chromatography (GLC) of FA methyl esters. Cholesterol, DMPC, DPPC, DSPC and EYPC were obtained from Sigma Chemical Co. (St. Louis, MO). Triolein was from Nu Chek Prep (Elysian, MN). Glycerol tri[9,10-^{3}H]oleate, from Research Products, Amersham Corp. (Arlington Heights, IL), was shown to be >99% pure by thin-layer chromatography of neutral lipid classes and liquid scintillation spectrometry. FA-free albumin (Fraction V) was from Sigma Chemical Co.

Statistical procedures. Data were analyzed by 2-way analysis of variance, Student's t-test or paired t-test, as appropriate.

RESULTS

Lipoprotein lipase. Lipolysis rates were essentially linear for at least 20 min for all 8 emulsion types (Fig. 1). Within the groups, the rates determined over 20 min were not significantly different from the rates determined over 10 min (paired t-test).

Overall, PC species significantly affected lipolytic rates ($p<0.05$). Saturated PC emulsions containing the lower cholesterol concentrations (2–3 mol % of the total emulsion) were hydrolyzed at rates similar to those of EYPC-LoC emulsions ($p=0.18$). When the cholesterol content was increased, TG lipolysis was reduced in saturated PC emulsions significantly more than in EYPC emulsions ($p<0.005$). In EYPC emulsions lipolysis was unaffected by an increase in the cholesterol content to above a UC/PC equimolar ratio ($p=0.89$).

When lipolysis was followed for 1 hr in duplicate experiments with each of the 8 emulsion types, further differences became apparent (Fig. 2, top). In emulsions made with EYPC, which contained either low (2.2) or high (14.0 mol%) cholesterol concentrations, and in the DMPC-LoC emulsion, lipolysis rates remained in excess of 80% of the initial rates after 1 hr. In contrast, lipolysis was negligible after 20–30 min in emulsions made with DPPC or DSPC. Lipolysis of the DMPC-HiC emulsion also slowed somewhat after about 30 min, and the overall rate was considerably slower than for the DMPC-LoC emulsion. Even for the most rapidly hydrolyzed emulsions, however, the maximum fraction of substrate triolein hydrolyzed during 1 hr was <8%. Less than 3% of TG was hydrolyzed when the emulsions were stabilized by DPPC or DSPC. Hence the reactions were essentially zero order, at least for 20 min.

The best substrates for LPL were the lymph chylomicrons, although EYPC-stabilized emulsions were almost as reactive.

Hepatic lipase. HL activities for the 8 types of emulsions are shown in Figure 3. As with LPL, the lipolytic rates obtained at 10 and 20 min were not significantly different within groups by paired t-test, although some appear slower at 20 min (Fig. 3). Emulsions made with saturated PCs did not differ significantly from one another, and all were significantly less effective substrates then EYPC stabilized emulsions

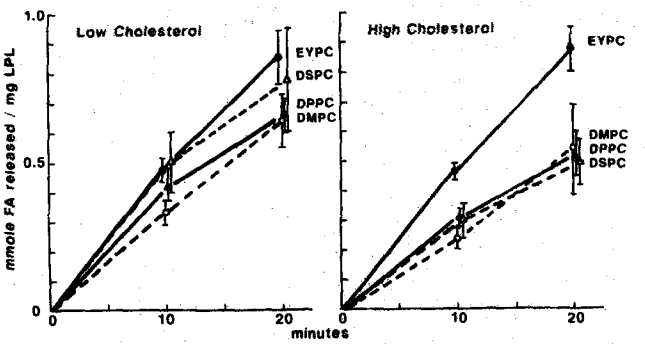


FIG. 1. Activity of purified bovine milk LPL on triolein emulsions with differing PC compositions and cholesterol contents, in the presence of apoC-II and albumin. Data are means and SEM from 5–7 separate preparations of each emulsion. Emulsion compositions are shown in Table 1. Conditions as described in Materials and Methods.

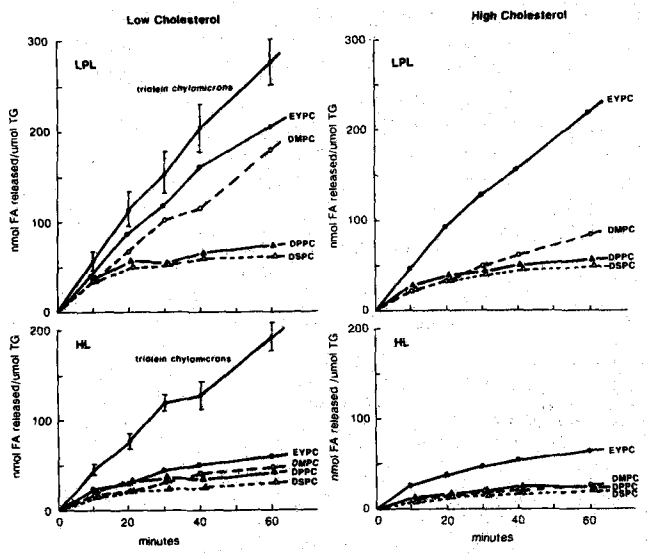


FIG. 2. LPL and HL activities on triolein emulsions and lymph chylomicrons over 1 hr. Assay conditions are described in Materials and Methods. Data points are means of duplicate emulsions, each assayed in duplicate. Chylomicrons ($\bar{X} \pm$ SEM, N=3) were obtained from a single pool of lymph from a triolein-fed rat, and duplicate assays were performed on each of 3 separate days.

($p<0.005$). Individual comparisons between EYPC and either DMPC, DPPC or DSPC all showed significant differences (p range 0.01 to 0.05 for 6 comparisons). Increasing the cholesterol concentration reduced HL activity overall ($p<0.005$); however, only for DPPC and DSPC were the individual comparisons (HiC *vs* LoC) significant ($p\leq0.05$). Thus, HL activity was more profoundly influenced by both the type of PC species and the cholesterol content of the emulsions than was LPL, although the overall trends were similar.

When the incubations were extended for 1 hr, TG lipolysis rates decreased with time for all the emul-

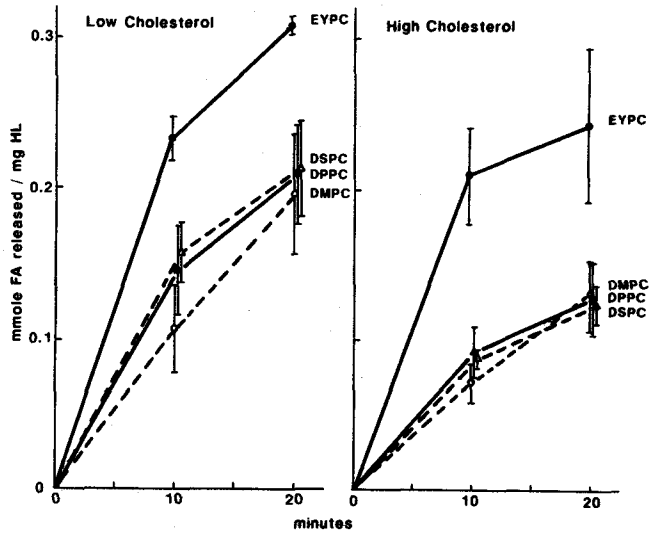


FIG. 3. Activity of purified rat hepatic lipase on triolein emulsions. Data are means and SEM of 5–7 separate preparations of each emulsion (Table 1). Conditions are described in Materials and Methods.

TABLE 2

Comparison of LPL and HL Activities on Triolein Emulsions[a]

	Emulsion Surface at 37°C[b]	μmol FA/hr/nmol enzyme	
		LPL[c]	HL[d]
EYPC-LoC	very fluid	236 (19)	281 (18)
EYPC-HiC	viscous fluid	232 (14)	253 (37)
DMPC-LoC	very fluid	166 (21)	128 (35)
DMPC-HiC	viscous fluid	120 (18)	87 (15)
DPPC-LoC	at transition	211 (28)	175 (34)
DPPC-HiC	viscous	157 (13)	111 (21)
DSPC-LoC	mainly solid	254 (53)	190 (24)
DSPC-HiC	partially solid	150 (31)	105 (7)
Chylomicrons	very fluid	225	450

[a]Data are means (SEM) of 5–7 emulsions in each group and a single chylomicron preparation, incubated for 10 min with LPL or HL, as described in Materials and Methods.
[b]Surface melting enthalpies were determined by differential scanning calorimetry (ref. 19, and Small, Steiner, Gantz, Derksen, and Bennett Clark, unpublished data).
[c]Molecular weight of dimer 83 kilodalton (KD) by sedimentation equilibrium ultracentrifugation as reported by Olivecrona and Bengtsson-Olivecrona (5).
[d]Molecular weight of tetramer 200 KD by gel fractionation as reported by Twu *et al.* (14).

sions (Fig. 2, bottom). The decrease was most marked for emulsions made with saturated PCs and higher concentrations of cholesterol. In contrast to LPL, no emulsions were as effective HL substrates as were lymph chylomicrons. Although the absolute lipolysis rates obtained with HL were less than with LPL when the same weights of both enzymes were used in the assays (100 ng per μmol TG), comparison of the enzymes on a molar basis revealed that HL activity approached that of LPL and in some cases exceeded it (Table 2). Emulsions made with EYPC and particularly chylomicrons, which have a similar PC composition to EYPC, were more rapidly hydrolyzed by HL than by LPL.

DISCUSSION

The present experiments have established that the physical state and the chemical composition of an emulsion surface both influence the rate and particularly the extent to which emulsified triolein is hydrolyzed by a purified lipoprotein lipase isolated from bovine milk and by purified hepatic lipase isolated from rat livers. All the 8 emulsion types studied were quite effective substrates for both enzymes in these isolated *in vitro* systems. For LPL, the initial lipolysis rates were significantly reduced when saturated PCs replaced mixed chain PCs and when the cholesterol content was increased. Increasing the cholesterol raises the viscosity of fluid PC monolayers. However, since lipolysis was unaffected by even a surface-saturating cholesterol content when EYPC was used, it is clear that a condensed, viscous surface alone does not necessarily inhibit triolein lipolysis by LPL. Similarly, fluidity of the emulsion surface is not, of itself, a determinant for lipolysis by LPL because DPPC- and DSPC-stabilized emulsions were better substrates when their surfaces were partially solid (low cholesterol) than when they were very viscous fluids (high cholesterol). HL, however, clearly was inhibited by a high surface viscosity alone. The inhibition was most evident with saturated PCs, but also occurred when the cholesterol concentration was increased in the presence of the unsaturated, mixed chain EYPC (see Figs. 2 and 3). The difference between the two lipases could be explained if the HL molecules require closer association with the emulsion surface monolayer in order to catalyze lipolysis than do LPL molecules. HL may even penetrate into the monolayer. If so, a highly viscous surface may inhibit HL activity by preventing penetration. LPL is thought not to attach directly to monolayers, but instead attaches as an enzyme-cofactor complex (5) in an optimum orientation for TG lipolysis. The present results might thus imply that apoC-II binding is less affected by emulsion surface composition than is the binding of a larger, higher molecular weight protein such as HL.

Although the assay conditions chosen ensured an excess of total TG substrate (about 1–4% was hydrolyzed in 20 min), the results suggested that TG available to the enzymes might, nevertheless, have become rate-limiting in certain instances. Thus, in certain emulsions lipolysis began to slow down within 20 min, while in others lipolysis was sustained beyond 60 min (see Fig. 2). This decrease in lipolytic rate over time could not have been caused by an increase in the back reaction rate for the following reasons. For LPL, the most effective emulsion substrates, which sustained rapid lipolysis rates (EYPC-LoC and EYPC-HiC), released about 200 nmol FA per μmol TG in 1 hr. This equates to about 130 nmol FA per 200 μL assay medium at 1 hr and about 40 nmol FA at 20 min. Since the BSA concentration was about 50 nmol/200 μL, the total FA released even after 1 hr was sufficient to occupy only about 3 of the 7 albumin binding sites. Moreover, the

reaction remained nearly linear for at least 1 hr not only for the EYPC emulsions, but also for the even more rapidly hydrolyzed lymph chylomicrons. Thus, TG lipolysis in DPPC- and DSPC-stabilized emulsions is most unlikely to have ceased after 20 min because of accumulation of product in the medium. That transfer of FA molecules from their formation site on the emulsion surface to BSA in the medium failed for some of the emulsions is theoretically possible, but is unlikely at pH 7.4 (22). Differences in co-factor binding or activation of LPL may play a role, as mentioned previously. However, the relative suppression of lipolysis by omission of apoC-II was indistinguishable among the 8 emulsions[3], which tends to suggest that neither differential apoC-II binding to the original emulsions nor enzyme activation was a major contributing variable in these systems. Nevertheless, co-factor binding may have differed among the emulsions once their surfaces had been further modified by the presence of other lipolytic products released during initial lipolysis of TG—diglycerides (DG), monoglycerides (MG), FA. More work is underway to clarify this possibility.

The differences in the reactivities of the substrate emulsions may relate in part to the surface TG concentration (23-25). No data are currently available that bear directly on triolein concentrations in emulsion surfaces of differing PC composition. Data obtained in pure PC vesicles (8), however, suggest that triolein may be present to a maximum of about 3 mol% of the surface irrespective of PC species (EYPC or DPPC). The number of surface TG molecules per emulsion particle were therefore calculated (Table 1) with the assumption that increasing the cholesterol in the surface inhibited TG incorporation similarly for all four PCs. It can be seen that surface TG concentration *per se* is not a predictor of lipolytic rate (compare Table 1 with Figs. 1-3). However, the decrease in lipolysis rate after 20 min observed in the partially solid-surfaced DPPC and DSPC emulsions, in particular, may have been caused by a failure to replace surface triolein molecules from the core as those originally present were removed. To date, no mechanism has been described for such a hypothetical failure, however. In sum, although very high (saturating) surface concentrations of cholesterol do reduce the emulsion surface triglyceride concentration to as little as 0.1 mol% or less (25), neither a decreased surface triglyceride concentration *per se* nor a high surface viscosity *per se* could have been major determinants of the failure to sustain lipolysis, because EYPC-HiC emulsions continued to be hydrolyzed at close to initial rates for at least an hour. Lipolysis of DMPC-HiC emulsions also remained roughly constant for 1 hr, albeit at a slower rate than DMPC-LoC.

The use of well-defined, chylomicron-sized emulsions as substrates revealed further differences between HL and LPL. Although EYPC emulsions were the best model substrates for both enzymes, even these were much less effective substrates for HL than were lymph chylomicrons. Chylomicrons were hydrolyzed twice as fast by HL as by LPL (see Table 2). With HL, unlike with LPL, triolein lipolysis in all of the emulsion substrates, including those made with EYPC, slowed considerably after 10-20 min. Only chylomicron lipolysis proceeded linearly for a full hour. However, with HL as with LPL, the emulsions whose lipolysis slowed down most over time contained DPPC or DSPC with high cholesterol concentrations. Thus, some similar, as yet unidentified, constraints probably affected both assay systems.

The pools of emulsified TG accessible to HL and LPL lipolysis also appear to differ, because the effects of emulsion surface composition on TG lipolysis were significantly more dramatic for HL than for LPL. Comparing Figures 1 and 3, it is clear that HL activity is more affected by the type of surface PC species and by cholesterol content than is LPL.

[3]Duplicate tubes lacking apoC-II were routinely included in all 20 min LPL assays (Bennett Clark, unpublished data).

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METHODS

Separation and Quantitation of Phospholipids in Animal Tissues by Iatroscan TLC/FID

Remi De Schrijver* and **Daniel Vermeulen**
Catholic University of Leuven, Laboratory of Nutrition, B-3001 Leuven, Belgium

A procedure has been developed to separate and quantitate phospholipids, including phosphatidylinositol and phosphatidylserine, from animal tissues by means of the Iatroscan TLC/FID technique. The method is based on the use of 0.01 M oxalic acid impregnated Chromarods-SII and stepwise resolution of the phospholipids in the presence of 1,2-dipalmitoyl-*sn*-glycero-3-phospho (*N,N*-dimethylethanolamine) as internal standard. To remove the neutral lipids, the rods are initially developed in a non-polar solvent mixture followed by partial scanning. Next, the rods are impregnated with oxalic acid, developed twice in $CHCl_3/CH_3OH/CH_3COOH/HCOOH/H_2O$ (80:35:2:1:3, v/v/v/v/v) and partially scanned for measuring lysophosphatidylcholine, sphingomyelin and phosphatidylcholine. The subsequent step involves double development in $CHCl_3/CH_3OH/30\%$ NH_4OH (60:35:0.9, v/v/v) to resolve cardiolipin, internal standard, phosphatidylethanolamine, phosphatidylinositol, phosphatidylserine and phosphatidic acid. For each phospholipid a linear calibration curve with a highly significant correlation coefficient was obtained. However, the calibration lines extrapolated to negative intercepts on the ordinate, indicating declining sensitivity at low phospholipid loads. ***Lipids 26,*** **74–76 (1991).**

The Iatroscan TH-10 system combines thin-layer chromatography (TLC) and flame ionization detection (FID) and has been shown to be a useful tool in lipid analysis (1,2). However, the separation and quantitation of certain phospholipids still poses problems. Analysis of blood plasma phospholipids with the solvent system $CHCl_3$/MeOH/H_2O (80:35:3, v/v/v) resulted in poor separation of cardiolipin (CL), phosphatidylserine (PS) and phosphatidylglycerol (PG) (3). Phosphatidylinositol (PI) and PS from lymphocytes were not separated by $CHCl_3$/MeOH/H_2O (60:30:3.5, v/v/v) (4). The major phospholipids from heart mitochondria, including PS and PI, were resolved by development in $CHCl_3$/MeOH/H_2O (80:35:3, v/v/v) (5). In the latter solvent system, the water concentration was reported to be critical. However, using the same experimental conditions in our laboratory, or changing the volume ratios of $CHCl_3$/MeOH/H_2O, did not result in satisfactory resolution of PI and PS. Banerjee *et al.* (6) demonstrated that rod impregnation with 0.25M oxalic acid in acetonitrile gave better and more reproducible separations. Nevertheless, oxalic acid treatment did not prevent concomitant elution of PI and PS. Except for lysophosphatidylcholine (LPC), the major phospholipids from trout gill could be separated using the solvent system $CHCl_3$/MeOH/H_2O (80:35:3, v/v/v), but quantitation of PI by the Iatroscan method did not agree with the results obtained by classical TLC combined with phosphorus determination (7). Kramer *et al.* (8) developed the rods twice in $CHCl_3$/MeOH/H_2O (68.5:29:2.5, v/v/v) with air drying between developments, which enhanced the separation of PI and PS, but PI was not reproducibly resolved from PE. The use of internal standards was recommended to reduce the variation in results attributed to the inconsistent behavior of individual rods (9,10).

The present paper describes a modification of existing TLC/FID methods permitting better resolution of phospholipids from blood plasma and animal tissues.

MATERIALS AND METHODS

Chemicals. The phospholipid standards PE, PC, PI, PS, LPC, SPH, PA and CL were obtained from Serdary Research Laboratories (London, Ontario, Canada). The internal standard 1,2-dipalmitoyl-*sn*-glycero-3-phospho-(*N,N*-dimethylethanolamine) was purchased from Koch-Light Laboratories (Colnbrook, England). Control for purity of the individual phospholipid standards revealed a single peak on the chromatogram. All other chemicals and organic solvents were of analytical grade.

Instrumentation. The Iatroscan TH-10 Mark II was used in combination with Chromarods-SII having a precoated active silica thin layer (Iatron Laboratories Inc., Tokyo, Japan). Peak areas were calculated with a Shimadzu RIA-integrator (Shimadzu Corporation, Tokyo, Japan). Rods were scanned under the following conditions: hydrogen pressure, 0.8kg/cm^2; air flow, 1300 mL/min; scanning speed, 3.8 cm/sec. In order to allow sufficient time for peak area integration after each rod scan, we installed a push-button in the Iatroscan instrument, making it possible to stop the rod holder between successive scannings.

Lipid extraction. Total lipids were extracted from blood plasma and animal tissues (liver, heart) using successive solvent mixtures: $CHCl_3$/MeOH/30% aqueous NH_4OH (35:5:2, v/v/v), $CHCl_3$/MeOH (2:1, v/v) and $CHCl_3$/MeoH (1:2, v/v). Each solvent system contained 0.01% butylated hydroxytoluene as antioxidant. Tissue samples were homogenized in the solvents with an Ultra Turrax mixer (Janke & Kunkel, Staufen, Germany). The homogenates were centrifuged and the supernatants from each extraction step were combined. Subsequently, the total lipid extract was washed with 0.2% $CaCl_2$. The organic phase

*To whom correspondence should be addressed at Catholic University of Leuven, Laboratory of Nutrition, 92 Kard. Mercierlaan, B-3001 Leuven, Belgium.

Abbreviations: CL, cardiolipin; FID, flame ionization detector; LPC, lysophosphatidylcholine; PA, phosphatidic acid; PC, phosphatidylcholine; PDME, phosphatidyl-*N,N*-dimethylethanolamine, 1,2-dipalmitoyl-*sn*-glycero-3-phospho(*N,N*-dimethylethanolamine); PE, phosphatidylethanolamine; PI, phosphatidylinositol; PS, phosphatidylserine; SPH, sphingomyelin; TLC, thin-layer chromatography.

was separated by centrifugation and solvents were almost completely removed in a rotary evaporator at 35°C. Finally, the lipid residue was evaporated to dryness under a stream of nitrogen at 35°C. Dried lipids were redissolved in chloroform containing the internal standard (4 mg/mL). The internal standard was intended to reduce the variability in FID response due to differences between rods as well as to the quantity applied to the rods (9,11).

Chromarod development and scanning. Chromarods-SII were routinely stored in 4.5M H_2SO_4. Prior to use, the rods were washed with distilled water, dried at 110°C for 15 min and activated by passing through the flame of the Iatroscan detector. One μL of total lipids, containing the internal standard, was spotted on each rod using a 10-μL Hamilton syringe. To separate total phospholipids from neutral lipids, rods were developed in hexane/diethyl ether/formic acid (70:30:1, v/v/v), dried at 70°C, followed by a partial burn, leaving the polar lipid fraction unpyrolyzed. Thus, scanning was stopped when the peak of free cholesterol reached baseline. The phospholipids were resolved in subsequent steps, starting with impregnating the rods with oxalic acid. For that purpose, the rods were placed upside down in a development chamber containing 0.01 M oxalic acid in acetone and allowed to develop over their full length in direction of the lipid spot. The reason for developing the rods in the upside down position was that oxalic acid impregnation appeared to diminish in the direction of the moving solvent and that higher impregnation levels facilitated the separation of PI and PS (see Results and Discussion). Acetone was chosen because phospholipids do not migrate in this solvent. Next, the rods were developed twice in $CHCl_3$/MeOH/CH_3COOH/HCOOH/H_2O (80:35:2:1:3, v/v/v/v/v) with drying at 70°C for 10 min before each development. The slower migrating components LPC, SPH and PC were very well resolved; the other phospholipids moved to 1 cm from the top of the rod and were poorly resolved, however well separated from PC. After drying (70°C, 10 min) and partial burning to quantitate LPC, SPH and PC, rods were developed twice in $CHCl_3$/MeOH/30% aqueous NH_4OH (60:35:0.9, v/v/v) with drying (70°C, 10 min) after each development. The remaining phospholipids CL, IS, PE, PI, PS and PA were then scanned.

Calibration curves. Regression equations were calculated between the phospholipid amounts (x) spotted on the rods and the area ratios (y) between phospholipid and internal standard. Data were used from analyses of eight different loads, each load being analyzed with 12 different Chromarods-SII. Phospholipids were applied in the 0.4–20 μg range, depending on the nature of the phospholipid (Table 1). Regression analyses was done for the following functions: $y = a + bx$, $y = a + bx + cx^2$ and $y = a.x^b$ (12).

RESULTS AND DISCUSSION

In the procedure given in the Materials and Methods section, only the optimal conditions for Chromarod development and scanning are reported. However, various oxalic acid concentrations, solvent systems, single *vs* multiple development procedures and different ways of partial burns were tested. Figure 1 shows a typical phospholipid chromatogram obtained from the lipid extract of chicken liver.

TABLE 1

Linear Regression Parameters for Phospholipid Calibration Curves

Phospholipid[a]	Range (μg)	Linear calibration curve[b] slope	Y-intercept	Correlation (R^2)
LPC	0.4-9.2	0.119	−0.026	0.986
SPH	1.0-9.4	0.185	−0.184	0.969
PC	1.9-20.0	0.302	−0.347	0.986
CL	1.2-10.0	0.193	−0.140	0.992
PE	1.2-20.0	0.415	−0.510	0.981
PI	1.5-10.0	0.133	−0.025	0.986
PS	1.5-10.0	0.225	−0.082	0.988
PA	1.7-10.0	0.220	−0.221	0.996

[a]For abbreviations, see footnote to title.

[b]According to the equation: area ratio between phospholipid and internal standard = slope.μg phospholipid + y-intercept.

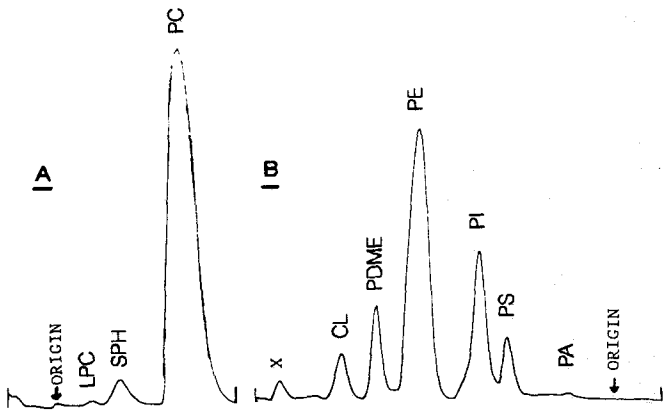


FIG. 1. TLC/FID separation of chicken liver phospholipids on Chromarods-SII impregnated with 0.01 M oxalic acid in acetone. A, first partial phospholipid scan after double development in $CHCl_3$/MeOH/CH_3COOH/HCOOH/H_2O (80:35:2:1:3, v/v/v/v/v). B, second partial phospholipid scan after double development in $CHCl_3$/MeOH/30% NH_4OH (60:35:0.9, v/v/v). For peak assignments, see abbreviations in footnote to title; X, probably monoglyceride. In A and B, scanning direction was from left to right.

Neutral lipids were eliminated from total lipid extract by rod development in hexane/diethyl ether/formic acid (70:30:1, v/v/v), followed by partial burning. The inclusion of hexane in the solvent system resulted in quick development and a greater difference in the retention times between free cholesterol and the polar lipid fraction, facilitating complete burning of the neutral lipids and leaving the phospholipids intact. However, this solvent mixture did not separate cholesteryl esters and triglycerides. Consequently, in case the neutral lipids are also to be determined, a less polar solvent system has to be used. Neutral lipids can be separated with the solvent system hexane/diethyl ether/formic acid (85:50:0.1, v/v/v).

Our experiments showed that oxalic acid impregnation of the rods improved peak shape, which is consistent with the observation of Banerjee *et al.* (6). However, we noted that the oxalic acid concentration in acetone is critical.

Concentrations lower than 0.01 M produced a non-symmetrical PS peak, while concentrations higher than 0.01 M gave rise to an additional spurious peak in front of CL. This extra peak was not a phospholipid, as demonstrated by spraying of the developed rod with molybdenum reagent (13), but had the same R_f value as a monoglyceride. Possibly, monoglycerides were not well separated from phospholipids in the first solvent system. The rods were impregnated upside down, with the remaining phospholipids on top, because impregnation in the other direction resulted in no baseline separation of PI and PS. The reason for this is not clear, but it may be due to the existence of an oxalic acid gradient along the rod. It was also necessary to perform oxalic acid impregnation just prior to the phospholipid separations. When the oxalic acid treatment was carried out after quantitation of LPC, SPH and PC, there was an insufficient separation between PE, PI and PS, while PDME and CL co-migrated. It has been suggested that oxalic acid may form a complex with the inorganic phospholipid moiety, improving phospholipid separation (6). If this were true, the formation of the complex may be pH-dependent, apparently being favored by acidic pH.

Satisfactory separations of phospholipids could not be achieved by using only one solvent system, even when the analyses were carried out with oxalic acid impregnated rods combined with repeated developments. This should not be surprising since also in ordinary TLC, two-dimensional procedures combined with two different solvent systems are the method of choice (13).

In general, neutral and acidic solvent mixtures gave satisfactory separations between LPC, SPH and PC, whereas the resolution of the remaining phospholipids was best achieved with basic solvent systems. The acidic solvent system was made rather polar in order to obtain baseline separation between LPC and SPH. In the composition of the basic solvent system the amount of NH_4OH was very critical. Using 0.5 instead of 0.9 volumes resulted in poor resolution of PS and PI. Both phospholipids remained separated up to 1.5 volumes of 30% NH_4OH, but then CL and the internal standard co-migrated. Increasing the NH_4OH content shifted the components towards the solvent front, except for PA.

Regression analysis revealed that for all phospholipids, linear as well as curvilinear calibration curves were obtained with excellent fits. For the linear regression lines, the coefficient of determination (R^2), the slope and y-intercept are presented in Table 1. The R^2 values for the curvilinear (data not tabulated) and the linear regressions were similar ($R^2>0.97$). The FID-response varied consistently among phospholipids, as indicated by the slope differences. In accordance with Hazel (7), the linear standard curves passed through the x-axis, the PE and PC curves showing the highest Y-intercept deviation. Thus the regression equations are not suitable for measurements of loads which are lower than the range studied. Consequently, the concentration of the lipid extract applied to the rods must be adjusted so that the quantity of each phospholipid is within the range for which its standard curve was calculated.

A drawback of the present procedure is that the Chromarods need to be impregnated each time before use. Also, the multiple developments are more time-consuming as compared to methods employing only a single developing solvent system. However, these disadvantages become less important in routine analyses, the more so since all phospholipids can be quantified by the present method.

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A Procedure for the Simultaneous Determination of Lipid and Protein in Biomembranes and Other Biological Samples

Felipe Rodríguez-Vico*, Marina Martínez-Cayuela, M. Flor Zafra, Eduardo García-Peregrín and Hilario Ramírez

Department of Biochemistry and Molecular Biology, University of Granada, 18071 Granada, Spain

Very small sample sizes frequently become the limiting factor in biochemical and biomembrane studies in which routine quantification of protein and bulk lipids are required. The procedure described here allows the simultaneous determination of protein and lipid without initial, multiple aliquots. The method is based on the quantitative precipitation of proteins from a defined hexane/isopropanol mixture. The liquid phase resulting after decanting and concentrating to dryness can then be used to assay the lipid content directly. Quantitative assay of protein can be achieved after resuspension of the pelleted material by addition of sodium dodecyl sulfate (0.1%) and deoxycholate (1%). The method is also applicable to other types of lipid- and protein-containing samples with a broad range of protein/lipid ratios and lipid compositions, as they occur, for example, in serum lipoproteins.
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The Lowry procedure is the most often used method for the routine assay of proteins (1). While the procedure was originally developed for working with soluble proteins, it is also extensively employed for the assay of hydrophobic proteins, such as those in membrane subcellular fractions.

When working with hydrophobic proteins, a number of difficulties can be encountered which can make it necessary to include a preliminary solubilization or delipidation step (1-3). Heat solubilization by holding samples at 37°C overnight, or at 100°C for 30 min, has been used by various investigators to dissolve protein from samples containing large amounts of lipid. Nevertheless, this treatment can give rise to significant errors due to lipid oxidation and subsequent reaction with the Folin-phenol reagent (4,5).

Delipidation methods based on acid precipitation and/or precipitation with organic solvents represent alternatives to achieve solubilization of protein from membrane preparations (3,6-8). These methods allow the removal of most lipids. Alternatively, the precipitation step involves protein concentration, which has been proven highly useful for very dilute samples (6-8). Lipid extraction with organic solvents can be used to determine proteins and lipids in the same sample aliquot simultaneously. The solvent mixture most frequently used to extract lipids is chloroform/methanol/water. However, this solvent system is not suited to precipitate proteins quantitatively prior to the Lowry assay (5,9,10).

The new procedure we describe in this communication lends itself very well to the determination of soluble as well as hydrophobic proteins and allows simultaneous extraction and determination of lipids on the same sample. The method is especially useful when very small volumes of membrane samples are to be analyzed for their protein and lipid contents.

*To whom correspondence should be addressed.

Abbreviations: DTT, dithiothreitol; HDL, high density lipoprotein; IDL, intermediate density lipoprotein; LDL, low density lipoprotein; SDS, sodium dodecyl sulfate; TCA, trichloroacetate; VLDL, very low density lipoprotein.

MATERIALS AND METHODS

Chemicals. HEPES, imidazole, dithiothreitol (DTT), sodium dodecyl sulfate (SDS), phospholipids (phosphatidylcholine from egg yolk), deoxycholate and leupeptine were purchased from Sigma Chemical Co. (St. Louis, MO); trichloroacetic acid (TCA), isopropanol and hexane were from Merck (Darmstadt, Federal Republic of Germany); Folin-Ciocalteu reagent was from Analema (Vigo, Spain). All other reagents used were of analytical grade.

Isolation of microsomes from chicken liver. White Leghorn male chicks (14-day-old) were used. Livers were removed, weighed, minced and homogenized with a motor-driven, all-glass Potter-Elvejhem homogenizer in 3 vol of 10 mM HEPES buffer, pH 7.4, containing 0.25 M sucrose, 50 mM NaCl, 20 mM EDTA, 2 mM EGTA, 40 mM leupeptine and 5 mM DTT. The broken cell preparation was centrifuged at 17,000 × *g* for 20 min. The resulting supernatant fraction was centrifuged at 105,000 × *g* for 60 min. The pellet containing microsomes was resuspended with a motor-driven mechanical Potter-Elvejhem homogenizer in 50 mM imidazole buffer, pH 7.4, containing 250 mM NaCl, 1 mM EDTA, 2 mM EGTA, 40 mM leupeptine and 5 mM DTT. The resuspended suspension was then centrifuged at 105,000 × *g* for 60 min to obtain a very clean microsomal fraction. All operations were done at 4°C.

Isolation of lipoproteins from chick plasma. Plasma from three-week-old chicks was used. Chicks were fed a standard diet (Sanders A-00) *ad libitum* from 0 to 14 days of age, followed by the standard diet supplemented with 20% coconut oil for seven days. Samples of plasma were adjusted to a density of 1.21 with solid KBr as described by Redgrave *et al.* (11). Ultracentrifugation was done on a density gradient according to the procedure of Hermier *et al.* (12). On completion of the centrifugation, fractions of 0.5 mL instead of 0.4 mL were collected from the bottom by using a peristaltic pump. This fractionation did not affect the final 2 mL at the bottom of the tube, which contained proteins almost exclusively and lacked lipids (12).

Procedure. An aliquot (50 μL) of the appropriate aqueous microsomal or lipoprotein fraction was added to 5 mL of hexane/isopropanol (3:2, v/v). Samples were vortexed and centrifuged at 4000 × *g* for 15 min. The liquid phase was decanted and concentrated to dryness under a stream of N_2. Pelleted protein was also dried under a stream of N_2 and then solubilized by addition of 200 μL of SDS (0.1%) and deoxycholate (1%) in NaOH (0.5 M). The amount of protein present was determined in each case by the standard Lowry method using 2.5 mL of Biuret reagent and 0.25 mL of Folin-Ciocalteu reagent

(1). Lipid phosphorus was determined by the method of Bartlett (13). Cholesterol and triglycerides were determined by enzymic colorimetric methods using test kits from Boehringer Mannheim GmbH (Mannheim, Federal Republic of Germany) with samples being dissolved in isopropanol.

Precipitation with trichloroacetic acid. Unless otherwise specified, an aliquot (500 μL) of trichloroacetic acid (20%) was added to 500 μL of sample. Samples were vortexed and centrifuged at 15000 × *g* for 5 min, and the supernatant was removed. The protein pellet was resuspended in water and assayed for protein content by the standard Lowry method (1).

RESULTS AND DISCUSSION

Quantitative analysis of the protein and lipid content of biological materials frequently requires an extraction procedure suitable for separating lipids from protein. Folch *et al.* (9) developed a procedure for the extraction of lipids based on a chloroform/methanol/water biphasic solvent system which has been used by numerous investigators (*e.g.*, 6,14–16). This method, however, was not optimized for determining proteins, and protein recoveries are normally lower than 80% (5,10). Additional drawbacks are encountered when working with subcellular fractions because of the small sample sizes which make it impossible to extract proteins without loss. All biphasic solvent systems that are in use seem to have the same disadvantage (10,14,15). Because monophasic methods have been shown to be more efficient for lipid extraction (16,17), we decided to use a single-phase solvent system in the present study.

Our method is based upon a precipitation step with a hexane/isopropanol mixture which allows separation of lipids from proteins. The solvent mixture, which is similar to mixtures suggested by others, has successfully been adapted and used for microsomal, mitochondrial and liposomal samples (14,18,19). After protein precipitation the supernatant is decanted to determine the lipid content, whereas the protein pellet is solubilized with SDS (0.1%) and deoxycholate (1%) in NaOH (0.5M) for the Lowry assay. Use of this solubilization mixture does not interfere with the Lowry method. In fact, we observed that use of the mixture can partially counteract the increase in absorbance at 640 nm produced by Triton X-100 or phospholipids when these are added before addition of the Biuret solution.

The purpose of the present procedure is not only to determine protein, but also to quantitatively extract lipids for assay. We have studied the effective ranges in which the method remains operative with respect to volume as well as lipid and protein concentrations.

As shown in Table 1, the assay of neither protein, nor phospholipid nor cholesterol was appreciably affected by the sample volume in the entire range assayed (5–50 μL). Standard errors of the mean for the protein, phospholipid or cholesterol values obtained were lower than 10%. We measured phospholipids and cholesterol in the organic phase because these typically constitute the bulk of membrane lipids and commonly require assay in biochemical studies.

When different concentrations of protein, phospholipid or cholesterol were measured in the same volume of sample (50 μL), the estimated concentrations were approximately linear in all the ranges assayed (Fig. 1).

TABLE 1

Effect of Sample Volume on Protein, Phospholipid and Cholesterol Determinations[a]

Volume (μL)	Protein (μg)	Phospholipid (μg)	Cholesterol (μg)
5	30.56 ± 1.08	1.508 ± 0.256	4.651 ± 0.141
10	32.54 ± 1.43	1.251 ± 0.049	4.250 ± 0.041
25	32.38 ± 0.58	1.279 ± 0.111	4.912 ± 0.496
50	33.19 ± 0.66	1.216 ± 0.061	4.530 ± 0.095

[a]Different volumes of samples ranging from 5 μL to 50 μL, but containing the same amount of microsomal protein, were assayed according to Lowry *et al.* (1) (28.22 μg). Phospholipid (1.32 μg) and cholesterol (3.64 μg) were measured after extraction by the Folch method (9) using the new procedure. Values are means ± SEM of five determinations.

Acidic precipitation is normally used on membrane preparations to eliminate multiple interferences and to separate proteins from lipids. This approach, however, does not guarantee quantitative recovery when used at protein levels lower than 5 μg (8). To demonstrate the higher efficiency of our procedure as opposed to the common acidic precipitation method, we have compared the operative ranges with regard to protein concentration and sample volume. With respect to the amount of protein, both methods showed an essentially linear relationship; nevertheless, values obtained by acidic precipitation were lower than those obtained by our new procedure (Fig. 2). Furthermore, the minimum amount of protein (1.88 μg) required was clearly higher for the acidic precipitation (5.51 μg) than for our procedure (1.64 μg). Likewise, values resulting from the acidic precipitation showed higher SEM at low protein concentrations, indicating a comparatively lower reproducibility. Standard deviations obtained for the new method were clearly lower.

Measurement of the same amount of protein at varying volumes of sample seemed to show poor efficiency of precipitation with trichloroacetic acid for volumes below 25 μL (Fig. 3). Precipitation with the hexane/isopropanol mixture was, by contrast, equally efficient over the entire range assayed.

In order to determine whether the method would also be applicable to the analysis of other types of lipid- and protein-containing samples within the range of protein/lipid ratios and for different lipid compositions in respect to phospholipids, cholesterol, cholesterol ester and triacylglycerol, chick lipoproteins were analyzed. Table 2 shows that the results obtained by direct measurement of each of the components (expected results) were quite similar to those obtained with our new method (measured results).

In summary, the new procedure reported here simplifies the assay of lipid and protein in membrane and other preparations, and permits their quantitative separation. The procedure allows the investigator to carry out the Lowry assay on the sedimented proteins, and to directly analyze the lipids present in the liquid phase. The method avoids the need for initial, multiple aliquots of the same sample for the separate measurement of proteins and lipids.

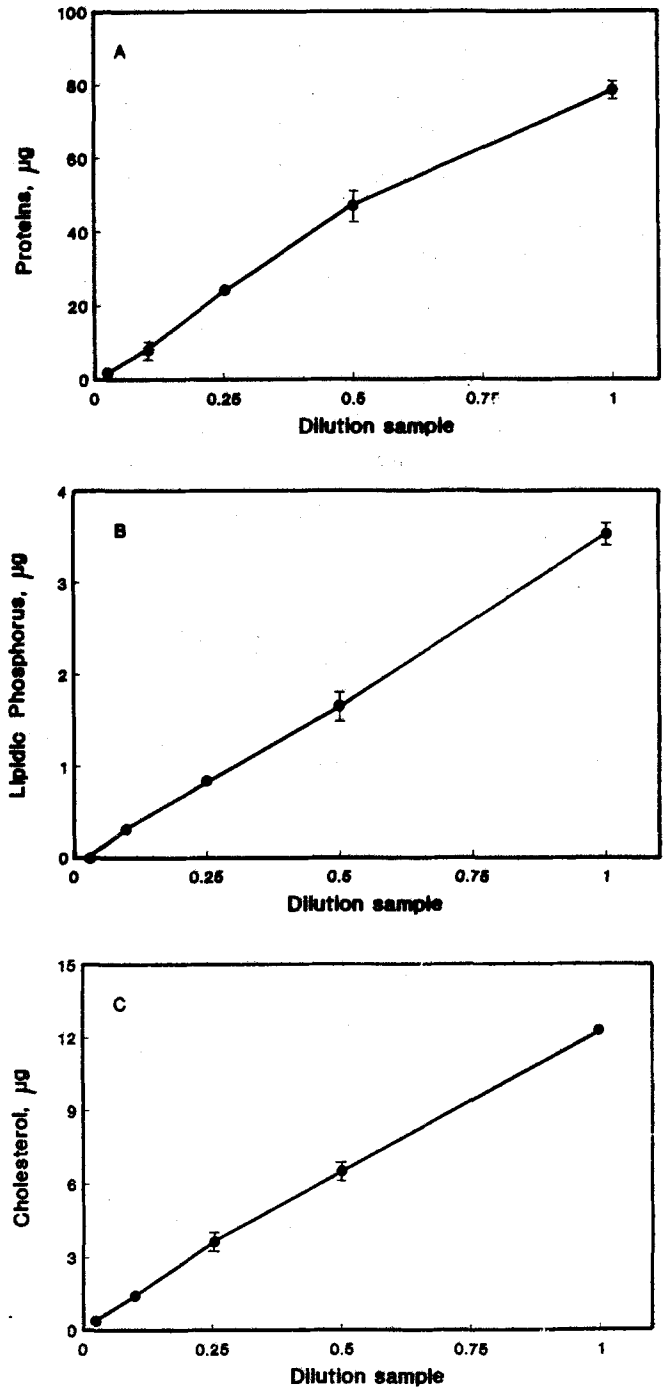
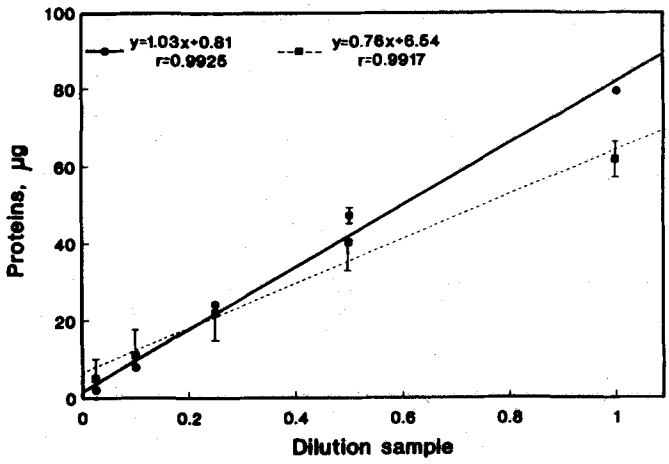


FIG. 1. Effect of the concentration of protein (A), phospholipid (B) and cholesterol (C) on the hexane/isopropanol precipitation method. A 50-µL sample containing different amounts of microsomal extract was assayed for protein, phospholipid and cholesterol using the new procedure. Values are means ± SEM of five determinations.



DIF. 2. Effect of protein concentration on the trichloroacetic acid and the hexane/isopropanol precipitation method for protein determination. A 50-µL sample containing different amounts of microsomal extract was assayed for protein using the new procedure (●), or the trichloroacetic acid precipitation method (■). Values are means ± SEM of five determinations.

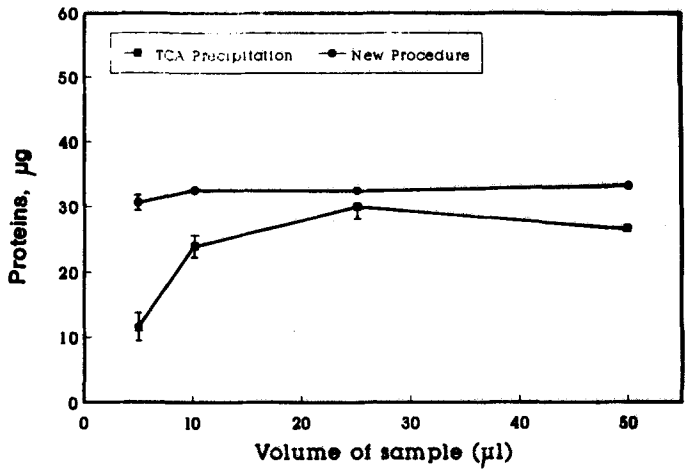


FIG. 3. Effect of volume of the sample on protein determination by the trichloroacetic acid and the hexane/isopropanol precipitation method. Different volumes of samples ranging from 5 µL to 50 µL but containing the same amount of microsomal extract, were assayed for protein using the new procedure (●), or the trichloroacetic acid precipitation method (■). Five hundred µL of 20% TCA were added to samples. Values are means ± SEM of five determinations.

TABLE 2

Plasma Lipoprotein Composition Determined by Different Procedures[a]

Component	Fraction	Expected (mg/mL plasma)	Measured (mg/mL plasma)
Cholesterol	HDL	1.579 ± 0.020	1.607 ± 0.028
	LDL	0.598 ± 0.015	0.580 ± 0.018
	IDL	0.055 ± 0.005	0.057 ± 0.005
	VLDL	0.111 ± 0.006	0.093 ± 0.005
Triglyceride	HDL	0.179 ± 0.010	0.185 ± 0.005
	LDL	0.108 ± 0.007	0.108 ± 0.006
	IDL	0.028 ± 0.004	0.025 ± 0.004
	VLDL	0.059 ± 0.004	0.055 ± 0.003
Phospholipid	HDL	2.498 ± 0.091	2.565 ± 0.096
	LDL	0.936 ± 0.010	0.781 ± 0.011
	IDL	0.090 ± 0.008	0.102 ± 0.012
	VLDL	0.059 ± 0.006	0.047 ± 0.009
Protein	HDL	2.186 ± 0.120	2.392 ± 0.111
	LDL	0.455 ± 0.021	0.365 ± 0.025
	IDL	0.033 ± 0.005	0.040 ± 0.006
	VLDL	0.093 ± 0.007	0.100 ± 0.010

[a]Different lipoprotein fractions were analyzed for cholesterol, triglyceride, phospholipid and protein contents by direct determination using the classical methods (expected results) or by the new procedure (measured results). Values are means ± SEM of three determinations.

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Absence of Sterol Biosynthesis in Oyster Tissue Culture

Marcia J. Holden[1] and **Glenn W. Patterson***
Department of Botany, University of Maryland, College Park, Maryland 20742

An oyster heart tissue culture was used to investigate the ability of the American oyster (*Crassostrea virginica*) to synthesize sterols from [^{14}C]acetate. Lipids were extracted from cultured oyster heart cells and no label was found in the purified fatty acid fraction, 90% of which was located in the saturated and monounsaturated fractions. Although these results cannot rule out the possibility of sterol synthesis in other oyster tissues, this and previous evidence are in support of the hypothesis that the American oyster is unable to synthesize sterols.
Lipids 26, 81-82 (1991).

The oyster has long been one of the most difficult of the challenges to the sterol biochemist. Approximately forty sterols have been identified from the oyster, *Crassostrea virginica* (1-3). Since it appeared that many of the sterols identified must be of dietary origin, the ability of the oyster to incorporate injected radioactive acetate was studied (4). Of the forty sterols naturally occurring in the oyster, only four were labeled by injection of labeled acetate: cholesterol, desmosterol, 24-methylene-cholesterol, and fucosterol. Most animals synthesize sterols, but the sterols produced are usually of the C-27 series only. Perhaps it is coincidental that aquatic fungi frequently produce the same four sterols (5-8), that were labeled in the oyster study (4). It is not possible at present to grow oysters aseptically. However, some success has been attained with the development of an oyster tissue culture. It was the objective of this work to determine whether actively metabolizing oyster tissue culture cells can convert acetate into sterols.

MATERIALS AND METHODS

Oyster heart tissue culture. The procedure for oyster heart tissue culture was adopted from Dr. Frank Hetrick (personal communication). Hearts were dissected from the pericardial sac of oysters (*Crassostrea virginica*), minced and washed with an antibiotic solution (125 μg/mL gentamicin sulfate, 100 μg/mL polymixin B sulfate, 200 U/μL penicillin, 200 μg/mL streptomycin, 100 U/mL mycostatin, 1% glucose, 1% galactose in filtered sea water) for 2 hr. This was followed by rinses in 1% zephalin chloride to eliminate possible protozoan contamination and digestion of the tissue with 0.05% trypsin for 10 min. Between treatments the heart pieces were washed with 1/2 strength sea water (pH 7.0) containing 1% each glucose and galactose. All solutions and media were filter-sterilized (0.2 μm Millipore filter). Heart pieces were transferred to wells of a culture dish each containing 2.5 mL of medium. The medium consisted of 40% NCTC-135 medium (Grand Island Biologicals, Grand Island, NY), 10% fetal calf serum, 47% 1/2 strength sea water, 1 mg/mL glucose, 1 mg/mL galactose, 1 mg/mL trehalose, 50 U/mL penicillin-streptomycin, 100 mg/mL gentamicin, 0.0025% phenol red, and 10 mM piperazine-*N,N'-bis*(2-ethanesulfonic acid) (PIPES), pH 7.0. Cultures were incubated at 11°C and the medium was changed every 3-5 days. The cultures were labeled after 7 to 10 days, when a confluent layer of cells was observed to cover the bottom on the 24-well dish. A total of 8-10 μCi of [^{14}C]acetate was added to the culture medium in this dish and incubation continued for 65 hr.

Lipid extraction. Tissue pieces were scraped from culture wells, collected on filter paper and washed. A crude lipid fraction was prepared by saponifying the tissue followed by acidification and extraction, twice with diethyl ether and then chloroform. Unlabeled oyster sterols and myristic acid were added to the combined crude lipid extract which was then chromatographed, along with standards, on a silica gel 60 plate using a solvent system of hexane/diethyl ether/acetic acid (80:20:1, v/v/v). The plate was developed twice and components were visualized with 2,7-dichlorofluorescein. Fatty acids and sterols were separately eluted from the silica gel with diethyl ether.

Chromatographic separation of fatty acids and sterols. Sterols were rechromatographed as above. This was followed by chromatography on an alumina column and elution of sterols with diethyl ether (4). Sterols were subsequently acetylated using pyridine/acetic anhydride (1:1, v/v), and the product was applied to an alumina column and eluted with hexane/benzene (1:1, v/v). At each step, the sterol fraction was identified using a Varian 3700 Gas Chromatograph (3% SE-30 column, 246°C) by comparing the relative retention times of the major sterol components with those of standards (1-3,9). Radioactivity was determined by scintillation counting.

Fatty acids were converted to methyl esters with

TABLE 1

Label Present in Fractions During Sterol Purification

Fraction	Purification step[a]	DPM
Total lipid	extraction	623700
Sterol	thin-layer chromatography	1647
Sterol	alumina column	1465
Sterol acetate	alumina column	0

[a]For details, see Materials and Methods.

[1]Present address: Foreign Disease-Weed Science, USDA Agricultural Research Service, Ft. Detrick, Frederick, MD 21701.
*To whom correspondence should be addressed.

Abbreviation: PIPES, piperazine-*N,N'-bis*(2-ethanesulfonic acid).

TABLE 2

Label Present in Fractions During Fatty Acid Purification[a]

Fraction	Purification step	DPM
Total lipid	extraction	623700
Fatty acid	thin-layer chromatography	140233
Fatty acid methyl ester	extraction	115099
Fatty acid methyl ester	alumina column	62059
Fatty acid methyl ester	$AgNO_3$ thin-layer chromatography	
Fraction 1—3 double bonds		402
Fraction 2—2 double bonds		3041
Fraction 3—0-1 double bonds		34698

[a]Data from the same experiment shown in Table 1.

10% boron trichloride/methanol. Fatty acid methyl esters were applied to an alumina column and eluted with hexane/benzene (1:1, v/v). Separation of the fatty acid methyl esters, based on type and number of double bonds, was accomplished by silver nitrate thin-layer chromatography (15 g $AgNO_3$/100 g silica gel 60) using 25% diethyl ether in hexane (v/v) as the developing solvent system. A known mixture of unlabelled fatty acids was added as carrier to the oyster fatty acids prior to chromatography to provide sufficient material for determining the double bond composition of the separated fractions. At each step, fatty acids were identified using a Varian 3700 gas chromatograph (15% Hi-EEF-1BP column, 160°C), and radioactivity was determined by scintillation counting as previously described (4).

RESULTS AND DISCUSSION

Previous results giving positive evidence of a low rate of synthesis of four oyster sterols were considered due to probable fungal contamination of wild oysters. An oyster tissue culture system, developed by Hetrick and co-workers (personal communication) was used to resolve the problem of contamination. While several oyster tissues have been cultured successfully, the heart tissue was chosen because it is initially free of microorganisms while enclosed in the pericardial sac. Heart tissue, exposed to potential contamination only during dissection, requires less drastic antibiotic and fungicidal treatment than other oyster tissues. The disadvantage of the oyster tissue culture is that the cells that form the confluent layer are mostly amoebocytes which migrate from the tissue pieces. The cultures can be maintained in a metabolically active state for up to a year, but show no real evidence of cell multiplication (Hetrick and co-workers, personal communication).

Between 3-4% of the total [^{14}C]acetate provided to the cultured cells was recovered in the crude lipid extract from the cells (Table 1), but only a very small fraction of that was left in the first sterol fraction. Acetylation of the sterol fraction, followed by chromatography on alumina, produced a sterol acetate fraction which was devoid of label. Analysis of the fatty acid components of the labeled crude lipid extract showed that 22% of the label in the total lipid was in the crude fatty acid fraction (Table 2). Subsequent separation of fatty acids, based on the number of double bonds, revealed that most of the label was in the saturated and monounsaturated fatty acids. Thus, oyster tissue culture is capable of synthesizing fatty acids which shows that biosynthetic activity is maintained in cell culture.

Previous work demonstrated the synthesis of sterols from acetate in intact oysters (4). The present results suggest that the incorporation of labeled acetate into four labelled sterols in intact oysters was likely due to contamination of the oysters by aquatic fungi which have been shown to contain the same series of sterols labelled in the intact oyster (5-8). Although the results of this study do not rule out the possibility of synthesis of sterols by other oyster tissues, it adds strength to previous data suggesting that the oyster cannot synthesize sterols from acetate.

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Enhanced β-Oxidative Utilization of [1-^{14}C]Palmitate During Active Myelinogenesis in Developing Rat Brain Under Nutritional Stress

S. Padmini and **P. Srinivasa Rao***
National Institute of Nutrition, Indian Council of Medical Research, Jamai Osmania P.O., Hyderabad 500 007, India

Food restriction was found to impair the incorporation of [1-^{14}C]palmitate into myelin membrane lipids of developing rat brain. An attempt was made to determine whether this phenomenon is due to differences in the rate of utilization of the labelled precursor or to its enhanced degradation *via* β-oxidative pathways. Undernutrition in pups was imposed by food restriction during gestation and lactation. β-Oxidation by brain region homogenates using [1-^{14}C]palmitate was monitored at days 7, 14 and 21 of postnatal age. There was a significant increase in β-oxidation in the brain regions of undernourished pups, with the cerebrum and cerebellum being more affected than the brain stem. Because developing brain possesses the enzymic potential to utilize ketone bodies, the data may indicate increased usage of palmitate as an energy source in the developing brain of undernourished animals.
***Lipids 26,* 83–85 (1991).**

Myelinogenesis in the developing nervous system is affected by diet and responds to subnormal nutriture with certain dietary components (1,2). Protein caloric restrictions that are imposed during gestation and lactation were found to significantly impair incorporation of [1-^{14}C]palmitate-associated radioactivity into myelin membrane phospholipids and cerebrosides (3) in young rat pups. It is known that fatty acids can serve as fuel to meet the energy requirements of the brain in suckling rats (4,5). The studies of Spitzer (6) revealed that brains of adult dogs do not utilize free fatty acids *in vivo*, but that in the brains of starved puppies up to 25% of the oxygen consumption is accounted for by utilization of palmitate. We hypothesized that a greater proportion of palmitate produced *in situ* might be used as an energy source rather than for the biosynthesis of lipids, *viz.* phospholipids and cerebrosides, in undernourished, growing rat brains.

The present report describes the capacity of various regions of the developing rat brain to degrade palmitate by β-oxidation. The results affirm that under conditions of food restriction, the rapidly developing brain displays a significantly higher potential for routing fatty acids into energy-generating pathways.

MATERIALS AND METHODS

[1-^{14}C]Palmitate (sp. act. 20.5 mCi/mmol) and chemicals for scintillation counting were purchased from BARC (Bombay, India). Radiopurity as assessed by thin-layer chromatography (TLC) analysis and scintillation counting was 95–98%. Solvents used were of analytical grade. Substrates and cofactors for assays were obtained from Sigma Chemical Co. (St. Louis, MO).

Undernutrition was imposed by food restriction during gestation and lactation. Ten-day pregnant Wistar strain rats fed a 22% protein diet *ad libitum* (adequate with respect to all other essential nutrients, *i.e.*, fats, carbohydrates, vitamins, and minerals) were used as controls, while animals fed 50% of the diet consumed by controls (measured as 50% of the previous day's control intake) were treated as the experimental (undernourished) group. Pups nursed by these two groups of dams were considered controls and experimentals, respectively. Litters were culled to a size of 8 at birth. Pups were used at 7, 14 and 21 days of postnatal age, in order to bracket the period of myelinogenesis.

The rats were decapitated by guillotine, while care was taken not to damage nervous tissue. The surface of the brain was washed free of blood in saline and blotted on filter paper. The time from decapitation to homogenization was kept constant. Brain tissues from pups nursed by control and food-restricted dams were pooled. Each pooled sample had 8 whole brains at day 7, whereas 4 cerebra or 8 cerebella or 8 brain stems were pooled at day 14 and day 21 per sample. Six whole brains or regional tissue samples were homogenized in 20 vol of 25 mM (hydroxymethyl)aminomethane (Tris)-HCl/1 mM ethylenediaminetetraacetic acid (EDTA) 0.32 M sucrose (pH 7.4) buffer. The crude homogenates were centrifuged at 800 × *g* for 10 min at 4°C (after reserving an aliquot of the homogenate in chloroform/methanol, 2:1, v/v, for extraction of lipids).

β-Oxidation of [1-^{14}C]palmitate was monitored according to the method by Menon *et al.* (7) using the 800 × *g* supernatant. Lipids of aliquots of brain region homogenates were extracted (8), and the nonpolar lipids were resolved by TLC on silica gel G using the solvent system petroleum hydrocarbon (40–60°C bp)/diethyl ether/acetic acid (80:20:1, v/v/v). The bands corresponding to free fatty acids were visible under ultraviolet (UV) light after spraying with 2′,7′-dichlorofluorescein. Bands were scraped off, and fractions were eluted with chloroform containing 0.22% formic acid. The eluates were taken to dryness and methylated (9). The methyl esters were resolved using a Varian Model 3700 gas chromatograph equipped with a flame ionization detector (FID). The stainless-steel chromatographic column (3 m length × 2mm i.d.) packed with 10% Silar 10c on Chromosorb-W was kept at 190°C. Nitrogen was used as carrier gas at a flow rate of 20 mL/min. Weight percentages of methyl palmitate in total fatty acid methyl esters were computed, using

*To whom correspondence should be addressed.

Abbreviations: EDTA, ethylenediaminetetraacetic acid; FID, flame ionization detector; TLC, thin layer chromatography; Tris, (hydroxymethyl)aminoethane; UV, ultraviolet light.

TABLE 1

Palmitate Pool Sizes in Free Fatty Acid Fractions from Different Regions of Developing Brain[a,*]

Age in days	Whole brain		Cerebrum		Cerebellum		Brain stem	
	C**	E**	C	E	C	E	C	E
7	11.69 ±1.186[b] (8.96)	16.11 ±1.651 (9.57)	—	—	—	—	—	—
14	—	—	13.75 ±0.531 (11.05)	19.08 ±0.323 (11.68)	11.28 ±1.032 (9.06)	14.93 ±0.409 (9.16)	10.93 ±0.710 (8.78)	15.38 ±1.412 (9.43)
21	—	—	4.83 ±0.294 (9.62)	6.59 ±0.511 (9.61)	4.91 ±0.922 (11.77)	7.16 ±0.631 (10.42)	4.36 ±0.647 (8.86)	5.94 ±0.983 (8.70)

[a]Expressed as μmoles methyl palmitate/g brain tissue. Values in parentheses indicate palmitate pool sizes as weight percentages of total free fatty acid methyl esters.
[b]Mean ± SE of six observations in each case.
*Free fatty acids fractions were separated by silica gel G TLC (see Materials and Methods).
**C—Control, E—Experimental.

an electronic integrator, based on the peak areas of known amounts of standard fatty acid methyl esters. The data on the concentration of free palmitate pools are expressed as μmoles/g brain tissue.

RESULTS AND DISCUSSION

It has been shown (10) that protein deficiency influences lactation quantitatively, but not qualitatively. Although no attempt was made in this study to measure milk output of lactating dams, body weights in experimental pups were found to be significantly lower (62% and 40% of controls at 14 and 21 days, respectively). Brain and brain region weights were less affected with 80–90% of control weights being reached at both these ages.

Quantitation of the free palmitic acid pool in brain tissues of control and experimental pups showed no difference between the two groups when expressed as weight percentages of total free fatty acid methyl esters. However, experimental rats showed relatively higher concentrations of palmitate pools (in terms of μmols/g wet weight of brain tissue) especially in the three regions of the brain of experimental rats on day 14 (Table 1). The β-oxidative degradation profiles of [1-^{14}C]palmitate, as reflected by $^{14}CO_2$ liberation, however, showed large differences between control and feed-restricted animals (Fig. 1). The rate of β-oxidation of palmitate in whole brain from experimental animals at day 7 was 142% of that in controls. At day 14, β-oxidation of palmitate in experimental cerebra, cerebella and brain stems was 283%, 197% and 323% of that in corresponding control regions, while by day 21 β-oxidation in experimental cerebra and cerebella reached 334% and 289% of corresponding control values ($P<0.001$). On the other hand, the β-oxidative capacity in control cerebra and cerebella at day 21 was lower than at day 14, with 78% and 68% at 14 days. This age-related decrease was not observed in brain tissues from feed-restricted pups.

Growth and development during the suckling period appears to place an exceptional demand for appropriately constituted nutrient sources. Fat as a major carbon source in rat milk appears to be earmarked to contribute to energy needs next to providing building

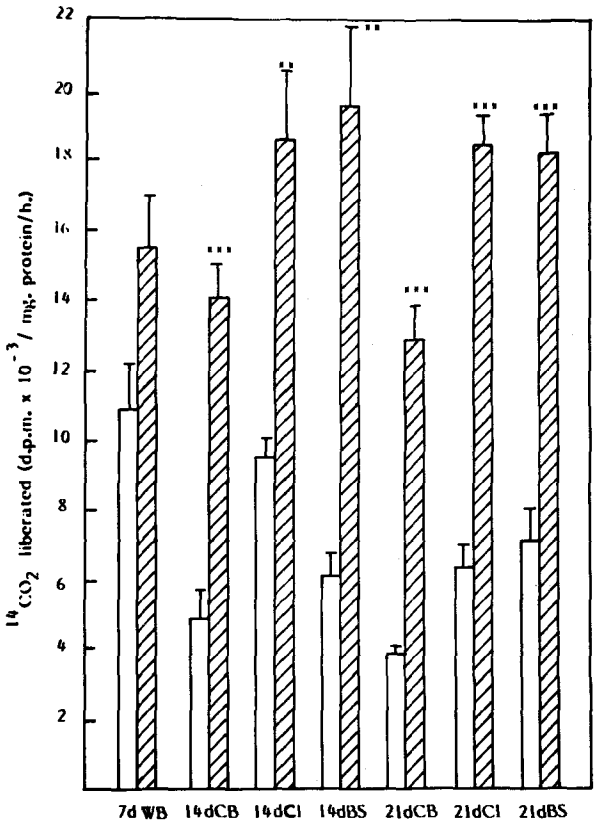


FIG. 1. β-Oxidation of [1-^{14}C]palmitate in developing brain regions from control *vs* feed-restricted animals. Open bars, control animals; hatched bars, undernourished animals. WB, whole brain; CB, cerebrum; Cl, cerebellum; BS, brain stem. Pair differences were assessed by Student's t-test. *P<0.05; **P<0.01; ***P<0.001.

units for lipid metabolism. Acetoacetate and 3-hydroxybutyrate, which are transition metabolites in the conversion of fat into energy and serve as substrates for lipogenesis, are produced by ketogenesis in liver mitochondria by fatty acid oxidation. The liver produces ketone bodies for export of acetoacetate and 3-hydroxybutyrate, but does not use them for respiration as precursors of mitochondrial acetyl-CoA (11), while the developing brain possesses the enzymic potential to do so.

Under the conditions of feed insufficiency imposed on growing rat pups, it is reasonable to speculate that subnormal intake of milk in such animals would affect the supply of fat to hepatic tissue and thereby diminish the levels of circulating ketone bodies. By inference, this could diminish fatty acid synthesis in the developing brain, which in turn, could down-regulate complex myelin membrane lipid synthesis.

The distinctly higher β-oxidative capacity of the brains of undernourished animals is indicative of an attempt by the rapidly growing brain to compensate for the deficit in energy substrates (milk-derived fats), albeit at the expense of lipogenesis. Another interesting observation (12) is the ability of ketone bodies at high concentrations to impair the *de novo* pathway of pyrimidine biosynthesis. Thus, higher rates of β-oxidation could be expected to impair rates of fetal brain growth under conditions of nutritional stress.

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The Effect of a Salmon Diet on Blood Clotting, Platelet Aggregation and Fatty Acids in Normal Adult Men

Gary J. Nelson[a,*], **Perla C. Schmidt**[a] and **Laurence Corash**[b]

[a]Western Human Nutrition Research Center, U.S. Department of Agriculture, ARS, Presidio of San Francisco, California 94129 and [b]Department of Laboratory Medicine, University of California School of Medicine, San Francisco, California 94143

This study was designed to measure the effect of dietary n-3 fatty acids (FA) on platelets and blood lipids. Healthy men (n=9), ages 31 to 65, were fed diets in which salmon was the source of n-3 fatty acids. They were confined in a nutrition suite at this Center for 100 days. Food intake and exercise levels were rigidly controlled. Initially they were placed on a stabilization diet for 20 days, then six men were fed the salmon diet for 40 days. The others remained on the stabilization diet. The two groups switched diets for the last 40 days of the study. Both diets were isocaloric [16% protein, 54% carbohydrate, and 30% fat by energy-% (En%)]. The salmon diet contained 7.5% of calories from n-6 FA and 2% from n-3 FA, primarily eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA) in a 40:60 ratio, while the stabilization diet contained 7.5% of calories from n-6 FA and less than 0.3% n-3 FA, mainly 18:3n-3. The bleeding time was unaffected by the diets in this study. The prothrombin time was shortened (11.6 sec. *vs.* 12.6 sec., $p < 0.01$) for the subjects consuming the salmon diet as compared to that measured after 20 days of the stabilization diet. Mean platelet volume increased significantly during the period in which the volunteers consumed the salmon diet compared to the baseline diet ($p<0.01$), while the mean platelet levels decreased. Platelet aggregation (PA) was measured in platelet rich plasma before, during, and after the salmon diet using collagen, ADP, arachidonic acid (AA), and thrombin agonists. The PA threshold for ADP was significantly increased for the subjects on the salmon diet ($p < 0.05$). No change in the PA threshold was detected for collagen or thrombin. The PA threshold for AA was unchanged also, but the platelets in subjects consuming the salmon diet had a prolonged time to maximum aggregation ($p<0.01$) with this reagent compared to platelets from men on the stabilization diet. Plasma, red cell, and platelet total FA composition was determined by capillary GLC. While the men consumed the salmon diets, there were marked increases (3 to 10-fold) in the EPA and DHA levels in all blood components with concomitant decreases in linoleic acid and AA levels. Thus, a salmon diet, high in n-3 FA, did not influence the bleeding times, but it decreased the sensitivity of platelets to ADP and AA, increased the mean platelet size, decreased the platelet count, and changed the FA composition of the plasma, RBC and platelet membrane lipids.

Lipids 26, 87-96 (1991).

Epidemiological studies have suggested that populations consuming fish regularly tend to have a lower incidence of morbidity and mortality from heart disease (1-4). Purified fish oils, usually consumed as a supplement to an *ad libitum* diet, influenced human blood clotting parameters in several previous studies (5-8). The hypothesis accepted by most investigators is that the long-chain, polyunsaturated n-3 fatty acids found in the marine food chain are the beneficial nutrient in a diet containing large amounts of fish or other foods of marine origin. Few studies have been reported, however, in which fresh fish was the only source of dietary n-3 fatty acids, although van Houwelingen *et al.* (8) and Singer and associates (9) have used canned fish instead of fish oil supplements. Goodnight *et al.* (10) used fresh salmon combined with a salmon oil supplement in a study with free-living volunteers eating meals prepared in a dietetic kitchen.

The beneficial effects of a fish diet or fish oil supplement is still controversial (11-15), and the mechanism by which a fish diet or fish oil supplement provides its purported beneficial effect is unproven (16-19). The main supposition is that the n-3 fatty acids incorporated in the platelet membrane reduce the tendency for platelets to aggregate *in vivo* (20,21) perhaps through alteration in the production of pro-aggregating eicosanoids (22,23). Even if dietary fish or fish oil have beneficial health effects, it is not practical to recommend that the public take fish oil supplement. Consuming fresh fish is a reasonable alternative, if the fish have a reasonable oil content and are oceanic varieties, not aquacultured unless the aquacultured species are fed n-3 fatty acids.

In an attempt to determine if short-term feeding of a moderate amount of fresh fish to normal subjects would produce similar effects as reported when subjects consumed large amounts of pure fish oils, a carefully controlled dietary study was designed. The study was intended to find, among other items, the effects of a fish diet on *in vivo* and *in vitro* hemostatic parameters and to correlate those changes with corresponding changes in the fatty acid composition of the blood.

Results of this study are reported here and in the companion paper (24). We fed normal, healthy, male volunteers a diet containing approximately 500 g daily of fresh salmon for 40 days. The volunteers were confined to a Nutrition Suite for the entire period of the study (100 days) and consumed only food prepared in the metabolic kitchen. All exercise and activity was rigidly controlled. Hemostatic parameters, *in vitro* platelet aggregation and fatty acid composition of plasma, red cells and platelets were analyzed at regular intervals during the study.

MATERIALS AND METHODS

Materials. All natural foods were purchased at local food markets. The salmon (Pacific, chinook, *Oncorhynchus tshawytscha*) was purchased fresh from a seafood wholesale supply company as a large batch from a single boat.

*To whom correspondence should be addressed.

Abbreviations: AA, arachidonic acid; ADP, adenosine diphosphate; DHA, docosahexaenoic acid; DPA, docosapentaenoic acid; EPA, eicosapentaenoic acid; FA, fatty acid; GLC, gas-liquid chromatography; PA, platelet aggregation; PPP, platelet poor plasma; PRP, platelet rich plasma; RBC, red blood cells; RDA, recommended daily allowance.

It was frozen until used for the meal preparation during the study. Fatty acid standards were purchased from Nu-Chek-Prep (Elysian, MN). Aggregating agents [adenosine diphosphate (ADP), collagen, thrombin, arachidonic acid (AA)] were obtained from Chrono-log Corporation (Havertown, PA). Organic solvents were obtained from Baxter Scientific, Burdick and Jackson Brand (McGaw Park, IL).

Subjects. The volunteers were recruited from the West Coast, and consisted of men between the ages of 30 and 65. Table 1 gives the physical characteristics of the final nine volunteers who completed the study. Initially twelve volunteers were included in the study, but three were unable to complete the protocol. The volunteers were given complete physical examinations. Body weights had to be within −10 to +10% of ideal body weights using the Metropolitan Life Insurance Company tables (Medium frame values from the 1983 edition). Evidence of existing illness or chronic disease was an exclusion criterion. Mild hypertesion was not an exclusion criterion, but the group that was recruited tended to have blood pressures slightly below average for men in this age group. Smoking, excessive alcohol consumption or evidence of narcotic abuse were also exclusionary.

Experimental design. All the volunteers were confined to the Nutrition Research Suite of the Western Human Nutrition Research Center for the duration of the study. A diagram of the study design is given in Figure 1. A crossover design was used. The subjects were fed a stabilization diet, without 20-carbon, polyunsaturated n-3 fatty acids for 20 days. On day 21 of the study, they were divided into two groups. The first was designated group A and placed on the salmon diet for 40 days. The other group, designated Group B, remained on the stabilization diet. After forty days, day 61 of the study, the groups switched diets for the remaining 40 days of the study.

TABLE 1

Characteristics of the Subjects upon Entry into the Study (All Males)

Parameter	Values
Subjects completing study	9
Number in group A	6
Number in group B	3
Age, yrs (n=9) (mean ± S.D.)	52.0 ± 12.7
Weight, kg (n=9) (mean ± S.D.)	84.0 ± 12.5
Body mass index, Kg/m^2 (n=9) (mean ± S.D.)	24.79 ± 2.33
Blood pressure, systolic (n=9) (mean ± S.D.)	118.5 ± 10.3
Blood pressure, diastolic (n=9) (mean ± S.D.)	78.9 ± 5.4
Smokers	None

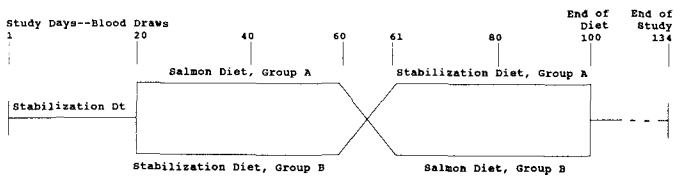


FIG. 1. A schematic presentation of the design of this dietary study showing the days on which blood samples wre drawn and the various dietary periods.

After 100 days, all the volunteers were discharged from the Nutrition Suite and returned to an *ad libitum* diet. They returned to the Center on day 134 of the study for a follow-up visit and final blood draw. During the confinement period of the study, there was a blood draw every 20 days. Statistical comparisons of the measured parameters were made using day 20 as the baseline values for each subject. Subjects served as their own controls.

Diets. The diets consisted of natural foods. No dietary supplements were given except α-tocopherol to bring the diet to 200% of the RDA for this vitamin. The macronutrient composition of the diets is given in Table 2. (A complete description of the diets, listing all the major and minor nutrients is available upon request.) There was a five-day menu cycle used throughout the study. The proximate analysis was made on five individual diet composite samples taken on each menu once during the study for both the stabilization diet and salmon diet diets. The results for the five composite samples were averaged to find the actual composition of the diets. No alcohol was included in these diets. The diets were designed around a prudent diet concept of 30:55:15 fat, carbohydrate, protein in energy percent (En%). The nutrient composition of the diet was calculated from a computerized nutrient data bank, and adjusted to provide at least the RDA for known essential nutrients.

Table 3 gives the measured fatty acid composition of both diets. The amount of saturated and n-6 fatty acids were held constant in these diets. The stabilization diet had less than 1% n-3 fatty acids as α-linolenic acid and approximately 50% monounsaturated fatty acids. The salmon diet had approximately 7.5% long chain polyunsaturated n-3 fatty acids and concomitant reduction in the level of monounsaturated fatty acids.

At the beginning of the study, each participant's caloric intake was estimated, and the caloric content of portions calculated to maintain current body weight. As the estimates were subject to error, the participants were weighed every day, and the caloric value of their meals adjusted daily, if necessary, to maintain their weight within the design limits of the study. Thus, the average weights of the participants did not vary significantly

TABLE 2

Composition of the Diets. Average Values from Diet Composite Samples (n=5)

	Stabilization diet (n-6)		Salmon diet (n-3)	
Dietary component	Calculated	Measured	Calculated	Measured
Protein[a]	16.41	18.83	16.02	18.53
Carbohydrate[a]	54.49	54.77	54.89	56.82
Fat[a]	29.00	26.20	29.09	24.67
Cholesterol, mg	321	309	321	335
Fatty acid distribution	Wt%	En%	Wt%	En%
Saturated	25	7.5	25	7.5
Monoenes	50	14.7	43	12.9
n-3 Polyunsaturated	<1	0.3	7	2.1
n-6 Polyunsaturated	25	7.5	25	7.5

[a]Values listed as energy percent of calories.

TABLE 3

Fatty Acid Composition of Actual Diets used in Stabilization (n-6) and Salmon (n-3) Diets

Fatty acid	Stabilization diet mean value	Salmon diet mean value
14:0	1.1	1.2
16:0	18.0	16.8
16:1n-7	1.2	2.1
18:0	7.0	5.6
18:1n-9*trans*	6.4	6.2
18:1n-9	33.6	24.3
18:1n-7	2.0	2.1
18:1n-5	—	1.4
18:2n-6	21.2	23.0
18:3n-3	1.1	1.1
20:1n-11	—	0.8
20:1n-9	0.1	1.0
20:4n-6	0.2	0.6
20:5n-3	—	2.0
22:0	0.3	—
22:5n-3	—	0.7
22:6n-3	—	2.8
Sum of identified fatty acids[a]	97.0	96.6
Subtotals,		
Saturated	26.5	23.6
Monounsaturated	43.2	37.9
Polyunsaturated, n-6	21.4	23.6
Polyunsaturated, n-3	1.1	6.7
n-3/n-6 ratio	0.1	0.3

[a]The listed components will not add to this total as the diet samples contained many minor components that are not shown here, but their contributions to the total recovery are included in the totals given on this line.

during the 100 days of the study. If all the intakes for each volunteer were averaged during the entire study, the mean energy intake was approximately 2800 Kcal/day.

As no two subjects ate the same amount of salmon each day the amount of n-3 fatty acids is best expressed in terms of percent of calories per day rather than grams of n-3 fatty acids consumed per day. Individuals who take fish oil capsules usually consume a set number of capsules per day, and, thus, the dosage varies from individual to individual depending on their specific weight. In this study each volunteer received 2.1% of their calories from long chain, n-3 polyunsaturated fatty acids. If, for example, an individual in this study was receiving 3000 Kcal/day, he would have consumed about 2.1 g of 20:5n-3, 0.8 g of 22:5n-3 and 3.0 g of 22:6n-3. This amounts to a total of about 6 g/day of n-3 polyunsaturated fatty acids.

Measurements. Blood was drawn according to the schedule shown in Figure 1 between 7:00 and 8:00 a.m. after an overnight fast. It was drawn into vacutainers with appropriate anticoagulants using a Teflon catheter (Angiocath, Deseret Medical, Sandy, UT) for fatty acid analysis, or syringes containing 1 mL of citrate solution for platelet aggregation studies. Platelet rich plasma (PRP) was prepared by low speed centrifugation for 10 min at 100 × *g*. Platelet poor plasma (PPP) was prepared by respinning the blood after removal of the PRP for 15 min at 400 × *g*. Red cells for fatty acid analysis were prepared from the residue by resuspending the cells in 10 mL of PBS and recentrifuging the cells at 400 × *g* for 15 min three times. The supernate was discarded. The red cells were frozen at −70°C until their lipids could be extracted.

Blood for platelet sizing was drawn into plastic 20-mL syringes and then transferred to polyethylene tubes containing the final concentrations as follows: sodium citrate 13mM, EDTA 1mM, 1.1 μg/mL PGE-1, theophylline 5.4 mg/mL (25). The blood for the prothrombin and partial thromboplastin times were drawn into vacutainers containing 3.2% sodium citrate.

Platelet aggregation. Solutions of the aggregating agents were prepared as recommended by Chrono-log as follows: ADP, 1 μM/μL; collagen, 0.5 μg/mL, AA, 0.05 μM; thrombin, 10 units/mL. The *in vitro* aggregation characteristics of the subjects' platelets were measured in a Chrono-log Model 560 dual channel aggregometer. Briefly, 0.45 mL of PRP were added to an aggregation tube containing a Teflon coated stirring bar. The blank was 0.5 mL of the individuals PPP, unstirred. The instrument was zeroed to 10% transmission of the sample *vs* the blank. An appropriate amount of aggregating reagent was added to the PRP and aggregation was measured as the increase in transmission of light through the PRP. Generally the sample was monitored until the curve became flat or, with some reagents, notably ADP, disaggregation occurred. The threshold for aggregation was determined when the amount of a particular reagent produced at least 50% of maximum aggregation within 15 min of its addition to PRP. All aggregation tests were performed within 4 hr of the initial blood draw.

Platelets. Platelet counts were determined with an electronic particle counter, a Baker-Serono Model 9000 Differential Cell Counter (Baker-Serono, Inc., Allentown, PA) electronic particle counter and cytometer using the manufacturers recommended procedures (Operations Manual, System 9000 Differential Cell Counter, Baker-Serono, Manual No. DS-014A). Mean platelet volume was measured by the procedure described by Corash *et al.* (26). Briefly, sizing was performed using a Particle Data System (Particle Data, Chicago, IL) equipped with a 48 μ orifice and a logarithmic amplifier. Platelet volume was expressed as the median volume derived from a probability plot distribution of platelet volume.

Bleeding time. Bleeding times were measured on the left or right proximal forearm of each subject using a 1 mm deep by 1 cm long incision using a Simplate II bleeding time device (Organon Teknika, Durham, NC). A wipe of the incision was made every 15 sec. Time was measured with a hand-held stop watch.

Clotting factors. Prothrombin time and the partial thromboplastin times were measured by the methods described by Houghie (27). Briefly platelet poor plasma was prepared as described and the PT and PTT were measured using the Bio/Data Corporation Model MCA 110 instrument (Bio/Data Corp., Hatboro, PA) with Organon Teknika coagulation reagents.

Lipid extraction and fatty acid analysis. Tissues were extracted by the procedures described by Nelson (28, 29) using chloroform/methanol (2:1, v/v). Samples were then transmethylated with methanolic HCl (7%, w/w) by the procedures described elsewhere (30,31). When the total lipid extract was transmethylated, the impurities,

TABLE 4

Hemostatic Parameters in All Subjects (n=9), Stabilization and Salmon Diet

Parameter	Pre-diet	Stabilization diet mean ± S.D.	Salmon diet
Prothrombin time (sec)	10.6 ± 0.9	12.6 ± 0.4[a]	12.00 ± 0.4[a]
Partial thromboplastin time (sec)	29.5 ± 1.7	29.5 ± 1.5	29.4 ± 4.1
Mean platelet volume (femtoliters)	5.3 ± 0.5	6.1 ± 0.3[a]	6.8 ± 0.4[a]

[a]Values significantly different from each other at P<0.01, paired *t*-test.

extracted into the hexane phase after termination of the reaction, were removed by thin-layer chromatography as described by Nelson (29). Because the amount of material available from human platelets for fatty acid analysis was limited, fatty acid methyl esters were obtained by direct transmethylation of the intact tissue without prior solvent extraction as described in an earlier publication (32). The conditions for capillary gas-liquid chromatography (GLC) have been described previously (30). Briefly, the column was a 30 meter by 0.025 mm fused quartz column coated with SP-2340 purchased from Supelco, Inc. (Bellefonte, PA). The GLC data were processed with a Perkin-Elmer Corp. (Norwalk, CT) Model 7500 data station using Perkin-Elmer Chrom 3 software.

Statistical analysis. The clotting data and aggregation data were analyzed using the paired *t*-test with a n=9. Analysis of variance was used to determine if Group A and Group B data were comparable. The fatty acid data were analyzed with a single tailed *t*-test. All the statistical analyses were performed with an IBM PS/2 Model 60 using the PC SAS data system, version 6.03, obtained under license from the SAS Institute, PC-SAS, version 6.03 (SAS PC Manual, SAS Institute, Chapel Hill, NC).

RESULTS

There were no significant differences in the bleeding time between subjects on or off the salmon diet (stabilization n-6 diet, 4.2 ± 0.5 min; salmon n-3 diet, 4.1 ± 1.1 min). The bleeding time for subjects on the salmon diet was slightly shorter than for those on the stabilization diet.

In this study, the prothombin time was significantly shorter for the subjects while they were consuming the salmon diet than on the stabilization diet (Table 4). There was no difference in the partial thromboplastin time but the platelets from subjects on the salmon diet were significantly larger than while they were consuming the stabilization diet (Table 4). The platelet counts were reduced when subjects consumed the salmon diet (Table 5). In Group A, after being off the salmon diet for 40 days, the platelet count increased significantly.

The salmon diet did not influence the aggregation characteristics of the subjects' platelets in response to collagen or thrombin, but there was a marked decrease in the sensitivity of the subjects' platelets to ADP on the salmon diet (Table 6). Although there was no difference in the threshold amounts of arachidonic acid necessary to induce aggregation, the time to reach maximum aggregation was significantly longer while the subjects were consuming the salmon diet (Table 6).

TABLE 5

Platelet Counts in Subjects, Stabilization and Salmon Diet

Diet period	Cells × 10^9/L × 10^3 Stabilization diet mean ± S.D.	Salmon diet mean ± S.D.
All subjects, pre-diet (n=9)	316.2 ± 110.6	
All subjects (n=9)	288.1 ± 80.9[a]	250.7 ± 76.5[a]
Group A, day 60 (n=6)		238.2 ± 84.9[b]
Group A, day 100 (n=6)	278.3 ± 62.0[b]	
Group B, day 60 (n=3)	290.6 ± 62.0	
Group B, day 100 (n=3)		249.0 ± 67.8
All subjects, post-diet (n=9)	275.6 ± 78.1	

[a]Values significantly different at P<0.01, paired *t*-test.
[b]Values significantly different at P<0.05, paired *t*-test.

Stearate, oleate and arachidonate levels in the platelets of subjects after 40 days on the salmon diet were markedly reduced while the eicosapentaenoate and docosahexaenoate levels were significantly increased (Table 7). Palmitate and linoleate levels were unchanged by the salmon diet. The fatty acid composition of the platelets from Group A were analyzed after 100 days in the study and those for Group B after 60 days in the study (Table 8). Group A had been removed from the salmon diet and placed on the stabilization diet for 40 days (Table 8), and Group B had consumed only the stabilization diet for 60 days. The fatty acid composition of their platelets was similar except that Group A still retained a significant amount of DHA. EPA apparently had been washed out of the platelets in Group A after this fatty acid was eliminated from their diet for 40 days.

Plasma fatty acid composition of subjects before and after the salmon diets were similar to those observed in the subjects' platelets (Tables 9 and 10). Palmitate and linoleate levels were essentially unchanged, while oleate and arachidonate levels decreased. EPA and DHA levels increased significantly, as expected after the subjects had consumed the salmon diet for 40 days. After Group A had returned to the stabilization diet for forty days (Table 10) the plasma showed no significant carryover of the n-3 fatty acids as compared to Group B who had been eating the stabilization diet for 60 days.

After 40 days on the salmon diet there was a marked change in the fatty acid composition of the erythrocytes in the subjects (Tables 11 and 12). EPA, DPA and DHA all showed marked increases while the oleate and linoleate

TABLE 6

Platelet Aggregation in All Subjects (n=9) Induced by ADP, Collagen, Thrombin, and Arachidonic Acid

Aggregating agent	Pre-diet	Stabilization diet mean ± S.D.	Salmon diet mean ± S.D.
ADP[a] (μmoles/mL)	—	0.334 ± 0.362[b]	0.845 ± 0.349[b]
Collagen[a] (μg/mL)	—	0.390 ± 0.219	0.238 ± 0.119
Thrombin[a] (units/mL)	—	0.515 ± 0.383	0.270 ± 0.087
Arachidonic acid[a] (μmoles/mL)	0.10 ± 0.09	0.11 ± 0.05	0.12 ± 0.07
Arachidonic acid, (min) time to maximum aggregation	9.3 ± 1.9	8.3 ± 1.3[b]	11.3 ± 1.6[b]

[a]Threshold for 50% aggregation.

[b]Values significantly different at P<0.01, paired t-test.

TABLE 7

Fatty Acid Composition of Platelets in All Subjects (n=9), Stabilization and Salmon Diets

	Stabilization diet study day 20 mean ± S.D.		Salmon diet study days 60 & 100 mean ± S.D.	
16:0	19.7	1.9	19.6	1.3
16:1n-7	0.5	0.3	0.5	0.1
18:0[a]	16.2	1.1	14.5	0.9
18:1n-9	13.1	2.0	12.3	0.7
18:1n-7	0.9	0.3	1.1	0.2
18:2n-6	6.9	1.2	6.9	0.9
18:3n-3	0.3	0.3	0.1	0.2
20:0	1.2	0.7	1.4	0.2
20:1n-9	0.4	0.3	0.7	0.2
20:4n-6[a]	22.9	1.5	18.4	1.5
20:5n-3[a]	0.1	0.2	2.5	0.8
22:0	2.4	0.9	2.5	0.2
22:4n-6	0.7	0.8	0.4	0.3
22:5n-3[a]	0.1	0.1	0.4	0.4
22:6n-3[a]	1.6	0.7	3.7	0.4
24:0	1.5	0.6	1.5	0.2
24:1n-9	4.5	1.6	3.4	1.8
Sum of identified fatty acids	95.9		91.8	
Unidentified	2.2	0.1	0.3	0.2
Sum of minor and unidentified fatty acids	4.1	2.8	8.2	4.1

[a]The letter designates fatty acid levels that are significantly different between the two diet groups with P < 0.05 or less.

content of the erythrocytes was reduced. The arachidonic acid content of the cells did not decrease significantly. After Group A was returned to the stabilization diet for 40 days, the fatty acid composition of their erythrocytes was still different from that observed in Group B after 60 days on the stabilization diet. Most of the EPA was eliminated from Group A's erythrocytes by study day 100, but the DHA content remained elevated. As erythrocytes have a 120-day half life in the circulation, neither *de novo* synthesis nor the removal of old cells could account for changes and disappearance of EPA from the erythrocytes of these subjects. Linoleate levels were unaffected by the diet, but arachidonic acid levels in the red cells were higher in the subjects consuming the stabilization diet for sixty days.

DISCUSSION

Most reports on the hematological effects of fish oils have indicated that these substances, when consumed as the purified oil, tend to prolong bleeding times in humans (10–12,21). In this study bleeding times were not prolonged after the volunteers consumed the salmon diet for 40 days. A possible explanation is that the amount of n-3 fatty acids present in the diet of these subjects was less than the amount necessary to elicit this response, or,

TABLE 8

Fatty Acids Composition of Platelets in Groups A (n=6) and B (n=3), Study Days 60 and 100

Fatty acid	Group A day 100 mean ± S.D.		Group B day 60 mean ± S.D.	
16:0	19.1	0.7	18.4	0.4
16:1n-9	0.4	0.4	0.3	0.2
18:0[a]	14.4	0.6	15.4	0.5
18:1n-9	13.7	1.1	10.0	6.7
18:1n-7	1.1	0.1	1.1	0.1
18:2n-6	7.1	0.8	6.2	0.5
20:0	1.4	0.2	1.5	0.2
18:3n-3	0.2	0.3	0.1	0.2
20:1n-9	0.5	0.1	0.6	0.2
20:2n-6	0.3	0.5	0.1	0.2
20:3n-6	1.2	0.2	1.3	0.3
22:0	2.6	0.2	2.6	0.2
20:4n-6	20.6	1.8	23.4	1.5
20:5n-3	0.1	0.3	0.1	0.2
22:4n-6	0.4	0.2	0.3	0.2
22:5n-6	1.1	0.1	10.0	0.7
22:6n-3[a]	2.1	0.2	1.5	0.4
24:0	1.4	0.2	1.4	0.2
24:1n-9	2.5	2.0	2.0	2.4
Sum of identified fatty acids	93.0		91.7	
Unidentified	2.4	0.1	3.0	0.5
Sum of minor and unidentified fatty acids	7.0	1.1	8.3	0.3

[a]The letter designates fatty acid levels that are significantly different between the two diet groups with $P < 0.05$ or less.

TABLE 9

Fatty Acid Composition of Plasma in All Subjects (n=9), Stabilization and Salmon Diets

Fatty acid	Stabilization diet study day 20 mean ± S.D.		Salmon diet study days 60 & 100 mean ± S.D.	
14:0	0.4	0.1	0.5	0.2
16:0	23.3	1.2	23.5	1.8
16:1n-7	1.8	0.5	1.6	0.5
18:0	5.8	0.3	6.7	0.7
18:1n-9	17.6	2.3	13.5	2.4
18:1n-7	1.7	0.1	1.7	0.1
18:2n-6	27.6	2.0	27.5	1.7
18:3n-3	0.4	0.1	0.4	0.1
20:0[a]	0.5	0.3	0.1	0.2
20:3n-6[a]	1.4	0.3	0.8	0.3
20:4n-6[a]	7.0	1.7	4.6	1.0
20:5n-3[a]	0.2	0.2	3.9	1.5
22:0	0.8	0.2	0.8	0.1
22:5n-6[a]	0.4	0.1	0.8	0.3
22:6n-3[a]	1.8	0.5	5.0	1.1
24:0	0.8	0.2	0.8	0.1
24:1n-9	1.5	0.3	1.1	1.0
Sum of identified fatty acids	94.0		94.2	
Unidentified	1.9	0.5	2.3	0.3
Sum of minor and unidentified fatty acids	6.0	0.8	5.8	0.8

[a]The letter designates fatty acid levels that are significantly different between the two diet groups with $P < 0.05$ or less.

TABLE 10

Fatty Acid Composition of Plasma in Groups A (n=6) and B (n=3), Stabilization Diet

Fatty acid	Group A day 100 mean ± S.D.		Group B day 60 mean ± S.D.	
14:0[a]	0.5	0.1	0.4	0.1
16:0	23.6	2.6	21.9	0.4
16:1n-7	1.6	0.8	1.6	0.2
18:0	6.1	0.5	6.1	0.5
18:1n-9	17.4	2.8	17.6	2.0
18:1n-7	1.6	0.1	1.7	0.2
18:2n-6	28.5	3.1	28.6	2.2
18:3n-3	0.3	0.1	0.3	0.3
20:0	0.6	0.1	0.5	0.4
20:3n-6	1.3	0.1	1.6	0.6
20:4n-6	6.3	2.0	6.9	1.3
20:5n-3	0.2	0.2	0.1	0.2
22:0	0.8	0.1	0.9	0.1
22:5n-6	0.2	0.2	0.5	0.1
22:6n-3	1.5	0.8	1.6	0.7
24:0	0.7	0.1	0.8	0.1
24:1n-9	0.8	0.1	1.6	0.2
Sum of identified fatty acids	91.1		93.7	
Unidentified	0.8	0.4	2.5	0.5
Sum of minor and unidentified fatty acids	8.9	0.8	6.3	0.5

[a]There were no significant differences between the two diet groups.

TABLE 11

Fatty Acid Composition of Red Blood Cells in All Subjects (n=9), Stabilization and Salmon Diets

Fatty acid	Stabilization diet study day 20 mean ± S.D.		Salmon diet study days 60 & 100 mean ± S.D.	
14:0[a]	0.2	0.2	0.1	0.1
16:0[a]	25.1	0.9	27.6	0.9
16:1n-7[a]	0.7	0.2	0.2	0.2
17:0	0.3	0.1	0.4	0.1
18:0[a]	8.6	0.7	9.5	0.5
18:1n-9[a]	12.7	1.4	10.9	0.7
18:1n-7[a]	1.4	0.1	1.3	0.1
18:2n-6[a]	14.5	2.1	8.5	0.7
20:3n-6[a]	1.1	0.2	0.7	0.1
20:4n-6[a]	9.8	1.4	8.2	0.9
20:5n-3[a]	0.4	0.2	2.3	0.3
22:0[a]	1.5	0.2	1.9	0.2
22:4n-6	0.4	0.1	0.4	0.1
22:5ω6	1.2	0.2	1.2	0.8
22:5n-3[a]	0.7	0.1	1.0	0.2
22:6n-3[a]	2.8	0.8	4.8	0.9
24:0[a]	4.0	1.1	5.1	0.5
24:1n-9[a]	5.6	0.5	7.3	0.5
Sum of identified fatty acids	91.0		90.6	
Unidentified	5.0	0.3	5.5	0.4
Sum of minor and unidentified fatty acids	9.0	2.4	9.4	1.9

[a]The letter designates fatty acid levels that are significantly different between the two diet groups with $P < 0.05$ or less.

TABLE 12

Fatty Acid Composition of Red Blood Cells in Groups A (n=6) and B (n=3), Study Days 60 and 100

Fatty acid	Group A day 100 mean ± S.D.		Group B day 60 mean ± S.D.	
14:0	0.1	0.1	0.2	0.1
16:0	26.5	0.4	27.0	0.8
16:1n-7	0.2	0.2	0.3	0.1
18:0	9.0	0.5	9.1	0.5
18:1n-9	11.3	0.8	10.4	0.3
18:1n-7	1.1	0.1	1.2	0.1
18:3n-6	9.4	0.7	9.7	0.2
20:0	1.8	0.2	1.9	0.1
20:3n-6	1.1	0.2	1.1	0.3
20:4n-6[a]	9.4	1.1	11.5	1.6
20:5n-3[a]	0.9	0.1	0.1	0.1
22:5n-6	1.0	0.1	0.8	0.1
22:5n-3[a]	1.6	0.1	0.9	0.8
22:6n-3[a]	4.4	0.7	2.9	0.8
24:0	4.9	0.4	4.6	0.4
24:1n-9	6.9	0.6	7.7	0.7
Sum of identified fatty acids	90.2		90.3	
Unidentified	6.0	0.4	6.0	0.4
Sum of minor and unidentified fatty acids	9.8	1.0	9.7	0.8

[a]The letter designates fatty acid levels that are significantly different between the two diet groups with $P < 0.05$ or less.

perhaps, the duration of the intervention period was not long enough. Volunteers consuming purified fish oil supplements can ingest as much as 50 g of oil containing up to 20 g of n-3 fatty acids such as EPA or DHA per day (33). Van Houwelingen *et al.* (8), using free-living volunteers fed a canned mackerel supplement, *ad libitum* diet, did report mild increases in bleeding times, but no changes in platelet aggregation responses. The amount of n-3 fatty acids consumed by the volunteers (1.7 g EPA and 3.0 g DHA) in the studies by Van Houwelingen *et al.* (8) was equal to or less than the amount fed to the volunteers in our study. The subjects in the study reported by Van Houwelingen *et al.* were consuming alcohol, but it is not known if this would influence the results.

In the present study the typical volunteer consumed approximately 2 g of 20:5n-3 and 3 g of 22:6n-3 daily. Still, it should be noted that the amount of n-3 fatty acids consumed by the volunteers in this study exceeded the amount consumed by most populations noted for consuming large amounts of fish in their diets. Japanese fishing villages have been reported to consume equivalent amounts of fish as the volunteers in this study (34). Only Eskimos routinely consume more fish oil than the volunteers in this study (4). Additionally, 40 days may be an insufficient period to manifest the prolongation of bleeding time noted by other investigators with this dietary level of n-3 fatty acids. Also, triglycerides containing n-3 fatty acids are hydrolyzed more slowly than other triglycerides without n-3 fatty acids (35,36). Thus, it is also possible that purified fish oils, when consumed as a bolus of oil, have pharmacological properties different from those manifested by n-3 fatty acids when they are consumed during a normal meal (37).

The threshold for platelet aggregation in response to ADP was significantly raised, confirming other investigator's reports (6-8,10). The anomalous response of the time to reach maximum aggregation when arachidonic acid was the agonist has not been reported previously. Until recently arachidonic acid was not used often as an aggregating agent in human platelet studies.

Houwelingen *et al.* (22) recently reported use of a mackerel paste (135 g/day) as a dietary supplement in a group of normal male volunteers. They measured platelet aggregation response to collagen and observed a small effect that was only apparent after sophisticated statistical analysis of the diets and was dependent on the amount of the supplement consumed. These observations are consistent with those reported here using a salmon diet, although the present experiment was not large enough to apply the same type of statistical analysis used by Houwelingen *et al.* (22).

A more consistent effect of the fish diet was observed on platelet size and count in the subjects consuming the salmon diet. Consumption of purified fish oil supplements does cause a decrease in platelet counts (6,8,11-12). The subjects in this study did have modest but statistically significant reductions in the platelet counts and a concomitant increase in platelet size. These observations suggest a decrease in the platelet life span in the circulation, although that parameter was not measured directly in this study.

Another anomalous result was that the prothrombin time in the subjects consuming the salmon diet decreased. (These data were statistically significant but may not be physiologically significant.). The prothrombin time does not correlate with the bleeding time *in vivo*, and the

prothrombin time is not related to platelet aggregation *per se,* so that a change in this parameter may have little relation to platelet function and thrombus formation *in vivo.*

Overall, the results of the coagulation tests and platelet aggregation studies in this investigation are consistent with earlier reports in the literature (8,9,21,38), in which larger amounts of fish oil were fed to human volunteers. It should be noted that the 2 g/day of 20:5n-3 fed on the average to the volunteers in our study was considerably less than the amount used in clinical trials, in which up to 20 MaxEPA capsules containing 3.6 g of 20:5n-3 were fed daily (12-14,21).

These data do not establish, however, that modest amounts of dietary n-3 fatty acids shift the thrombotic properties of the blood toward a less thrombotic condition. Still, epidemiological reports (3,39,40) have suggested that very modest amounts of fish, if consumed regularly over extended time periods, will reduce mortality and morbidity from cardiovascular disease. The role of the eicosanoids in this picture is currently unclear (18,23,40,41).

This study also found that the n-3 fatty acids from fish are rapidly incorporated into plasma, platelets and red cells, as reported by other investigators (7,10,17,18,38, 42-44). We found that equilibrium between the diet and the blood tissues was reached after 20 days for plasma and platelet (data not shown); red bloods cells (RBC) also equilibrated more rapidly than could be accounted for by *de novo* synthesis of new cells in the marrow. Without plasma exchange the presence of 20:5n-3 in RBC after 20 days on the salmon diet would be barely detectable, as only 15% of the red cells have been replaced in that period. Thus, exchange in the plasma must account for the initial equilibrium that was observed in this study. It also appeared that in erythrocytes arachidonic acid can be replaced by EPA and DHA. No conservation of n-3 fatty acids was detected in plasma in this study.

Dyerberg and Bang have reported the fatty acid composition of Eskimo platelets (5). It is interesting that the amount of n-3 fatty acids in the Eskimo platelets was not much higher than that found in the volunteers in this study after 20 days on the salmon diet. Presumedly the Eskimos had been eating their normal diet from childhood, yet their platelets contain about the same amounts of n-3 fatty acids as men who had consumed n-3 fatty acids in modest amounts for only 40 days. This would suggest that it is not simply the fatty acid composition of the platelet membrane that prolongs the bleeding time nor the primary cause of the purported protection against cardiovascular disease.

There was no significant persistence of the n-3 fatty acids in the plasma or platelet membranes after the volunteers stopped consuming the salmon diet. After 40 days of n-3 devoid diet, the subjects plasma and platelet fatty acid composition was similar to that found in the plasma and platelets of volunteers who had consumed a n-3 deficient diet for 60 days. There was some persistence of n-3 fatty acids in the erythrocytes of these volunteers, presumedly reflecting the 120-day life-span of red cells. It is noteworthy that the EPA content of the erythrocyte did decrease more than expected from red cell turnover. Hence, exchange processes in the circulation probably influence removal of EPA from red cells, just as they apparently increase its uptake when it is present in the diet. Holub has speculated that the lecithin:cholesterol acyltransferase is an important mediator of the exchange of platelet fatty acids when fish oil is consumed (44). It may be equally important in exchanging red cell fatty acids.

Generally, this study confirmed that eating a diet in which fresh fish is the only source of n-3 fatty acids does induce similar changes to those observed when people consume fish oil supplements. The fatty acid composition of the blood components shows rapid incorporation of the n-3 fatty acids. Platelet aggregation changes are small but in the direction of less aggregation. Yet the bleeding times in these volunteers did not change, an observation in sharp contrast to previous studies using fish oil supplements or mackerel paste (8,10-12). The subjects in the study conducted by van Houwelingen *et al.* (8) consumed roughly the same amount of n-3 polyunsaturated fatty acids as those in our study. No explanation for the difference observations between the two studies is readily apparent. Also, the prothrombin time was statistically shorter, but not beyond the normal, laboratory range, in the volunteers on the salmon diets. Platelet counts in the volunteers consuming the salmon diet did decrease, a phenomenon also observed when individuals consumed mackerel (8) or fish oil supplements (10). Finally, there was little persistence of the n-3 fatty acids in the blood 40 days after cessation of the salmon diet.

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Effect of a Salmon Diet on the Distribution of Plasma Lipoproteins and Apolipoproteins in Normolipidemic Adult Men

Frank T. Lindgren[a,*], Gerald L. Adamson[a], Virgie G. Shore[b], Gary J. Nelson[c] and Perla C. Schmidt[c]

[a]Research Medicine and Radiation Biophysics Division, Lawrence Berkeley Laboratory, University of California, Berkeley, California 94720, [b]Biomedical Sciences Division, Lawrence Livermore National Laboratory, Livermore, California 94550 and [c]Western Human Nutrition Research Center, U.S. Department of Agriculture, ARS, Presidio of San Francisco, California 94129

The effects of n-3 fatty acids on plasma lipids, lipoproteins and apoproteins have usually been studied in humans after feeding of purified fish oil. This study describes the effect of a natural diet, containing salmon as the source of n-3 fatty acids, on these parameters as compared to a diet very low in n-3 fatty acids. The subjects were nine normolipidemic, healthy males who were confined to a nutrition suite for 100 days. During the first 20 days of the study the participants were given a stabilization diet consisting of 55% carbohydrates, 15% protein, and 30% fat. The n-3 content of this diet was less than 1%, and it contained no 20- or 22-carbon n-3 fatty acids. After the stabilization period the men were split into two groups, one group continued on the stabilization diet while the other received the salmon diet that contained approximately 2.1 energy percent (En%) of calories from 20- and 22-carbon n-3 fatty acids. Both diets contained equal amounts of n-6 fatty acids. This regime continued for 40 days, then the two groups switched diets for the remainder of the study. Plasma triglycerides were lowered significantly ($p<0.01$) and high density lipoprotein cholesterol (HDL-C) was significantly elevated ($p<0.01$) after the men consumed the salmon diet for 40 days. The very low density lipoproteins (VLDL) were lowered, but the trend did not reach statistical significance during the intervention period. The total plasma cholesterol, total low density lipoprotein (LDL) and the total high density lipoprotein (HDL) levels were not influenced by the salmon diet. Within the HDL fraction, however, the larger HDL_2 subfractions were significantly elevated ($p<0.002$), and the smaller, more dense HDL_3 was lowered ($p<0.002$) by the salmon diet. These significant changes were detected by analytic ultracentrifugation and confirmed by gradient gel electrophoresis. Analysis of the apolipoproteins (apo) AI, AII, B, and E, and Lp(a) indicated only significant lowering of apoAI, consistent with the increased HDL_2, which is higher in cholesterol but lower in the major HDL apolipoprotein, apoAI. Thus, the purported beneficial cardiovascular effects of consumption of n-3 fatty acids by humans may, in part, be attributable to changes in the HDL distribution, *i.e.*, the lowering of the more dense HDL_3 and the elevation of the larger, less dense HDL_2.

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There is considerable evidence that fish oils containing a high content of n-3 polyunsaturated fatty acids lower plasma triglycerides and very low density lipoprotein (VLDL) (1-3). However, the effect of fish oil supplements high in n-3 fatty acids is not consistent with many reports of either lowering or raising both low density lipoprotein (LDL) cholesterol (LDL-C) and high density lipoprotein (HDL)-C. The recent comprehensive review by Harris (4) compares the results obtained with both normolipidemic and the major types of hyperlipidemic patients. Certainly, part of the discrepancies result from the different types of fish oil fed, the dosage, the subjects or patients used, and the design of the experiment. Also, whatever effects observed appear to be the result of the fish oils high in n-3 polyunsaturated fatty acids. Thus, early dietary studies of defatted fish showed no effects on cholesterol levels but the extracted fish oil did lower plasma cholesterol in rats (5). The early epidemiologic studies of Greenland Eskimos (5-7) indicated a very low incidence of ischemic heart disease, and these findings initiated interest in the potential beneficial effects of n-3 polyunsaturated fatty acids. However, these studies were of a normal population whose food consisted of chronic ingestion of marine foods rich in n-3 polyunsaturated fatty acids, estimated to be of the order of about 7 g/day of these specific marine n-3 polyunsaturated fatty acids.

Thus, while it is clear that pharmacological dosages of purified fish oil in man produce marked changes in blood lipid and lipoprotein levels, fatty acid distributions, and lipid metabolism (8-10), it is not clear whether similar changes, particularly in the distribution of lipoproteins and apolipoproteins, occur when whole fish are consumed as a significant part of the diet. This study, the protocol of which is described in more detail in the companion paper (11), fed healthy volunteers all natural diets where fresh salmon supplied 2.1 En% of calories as n-3 fatty acids. The results are compared to a diet containing little (<1 En% of calories) n-3 fatty acids but a similar amount of n-6 fatty acids in a totally controlled environment, at the nutrition suite at USDA's Western Human Nutrition Research Center (San Francisco, CA). This study using marine (salmon) and terrestrial polyunsaturated food seems appropriate and practical to help resolve some of the discrepancies in results of the lipid and lipoprotein effects of n-3 polyunsaturated fatty acids. Of particular interest is the quantitative change, or no change, in the subfractions of all major VLDL, LDL, and HDL lipoprotein classes.

METHODS

Materials. All natural foods were purchased at local food markets. The salmon was purchased fresh from a seafood wholesale supply company as a large batch from a single boat, and was frozen until used for meal preparation during the study.

Subjects. The volunteers were recruited from the West Coast and consisted of men between the ages of 30 and 65. The physical characteristics of the final nine

*To whom correspondence should be addressed.

Abbreviations: AnUC, analytic ultracentrifugation; apo, apolipoprotein; En%, energy percent; GGE, gradient gel electrophoresis; HDL, high density lipoprotein; HDL-C, high density lipoprotein cholesterol; IDL, intermediate density lipoprotein; LDL, low density lipoprotein; LDL-C, low density lipoprotein cholesterol; VLDL, very low density lipoprotein.

TABLE 1

Plasma Lipids in All Subjects (N = 9) on Stabilization and Salmon Diets

Lipid	Stabilization diet Mean ± S.D. (mg/dL)	Salmon diet Mean ± S.D. (mg/dL)
Total cholesterol	187.1 ± 37.7	192.8 ± 44.3
LDL-C	137.6 ± 34.7	140.6 ± 41.0
HDL-C	30.4 ± 10.0[a]	36.8 ± 8.7[a]
Triglycerides	94.2 ± 33.7[a]	77.2 ± 36.7[a]

[a]Values significantly different at $p < 0.01$, paired t-test.

volunteers who completed the study are given in Table 1 of the companion paper (11).

All volunteers were confined to the Nutrition Research Suite of the USDA Western Human Nutrition Research Center for the duration of the study. The study protocol is described in the companion paper (11). Statistical comparison of the measured parameters were made using day 20 as the baseline values for each subject. Subjects served as their own controls.

Diets. A complete description of the diets is given in the companion paper (11). The diets consisted of natural foods. No dietary supplements were given except α-tocopherol to bring the diet to 200% of the RDA for this vitamin. No alcohol was included in these diets. The diets were designed around a prudent diet concept of 30:55:15, fat/carbohydrate/protein En%.

The amount of saturated and n-6 polyunsaturated fatty acids (7.5 En%) were held constant in these diets. The stabilization diet had <1% n-3 fatty acids as α-linolenic acid and approximately 50% monounsaturated fatty acids (oleic acid). The salmon diet had approximately 2.1 En% long-chain polyunsaturated n-3 fatty acids and a concomitant reduction in the level of monounsaturated fatty acids.

Measurements. Blood was drawn between 7:00 a.m. and 8:00 a.m. after an overnight fast. It was drawn into vacutainers with EDTA as the anticoagulant using a Teflon catheter (Angiocath, Deseret Medical, Sandy, UT). Plasma was separated from the cellular components of the blood by centrifugation at 400 × *g* for 15 min. Plasma samples were drawn using EDTA, gentamicin sulfate, and ε-amino-n-caproic acid (12) at the end of each of the three diet periods, the 20-day stabilization diet, the 40-day n-3 salmon diet, and the 40-day n-6 diet. Plasma triglycerides, total cholesterol, LDL-C, and HDL-C were done on a Cobas-Fara centrifugal analyzer (Hoffman-La Roche, Nutley, NJ). Preparative isolation of the total low density fraction, $d < 1.063$ g/mL and the total lipoprotein fraction, $d < 1.21$ g/mL) were as described earlier (13). Also, analytical ultracentrifugation (AnUC) of both total LDL and HDL with complete computer analysis of the schlieren films were as described previously (13,14) using a sonic digitizer and a VAX Station 3200. HDL subfractions by AnUC, HDL_{2b}, HDL_{2a}, and HDL_3, were resolved as described elsewhere (15).

Nondenaturing quantitative gradient gel electrophoresis (GGE) was performed using Pharmacia 2-16% gels for total LDL and 4-30% gels for HDL as described by Nichols *et al.* (16). The LDL gels were stained with Coomassie Brilliant Blue R-250, and scanned at 555 nm using an RFT Transidyne grating densitometer (17). HDL gels were similarly scanned and HDL subfractions of HDL_{2b}, HDL_{2a}, HDL_{3a}, HDL_{3a}, and HDL_{3c}, were calculated as percent of total HDL area as described previously (16).

Plasma apolipoprotein concentrations. Plasma concentrations of apolipoproteins AI, AII, B and E and Lp(a) were measured in plasmas that had been stored at −70°C and thawed just before analysis. Samples from all the diet periods were analyzed simultaneously. Competitive ELISA tests (18) were used for all assays except apoAI, which was measured by RID (19) with commercially available plates (TAGO Inc., Burlingame, CA, and Kent Laboratories, Inc., Redmond, WA). Controls included purified apoAI, isolated HDL at several dilutions, pooled plasmas including five (frozen) from the Northwest Lipid Research Center and our freshly prepared pools. In the competitive ELISA tests, anti-apolipoprotein was immobilized in 96-well NUNC high-binding, flat-bottomed microtiter plates (Alameda Chemical and Scientific, Oakland, CA) by a capture antibody, anti IgG Fc (USB Immunochemicals, Cleveland, OH) that had been precoated, 1 μg/well, in carbonate buffer, pH 9. The apoB assay used a mixture of high-affinity monoclonal antibodies (MEDIX Biotech Inc., Foster City, CA) at a total concentration of 0.0045 μg/well; the apoAII assay used polyclonal goat antiserum (International Immunology Corp., Murrieta, CA), 0.045 μg IgG/well; the apoE assay used a mixture of monoclonal antibodies totaling 0.035 μg/well (Chemicon clones 16 and 17, Chemicon International Inc., El Segundo, CA); the Lp(a) assay used 100 μL of a 1/300 dilution of an Lp(a)-specific polyclonal rabbit antiserum (Calbiochem-Behring Corp., La Jolla, CA). Standards and samples were premixed with a fixed amount (antibody-saturating but not excess) of the appropriate biotinylated antigen in sample diluter containing 0.375% Tween 20, 1% bovine albumin (RIA grade), 1% casein acid hydrolysate, and 0.5 mM EDTA in phosphate-buffered saline, pH 7.2, with gentamicin sulfate (0.05 mg/mL) and 0.1 mM thimerosal as preservatives. Samples, 100 μg/well, at two different dilutions, each in triplicate, were incubated with antibody for 4 hr at room temperature. After washing the plates, bound labeled antigen was detected with streptavidin-horseradish peroxidase and *o*-phenylenediamine substrate (18). A standard curve of B/B° *vs* ng antigen/well was constructed, with B and B° the optical densities at 492 nm in the presence and absence, respectively, of competing, unlabeled antigen.

RESULTS

Table 1 shows the plasma lipid data for both the stabilization and salmon diets. No significant differences were observed in plasma cholesterol or LDL-C concentrations. However, HDL-C levels were significantly increased ($p<0.01$) and plasma triglycerides were decreased significantly ($p<0.01$) in subjects consuming the salmon diet as compared to those consuming the stabilization diet. Table 2 shows the AnUC concentrations of the major low density subfractions, total HDL, and HDL subfractions. The data show a lower VLDL level (not significant) in subjects on the salmon diet, but otherwise the

TABLE 2

Lipoprotein Concentrations (mg/100 mL ± S.D., N = 9) from Analytical Ultracentrifugation

S_f^o interval				$F_{1.20}^o$ interval		
(VLDL) 100–400	(VLDL) 20–400	(IDL) 12–20	(LDL) 0–12	(HDL) 0–9	(HDL_2) 3.5–9	(HDL_3) 0–3.5
			Stabilization diet (20 days[a] mean)			
13.2 ± 16.1	65.8 ± 28.7	35.0 ± 16.1	293.8 ± 69.4	202.7 ± 22.9	31.0 ± 18.4	171.7 ± 13.3
			Salmon diet (40 days[a])			
8.1 ± 15.1	45.6 ± 30.2	25.9 ± 19.6	282.4 ± 73.3	202.8 ± 27.2	54.7 ± 21.6	148.1 ± 21.8
			Stabilization diet (40 and 60 days[a])			
15.8 ± 24.9	49.0 ± 20.5	23.7 ± 12.4	272.6 ± 69.8	218.8 ± 35.4	41.7 ± 24.4	177.1 ± 19.4

[a]The time periods denote the length of the diet period. Samples were drawn at 20 days, 60 days (salmon diet n = 6 and stabilization diet n = 3), or 100 days (salmon diet n = 3, and stabilization diet n = 6).

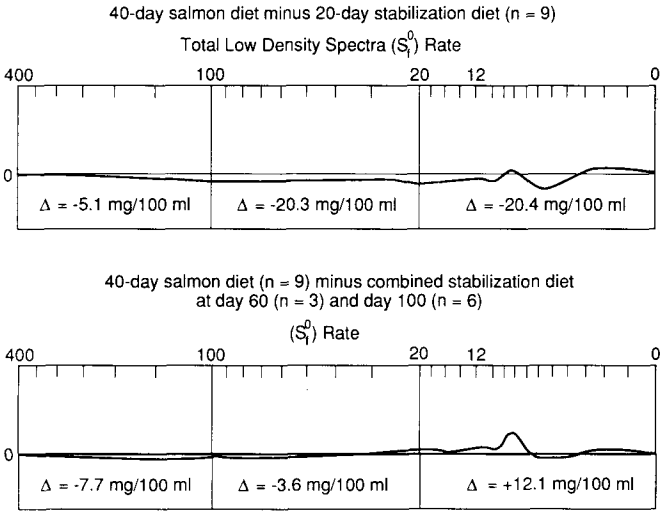


FIG. 1. Difference spectra for Schlieren patterns of total low density lipoprotein distribution profiles obtained by analytic ultracentrifugation.

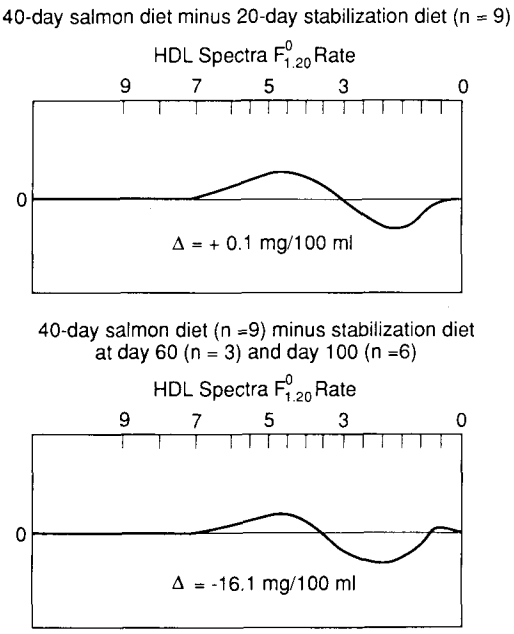


FIG. 2. Difference spectra for high density lipoprotein distribution profiles obtained by analytic ultracentrifugation.

levels of each major class are comparable for the initial stabilization diet, the salmon diet and the final stabilization diet periods. However, examination of the AnUC HDL subfractions HDL_{2b}, HDL_{2a}, and HDL_3, reveals differences in the subjects' patterns between the salmon and the stabilization diet periods, both on 40-day and 20-day duration, respectively. Plasma HDL_{2b} increased by 15.1 ± 2.9 mg/dL on the salmon diet, while HDL_{2a}, HDL_3 and total HDL decreased by 13.3 ± 8.0, 17.7 ± 8.5 and 16.1 ± 13.1 mg/dL, respectively. These decreases were not significant in paired t-tests; however, the HDL_{2b} increase was significant (P=0.0009). A more complete visual picture of the AnUC data are provided by mean Schlieren difference plots (Figs. 1 and 2). Plasma VLDL was consistently lower on the salmon diet (not significantly) while LDL and IDL varied, but neither systematically nor significantly. However, the HDL Schlieren difference plots for subjects on the 40-day salmon diet *vs* the stabilization diet at 20 and 60 days showed a consistent redistribution of HDL with an elevation of the

TABLE 3

Comparison of HDL Subfractions from Densitometric Scans of Gradient Gel Electrophoresis Patterns: Salmon Diet Minus Stabilization Diet[a] (Paired t-test, N = 9 each)

Subfraction	Δ% Area ± SEM	T	P
HDL_{2b}	9.1 ± 1.3	7.28	0.0001
HDL_{2a}	−0.7 ± 0.9	−0.71	(NS)
HDL_{3a}	−6.1 ± 0.9	−6.65	0.0002
HDL_{3b}	−2.4 ± 0.9	−2.82	0.023
HDL_{3c}	−0.4 ± 0.4	−1.18	(NS)

[a]The definition of the HDL Subfractions and the area measurements were done as described in detail in ref. 16.

TABLE 4

Plasma Concentrations (Average ± S.D. in mg/mL for Nine Subjects) of Apolipoproteins and Lp(a) After the Stabilization (20-day), Salmon (40-day), and Stabilization (40- or 60-day) Diet Periods

Diet	AI	AII	B	E	Lp(a)	AI/AII	AI/B
Stabilization	1.04 ± 0.07	0.28 ± 0.04	0.81 ± 0.20	0.086 ± 0.007	0.23 ± 0.14	3.84 ± 0.56	1.35 ± 0.31
Salmon	1.01 ± 0.05	0.26 ± 0.05	0.77 ± 0.23	0.087 ± 0.008	0.23 ± 0.14	3.90 ± 0.56	1.40 ± 0.37
Stabilization (40 or 60 day)	1.11 ± 0.05	0.31 ± 0.04	0.79 ± 0.22	0.088 ± 0.008	0.23 ± 0.15	3.59 ± 0.43	1.48 ± 0.37

$F^{o}_{1.20}$ 3.5-9 (HDL_2) and depression of the $F^{o}_{1.20}$ 0-3.5 (HDL_3) AnUC subclasses. Comparisons were significant at $p<0.002$, $p<0.002$, and $p<0.04$, $p<0.02$, respectively.

The percentage distribution of HDL in GGE confirmed the redistribution of smaller HDL_3 to larger HDL_2 subclasses noted in quantitative AnUC. Table 3 compares by paired t-test the five major subfractions obtained by GGE of HDL from the subjects on the salmon and the stabilization diets. Since resolution is higher with GGE and total HDL relatively constant, it is not unexpected that the elevation of HDL_{2b} and depression of the smaller HDL_{3a} and HDL_{3b} were highly significant.

The plasma concentrations of apolipoproteins AI, AII, B, and E and Lp(a) are given in Table 4. The ELISA Lp(a) measurements were independently confirmed by a quantitative GGE method (20) which yielded plasma concentrations in mg/mL of 0.246 ± 0.135 (salmon diet, r= 0.911), 0.237 ± 0.119 (20-day stabilization diet, r=0.957) and 0.242 ± 0.118 (40- or 60-day stabilization diet, r= 976). Comparison of ELISA and GGE values for Lp(a) yielded r=0.943 in all 27 cases. Table 5 gives the corresponding comparison by paired t-test of apolipoprotein levels in subjects as a function of diet. In all individuals, apoAI concentration was lower after the salmon diet than after the 40 or 60 day stabilization diet ($p=0.0004$). ApoAII was also lower in six subjects (those with salmon diet preceding the stabilization diet) and similar in two, for $p<0.053$ for all subjects. The ratio of apoAI to apoAII was higher by 24% on the average in the six subjects who ate the salmon diet before the stabilization diet, and approximately 20% lower in the other three subjects. ApoAI values, ranging from 0.95 to 1.18 mg/mL, were on average 15% lower than pooled male plasma values (approximately 1.20 mg/mL) obtained with the same ELISA reagents and conditions. Concentrations of apoB, apoE and Lp(a) were similar on average after all the diets. Individual changes, generally <10%, were uncorrelated with diet, total plasma cholesterol, or apolipoprotein concentration, which ranged from 0.59 to 1.25 mg/mL for apoB and 0.033 to 0.41 mg/mL for Lp(a). Even though apoAI was lower in all subjects, on the salmon diet, only five subjects had lower apoAI to apoB ratios. ApoE concentrations, 0.076 to 0.102 mg/mL, were somewhat higher than the average (0.066 ± 0.031 mg/mL) for a random group of 70 plasmas that were assayed under the same conditions. By chance, Lp(a) concentrations proved to be high in six of the nine subjects, so the average concentrations in Table 4 is twice that (0.115 ± 0.140 mg/mL) observed in the 70 control plasmas.

TABLE 5

Comparison of Apolipoprotein Data: Salmon Diet *vs.* Stabilization Diet (Paired t-test)

Apolipoprotein	n	Mean Δ mg/100 mL±SEM	T	P
AI	9	−9.7 ± 1.7	−5.74	0.0004
AII	9	4.8 ± 2.1	−2.27	0.053
B	9	1.9 ± 16	−1.24	(NS)
E	9	0.17 ± 0.24	−0.69	(NS)
Lp(a)(ELISA)	9	−0.6 ± 1.2	−0.48	(NS)
Lp(a)(GGE)	9	−0.5 ± 2.0	−0.25	(NS)

DISCUSSION

Although the dietary effects of marine (20 and 22 carbon) n-3 fatty acids in humans have been studied extensively, there are conflicting results with regard to LDL-C and HDL-C (4). However, in nearly all previous studies, plasma triglycerides and VLDL were lowered. In the normolipidemic human studies reported in the Harris review (4), there was essentially no change in total or LDL cholesterol, a slight rise (3%) in HDL-C, and a 25% decrease in plasma triglycerides. Our data and that of the previous paper (11) are consistent with these earlier studies, and also explain the nature of the increased HDL-C as a significant shift in particle size and density of the HDL subfractions. Other recent studies of three groups of volunteer normal males in the Netherlands and Norway by Van Houwelingen *et al.* (21) also showed significantly increased HDL-C after six weeks of long-chain n-3 polyunsaturated fatty acid consumption. Triglycerides were significantly lowered in these studies. Another recent six-week n-3 study of mildly hypercholesterolemic males by Abbey *et al.* (22) also observed a substantial fall in VLDL and triglycerides, an increase in LDL-C, and a slight fall in HDL-C. However, despite the decrease in HDL-C, there was a consistent increase in the HDL_2/HDL_3 ratio as measured by GGE. In our study, the larger, more lipid-rich HDL_2 were increased and the smaller, more protein-rich HDL_3 were significantly reduced, consistent with the results of Abbey *et al.* (22). Since the total HDL concentration was not significantly changed, this redistribution from small to large HDL would tend to raise the HDL-C and lower the major HDL apolipoproteins, AI and AII.

In the study of Abbey *et al.* (22), apoAI was lower by 10% on average in 11 mildly hypercholesterolemic men

after six weeks on a fish-oil-supplemented diet than after the preceding 3-week diet supplemented with safflower oil. ApoB and apoAII were also lower by 6% and 14%, respectively, and the apoAI/apoAII ratio was higher. In our study with normolipidemic men, significant differences in apoAI (lower with the salmon diet) were observed only after the maximum diet periods (40-day salmon diet *vs* 40- or 60-day stabilization diet); apoB did not change. Although our results on apoAII and apoAI/apoAII ratios did not reach the significance of changes in the study of Abbey *et al.* (22), our results tend to confirm theirs. Perhaps we needed more than nine subjects to establish this effect. In retrospect, 20 days on the stabilization diet may have been insufficient for equilibration of plasma apoAI concentration; increased values were observed in six subjects after 40 or 60 days on the diet.

Reductions in plasma apoE and/or Lp(a) concentrations could be anti-atherogenic. However, no changes during this study were observed, irrespective of whether the subject's plasma Lp(a) was high or low. The potentially anti-atherogenic effect of n-3 fatty acid consumption may include a profound redistribution of HDL with a selective increase in HDL_2. The larger, less dense HDL_2 appear to be the most variable and the anti-atherogenic subfraction in the HDL spectrum (23,24). Possibly this change in HDL profile is related to a reduction in plasma VLDL, which has been reported after fish-oil diets (22), but did not reach significance in this study. Offsetting this apparently beneficial effect on HDL, the salmon diet lowered plasma apoAI, and the apoAI/apoB ratio was reduced by 5% or more, up to 21%, in four subjects.

The above findings are more realistic in terms of human dietary modification because they included n-3 fatty acids fed as natural marine food as opposed to fish oil supplements. The 2.1 En% of n-3 fatty acids in our salmon diet compared quantitatively to the mean n-3 supplements cited in the Harris review (4). Recently Harris *et al.* (25) found similar results with a diet containing 4.5 g/day of n-3 fatty acids. While it is possible to consume large amounts of n-3 fatty acid-containing marine food over an extended period of time, it is more realistic to evaluate the chronic dietary effect in humans of a lower n-3 intake, *e.g.*, 1.5–3 g/day. This amount of n-3 fatty acid was effective when fed as fish oil supplements (4).

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The Participation of Soluble Factors in the ω-Oxidation of Fatty Acids in the Liver of the Sheep

W. Robert Hare* and **Klaus W.J. Wahle**
Rowett Research Institute, Bucksburn, Aberdeen, AB2 9SB, United Kingdom

The removal of soluble components from an ovine hepatic microsomal preparation decreased the ω-hydroxylation of dodecanoic and hexadecanoic acids. The results suggest that one or more soluble components play a role in the microsomal ω-hydroxylation of fatty acids. The possible roles in the reaction of catalase (known to stimulate the microsomal desaturations of fatty acids and alkylglycerols) and superoxide dismutase were investigated. The addition of these enzymes to the complete (but not the washed) microsomal preparation stimulated both the initial ω-hydroxylation reaction and the subsequent dehydrogenation reactions of the ω-oxidation pathway. The similarity of the effects of catalase and superoxide dismutase and stimulation of two different steps of the ω-oxidation pathway suggest that these agents are acting indirectly by removing active oxygen species rather than directly on the enzymes of microsomal fatty acid ω-hydroxylation.
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Three separate reactions constitute the ω-oxidation of fatty acids in mammalian tissues: i) the hydroxylation[1] of the ω1- or ω2-carbons of a fatty acid (1,2); ii) the dehydrogenation of the resulting ω1- or ω2-hydroxyl group to produce an aldehyde or ketone group, respectively; and iii) the dehydrogenation of the aldehyde (but not the ketone) group to yield a carboxyl group (2–4). The pathway thus converts monocarboxylic into dicarboxylic acids. The complete β-oxidation of an even carbon number dicarboxylic acid produces succinate which can enter the tricarboxylic acid cycle (5). The ω-oxidation of fatty acids is, therefore, able to spare or allow the synthesis of glucose and to reduce ketosis (5). This could be of importance to domestic ruminants, particularly to high-yielding dairy cows and twin-bearing ewes, in which ketosis due to hypoglycemia causes economically significant losses.

The ω-hydroxylation reaction is catalyzed by cytochrome P-450 of the endoplasmic reticulum using electrons supplied by NADPH cytochrome P-450 reductase. Reconstitution of the ω-hydroxylation systems of the rat (6) and mini-pig (7) has been achieved using purified cytochrome P-450, the reductase and lipids. However, the reconstitution of a functional enzyme system from these components does not exclude the participation of other components in the reaction. In the intact microsomal system, cytochrome b_5 and, by implication, NADH cytochrome b_5 reductase have been shown to play a role in supplying electrons from NADH to cytochrome P-450 for fatty acid ω-hydroxylation (8).

Similar results have been obtained for the fatty acid desaturase systems of hepatic microsomes. There are several desaturase systems, each consisting of a desaturase enzyme which obtains electrons from NADH-cytochrome b_5 reductase. They may be reconstituted using only these two enzymic components and lipid (9). However, in microsomal desaturation systems *in vitro* the participation of soluble factors such as catalase and various fatty acid binding proteins including bovine serum albumin, ligandin and Z-protein (10,11), and an additional unidentified protein which appeared to act directly on the desaturase system (12), has been demonstrated. Catalase has also been shown to stimulate the microsomal desaturation of 1-*O*-alkyl-2-acyl-*sn*-glycero-3-phosphoethanolamine in the synthesis of ethanolamine plasmalogen (13).

Here we report findings which support the participation of one or more soluble stimulatory factors in optimizing the ω-hydroxylation of fatty acids by sheep liver microsomes.

MATERIALS AND METHODS

Animals and preparation of tissue fractions. Samples of liver tissue from adult male and female sheep (Ovis aries) were obtained from a local abattoir. Animals were killed by captive bolt and immediately exsanguinated. Samples of liver tissue were quickly removed, placed in ice-cold homogenizing medium, and transported to the laboratory within 30 min. The homogenizing medium contained sucrose, 200 mM; *N*-2-hydroxyethylpiperazine-*N*-2-ethanesulfonic acid (HEPES), 40 mM; ethylenediaminetetraacetic acid (EDTA), 1 mM; and dithiothreitol, 1 mM, adjusted to pH 7.2. Tissue was coarsely chopped, washed, then homogenized (15% tissue w/v) at 0°C using a Potter-Elvehjem type homogenizer (Norlab Instruments, Dyce, Aberdeen, U.K.). Mitochondria, nuclei and cell debris were sedimented by centrifugation of the homogenate at 10,000 × *g* for 10 min at 4°C, and then the resulting supernatant was centrifuged at 140,000 × *g* for 60 min. The supernatant from the second centrifugation was termed the particle-free supernatant (PFS). The sediment from the second centrifugation was crude microsomes. Half of the crude microsomes were resuspended in homogenizing medium to a concentration of about 20 mg protein/mL (the non-washed or "complete" preparation). A "washed" microsomal fraction was prepared by gently resuspending the other half of the crude microsomal fraction in 15 mL of ice-cold homogenizing medium and recentrifuging (at 140,000 × *g* for 60 min). This washing step was repeated. During the washing procedures, the non-washed microsomal preparation was maintained at 0°C. Protein concentrations were measured by the method of Lowry *et al.* (14), using bovine serum albumin as the standard.

Incubations, and measurement of ω-oxidation activity. The microsomal activities of the ω-oxidation of dodecanoic and hexadecanoic acids were measured in a final volume of 1.0 mL of medium containing HEPES, 50 mM;

*To whom correspondence should be addressed at the Environment Unit, 639 High Road, London, N17 8BD, United Kingdom.

[1]The term ω-hydroxylation, without specification of position of hydroxylation, refers to ω1-hydroxylation plus ω2-hydroxylation.

Abbreviations: EDTA, ethylenediaminetetraacetic acid; HEPES, *N*-2-hydroxyethylpiperazine-*N*-2-ethanesulfonic acid; PFS, particle-free supernatant.

$MgCl_2$, 5 mM; $MnCl_2$, 5 nM; NAD, NADH, and NADPH all at 1 mM; glucose-6-phosphate dehydrogenase, 0.4 I.U./mL, pH 7.5. Radiolabeled substrate was added in propylene glycol; final concentrations were 300 μM (specific activity 0.33 mCi/mmol) for [1-^{14}C]dodecanoic acid and 100 μM (2.0 mCi/mmol) for [1-^{14}C]hexadecanoic acid in all incubations except the investigation of the time course, in which the final concentration of [1-^{14}C]dodecanoic acid was 500 μM (5.0 mCi/mmol). Unless otherwise indicated, the concentration of microsomal protein was 1 mg/mL in all incubations. If present, the concentration of PFS protein was 1 mg/mL; catalase (bovine liver, Boehringer Corporation Ltd., Lewes, U.K.), 8.000 ("low") or 80,000 ("high") I.U./mL; superoxide dismutase (bovine blood, type I, Sigma Chemical Co. Ltd., Poole, U.K.), 4 ("low") or 160 ("high") I.U./mL; sodium azide, 100 mM. Incubations were at 37°C and aerobic, and unless otherwise indicated, for 30 min. Incubations were stopped by the addition of 1 mL KOH in methanol (0.5 M), carrier fatty acids were added, the mixture saponified, and substrate and products extracted into diethyl ether, methylated, separated by thin-layer chromatography on silica gel G plates (15) and quantitated by liquid scintillation counting. The conversions of dodecanoic and hexadecanoic acids to products of ω-hydroxylation in the complete microsomal incubation system were typically about 10% and 2%, respectively, and recoveries of products typically greater than 97%. The identities of the products were confirmed by gas-liquid chromatography and mass spectrometry (15).

RESULTS AND DISCUSSION

Previous studies have shown that the microsomal fraction of sheep liver carries out the ω1- and ω2-hydroxylations of both medium- and long-chain fatty acids (15,16). The rate of the ω-hydroxylation of dodecanoic acid by a complete hepatic microsomal preparation was linear for more than 40 min (Fig. 1), and proportional to the concentration of microsomal protein up to 3 mg/mL (Fig. 2).

Washing procedure. Measurements of the protein present in the wash solutions showed that the washing procedures had removed 58% of the total protein of the crude microsomal preparation. The non-washed (complete) preparation was kept at the same temperature as the preparation being washed for the duration of the washing process. This was to limit, as far as possible, the differences between the preparations to those caused by the losses of soluble components. It should be noted that measurements of microsomal protein in the complete preparation represent both microsomal and soluble proteins; in contrast, those for the washed preparation represent microsomal protein with only traces of soluble protein.

Effect on ω-hydroxylation activity of removal of soluble material from the microsomal preparation. Figure 2 shows the relationships between the protein concentrations of the washed and the complete microsomal preparations and the rate of ω-hydroxylation of dodecanoic acid. At concentrations of microsomal protein of 1.0 mg/mL or less there were no significant differences in the specific activities of the ω-hydroxylation of dodecanoic acid. It follows that, because the washing process had removed about 60% of the protein present in the complete microsomal preparation, a loss of approximately 60% of the ω-hydroxylation activity originally present in the complete preparation had occurred. The dotted line shows the relation between specific activity and microsomal protein concentration which would have been obtained if no loss of activity had occurred on washing. Comparison of this relation with that for the washed preparation demonstrates the extent of the loss of activity.

The loss of activity became greater as the concentration of microsomal protein increased. At 3 mg microsomal protein/mL, the specific activity of the ω-hydroxylation of dodecanoic acid by the washed preparation was 58% of that of the complete preparation (Fig. 2). A similar variation in specific activity with microsomal protein concentration was observed for hexadecanoic acid. At a concentration of microsomal protein of 1.0 mg/mL, the specific activity of the ω-hydroxylation of hexadecanoic acid

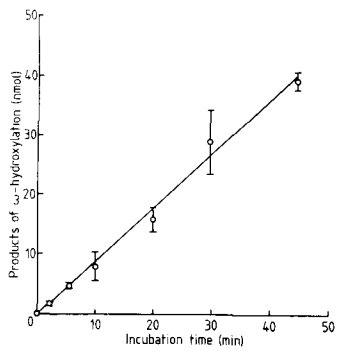


FIG. 1. Time course of the ω-oxidation of dodecanoic acid by an ovine hepatic microsomal preparation. The concentration of microsomal protein was 1 mg/mL, and of dodecanoic acid was 500 μM (specific activity 5.0 mCi/mmol). Values are the mean ± S.D. of three results.

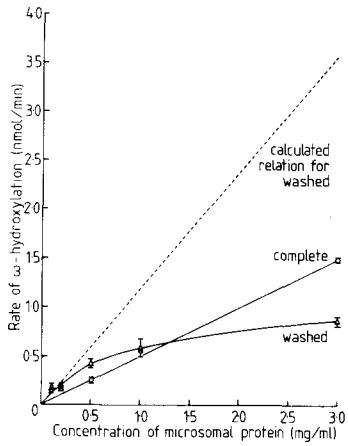


FIG. 2. Effect of washing an ovine hepatic microsomal preparation on the relation between the rate of ω-hydroxylation of dodecanoic acid and the concentration of microsomal protein. Results for the complete microsomal preparation are shown as circles, and for the washed preparation are shown as triangles. Values are the mean ± S.D. of three determinations, or mean ± range of two determinations (about half of the points for each preparation were from three determinations). The calculated relation between the expected activity after washing (based on the amount of protein removed during washing) and the concentration of microsomal protein is shown as a dotted line.

by the washed preparation was little different from that of the complete preparation (Fig. 3). At 3 mg microsomal protein/mL, the specific activity of the ω-hydroxylation of hexadecanoic acid was 54% of that of the complete preparation (Table 1). Thus, at the higher concentration of microsomal protein, the results for both dodecanoic and hexadecanoic acids suggest a loss due to washing of approximately 80% of ω-hydroxylation activity.

Figure 2 shows that the relationship between specific activity and microsomal protein concentration was linear for the complete preparation, but not for the washed preparation. This suggests that, at higher ratios of protein to the substrate fatty acid, the binding of the substrate to microsomal proteins reduces its access to the ω-hydroxylation system to a greater extent than does binding to soluble proteins (the latter may include specific fatty acid binding proteins). A limitation of reaction rate due to lack of the known participating agents such as NADPH or oxygen is improbable, since the total ω-hydroxylation activity in the incubations with the washed microsomal preparations was less than that with the complete preparations for both dodecanoic and hexadecanoic acids.

It is unlikely that an inherent instability of the ω-hydroxylation system could have caused the observed differences between the complete and washed microsomal preparations. Studies on the rat hepatic microsomal system have shown that it is quite stable. Okita *et al.* (17) found that storage for 24 hr at 4°C caused a loss of only 34% of ω-hydroxylation activity. In our studies, dithiothreitol (for protection of protein −SH groups) was present in the media for both microsomal preparations. Furthermore, the experimental design minimized differences caused by temperature and time in the enzyme activities of the washed and complete microsomal preparations. If the principal cause of the loss of activity was exposure to degradative (*e.g.*, lysosomal) enzymes, then the complete preparation would have been expected to show the largest decrease. Our results are consistent with the observed losses of activity being due to the removal by the washing process of one or more soluble components of the crude microsomal preparation.

Effect on dehydrogenation activity of removal of soluble material from the microsomal preparation. The conversion of the ω-hydroxy fatty acids to dicarboxylic acids occurs through the actions of alcohol and aldehyde dehydrogenases. Each of these enzymes is present in both the cytosol and in the microsomal membrane (18–20). Figure 4 shows that the removal, by washing, of the alcohol and aldehyde dehydrogenases present in the soluble fraction of a crude microsomal preparation did not significantly reduce the rate of production of dicarboxylic acid per mg microsomal protein. This suggests that approximately 60% of the total dehydrogenase activity of the crude microsomal preparation was present in the soluble fraction, and about 40% was associated with the microsomal membrane. The removal of the soluble dehydrogenases, together with around 60% of the total protein would result in little observable change in the dehydrogenase specific activity of the microsomal preparation.

TABLE 1

The Specific Activity of the ω-Hydroxylation of [1-^{14}C]Hexadecanoic Acid by Washed and Complete Preparations of Hepatic Microsomes[a]

Treatment	15- plus 16-hydroxy-hexadecanoic acids	Hexadecanedioic acid
Washed	26.7 ± 2.3	2.3 ± 0
Complete	44.0 ± 1.7	10.0 ± 9.3

[a]The concentration of microsomes was 3.0 mg/mL. The tabulated values represent the mean ± range of two determinations. The rates of formation of the different products are expressed as pmol/min/mg microsomal protein.

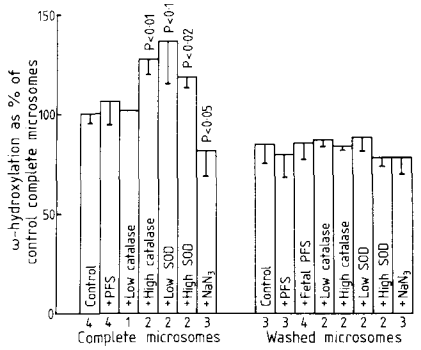


FIG. 3. The effects of adding 1 mg PFS, catalase at "low" (8,000 I.U./mL) or "high" (80,000 I.U./mL) concentrations, superoxide dismutase (SOD) at "low" (4 I.U./mL) or "high" (160 I.U./mL) concentrations or azide (100 mMol) to either the complete or washed ovine hepatic microsomal preparations on the ω-hydroxylation of hexadecanoic acid. Results were calculated as specific activities, and expressed as percentages in relation to the result for the control complete incubation. The values shown are means ± S.D. (three or more determinations) or means ± ranges (two determinations). The number of determinations is shown below each treatment. The significances of differences (by t-test) from the appropriate complete or washed control are shown. It should be noted that, if no activity had been lost on washing, the activity of the control washed microsomal preparation would have been about 240% of that of the control complete preparation.

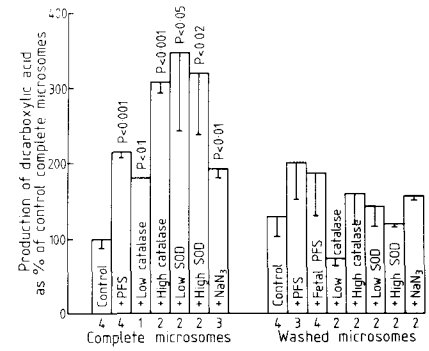


FIG. 4. The effects of the additions detailed for Figure 3 on the production of hexadecanedioic acid from hexadecanoic acid by complete or washed ovine hepatic microsomal preparations. Results were calculated as specific activities, and expressed as percentages in relation to the result for the control complete incubation. The values shown are means ± S.D. of three or more determinations or means ± ranges of two determinations. The number of determinations is shown below each treatment. The significances of differences from the appropriate complete or washed control are shown.

Effect on ω-hydroxylation activity of adding back a soluble fraction. No effect of the PFS on the ω-hydroxylation of hexadecanoic acid by either the washed or the complete microsomal preparations was observed (Fig. 3). This negative finding may simply reflect the large dilution of soluble components which occurred on homogenization and the further dilution occurring when the necessarily limited volume of PFS was added to the incubation system (overall dilution was about 250-fold). Alternatively, if the lowered activity was the result of the removal of a protective factor, then it would suggest that damage had occurred to either the enzyme system or its membrane environment, and was not reversible by replacement of the factor.

Effect on dehydrogenation activity of adding back a soluble fraction. For both the complete and the washed microsomal preparations, the addition of the soluble cytosolic fraction of liver (PFS) approximately doubled the rate of conversion of 16-hydroxy-hexadecanoic acid to dicarboxylic acid (Fig. 4). This is consistent with the presence in the PFS of soluble forms of the alcohol and aldehyde dehydrogenases which act in concert with the microsomal membrane bound dehydrogenases.

PFS from fetal liver was shown to have the same stimulatory effect on the dehydrogenation activity as the adult PFS (Fig. 4). This is of interest in the light of our previous observation that the ω-hydroxylation activity of fetal liver microsomes is negligible compared with that of adult preparations (15), consistent with findings on other fetal hydroxylation reactions (21). This suggests that dehydrogenases capable of acting on ω-hydroxy fatty acids develop earlier in ovine fetal life than the capacity to hydroxylate fatty acids.

Effects of catalase, superoxide dismutase and azide. The effects of two concentrations of catalase (8,000 = "low" and 80,000 I.U./mL="high") and superoxide dismutase (4 and 160 I.U./mL, "low" and "high") were investigated in incubations with both the washed and complete microsomal preparations. The concentrations of catalase were 10 ("low") and 100 times ("high") greater than the amount per mg microsomal protein found to give maximal stimulation of the alkylglycerol desaturation reaction (13). The concentrations of superoxide dismutase were similar to ("low") and about 50 times ("high") the concentration of this enzyme in mouse liver (22). The concentration of azide (100 mM) added to the incubations was sufficient to completely inhibit catalase activity, to inhibit 80% of superoxide dismutase activity and to quench singlet oxygen (23-25).

Both catalase and superoxide dismutase can interrupt the sequences of reactions which create "active oxygen" species. The latter are produced in large quantities by microsomal systems *in vitro* in the presence of NADPH (26). Since active oxygen species can damage both enzymes and membrane structures, they readily inhibit the functioning of enzyme systems. A well supported sequence of synthesis of active oxygen species begins with the production of the superoxide radical (27,28). This decomposes to give hydrogen peroxide, and the latter gives rise to the highly reactive hydroxyl radical (29). The hydroxyl radical can initiate fatty acid peroxidation which can produce singlet oxygen (30). Any of these active oxygen species could reduce ω-oxidation activity, either directly by reacting with the microsomal electron transport or cytosolic enzymes, or indirectly by attacking the lipid or protein of the membrane which contains the enzymes (31).

Catalase and superoxide dismutase both increased the rate of the ω-hydroxylation of hexadecanoic acid by the complete microsomal preparation (Fig. 3). Additionally, the proportion of products recovered as the dicarboxylic acid from the complete microsomal preparation in the presence of catalase or superoxide dismutase was about three times that found in the control incubation (Fig. 4). However, no effects of either catalase or superoxide dismutase were observed on the hydroxylation or dehydrogenation reactions carried out by the washed microsomal preparation (Figs. 3 and 4).

The dual stimulation of the electron-transporting enzymes of the microsomal membrane, and of the soluble and/or membrane bound dehydrogenases, strongly suggests that catalase and superoxide dismutase are acting non-specifically to increase the activity of these enzymes. Moreover, it is difficult to visualize a direct role for catalase or superoxide dismutase in the electron transport or dehydrogenation reactions of the ω-oxidation enzyme sequence. It seems likely, therefore, that the stimulatory effects of the additions of catalase and superoxide dismutase to the complete microsomal incubation system were due to the protection given to the microsomal and soluble enzymes from the damaging effects of active oxygen species produced by microsomes in the presnce of NADPH. In the cyclooxygenase enzyme system, self-destruction due to its associated peroxidase activity has been demonstrated by Lands and Byrnes (32), and may have some similarities to the effects we have described. Our results complement the previous findings that catalase stimulates the desaturations of fatty acids (10) and alkylglycerols (13).

The lack of effect of catalase and superoxide dismutase in the washed preparation could be explained by the production of much smaller quantities of active oxygen species by the washed microsomal preparation, or by the involvement of one or more soluble components in the complete preparation in the processes leading to membrane damage by active oxygen species.

Azide greatly stimulated the dehydrogenation reactions but inhibited the initial ω-hydroxylation reaction in the incubations with the complete microsomes. The stimulation may well reflect the ability of azide to quench singlet oxygen. The inhibitory effects may be exerted directly on the microsomal electron transporting enzymes or indirectly through inhibition of endogenous catalase and superoxide dismutase.

In conclusion, a large loss of fatty acid ω-hydroxylation activity is caused by the removal of the soluble components of a crude microsomal preparation. This suggests that one or more soluble factors may participate in or optimize the ω-hydroxylation of fatty acids by liver preparations from sheep. Possible identities of the soluble factor include: i) a fatty acid binding protein (or more than one) which functions, at least *in vitro,* as a carrier of substrate or products in the ω-hydroxylation reaction; ii) a component with direct stimulatory action on the ω-hydroxylase system; and iii) a factor (or more than one) which protects the ω-hydroxylase system from damage by oxygen radicals or other agents.

Both catalase and superoxide dismutase were able to

stimulate the ω-hydroxylation and dehydrogenation reactions carried out by crude microsomal plus soluble fractions *in vitro*. This finding implicates active oxygen species as agents which reduce the activity of the microsomal ω-hydroxylation system, either by acting directly on the enzymes, or by lipid peroxidation reactions which result in a disruption of the membrane environment of the ω-hydroxylation system.

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The Effects of Dietary Protein on the Fatty Acid Composition and Δ6 Desaturase Activity of Rat Hepatic Microsomes

Marie Lindholm and **Anders Eklund***
Department of Medical and Physiological Chemistry, University of Uppsala, Uppsala, Sweden

Recently, significant differences between rats fed a casein diet and rats fed a soybean protein diet have been observed in hepatic phospholipid fatty acid patterns (Sjöblom, L., and Eklund, A., *Biochim. Biophys. Acta 1004,* 187–192, 1990). The influence of these two diets on the Δ6 desaturase activity was investigated in the present study because the hepatic desaturase system is a source of unsaturated fatty acids. The rats fed a casein diet showed higher desaturase activity than those fed soybean protein when using either linoleic acid ($P<0.005$) or oleic acid ($P<0.05$) as substrates. The phosphatidylcholine fraction of hepatic microsomes showed increases in oleic acid ($P<0.005$) and 20:3ω9 ($P<0.001$) levels as well as decreases in stearic acid ($P<0.001$), linoleic acid ($P<0.005$) and arachidonic acid ($P<0.005$) levels in rats which were fed casein rather than soybean protein. Similar differences between the two groups were also observed in the phosphatidylethanolamine and phosphatidylinositol fractions. These data indicate that the qualitative properties of the dietary protein source may influence the fatty acid pattern of rat hepatic microsomes by interfering with Δ6 desaturase activity.
***Lipids 26,* 107–110 (1991).**

Dietary protein exerts a profound influence on the level and composition of the different plasma lipid and lipoprotein fractions in various experimental animals (1–4). Recently, it has been shown that the source of protein also influences the fatty acid pattern in various lipids in the liver, aorta and plasma of rats fed cholesterol-containing and cholesterol-free diets (5–7).

Within the lipid fractions of casein-fed rats, reduced levels of linoleic (18:2ω6) and arachidonic (20:4ω6) acids and raised levels of oleic (18:1ω9) and vaccenic (18:1ω7) acids were found compared to rats fed soybean protein. When the dietary fat solely consisted of olive oil, casein-fed rats showed markedly increased hepatic levels of Mead acid (20:3ω9) compared to rats fed soybean protein (7).

A possible explanation for the changes in fatty acid patterns could be a stimulation or inhibition of fatty acid desaturase activities in the rat liver, induced by the source of dietary protein. Choi *et al.* (8) have recently demonstrated increased Δ6 desaturase activity in rats fed casein compared to rats fed soybean protein, when both proteins were administered in a cholesterol-free diet. These authors did not report Δ6 desaturase activity in rats fed cholesterol-containing diets, and the dietary fat source and amount used were different from those used in the present study; we therefore undertook these experiments to compare the Δ6 desaturase activities in liver microsomes from rats fed casein or soybean protein in combination with cholesterol and olive oil.

*To whom correspondence should be addressed at the Department of Medical and Physiological Chemistry, University of Uppsala, P.O. Box 575, S-751 23 Uppsala, Sweden.

Abbreviations: BHA, butylated hydroxyanisole; CAS-rat, rat fed a casein diet; GLC, gas-liquid chromatography; PC, phosphatidylcholine; PE, phosphatidylethanolamine; PI, phosphatidylinositol; SOY-rat, rat fed a soybean protein diet; TLC, thin-layer chromatography.

MATERIALS AND METHODS

Materials. [1-^{14}C]Linoleic acid (specific activity 59 mCi/mmol) and [1-^{14}C]oleic acid (specific activity 57 mCi/mmol) were purchased from DuPont (Boston, MA); unlabeled linoleic acid, oleic acid and fatty acid standards were from Nu-Chek-Prep (Elysian, MN). KF was obtained from Aldrich-Chemie (Steinheim, Germany). Butylated hydroxyanisole (BHA) was purchased from May & Baker LTD (Dagenham, England). Adenosine triphosphate (ATP), Coenzyme A (CoA), reduced nicotinamide adenine dinucleotide (NADH) and N-acetyl-L-cystein were obtained from Sigma (St. Louis, MO). Other chemicals and reagents were obtained from Merck (Darmstadt, Germany).

Preparation of microsomes. Male 70 g Sprague-Dawley strain rats (Alab, Sollentuna, Sweden) were fed either a casein or a soybean protein-based diet, containing 20% protein, 10% olive oil, 0.5% cholesterol, vitamins, minerals, cellulose and cornstarch for four weeks as previously described (2). The rats were housed in a temperature- and light-controlled room (22°C, lights on 0600–1800). Water and food were provided *ad libitum.* The animals were killed in pairs, one from each diet on each occasion, and the livers were immediately excised and rinsed in ice-cold physiological saline solution. The livers were homogenized in 0.25 M sucrose, 0.15 M KCl, 4.9 mM $MgCl_2$ and 1.4 mM N-acetyl-L-cysteine in 40 mM KH_2PO_4/K_2HPO_4, pH 7.2. The homogenate was layered above an equal volume of 0.34 M sucrose, 0.15 M KCl, 4.9 mM $MgCl_2$ and 1.4 mM N-acetyl-L-cysteine in 40 mM KH_2PO_4/K_2HPO_4, pH 7.2 and was centrifuged 10 min at 800 × *g*. The supernatant was collected and centrifugated for 10 min at 17,000 × *g* (RC2-B centrifuge, Sorvall, Newtown, CT). The supernatant obtained was collected and centrifugated for 1 hr at 100,000 × *g* (Prepspin 50 ultracentrifuge with a fixed angle rotor, cat. no. 43114-118, MSE, Crawley, England). The remaining pellet was resuspended in 2 × 750 μL of the homogenization buffer. This suspension is referred to as the microsomal fraction.

Protein content was determined by the Lowry method (9). NADPH-cytochrome C-reductase was assayed as a microsomal marker (10).

Δ6 Desaturase assay. The optimal concentration of microsomes was determined in a series of incubations with different amounts of microsomes to assure a non-saturating protein level. Microsomal suspensions (300 μL) were mixed with 500 μL of incubation medium to a final microsomal concentration of 5 mg protein/mL. The homogenization buffer with 63.5 mM KF, 2.08 mM ATP, 97.7 μM CoA and 1.39 mM NADH added was used as incubation medium. After the addition of 5 μL 5.5 mM

[1-^{14}C]linoleic acid with a final specific activity of 1.14 mCi/mmol, the samples were incubated for 15 min at 37°C. The experimental procedure was repeated with 5 μL [1-^{14}C]oleic acid (1.14 mCi/mmol, 5.5 mM). The incubations were terminated by addition of 650 μL of 10% KOH in ethanol. BHA was added to suppress oxidation (final concentration 0.005%). The sample was saponified for 20 min at 100°C, cooled to room temperature and neutralized with 650 μL 2 M H_2SO_4.

The fatty acids were extracted with 2 × 5 mL of n-hexane, dried under a stream of N_2, and methylated with 1 mL of H_2SO_4/dry methanol (0.5:20, v/v) for 1 hr at 67°C. After addition of 1.0 mL of distilled water, the fatty acid methyl esters were extracted with 3 × 1.5 mL of petroleum ether (boiling range 40–60°C), concentrated by evaporation, and purified by thin-layer chromatography (TLC) on a prewashed silica gel plate (20 × 20 cm, Merck DC-Fertigplatten, cat. no. 5721) which was developed with hexane/toluene (1:1, v/v). After extraction, the purified fatty acid methyl esters were separated according to the degree of unsaturation by TLC on a $AgNO_3$ impregnated plate developed with hexane/diethyl ether/acetic acid (80:20:1, v/v/v). For the purpose of identification, pure standard fatty acids were run in parallel. The plates were scanned for radioactivity using an LB 2842 Automatic TLC-linear analyzer (Berthold, Wildbad, Germany).

Fatty acid analysis. The amount of total lipid in the microsomes was determined by Folch-extraction, as previously used by us (11). Phosphatidylcholine (PC), phosphatidylethanolamine (PE) and phosphatidylinositol (PI) were isolated by TLC as described previously (7). The fatty acid composition of the phospholipid classes in the hepatocyte microsomes was determined by gas-liquid chromatography (GLC) (7).

Statistical data treatment was by Student's t-test with NADPH-cytochrome C-reductase and desaturase activity values grouped in pairs (12).

RESULTS AND DISCUSSION

Fatty acid composition. As shown in Figures 1 and 2, the fatty acid composition of the two predominant phospholipid classes present in hepatic microsomes differed significantly depending on the source of dietary protein consumed by the rats. Oleic acid ($P<0.005$), vaccenic acid ($P<0.005$), and 20:3ω9 (Mead acid, $P<0.001$) reached higher levels in the microsomal PC fraction of rats fed a casein diet (CAS-rats) than in the corresponding fraction from rats fed a soybean protein diet (SOY-rats)

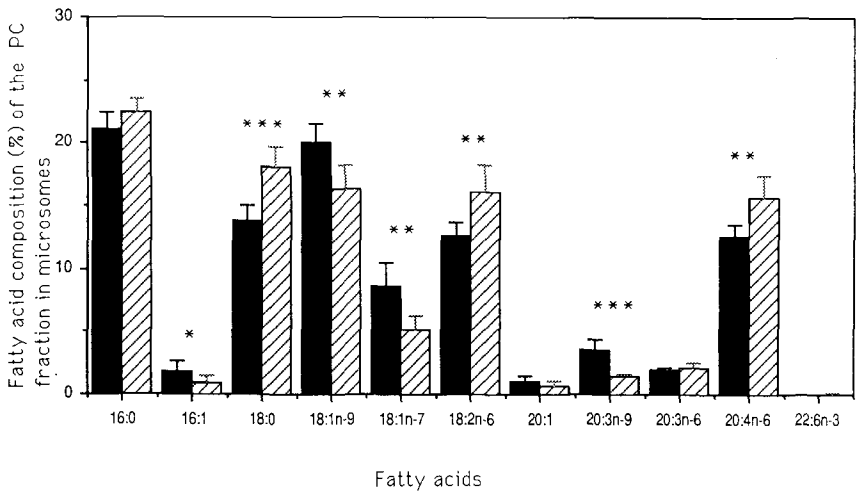


FIG. 1. Fatty acid composition of the PC fraction in hepatic microsomes from rats fed a casein- (solid bar) or a soybean protein- (hatched bar) based diet. Mean values ± SD. Significance levels: *P<0.05, **P<0.005, ***P<0.001.

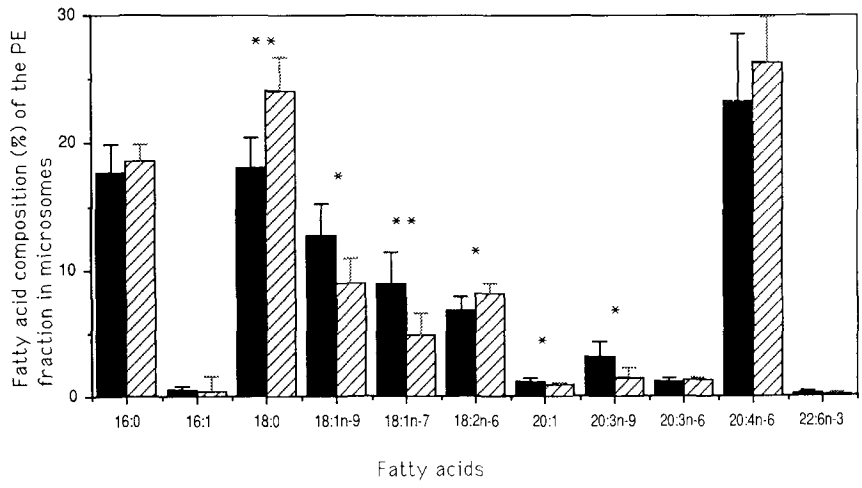


FIG. 2. Fatty acid composition of the PE fraction in hepatic microsomes from rats fed a casein- (solid bar) or a soybean protein- (hatched bar) based diet. Mean values ± SD. Significance levels: *P<0.05, **P<0.005, ***P<0.001.

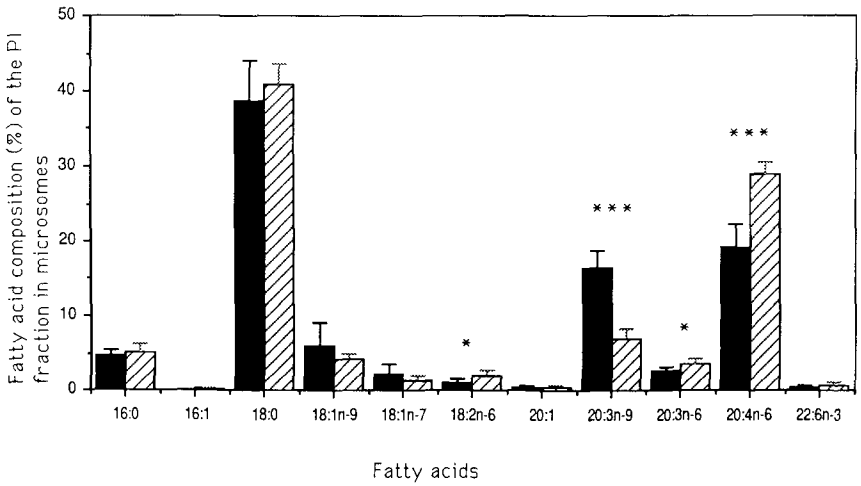


FIG. 3. Fatty acid composition of the PI fraction in hepatic microsomes from rats fed a casein- (solid bar) or a soybean protein- (hatched bar) based diet. Mean values ± SD. Significance levels: *P<0.05, **P<0.005, ***P<0.001.

TABLE 1

Effects of Diet on Δ6 Desaturase and NADPH-cytochrome C-Reductase Activities[a]

	Δ6 Desaturase activity of hepatic microsomes		
Dietary groups	Linoleic acid as substrate μmol/sec/mg protein	Oleic acid as substrate μmol/sec/mg protein	NADPH-cyt C-reductase activity μmol/sec/mg protein
Casein	4.14 ± 1.07	0.69 ± 0.24	30.6 ± 5.9
Soybean protein	2.90 ± 1.10	0.43 ± 0.15	39.4 ± 8.3
Significance level	P < 0.005	P < 0.05	P < 0.025

[a]The values represent mean values and SD of 10 rats (NADPH-cytochrome C-reductase and Δ6 desaturase with linoleic acid as substrate) or 7 rats (Δ6 desaturase with oleic acid as substrate). Three rats were not analyzed with oleic acid as substrate due to insufficient sample size.

(Fig. 1). In contrast, the levels of stearic (P<0.001), linoleic (P<0.005) and arachidonic (P<0.005) acids were reduced in the hepatic microsomal PC fraction of CAS-rats compared to SOY-rats. Thus CAS-rat hepatic microsomes contained increased levels of fatty acids of the ω9 series and decreased levels of ω6 series acids when compared to SOY-rat hepatic microsomes. The hepatic microsomal PE fraction displayed a similar pattern of differences in fatty acid composition between groups, as shown in Figure 2. The differences between CAS- and SOY-rats are similar to the patterns previously observed in whole livers (7).

Liver cells can produce oleic acid and a precursor of vaccenic acid by Δ9 desaturation of stearic and palmitic acid, respectively. Therefore, variations in fatty acid patterns observed between CAS-rats and SOY-rats could in part be explained by differences in Δ9 desaturase activity between groups. However, since the level of 20:3ω9 in the CAS-rat hepatic microsomal phospholipids was more than twice that in the SOY-rat microsomes, the fatty acid patterns show a difference in Δ6 desaturation of oleic acid as well.

In the present study, the PI fraction was also collected and analyzed, in addition to PC and PE, because in our previous work (7) the fatty acid patterns of the hepatic PI fractions were shown to differ significantly between rats fed casein and soybean protein diets. In CAS-rats, the level of 20:3ω9 in the PI fraction was more than twice as high as in the corresponding fraction of the SOY-rats (137% increase), while the level of arachidonic acid was reduced by 34% (Fig. 3). The results imply that casein feeding causes increased Δ6 desaturation of oleic acid, possibly due to reduced availability of linoleic acid (see below).

Δ6 Desaturase activity. As shown in Table 1, there was a significant difference in microsomal Δ6 desaturase activity between the two groups when either linoleic acid (P<0.005) or oleic acid (P<0.05) was used as substrate. With both substrates, the CAS-rats showed a higher activity than was observed in SOY-rats (43% higher with linoleic acid as substrate, 60% higher with oleic acid). In contrast, the activity of the microsomal NADPH-cytochrome C-reductase was on the average 29% higher in SOY-rats than in CAS-rats (P<0.025) which strengthens the significance of the difference in Δ6 desaturase activity between the two groups.

Provided the dietary supply of linoleic acid is limited, an increase in Δ6 desaturase activity could produce the changes in hepatic microsomal fatty acid pattern observed in the CAS-rats as compared to the SOY-rats by causing a depletion of the preferred substrate linoleic acid. In both experimental diets the linoleic acid content was approximately 2 energy percent (7). The recommended minimum level in rat diet is 1.3 energy percent (13). Although no visible signs of essential fatty acid deficiency became apparent in rats fed corresponding diets for 9 mon (Eklund, Anders, Sjöblom, Linnéa, and Lindholm, Marie,

unpublished observations), the present level of dietary linoleic acid may be insufficient provided that an increased metabolic need arises by increased Δ6 desaturase activity.

Thus, if a casein diet causes increased release and metabolism of arachidonic acid, this may accelerate the desaturation of linoleate to an extent that the hepatic linoleic acid pool becomes depleted. If so, the Δ6 desaturase system will use the normally less preferred substrate oleic acid and the level of 20:3ω9 will rise, as seen in the hepatic microsomes of the CAS-rats.

The mechanism behind the variable Δ6 desaturase activity is unknown. However, one possibility would be that the inability of CAS-rat hepatic microsomes to replace the metabolized arachidonic acid by Δ6 desaturation of linoleic acid, when the supply of the latter is inadequate, may cause a secondary increase in Δ6 desaturase activity.

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Triacylglycerol Synthesis in the Oleaginous Yeast *Candida curvata* D

Jane E. Holdsworth[1] and **Colin Ratledge***
Department of Applied Biology, University of Hull, Hull HU6 7RX, United Kingdom

Low rates of triacylglycerol (TAG) biosynthesis were observed in cell-free extracts of *Candida curvata*, but rates were increased up to 10-fold by adding either α- or β-cyclodextrins. Spheroplasts, whole or gently disrupted, had higher rates of incorporation of both [*U*-^{14}C]glycerol 3-phosphate or [1-^{14}C]oleate into triacylglycerol and the intermediates of its biosynthesis: lysophosphatic acid, phosphatidic acid and diacylglycerol. Fatty acyl-CoA synthetase was highest with palmitate, oleate and linoleate but was some 6- to 8-fold lower with stearate. Stearate and stearoyl-CoA were poorly incorporated into lipids. Subcellular fractionation of the spheroplasts into mitochondrial, microsomal, lipid bodies and supernatant fractions diminished the rates of ^{14}C incorporation of oleate into triacylglycerol. By comparing the relative specific activities for each activity in each fraction, the fatty acyl-CoA synthetase activity appeared mainly in the lipid bodies, and that for phosphatidic acid formation was mainly in the mitochondrion; other activities were too weak and too dispersed for accurate assessment of their location. Recombining all the subcellular fractions restored triacylglycerol synthesizing activity. Omitting any single fraction from the mixture did not result in restoration of triacylglycerol synthesizing activity. Starvation of the yeast, which leads to utilization of the endogenous lipid reserves, stimulated fatty acyl-CoA synthetase activity, but diminished phosphatidic acid and triacylglycerol biosynthesis indicating probable induction of β-oxidation in the peroxisomes and repression of lipid biosynthesis.
Lipids 26, **111-118 (1991).**

The biosynthesis of lipids in oleaginous yeasts, species possessing adenosine triphosphate (ATP):citrate lyase activity and producing more than 25% of biomass as lipids, has been studied extensively in our laboratories (1,2). Previous work concentrated on the events surrounding the hyperproduction of acetyl-CoA in these organisms. The events involving the conversion of fatty acyl-CoA esters into triacylglycerol have only been studied in the non-oleaginous yeasts *Saccharomyces cerevisiae* and *Candida tropicalis* (3,4). The pathways in non-oleaginous yeasts are broadly similar to those in plant and mammalian systems where the steps involved are well-established and the endoplasmic reticulum is considered the site for triacylglycerol synthesis. The pathway to triacylglycerol formation may be summarized as follows:

glycerol 3-phosphate + RCO-CoA → lysophosphatidic acid [1]

lysophosphatidic acid + RCO-CoA → phosphatidic acid [2]

phosphatidic acid → diacylglycerol + P_i [3]

diacylglycerol + RCO-CoA → triacylglycerol [4]

Using *S. cerevisiae,* Christiansen (5) reported that so-called "lipid particles" were associated with triacylglycerol-synthesizing enzymes in this yeast. Lipid particles are described as containing a mixture of triacylglycerols (45 to 50%) and may correspond to the larger, and more extensive, lipid droplets found as lipid storage bodies in oleaginous yeasts (6). The purpose of this study was to establish the pathway for triacylglycerol biosynthesis in *Candida curvata* and to identify the site of its synthesis.

MATERIALS AND METHODS

Media and growth of yeast. Candida curvata D (*Apiotrichum curvatum* ATCC 20599) was grown in batch culture at 30°C in 1-L, vortex-aerated bottles (7) containing 700 mL of nitrogen-limiting medium containing (g/L): glucose, 10; NH_4Cl, 0.5; KH_2PO_4, 7.0; Na_2HPO_4, 2.0; $MgSO_4 \cdot 7H_2O$, 1.5; yeast extract, 1.5; $CaCl_2 \cdot 2H_2O$, 0.1; $FeCl_3 \cdot 6H_2O$, 0.008; $ZnSO_4 \cdot 7H_2O$, 0.001; $CuSO_4 \cdot 5H_2O$, 0.0001; $Ca(NO_2)_2 \cdot 6H_2O$, 0.0001; $MnSO_4 \cdot 5H_2O$, 0.0005 with the pH adjusted to 5.5. Media were sterilized *in situ* by autoclaving at 121°C for 15 min.

Preparation of spheroplasts. Exponential phase cultures were harvested by centrifuging at 5000 × *g* for 10 min at 4°C. The cells were washed twice in citrate/phosphate buffer (0.01 M), pH 5.85, containing 0.6 M KCl and 5 mM dithiothreitol, resuspended in 20 mM MES buffer, pH 6.0, containing 2 M sorbitol and 2 mg Novozym 234 (Novo Industri, Bagsvaerd, Denmark) per mL, to give about 80 mg wet wt cells per mL. The cells were then incubated at 30°C for 45 min and spheroplast formation was followed by phase-contrast light microscopy. After complete conversion of whole cells to spheroplasts, the Novozym was removed by two washes of 20 mM MES buffer, pH 6.0, containing 2 M sorbitol.

Lysis of spheroplasts. Spheroplasts were lysed by diluting the sorbitol concentration of the suspending medium from 2 M to 0.25 M. The resultant suspension was centrifuged (2000 × *g* for 5 min) to remove whole cells and debris and the supernatant was used in the enzyme assays.

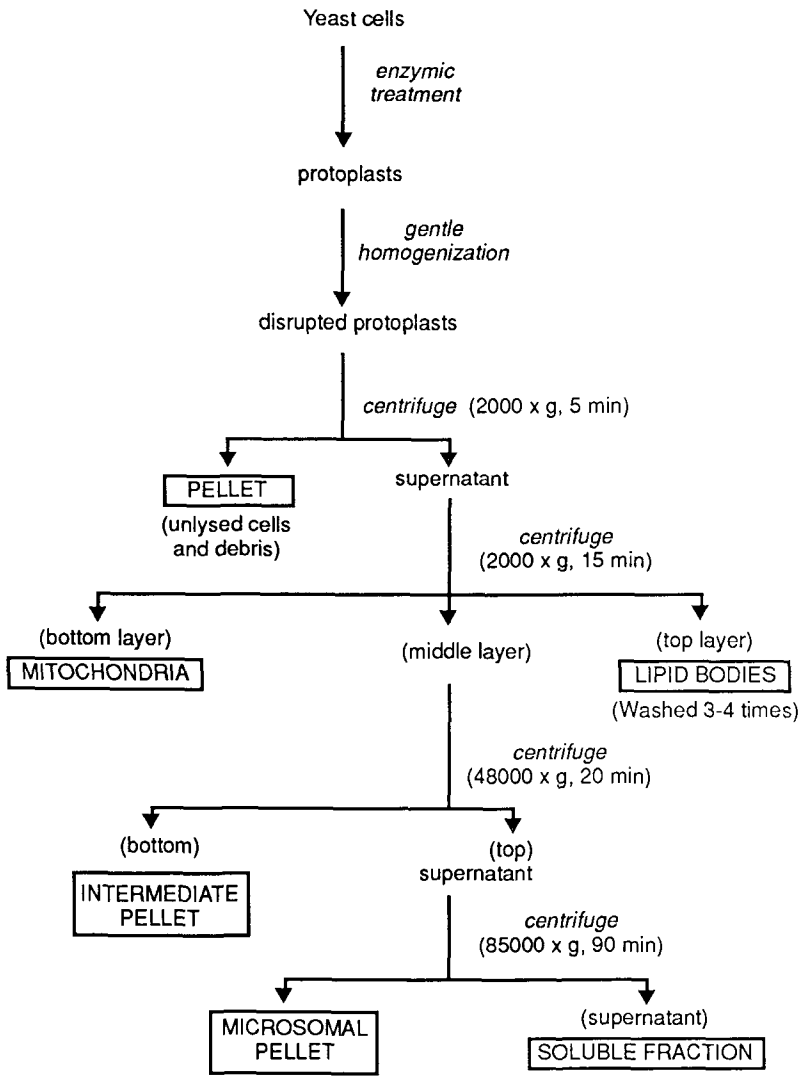
Subcellular fractionation of yeast cells (see Scheme 1). Complete rupture of the spheroplasts was accomplished using a Potter glass homogenizer. The resultant suspensions were centrifuged at 2000 × *g* for 5 min to remove any whole unlysed cells and debris. The supernatant was fractionated by centrifuging at 20,000 × *g* for 15 min to yield a crude mitochondrial pellet and a crude lipid fraction as top layer. The intermediate (middle) layer was centrifuged further at 48,000 × *g* for 20 min giving a pellet (termed the intermediate pellet) and a turbid supernatant which was re-centrifuged at 85,000 × *g* for 90 min to yield a microsomal pellet and a clear supernatant.

Purification of lipid globules. Lipid bodies or globules were purified by removing them from the top layer arising

[1] Present address: Industrial Processing Division, Box 31-310, Lower Hutt, DSIR, New Zealand.

*To whom correspondence should be addressed.

Abbreviations: ATP, adenosine triphosphate; DAG, diacylglycerol; G3P, glycerol 3-phosphate; lysoPA, lysophosphatidic acid; PA, phosphatidic acid; RSA, relative specific activity; TAG, triacylglycerol.



SCHEME 1. Subcellular fractionation of yeast cells. Fractions given in capital letters were tested for enzyme activity (see Table 3).

from the 20,000 × *g* centrifugation and resuspending them in 20 mM MES buffer, pH 6.0, containing 1 M sorbitol and re-centrifuging at 7000 × *g* for 5 min at 4°C. This washing procedure was repeated three or four times until the final top layer consisted only of purified oil globules.

Radiochemical assay procedure for enzymes involved in triacylglycerol biosynthesis. Assay systems were as described by Christiansen (5). All assays were performed in triplicate on two different extracts. Results shown are average values. Soluble protein was estimated by the method of Bradford (8) with bovine serum albumin as standard. In some assays whole spheroplasts were used as protein source, in others spheroplasts were lysed prior to use. Assays were also performed using subcellular fractions. The basic incubation mixture for all assays contained the following constituents: Tris/HCl, pH 7.4, 100 mM; ATP, 15 mM; $MgCl_2$, 15 mM; β-mercaptoethanol, 8 mM; and oleic acid, 0.4 mM, bound to 1mg/L defatted bovine serum albumin (fraction V, Sigma Chemical Co., St. Louis, MO). When added, glycerol 3-phosphate was at 5 mM. The reactions were carried out in a shaking water-bath at 27°C and initiated by adding the appropriate cell fractions. The total assay volume was 1 mL and reactions were carried out for 1 hr unless otherwise stated.

Assay of acyl-CoA synthetase. The conversion of 0.4 μmol [1-^{14}C]oleate (sp. act. 0.64 Ci/mol) to [1-^{14}C]oleoyl-CoA was measured in a total reaction volume of 0.25 mL basic incubation mixture. The reaction was started by the addition of protein (10 to 40 μg), carried out for 4 min and stopped by adding 1 mL Dole reagent. (propan-2-ol/n-heptane/1 M H_2SO_4, 40:10:2, v/v/v), 0.35 mL H_2O and 0.6 mL n-heptane (see ref. 5). The specific activity of the enzyme was determined with two different concentrations of protein. The extraction and scintillation counting of the product, acyl-CoA, was carried out as described by Lloyd-Davies and Brindley (9). The tube contents were mixed and separated and the heptane layer removed. The lower phase was washed 3 times with 0.6 mL portions of n-heptane. The radioactivity, in a 0.5 mL sample of the lower phase, was measured by scintillation counting after

adding 0.1 mL H_2O and 5 mL Triton X100/xylene (1:2, v/v) containing 2,5-diphenyloxazole (5.5 g/L), and 1,4-*bis*(5-phenyloxazol-2-benzene) (0.1 g/L).

sn-*Glycerol 3-phosphate acylation assay.* The biosynthesis of triacylglycerol was determined by measuring the rate of incorporation of *sn*-[2-^{3}H]glycerol 3-phosphate (sp. act. 0.1 Ci/mol) or [*U*-^{14}C]glycerol 3-phosphate (sp. act. 0.2 Ci/mol) into lipids in a total reaction volume of 1 mL. The reaction was started by the addition of 0.1 to 0.3 mg protein and carried out for 60 min. The reaction was terminated by the addition of 1.5 mL n-butanol followed by 2 mL H_2O. After shaking, the mixture was centrifuged and the butanol phase pipetted off and washed once with 3 mL water saturated with butanol to remove unreacted glycerophosphate. The butanol extract was removed and the solvent evaporated under reduced pressure. The residual lipid was dissolved in a known amount of chloroform, samples of which were taken for counting total radioactivity and for chromatography. Neutral lipids were analyzed by thin-layer chromatography on silica gel plates (Silica Gel G, 250-μm) using petroleum hydrocarbon (b.p. 60–80°C)/diethyl ether/acetic acid (60:40:1, v/v/v). In this system triacylglycerol, free fatty acids, diacylglycerol and monoacylglycerols are separated from phospholipid and acyl-CoA esters which remain at the origin. Phospholipids were separated from the neutral acyl-CoA using chloroform/methanol/acetic acid/water (80:15:2:2, by vol). Lipid classes were detected by exposure to iodine vapor and their R_F values compared with those of standards (Sigma). After the iodine had evaporated, the spots were scraped from the plates and the radioactivity determined by counting in 7.5 mL of a scintillation mixture containing 0.3 g 1,4-*bis*(5-phenyloxazol-2-yl benzene), 7.0 g 2,5-diphenyloxazole, 100 g naphthalene, 200 mL of water and 1 L dioxan.

Oleate esterification with added glycerol 3-phosphate as acyl acceptor. Oleate esterification was measured by incubating 0.4 μmol [1-^{14}C]oleic acid (sp. act. 0.64 Ci/mol) with 5 μmol glycerol 3-phosphate in a total reaction volume of 1 mL of basic incubation mixture. The reaction was stopped by adding 20 mL chloroform/methanol (2:1, v/v). After standing for 15 min, precipitated protein was removed by filtering the extract through Whatman No. 1 filter paper. The test tube and the filter were rinsed with 15 mL chloroform/methanol (2:1, v/v), followed by 15 mL chloroform/methanol (1:2, v/v). The combined extracts (50 mL) were evaporated in a rotary evaporator. The residue was extracted with 0.5 mL chloroform and the insoluble remainder dissolved in 1 mL chloroform/methanol/water, (4:10:2, v/v/v). For determination of total radioactivity, equal volume percentages of the two solutions were placed in the same vial, the solvent removed under a stream of nitrogen and then counted as for the glycerol 3-phosphate studies. Lipids in the two solutions were separated by thin-layer chromatography by applying aliquots representing equal-volume percentages as a single spot to the chromatogram which was developed and counted as in the glycerol 3-phosphate studies.

Oleate esterification with endogenous acyl acceptor. [1-^{14}C]Oleate was incubated under exactly the same conditions as stated above, except that glycerol 3-phosphate was not added. The extraction of the incubation mixture and the radioactivity assay were also as above.

RESULTS

Lipid synthesizing activities in cell-free extracts and spheroplasts. Initial experiments for assaying oleoyl-CoA synthetase and for following incorporation of [1-^{14}C]-oleate into intermediates of triacylglycerol biosynthesis using various acceptor molecules used unfractionated cell-free extracts (Table 1). Activities were lower than could account for the calculated rate of lipid synthesis in growing cells (see ref. 10). Addition of pre-formed lipids (di- and triacylglycerols), protease inhibitors or alternative acyl acceptors to glycerol 3-phosphate all failed to increase these activities. Some stimulation of oleate esterification activity, particularly of the formation of oleoyl-CoA and of phosphatidic acid, occurred in the presence of α- and β-cyclodextrins which are thought to increase the hydrophilicity of the substrate (Table 1). Cyclodextrins were, however, not included in subsequent experiments. Similar rates of incorporation of oleate into lipids occurred at both pH 6.0 and 8.0 (results not shown); subsequent experiments always were done at pH 7.4.

When assays were carried out using whole spheroplast preparations as protein source, considerable stimulation (increases between 5- and 10-fold) occurred for all activities being measured (Table 1). As such preparations of spheroplasts would have been lysed in the assay mixtures due to osmotic shock, the observed enzyme activities did not depend upon the spheroplasts remaining intact. Spheroplasts deliberately lysed before assay showed similar activities to preparations not lysed before commencement of the assay (Fig. 1). Spheroplast preparations also showed substantially improved ability to catalyze the acylation of *sn*-[*U*-^{14}C]glycerol 3-phosphate compared to cell-free extracts (Table 1).

The rates of biosynthesis of the various lipid components with [1-^{14}C]oleate as substrate (Fig. 1) showed that formation of oleoyl-CoA and of phosphatidic acid was most rapid and would not limit the incorporation of ^{14}C into intermediates later in the pathway. Formation of both di- and triacylglycerols was essentially linear over the 45-min incubation period. A small but apparently constant amount of label was incorporated into lysophosphatidic acid. A comparison of the data given in Table 1 and Figure 1 indicates, within the limits of experimental

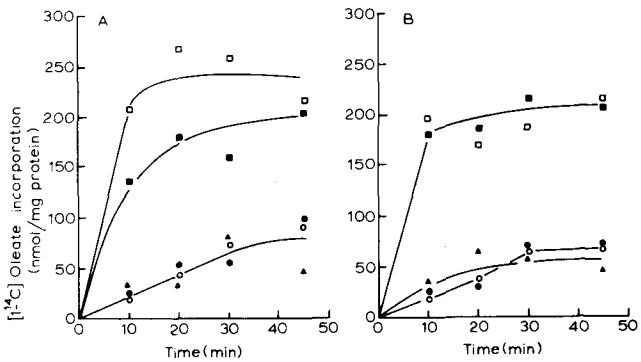


FIG. 1. Time course of [1-^{14}C]oleate incorporation into intermediates of triacylglycerol biosynthesis (A) and lysed (B) spheroplasts of *C. curvata*. Formation of acyl-CoA, (□); phosphatidic acid, (■), lysophosphatidic acid, (△); diacylglycerol, (○); and triacylglycerol, (●).

TABLE 1

Fatty Acyl-CoA Synthetase and Triacylglycerol and Intermediate Biosynthesis in Cell-Free Extracts and Spheroplasts of *C. curvata*[a]

(a) Oleoyl-CoA synthetase

Preparation	Sp. act. (nmol ^{14}C incorporated/hr/mg protein)
Cell-free extract	180
Whole spheroplasts	510

(b) Oleate esterification

Preparation	Additions to assay	nmol ^{14}C incorporated/hr/mg protein into Acyl-CoA	LysoPA	PA	DAG	TAG
Cell-free extract	none	120	0	14	1	7
	G3P	122	0	49	30	15
	G3P + α-cyclodextrin	735	0	97	0	1
	G3P + β-cyclodextrin	499	0	158	6	1
Whole spheroplasts	none	630	39	23	0	37
	G3P	505	295	339	148	117

(c) Glycerol 3-phosphate acylation

	nmol [*U*-^{14}C]glycerol 3-phosphate incorporated/hr/mg protein LysoPA	PA	DAG	TAG
Cell-free extract	—	4	0	2
Whole spheroplasts	120	121	74	61

[a]Abbreviations: G3P, glycerol 3-phosphate; PA, phosphatic acid; DAG, diacylglycerols; TAG, triacylglycerols.

error, that the ratio of incorporation of [1-^{14}C]oleate and [*U*-^{14}C]glycerol 3-phosphate into the various lipids is stoichiometrically compatible. Similar rates of biosynthesis occurred at 25°C; subsequent assays always were at 27°C.

Although no significant difference was seen between the triacylglycerol (TAG) synthesizing activities in spheroplasts and lysed spheroplasts, low-speed centrifuging (2000 × *g* for 5 min) of the lysed protoplasts led to substantial loss of activity in the supernatant, but recombining the pellet and supernatant restored activity.

It thus appeared that intact spheroplasts were not essential to achieve biosynthesis, but subsequent work showed that mechanical disruption of the spheroplasts using a Potter homogenizer was very critical if active cell-free extracts were to be obtained. Disruption of spheroplasts in a Potter homogenizer with 20 strokes of the rotor led to virtually complete loss of di- and triacylglycerol synthesizing ability in the supernatant following low-speed centrifugation. Ten strokes of the Potter rotor disrupted the spheroplasts without loss of enzyme activity which remained in the supernatant. Thus lipid-synthesizing activity could be obtained in a cell-free extract devoid of any residual whole cells, spheroplasts or large organelles (*e.g.*, nuclei).

Such preparations of lysed spheroplasts readily formed fatty acyl-CoA esters with [1-^{14}C]-labelled linoleic, palmitic and oleic acids but less readily with stearic acid (Table 2). Incorporation of the former three fatty acids into triacylglycerol and intermediates was also rapid and that of stearic acid also slow (Table 2). When stearoyl-CoA and [*U*-^{14}C]glycerol 3-phosphate were used to follow lipid biosynthesis (instead of labelled stearate and unlabelled glycerol 3-phosphate), there was no significant increase in activity and, indeed, no incorporation into triacylglycerol could be detected.

Lipid synthesis in subcellular fractions. Subcellular fractionation of the spheroplasts from *C. curvata* was carried out as detailed in Materials and Methods (see Scheme 1). Each fraction was used immediately in assays for fatty acyl-CoA synthetase and for the enzymes of triacylglycerol biosynthesis. The latter were assayed by following the incorporation of either [1-^{14}C]oleate (oleate esterification) or [*U*-^{14}C]glycerol 3-phosphate (glycerol 3-phosphate acylation) into the intermediates of the phosphatidic acid pathway. The results are shown in Table 3. The highest rates of incorporation of ^{14}C into acyl-CoA and phosphatidic acid pathway intermediates were in the pellet fraction which would have contained whole spheroplasts and whole cells. However, the highest activity of fatty acyl-CoA synthetase was found in the lipid bodies but, judging from the relative specific activity values which indicate if an enzyme has become located in particular fraction by relating its activity to how much protein is recovered in that fraction, no obvious location could be identified for the remaining enzymes of the triacylglycerol pathway. Two marker enzymes were assayed simultaneously: citrate synthase, as a mitochondrial enzyme, was recovered at a very high relative specific activity in this fraction though reduced nicotinamide adenine dinucleotide phosphate (NADPH)-cytochrome *c* reductase, as a microsomal enzyme, was recovered slightly preferentially in this fraction but it also occurred in the final soluble fraction. NADPH-cytochrome *c* reductase has been previously used as a marker for the microsomal fraction of baker's yeast (11). The nonexclusive location of the enzyme within *C. curvata* is in keeping with results found for other yeasts (12,13). Thus it appears that triacylglycerol biosynthesis only occurs maximally in extracts containing all cell components. Recombining all six fractions in their original proportions (by protein content) after fractionation restored the

TABLE 2

Incorporation of Various [1-^{14}C]-labelled Fatty Acids into Fatty Acyl-CoA and Triacylglycerols and Biosynthetic Intermediates in Lysed Spheroplast Preparations of *C. curvata*

(a) Fatty acyl-CoA synthetase

[1-^{14}C]-fatty acid	nmol fatty acid incorporated into acyl-CoA/hrmg protein
Linoleate	720
Oleate	510
Palmitate	750
Stearate	90

(b) Fatty acid esterification

Substrate	Addition to assay	nmol [1-^{14}C]-labelled fatty acid incorporated/hr/mg protein into Acyl-CoA	LysoPA	PA	DAG	TAG
Linoleate	G3P	120	79	214	16	90
	none	47	41	11	22	15
Oleate	G3P	51	50	218	59	70
	none	297	97	7	5	11
Palmitate	G3P	154	82	178	4	70
	none	391	56	34	7	38
Stearate	G3P	23	8	0	5	11
	none	5	0	1	10	2

Abbreviations: G3P, glycerol 3-phosphate; PA, phosphatic acid; DAG, diacylglycerols; TAG, triacylglycerols.

activity to the order seen with whole spheroplasts. Combining two or more of the fractions did not enhance the rate. Therefore it was concluded that all fractions were necessary for triacylglycerol biosynthesis to occur.

Assay for the enzyme of triacylglycerol biosynthesis in spheroplast preparations from C. curvata *grown under carbon starvation conditions. Candida curvata* was starved in a glucose-less medium (14) for 24 hr following growth on nitrogen-limited media prior to preparation of spheroplasts. Assays were carried out using the entire spheroplasts with and without added glycerol 3-phosphate as acyl acceptor and using [1-^{14}C]oleic acid esterification as a measure of activity. The results are shown in Table 4. The activity of fatty acid acyl-CoA synthetase was much higher in these starved spheroplasts than it had been in those undergoing accumulation. However, as expected, rates of incorporation of label into the intermediates of triacylglycerol biosynthesis were now much slower than those observed in preparations grown under nitrogen-limitation and hence accumulating lipid.

DISCUSSION

Previous studies on the location of the enzymes involved in lipid biosynthesis in yeasts have used almost exclusively *Saccharomyces cerevisiae* and have concentrated primarily upon the enzymes of phospholipid biosynthesis. These enzymes are associated with the membranes of the mitochondria and microsomes (15). Enzymes for triacylglycerol synthesis (see Introduction), of which the first two are the same as those involved with phospholipid biosynthesis, also seem to be membrane associated (16,17) though Christiansen's work (5,18) indicated the site of triacylglycerol biosynthesis to be the "lipid particles" of baker's yeast.

Saccharomyces cerevisiae is not a particularly good yeast for synthesizing triacylglycerols as these lipids usually do not constitute more than 5 or 6% of the cell's biomass (12) and only account for 50% of the lipids within the "lipid particles" (5). Oleaginous yeasts, such as *C. curvata* as used here, would seem more useful as they can readily accumulate over 50% of the entire biomass as triacylglycerol. In spite of the conceptual advantage of using an oleaginous yeast to study triacylglycerol biosynthesis, it has proved very difficult to elucidate the exact sites of synthesis principally because of the inherent instability of the enzyme systems.

Using whole spheroplasts, incorporation of both ^{14}C-labelled oleoyl-CoA and glycerol 3-phosphate into triacylglycerol and intermediates was achieved. The maximum rate of incorporation was about 100 to 110 nmol oleate/hr per mg protein. Previous work (10) indicated that a rate of about 250 nmol oleate should be incorporated per hr and mg protein to account for the observed rate of lipid biosynthesis in growing cells. Although the observed rate was less than it should have been it was considerably higher than previously reported by Christiansen (18) and is comparable to the rates reported for phospholipid biosynthesis in *S. cerevisiae* (15,19).

A linear rate of incorporation of label into diacylglycerol and triacylglycerol was observed over 30 min (Fig. 1). Incorporation of label into acyl-CoA and other intermediates of the pathway was not linear over this time but did not limit flow of carbon through the pathway. This finding was consistent with phosphatidic acid phosphatase being the rate-limiting step of lipid biosynthesis found in *S. cerevisiae* by Hosaka and Yamashita (16,17).

Assays using whole spheroplasts gave similar results to those using lysed spheroplasts but considerable activity was lost upon slow speed centrifugation (2000 × *g* for 5 min) of the lysed spheroplasts indicating that a subcellular component, necessary for biosynthetic activity, was being removed. Gentle homogenization (10 strokes of a homogenizer) could, however, be used to prepare

TABLE 3

Specific and Relative Specific Activities (RSA)[a] of Fatty Acyl-CoA Synthetase and of the Enzymes of Triacylglycerol Biosynthesis in Subcellular Fractions of *C. curvata*

Subcellular fraction (see Scheme 1)	Spec. act. (nmol/min/mg protein) marker enzymes			
	Protein content (mg)	NADPH-cytochrome *c* reductase	Citrate synthase	Fatty acyl-CoA synthetase (nmol oleoyl-CoA formed/hr/mg protein
Pellet (unlysed cells and debris)	220	10	162	8760
Mitochondria	25	9	439	1830
Lipid bodies	53	8	9	6300
Intermediate pellet	2	9	20	1290
Microsomal pellet	13	24	24	75
Soluble fraction	101	24	8	240
	% total protein in fraction	RSA	RSA	RSA
Pellet	53.1	0.74	0.74	1.55
Mitochondria	6.0	0.66	22.7	0.33
Lipid bodies	12.8	0.59	0.99	1.12
Intermediate pellet	0.5	0.64	0.08	0.22
Microsomal pellet	3.1	1.8	0.65	0.01
Soluble fraction	24.3	1.78	1.67	0.04

Subcellular fraction (see Scheme 1)	Biosynthesis of acyl lipids using: nmol [1-^{14}C]oleate incorporated/hr/mg protein into:										[U-^{14}C]glycerol 3-phosphate incorporated/hr/mg protein into:			
	Acyl-CoA + G3P	Acyl-CoA − G3P	LysoPA + G3P	LysoPA − G3P	PA + G3P	PA − G3P	DAG + G3P	DAG − G3P	TAG + G3P	TAG − G3P	LysoPA	PA	DAG	TAG
Pellet (unlysed cells and debris)	96	404	359	47	134	7	121	92	77	11	97	121	27	147
Mitochondria	305	259	159	29	112	5	37	11	17	12	0	20	3	3
Lipid bodies	545	238	60	38	35	6	4	0	4	4	9	0	4	0
Intermediate pellet	27	217	54	9	0	3	30	15	6	9	13	46	6	2
Microsomal pellet	33	33	66	5	61	6	0	27	2	3	30	0	0	0
Soluble fraction	66	80	17	16	32	35	3	17	4	2	0	0	16	0
	RSA	RSA	RSA		RSA		RSA		RSA		RSA	RSA	RSA	RSA
Pellet	0.61	1.43	1.67		1.45		1.98		1.74		1.81	1.84	1.4	1.88
Mitochondria	1.97	1.09	0.73		1.22		0.55		0.4		—	0.3	0.16	0.03
Lipid bodies	3.48	0.84	0.27		0.38		0.06		0.1		0.17	—	0.21	—
Intermediate pellet	0.16	0.74	0.24		0		0.42		0.14		0.24	0.66	0.3	0.08
Microsomal pellet	0.21	0.12	0.38		0.67		0		0.05		0.56	—	—	—
Soluble fraction	0.42	0.28	0.08		0.35		0.04		0.1		—	—	0.86	—

[a]RSA, relative specific activity is the % total enzyme activity in a fraction/% total protein in that fraction.
Abbreviations as given in Table 1.

TABLE 4

Fatty Acyl-CoA Synthetase and Triacylglycerol and Intermediates Biosynthesis in Spheroplast Preparations from *C. Curvata* Held Under Carbon Starvation Conditions[a]

(a) Fatty acyl-CoA synthetase

nmol [1-^{14}C]oleate incorporated into acyl-CoA/hr/mg protein

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(b) Oleate esterification

Addition to assay	[1-^{14}C]oleate incorporated (nmol/hr/mg/protein) into:				
	Acyl-CoA	LysoPA	PA	DAG	TAG
G3P	76	19	0	0	0
none	21	18	5	0	8

[a]Abbreviations: G3P, glycerol 3-phosphate; PA, phosphatic acid; DAG, diacylglycerols; TAG, triacylglycerols.

extracts from the spheroplasts which still retained triacylglycerol biosynthesizing activity. However great care had to be taken when using this technique as further homogenization (*e.g.*, 20 strokes) led to partial loss of activity. A possible explanation for this is that the lesser number of strokes led to gentle separation of the subcellular components and that the greater number led to actual disruption or dislocation of one or more subcellular components. Such a component, which may not be a protein, might be involved in the presentation of the substrate, oleoyl-CoA, to the appropriate enzyme. This speculation is supported by observing that triacylglycerol biosynthesis could be stimulated by adding α- and β-cyclodextrins to crude cell-free extracts. These molecules cyclohexa- and cyclohepta-amylose can bind lipids below their critical micelle concentration ($\sim$3 μM for oleoyl-CoA) and probably provide a more conducive (*i.e.*, hydrophilic) environment for the substrate to approach the enzyme's active site. By using whole or gently broken spheroplasts for triacylglycerol biosynthesis, the dependency upon cyclodextrins disappeared possibly indicating that correct orientation of the substrate was now being achieved *in situ*.

Subcellular fractionation of *C. curvata* resulted in six fractions including the first pellet. Mitochondrial and microsomal fractions were characterized by specific activities of marker enzymes. The highest relative specific activity of fatty acyl-CoA synthetase was in the pellet and lipid body fractions. That the lipid bodies could contain such activity possibly corresponds with the previous reports of Christiansen (5,18) for the location of triacylglycerol biosynthesis being in the "lipid particles" of baker's yeast. The final step(s) of triacylglycerol biosynthesis in the present yeast did not appear to be similarly located even though the lipid bodies of *C. curvata* would be composed primarily of triacylglycerol. However so much activity was lost during the preparation of the subcellular fractions, apart from the pellet which retained activity probably because of the presence of whole spheroplasts and unbroken cells, that it is not possible to ascribe with any certainty the possible location of the other enzymes for triacylglycerol biosynthesis. Nevertheless incorporation of both glycerol 3-phosphate and oleate into phosphatidic acid (*i.e.*, as a common precursor of both phospholipids and triacylglycerols) appeared to occur in the mitochondrial fraction with some activity being recovered in the intermediate fraction (for oleate incorporation) and microsomal fraction (for glycerol 3-phosphate incorporation). Such results would then be in agreement with the previous conclusions of Kuchler *et al.* (15).

Similar activities of fatty acyl-CoA synthetase(s) were observed with linoleic (18:2), oleic (18:1) and palmitic (16:0) acids in *C. curvata* though activity with stearic acid (18:0) was very low. (Such a difference was not seen when the specificity of the fatty acyl-CoA synthetase from another oleaginous yeast, *Lipomyces starkeyi* CBS 1809, was examined; ref. 20.) Rates of incorporation of linoleate, oleate and stearate into lipid intermediates using [*U*-^{14}C]-glycerol 3-phosphate were similar, but again little incorporation was seen using stearic acid. Although this was not surprising due to the low activities of stearoyl-CoA synthetase, very little improvement occurred when stearoyl-CoA itself was used as substrate. The reason for this is not clear as *C. curvata* has probably the highest content of stearic acid in its triacylglycerols of any yeast (1,21).

The most detailed knowledge of the long-chain fatty acyl-CoA synthetase of yeasts comes from work on *Yarrowia (Candida) lipolytica* (see ref. 22 for a review). These studies have indicated the presence of two long-chain fatty acyl-CoA synthetases, I and II. Synthetase I was present in cells grown on glucose and oleate, and was distributed between mitochondrial and microsomal fractions being responsible for the production of acyl-CoA in the synthesis of cellular lipids. Its activity toward stearic acid was half of that observed for palmitic, oleic or linoleic acids. Synthetase II, immunologically distinct from synthetase I, was induced by growing the yeast on oleic acid, and was located in peroxisomes, its main function being to produce acyl-CoA prior to degradation *via* β-oxidation. In the present study, the specific activity of fatty acyl-CoA synthetase for oleic acid increased 40-fold when *C. curvata* was starved, and thus was induced to consume its own intracellular storage lipid (6,14). This increase in activity then corresponds to the induction of synthetase II in the peroxisomes of *Y. lipolytica* as starvation of an oleaginous yeast also induces formation of peroxisomes (6). This, however, remains to be examined on some future occasion.

Simultaneously with this increase in fatty acyl-CoA synthetase activity in starved yeasts, enzymes for phospholipid and triacylglycerol biosynthesis were almost completely absent. This would again be in keeping with our previous observation that utilization of endogenous (or exogenous) lipid quickly leads to cessation of lipid biosynthesis (14). Thus the entire pathway of lipid biosynthesis, from citrate translocation across the mitochondrial membrane and cleavage of citrate by ATP:citrate lyase (see ref. 2) to the final steps of lipid synthesis, could be under strong regulatory control in oleaginous yeasts.

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The Comparative Effects of Dietary α-Linolenic Acid and Fish Oil on 4- and 5-Series Leukotriene Formation *in Vivo*[1]

J. Whelan*, K.S. Broughton[2] and J.E. Kinsella[3]
Lipids Research Laboratory, Institute of Food Science, Cornell University, Ithaca, New York 14853

The comparative effects of dietary α-linolenic acid and fish oil on eicosanoid metabolism was studied *in vivo*. Resident murine peritoneal cells were stimulated *in vivo* with opsonized zymosan in animals maintained on diets containing increasing amounts of α-linolenic acid or fish oil concentrate with projected n-3/n-6 ratios of 0.2, 0.4 and 1.0. While fish oil feeding resulted in significant changes in eicosapentaenoate tissue levels, α-linolenic acid was preferentially metabolized to docosahexaenoate. High performance liquid chromatographic analysis revealed the formation of leukotriene E_5 (LTE_5) in all the fish oil groups (19.8 ± 3.5 ng/mouse to 83.3 ± 13 ng/mouse), but only in the highest linolenic acid group (6.0 ± 3.2 ng/mouse). Concomitantly, the 4-series sulfidopeptide leukotrienes and PGI_2 were significantly reduced in the two highest fish oil containing dietary groups. Similar reductions were observed in the highest linolenic acid group, but the changes were not statistically different from the control values. In summary, this paper reports the *de novo* synthesis of 5-series sulfidopeptide leukotrienes in animals consuming α-linolenic acid. It also reveals that dietary fish oil is 2.5 to 5 times more effective than α-linolenic acid in modulating eicosanoid metabolism and altering tissue phospholipid fatty acid composition.
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The physiological significance of dietary polyunsaturated fatty acids (PUFA) in relation to lipid and eicosanoid metabolism is being elucidated (1-3). The role of n-3 PUFA, particularly those of marine origin, generate interest because they appear to possess distinct biological effects separate from the n-6 analogues. For example, n-6 unsaturated fatty acids of vegetable origin are effective in decreasing plasma cholesterol, while n-3 PUFA from fish oils can significantly depress plasma triglyceride levels (1,4). Additionally, n-3 long-chain PUFA have been shown to be involved in decreasing the pathology of diet-induced vascular lesions, parameters associated with carcinogenesis and modulate inflammatory indices (5-8). It has been postulated that while dietary n-6 unsaturated fatty acids are essential and beneficial, elevated intakes may contribute to pathologies associated with a number of chronic diseases, and it has been suggested that long-chain n-3 PUFA, *viz.*, eicosapentaenoic acid (20:5n-3), may ameliorate some of these effects by influencing eicosanoid metabolism (8,9).

While 20:5n-3 is exclusively derived from marine sources, α-linolenic acid (α-LNA, 18:3n-3), a precursor of 20:5n-3 and docosahexaenoic acid (22:6n-3), can be obtained from commonly consumed vegetable oils, such as soybean and canola oils. Dietary α-LNA can inhibit eicosanoid metabolism (10-13). Additionally *in vitro* studies have demonstrated that 20:5n-3 derived lipoxygenase and cycloxygenase products can be formed from α-LNA. Hwang *et al.* (14) observed 5-HEPE and 12-HEPE formation from lung homogenates, and TXB_3 and 12-HEPE from stimulated platelet preparations; however, these products were not quantified. Similarly, LTB_5 formation has been observed following stimulation of isolated leukocytes from rats maintained on a high α-LNA based diet (13).

These previous studies evaluated eicosanoid metabolism utilizing *in vitro* conditions. The present study investigates the effects of dietary α-LNA on eicosanoid synthesis *in vivo*, emphasizing sulfidopeptide leukotriene (SP-LT) formation. SP-LT are collectively known as the slow reacting substances of anaphylaxis and are involved in inflammatory response (15-18). At sites of inflammation, leukocytes such as neutrophils, lymphocytes and macrophages can produce significant quantitites of SP-LT upon stimulation. SP-LT have potent ionotropic properties, can increase postcapillary venule permeability, are potent stimulators of airway smooth muscle cells, and are mediators of pulmonary asthmas (19-22). Therefore, if long-chain n-3 PUFA possess beneficial properties, evaluating the effects of dietary α-LNA on tissue lipid composition and SP-LT formation *in vivo* is of great importance.

MATERIALS AND METHODS

Experimental design. Eighty-four CD-1 male mice (20 g) were randomly divided into seven dietary groups, 12 animals per group (each group contained two control animals) (Table 1). The control diet (group 1) contained 18:2n-6 as the sole dietary source of polyunsaturated fatty acids (PUFA). In addition to 18:2n-6, the experimental diets contained graded amounts of either α-LNA in the form of trilinolenin (NuChek Prep, Elysian, MN) or a fish oil concentrate as the sources of n-3 PUFA.

Diets. The mice were maintained on Prolab chow diet (Agway, RMF1000, Syracuse, NY) for 1 week, followed by a fat free diet (ICN Biochemicals, Clevelad, OH) for one week, at which time they were placed on their respective experimental or control diets for two weeks.

All diets (experimental and control) contained 10 wt% fat. The linoleic acid (18:2n-6) content of all the diets, as supplied by safflower oil (75%, 18:2n-6) was maintained at 1.5 wt%. The filler oils consisted of equal amounts of a high oleic safflower oil (73%, 18:1n-9) (SVO, Eastlake, OH) and tripalmitin (99%, 16:0) (Sigma, St. Louis, MO). Fish oil concentrate (NSK, Ltd., Japan) contained 20:5n-3 (27%), and docosahexaenoic acid (22:6n-3, 12%) as the major long-chain n-3 PUFA, with 18:2n-6 and 18:3n-3 comprising less than 1% of the total.

*To whom correspondence should be addressed.

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[2] Current address: Program in Nutrition, University of Wyoming, Laramie, WY 82071.

[3] Current address: College of Agriculture and Environmental Sciences, 200 Mrak Hall, University of California, Davis, CA 95616.

Abbreviations: α-LNA, α-linolenic acid; EDTA, ethylenediamine tetraacetic acid; HEPE, hydroxyeicosapentaenoate; HUFA, highly unsaturated fatty acids; LT, leukotriene; PG, prostaglandin; PUFA, polyunsaturated fatty acids; RP-HPLC, reverse phase high-performance liquid chromatography; SP-LT, sulfidopeptide leukotrienes; TLC, thin-layer chromatography; TX, thromboxane; UV, ultraviolet.

TABLE 1

Experimental Design

	Dietary groups						
	1	2	3	4	5	6	7
Diet	Control	α-LNA[a]	α-LNA	α-LNA	Fish oil[b]	Fish oil	Fish oil
n	12	12	12	12	12	12	12
Dietary[c] 18:2n-6	1.58	1.47	1.49	1.44	1.49	1.56	1.57
Dietary[c] n-3 FA	0	0.29	0.58	1.25	0.28	0.61	1.59
Dietary n-3/n-6 ratio	0	0.20	0.39	0.84	0.19	0.39	1.01

[a]Trilinolenin contained 97% 18:3n-3.

[b]Fish oil concentrate contained 27% 20:5n-3, 12% 22:6n-3.

[c]Weight %.

TABLE 2

Fatty Acid Composition of Diets[a]

	Dietary groups						
	Control	α-Linolenic acid			Fish oil		
Fatty acid	1	2	3	4	5	6	7
14:0	—[b]	—	—	—	—	—	2.3
16:0	43.6	44.0	48.5	41.3	48.8	41.7	33.6
16:1	—	—	—	—	—	1.3	3.4
18:0	3.8	3.9	3.3	3.3	3.2	4.0	3.4
18:1	36.5	34.7	27.5	28.3	29.0	30.4	25.7
18:2	15.8	14.7	14.9	14.4	14.9	15.6	15.7
18:3n-3	—	2.9	5.8	12.5	—	—	—
20:3n-3	—	—	—	—	—	—	—
20:5n-3	—	—	—	—	1.9	3.5	9.5
22:0	—	—	—	—	—	—	—
22:5n-3	—	—	—	—	—	—	—
22:6n-3	—	—	—	—	—	1.7	4.4

[a]g/kg Diet. [b]Less than 1 g/kg diet.

The purified trilinolenin contained 97% α-LNA. The experimental diets were designed to provide incremental levels of n-3 PUFA to generate n-3/n-6 ratios of 0.2, 0.4 and 1.0. The fatty acid composition of the diets are presented in Table 2.

The diets were prepared in bulk and prepackaged in separate Whirl-Pak bags (Nasco, Fort Atkinson, WI) and stored under nitrogen at 4°C. Water and food were provided *ad libitum* for two weeks. To minimize exposure to air prior to consumption, fresh diets were provided daily, with uneaten food being discarded. Gas chromatographic analysis demonstrated that storage of the diets did not alter their fatty acid composition.

In vivo generation and isolation of eicosanoids. Following two weeks on the control and experimental diets, opsonized zymosan (1 mg in 0.5 mL saline) was injected intraperitoneally to induce an inflammatory response (23–25). After 30 min, the animals were sacrificed by ether inhalation and the peritoneal cavity was washed with 3 mL saline (0.9%) containing 1 mM EDTA and 100 ng of prostaglandin B_1 (PGB_1) as internal standard, followed by a second wash of EDTA-saline (2 mL). The peritoneal washes were pooled and centrifuged (700 × *g* for 4 min at 25°C) to pellet the cells. The supernatant was made up to a final volume of 10 mL containing 10% methanol and 1.5 mM formic acid. One mL was used for 6-keto $PGF_{1\alpha}$ analysis and the remaining 9 mL for leukotriene analyses. The pelleted cells were analyzed for phospholipid fatty acid composition.

Leukotriene analysis. The leukotrienes were isolated by solid phase extraction using a C-18 cartridge (Burdick and Jackson, Muskegon, MI), sequentially washed with distilled water, then hexane, and eluted off the cartridge with methanol. Following evaporation, the residue was reconstituted in the HPLC solvent system of methanol/water (65:35, v/v), pH 5.6, containing 5 mM ammonium acetate and 1 mM EDTA. The leukotrienes were separated by reversed phase (RP)-HPLC on a Whatman (Hillsboro, OR) Partisphere C-18 column (6 mm × 12.5 cm) with a flow rate of 0.9 mL/min and quantified

TABLE 3

The Fatty Acid Composition of Liver Phospholipids From Mice Maintained on Diets Containing Increasing Amounts of α-Linolenic Acid or Fish Oil[a]

Fatty acid	Dietary groups						
	Control	α-Linolenic acid			Fish oil		
	1	2	3	4	5	6	7
14:0	0.36[a] ± 0.15	0.36[a] ± 0.13	0.46[a] ± 0.19	0.31[a] ± 0.11	0.26[a] ± 0.10	0.32[a] ± 0.10	0.36[a] ± 0.16
16:0	17.65[a] ± 1.36	20.37[a,b] ± 1.11	19.57[a,b] ± 1.09	21.80[a,b] ± 1.17	22.16[b] ± 1.45	22.63[b] ± 1.18	23.81[b] ± 1.87
16:1	1.55[a] ± 0.10	1.78[a] ± 0.13	1.64[a] ± 0.18	1.72[a] ± 0.19	1.68[a] ± 0.18	1.59[a] ± 0.14	1.45[a] ± 0.21
18:0	6.94[a] ± 1.47	8.65[a] ± 1.64	8.72[a] ± 1.79	8.41[a] ± 1.51	7.79[a] ± 1.60	8.79[a] ± 1.56	8.70[a] ± 1.71
18:1	24.77[a] ± 0.72	19.29[b] ± 1.05	17.94[b,c] ± 1.38	16.48[b,c] ± 1.37	15.31[c,d] ± 1.42	12.56[d,e] ± 0.76	9.79[e] ± 0.54
18:2	18.43[a] ± 0.79	16.27[a,b] ± 1.09	19.12[a] ± 0.92	17.14[a,b] ± 1.20	16.87[a,b] ± 1.32	14.11[b,c] ± 0.95	12.67[c] ± 0.63
18:3n-3	0.00[a] ± 0.00	0.14[b] ± 0.04	0.28[c] ± 0.03	0.61[d] ± 0.11	0.02[a,b] ± 0.02	0.05[a,b] ± 0.03	0.01[a,b] ± 0.00
20:3n-9	1.87[a] ± 0.20	1.49[a,b] ± 0.18	1.01[b,c] ± 0.14	0.82[c] ± 0.13	0.55[c,d] ± 0.11	0.27[d] ± 0.04	0.16[d] ± 0.02
20:3n-6	3.33[a] ± 0.18	2.91[a,b] ± 0.18	2.50[b,c] ± 0.11	2.32[c] ± 0.14	1.79[d] ± 0.18	1.14[e] ± 0.11	0.81[e] ± 0.12
20:4n-6	19.17[a] ± 1.03	14.53[b] ± 1.05	11.76[c] ± 0.46	9.14[d] ± 0.44	10.30[c] ± 0.58	8.10[d] ± 0.28	7.57[d] ± 0.40
20:5n-3	0.02[a] ± 0.01	0.73[a,b] ± 0.09	1.89[b] ± 0.13	4.30[c] ± 0.25	4.25[c] ± 0.37	8.06[d] ± 0.60	10.46[e] ± 0.81
22:4n-6	0.38[a] ± 0.10	0.24[a,b] ± 0.04	0.16[b] ± 0.02	0.11[b] ± 0.02	0.11[b] ± 0.02	0.08[b] ± 0.01	0.04[c] ± 0.01
22:5n-6	2.12[a] ± 0.44	0.57[b] ± 0.16	0.21[b] ± 0.04	0.09[b] ± 0.02	0.11[b] ± 0.03	0.08[b] ± 0.01	0.08[b] ± 0.01
22:5n-3	0.02[a] ± 0.01	0.43[a] ± 0.07	0.85[a] ± 0.12	1.28[b] ± 0.17	1.29[b] ± 0.22	2.37[c] ± 0.27	2.66[c] ± 0.29
22:6n-3	3.38[a] ± 0.26	12.25[b] ± 0.73	13.88[b,c] ± 0.78	15.46[c,d] ± 1.10	17.51[d,e] ± 1.25	19.85[e,f] ± 0.93	21.42[f] ± 1.34
n-3/n-6[b]	0.14[a] ± 0.01	0.74[b] ± 0.02	1.16[c] ± 0.04	1.86[d] ± 0.05	1.88[d] ± 0.08	3.23[e] ± 0.06	4.06[f] ± 0.12

[a] All values are expressed as means (mol%) ± SEM. Means with the same letter within the same fatty acid group are not significantly different at $p < 0.05$.

[b] Σ (18:3n-3, 20:5n-3, 22:5n-3, 22:6n-3)/Σ (20:3n-6, 20:4n-6, 22:4n-6, 22:5n-6).

based on their molar extinction coefficients (26) using the internal standard PGB_1, with a Hewlett-Packard (Liverpool, NY) 1040A Diode Array scanning spectrophotometer, monitoring at 280 nm. All compounds were identified by their distinct ultraviolet (UV) absorption spectra and retention times were compared to known standards.

Prostaglandin analysis. The prostaglandins were extracted (three times) from the aliquot of the peritoneal wash with equal volumes of ethyl acetate. The pooled extracts were evaporated to dryness under nitrogen and the prostaglandins were resuspended in phosphate buffered saline (0.9%) containing 0.1% (w/v) gelatin. Each sample was then analyzed for 6-keto-$PGF_{1\alpha}$ by radioimmunoassay (Advanced Magnetics, Cambridge, MA) according to the manufacturer's instructions. The reported antiserum crossreactivities (at 50% B/B_0) for $PGF_{1\alpha}$, 6-keto-PGE_1, and $PGF_{2\alpha}$ are 7.8%, 6.8% and 2.2%, respectively.

Fatty acid analysis. Livers were perfused with saline (0.9%), removed, and quick frozen in liquid nitrogen. For fatty acid analysis, the livers were thawed and homogenized in saline (0.9%). The liver homogenates and pelleted peritoneal cells were resuspended in equal volumes of saline (0.9%) and chloroform/methanol (1:2, v/v), and the lipids were extracted sequentially with chloroform/saline (1:1, v/v) and chloroform (×2). The pooled organic extracts were evaporated to dryness under nitrogen and redissolved in chloroform. The macrophage phospholipids were separated by TLC, using a chloroform/methanol (8:1, v/v) solvent system, and visualized with 0.01% 8-hydroxy-1,3,6-pyrene-trisulfonic acid trisodium salt (Eastman Kodak Co., Rochester, NY) in anhydrous methanol. The phospholipids were recovered from the TLC plates by scraping the appropriate bands and resuspended in toluene. They were then saponified with 0.5 N KOH in ethanol for 8 min at 86°C. Following acidification with 0.7 N HCl, the free fatty acids were extracted with equal volumes of hexane (×2), evaporated under nitrogen and methylated with ethereal diazomethane. Following evaporation, the fatty acid methyl esters were resuspended in hexane and analyzed by gas chromatography on a DB23 capillary column (0.25 mm × 30 m) (J&W Chromatography, Folsom, CA) with hydrogen as the carrier gas. The fatty acids were quantified using pentadecanoic acid as an internal standard. Fatty acid composition of the diets were determined using the same methods described above, excluding the TLC step.

Statistical analysis. The data from all diets were analyzed by randomized block design. The results were statistically evaluated by Duncan's Protected Least Significant Difference. Significance was determined at $p < .05$.

RESULTS

Tissue phospholipid fatty acids. Liver and peritoneal cell phospholipids contained equivalent amounts of 18:2n-6, respectively, with the exception of the liver phospholipids from mice consuming the fish oil concentrate, where 18:2n-6 progressively declined with increasing dietary fish oil (Tables 3 and 4). The levels of 20:4n-6 in hepatic and peritoneal cell phospholipids progressively declined in animals consuming the experimental diets compared to the animals on the control diet. The diets containing fish oil were more effective than those containing α-LNA in depressing 20:4n-6.

The 20:5n-3, 22:5n-3 and 22:6n-3 levels progressively increased in hepatic and peritoneal cell phospholipids in animals on all experimental diets (Tables 3 and 4). However, alterations of n-3 PUFA levels in the tissue

TABLE 4

The Fatty Acid Composition of Phospholipids Extracted From Resident Peritoneal Cells of Mice Maintained on Diets Containing Increasing Amounts of α-Linolenic Acid or Fish Oil[a]

Fatty acid	Dietary groups						
	Control	α-Linolenic acid			Fish oil		
	1	2	3	4	5	6	7
14:0	$1.03^{a} \pm 0.30$	$0.83^{a,b} \pm 0.16$	$0.81^{a,b} \pm 0.15$	$0.59^{a,b,c} \pm 0.08$	$0.42^{b,c} \pm 0.12$	$0.32^{c} \pm 0.12$	$0.85^{a,b} \pm 0.14$
16:0	$25.28^{a,b} \pm 1.61$	$25.70^{a,b} \pm 0.98$	$26.59^{a,b} \pm 0.62$	$28.39^{b,c} \pm 0.89$	$24.50^{a} \pm 0.55$	$24.72^{a,b} \pm 0.95$	$30.61^{c} \pm 2.05$
16:1	$7.10^{a} \pm 1.22$	$6.98^{a} \pm 1.03$	$6.86^{a} \pm 0.36$	$3.31^{a} \pm 0.81$	$4.87^{a} \pm 0.71$	$5.32^{a} \pm 0.55$	$5.27^{b} \pm 0.78$
18:0	$14.09^{a} \pm 0.86$	$13.98^{a} \pm 0.36$	$14.98^{a} \pm 0.39$	$11.79^{b} \pm 0.70$	$13.88^{a} \pm 0.49$	$15.08^{a} \pm 0.74$	$15.06^{a} \pm 0.48$
18:1	$29.08^{a,b} \pm 1.50$	$27.01^{a,b} \pm 1.44$	$24.53^{b,c,d} \pm 0.75$	$29.33^{a,b} \pm 2.95$	$31.95^{a} \pm 2.13$	$27.60^{b,c} \pm 2.62$	$19.96^{d} \pm 1.10$
18:2	$8.13^{a} \pm 0.29$	$8.41^{a} \pm 0.86$	$9.92^{a} \pm 0.76$	$10.45^{a} \pm 1.42$	$8.34^{a} \pm 0.93$	$8.48^{a} \pm 0.77$	$7.77^{a} \pm 0.33$
18:3n-3	$0.00^{a} \pm 0.00$	$0.00^{a} \pm 0.00$	$0.03^{a} \pm 0.03$	$0.44^{b} \pm 0.10$	$0.00^{a} \pm 0.00$	$0.00^{a} \pm 0.00$	$0.00^{a} \pm 0.00$
20:3n-9	$0.83^{a} \pm 0.17$	$0.78^{a,b} \pm 0.15$	$0.54^{a,b,c} \pm 0.09$	$0.48^{b,c} \pm 0.07$	$0.38^{c} \pm 0.07$	$0.26^{c,d} \pm 0.07$	$0.12^{d} \pm 0.05$
20:3n-6	$1.59^{a} \pm 0.22$	$1.56^{a} \pm 0.14$	$1.41^{a,b} \pm 0.10$	$1.20^{a,b} \pm 0.14$	$1.06^{b,c} \pm 0.13$	$0.75^{c,d} \pm 0.13$	$0.58^{d} \pm 0.03$
20:4n-6	$8.79^{a} \pm 1.21$	$8.50^{a} \pm 0.59$	$7.41^{a,b} \pm 0.28$	$5.92^{b,c} \pm 0.81$	$5.19^{c} \pm 0.32$	$4.79^{c} \pm 0.64$	$4.41^{c} \pm 0.47$
20:5n-3	$0.04^{a} \pm 0.03$	$0.12^{a} \pm 0.04$	$0.47^{a} \pm 0.03$	$1.30^{b} \pm 0.20$	$1.56^{b} \pm 0.10$	$3.08^{c} \pm 0.26$	$4.62^{d} \pm 0.34$
22:4n-6	$2.20^{a} \pm 0.31$	$1.74^{a,b} \pm 0.13$	$1.33^{b} \pm 0.16$	$0.71^{c,d} \pm 0.07$	$0.70^{c,d} \pm 0.06$	$0.41^{d} \pm 0.07$	$0.21^{d} \pm 0.08$
22:5n-6	$0.79^{a} \pm 0.14$	$0.28^{b} \pm 0.09$	$0.16^{b,c} \pm 0.04$	$0.21^{b,c} \pm 0.07$	$0.10^{b,c} \pm 0.03$	$0.04^{c} \pm 0.03$	$0.05^{c} \pm 0.03$
22:5n-3	$0.05^{a} \pm 0.04$	$0.83^{a,b} \pm 0.08$	$1.60^{a,b} \pm 0.12$	$2.03^{a,b,c} \pm 0.21$	$2.45^{b,c} \pm 0.18$	$3.38^{d} \pm 0.66$	$3.76^{c} \pm 0.40$
22:6n-3	$1.01^{a} \pm 0.10$	$3.27^{b} \pm 0.26$	$3.33^{b} \pm 0.23$	$3.83^{b,c} \pm 0.57$	$4.60^{c} \pm 0.32$	$5.76^{c} \pm 0.48$	$6.73^{e} \pm 0.43$
n-3/n-6[b]	$0.08^{a} \pm 0.01$	$0.35^{b} \pm 0.02$	$0.53^{c} \pm 0.02$	$0.94^{d} \pm 0.08$	$1.22^{e} \pm 0.06$	$2.17^{f} \pm 0.15$	$2.87^{f} \pm 0.28$

[a] All values are expressed as means (mol%) ± SEM. Means with the same letter within the same fatty acid group are not significantly different at $p < 0.05$.

[b] Σ (18:3n-3, 20:5n-3, 22:5n-3, 22:6n-3)/Σ (20:3n-6, 20:4n-6, 22:4n-6, 22:5n-6).

phospholipids were more dramatic in animals consuming the n-3 highly unsaturated fatty acids (HUFA) in fish oil than the animals consuming the α-LNA. The lowest fish oil diet (diet 5) was as effective in increasing tissue n-3/n-6 ratios as the highest α-LNA diet (diet 4) (Fig. 1).

Leukotrienes. Mice fed diets devoid of n-3 PUFA produced only LTC_4 and LTE_4, with LTE_4 being the major SP-LT formed (Fig. 2a). There was no evidence of LTD_4 formation. Upon introduction of fish oil to the diet, and to a lesser extent, α-LNA, LTE_5 was also formed following *in vivo* stimulation of peritoneal cells with opsonized zymosan (Fig. 2b and 2c). All of the SP-LT, 4- and 5-series, exhibited characteristic triplet UV absorption spectra indicative of conjugated triene formation, with absorption maxima at 280 nm (Fig. 3). LTE_5 formation was confirmed previously (27), based on i) co-chromatography with authentic standards; ii) equivalent elution profiles with previously published values (26,28,29); and iii) characteristic UV absorption spectra. The identity of LTE_5 was further confirmed enzymatically following conversion to LTF_5 *via* transpeptidation using γ-glutamyl transpeptidase (27). Previous studies indicated that LTE_5 was the major (80%) 5-series SP-LT formed (27,30).

In contrast to n-3 HUFA of fish oil, dietary α-LNA had very little effect on leukotriene formation even at the highest levels. No significant differences in 4-series SP-LT production were observed in any of the α-LNA dietary groups when compared to animals on the control diet (Fig. 4). While 4-series SP-LT production was depressed in the highest α-LNA group (diet 4), the decline was not statistically significant ($p < .05$).

Changes in leukotriene formation were much more responsive to fish oil feeding. Animals maintained on diets 6 and 7 produced approximately 50% less tetraenyl SP-LT than animals on the control diet. Additionally, as

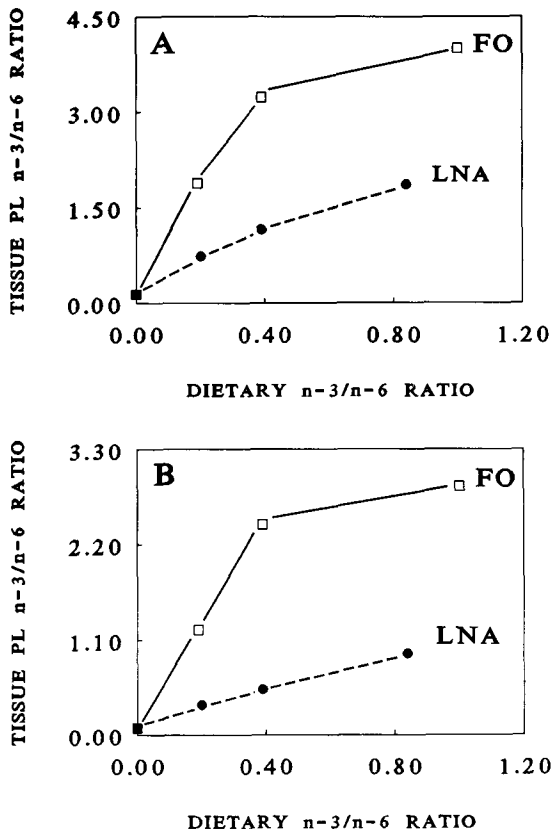


FIG. 1. Relationship between dietary n-3/n-6 ratio and the n-3/n-6 ratio of A) liver and B) peritoneal cell phospholipids. FO: fish oil (solid line); LNA: α-linolenic acid (broken line).

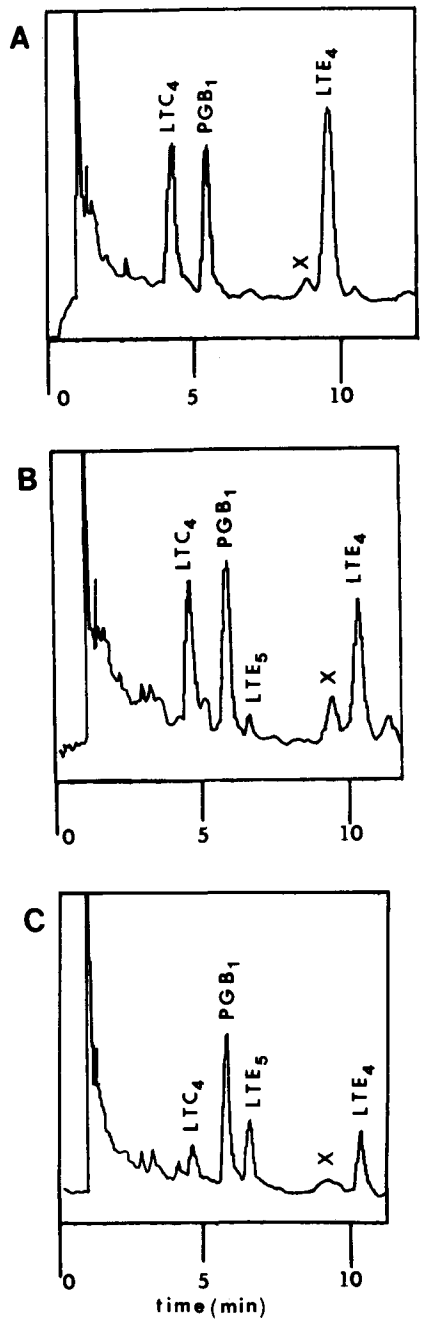


FIG. 2. RP-HPLC chromatograms of sulfidopeptide leukotrienes synthesized *in vivo* using animals maintained on diets A) devoid of n-3 PUFA (diet 1); B) containing α-linolenic acid (diet 4); or C) containing fish oil concentrate (diet 7). Conditions for RP-HPLC: Whatman partisphere C-18 column (6 mm × 12.5 cm); mobile phase, methanol/water (65:35, v/v) containing 5 mM ammonium acetate and 1 mM EDTA, adjusted to PH 5.6 with glacial acetic acid; flow rate, 0.9 mL/min.; diode array detector monitoring at 280 nm.

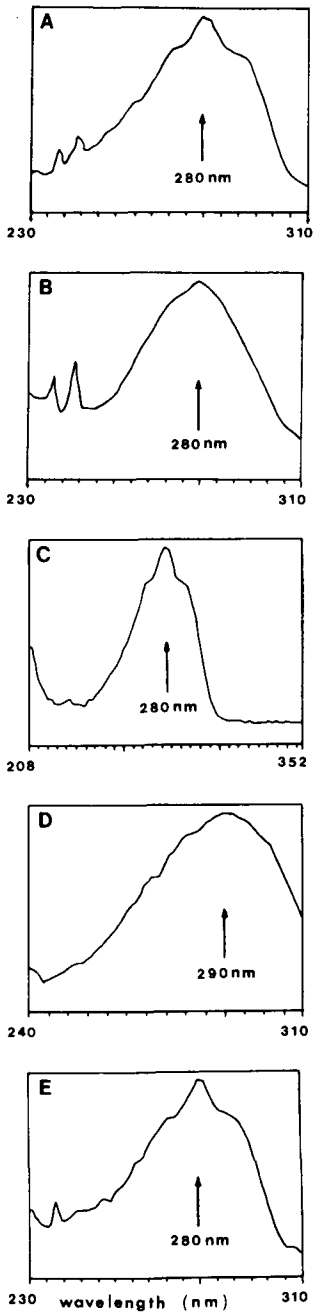


FIG. 3. UV absorption spectra of compounds isolated from mouse peritoneal exudates. The compounds were separated by RP-HPLC (see Fig. 2) and their spectra generated using a Hewlett-Packard 1040 diode array detector: A) LTC_4; B) PGB_1; C) LTE_5; D) X (unknown); and E) LTE_4.

the n-3/n-6 dietary ratio increased, SP-LT of the 5-series progressively increased in all animals consuming the fish oil diets (Fig. 5). In fact, LTE_5 production equaled LTE_4 formation in mice consuming a fish oil diet with an n-3/n-6 ratio of 1.0 (diet 7). In contrast, only the highest α-LNA dietary group (diet 4) demonstrated pentaenyl-LT formation. LTE_5 production in diet 4 was very low (6.0 ± 2.3 ng/mouse), even when compared to the LTE_5 produced (19.8 ± ng/mouse) by animals consuming the lowest fish oil diet (diet 5) with an n-3/n-6 ratio of 0.2.

Prostaglandins. The production of 6-keto-$PGF_{1\alpha}$ by the peritoneal cells significantly declined as a result of fish oil feeding (diets 6 and 7) when compared to the control group (Fig. 6), whereas diet 6 was as effective as diet 7. At the lower dietary intakes, α-LNA had no effect on 6-keto-$PGF_{1\alpha}$ production, but 6-keto-$PGF_{1\alpha}$ production declined 46% in diet 4. However, because of an outlying value (37 ng/mouse), this decrease was not significant.

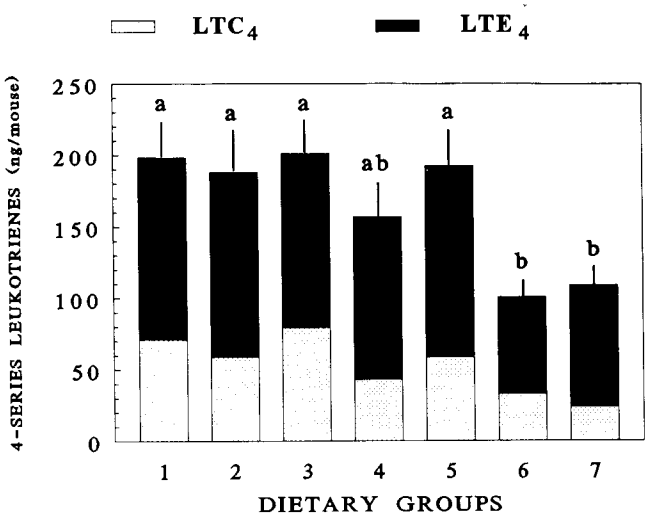


FIG. 4. Leukotriene C_4 (dotted bar) and leukotriene E_4 (solid bar) production *in vivo* by resident peritoneal cells following stimulation with opsonized zymosan from mice maintained on a diet devoid of n-3 PUFA (diet 1), progressive amounts of α-linolenic acid (diets 2–4) or fish oil (diets 5–7). Results are mean ± SEM of 10 experimental values. Bars with the same letter are not significantly different at $p < 0.05$.

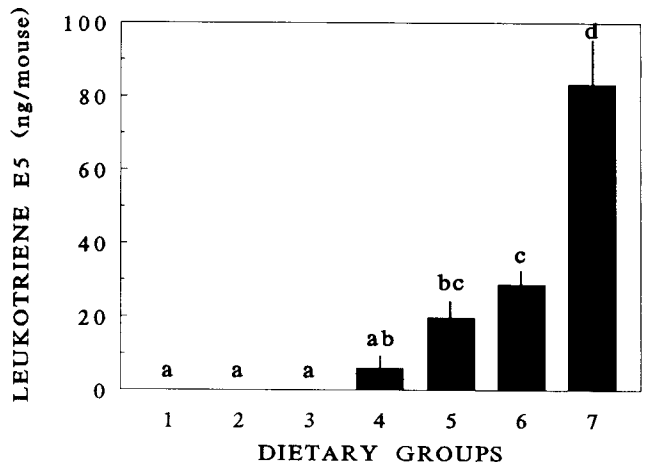


FIG. 5. Leukotriene E_5 formation *in vivo* by resident peritoneal cells following stimulation with opsonized zymosan from mice maintained on a diet devoid of n-3 PUFA (diet 1), progressive amounts of α-linolenic acid (diets 2–4) or fish oil (diets 5–7). Results are mean ± SEM of 10 experimental values. Bars with the same letter are not significantly different at $p<0.05$.

DISCUSSION

This study provides new information with regard to the relative effects of dietary n-3 PUFA, in the form of α-LNA or fish oil, on eicosanoid metabolism. Recently, we reported that LTE_5 was produced *in vivo* in peritoneal cells from mice consuming a diet containing fish oil (27). In this previous paper, LTE_5 formation *in vivo* was confirmed utilizing spectrophotometric, chromatographic and enzymologic techniques. A few studies have reported LTE_5 production *in vitro* from exogenous sources of 20:5n-3 (26,28,29), with only one report suggesting *in vivo* formation (31). However, with the exception of Örning

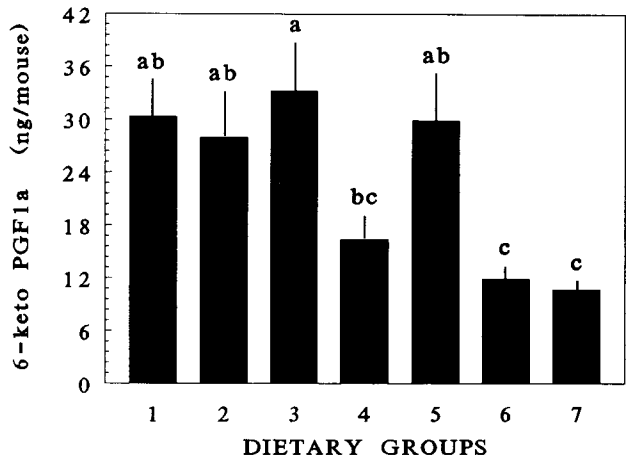


FIG. 6. 6-keto-$PGF_{1\alpha}$ formation *in vivo* by resident peritoneal cells following stimulation with opsonized zymosan from mice maintained on a diet devoid of n-3 PUFA (diet 1), progressive amounts of α-linolenic acid (diets 2–4) or fish oil (diets 5–7). Results are mean ± SEM of 10 experimental values. Bars with the same letter are not significantly different at $p<0.05$.

et al. (26), these studies could not verify the identity of LTE_5 and quantitate its production because of incomplete RP-HPLC resolution and the use of nonspecific radioimmunological binding assays.

The present study confirms that when fish oil is consumed in the diet, the 5-series SP-LT, produced by stimulated peritoneal cells, can significantly contribute to the total LT pool *in vivo*. This production is positively correlated with dietary n-3/n-6 ratios and as this ratio approaches 1.0, the contribution of 5-series SP-LT can approach the contribution by the 4-series SP-LT. A number of studies have shown that 20:5n-3 is a very good substrate for lipoxygenases (32–34) and LTA_5 is a substrate for the LTA hydrolase (35). Our results reveal that 20:5n-3 and its lipoxygenase-derived metabolites are also substrates for those enzymes responsible for SP-LT formation *in vivo*. Thus, the data presented here emphasize the need to account for SP-LT derived not only from n-6 PUFA, but also those generated from n-3 PUFA.

We also report, for the first time, that α-LNA, as a dietary component, can contribute to 5-series SP-LT formation, although not to the same extent as fish oil under the experimental conditions used. LTE_5 production was not detected in animals maintained on diet 2 and diet 3, with n-3/n-6 ratios of 0.2 and 0.4, respectively. Only animals consuming the highest levels of α-LNA (diet 4) produced detectable quantitites of LTE_5. Concomitantly, a reduction in 4-series SP-LT formation was observed only in animals on diet 4.

These findings agree with previous reports, indicating that α-LNA may have a significant effect on the lipoxygenase pathway, but only with elevated intakes (36). Hwang *et al.* (14), however, observed a progressional reduction in rat platelet 12-lipoxygenase activity *(in vitro)* with increasing dietary α-LNA, but a significant reduction in lung 5- and 12-lipoxygenase activities occurred only in animals maintained on a dietary regimen similar to the highest α-LNA intake in the present study.

In contrast, consumption of fish oil resulted in 5-series SP-LT formation in all dietary groups. In addition,

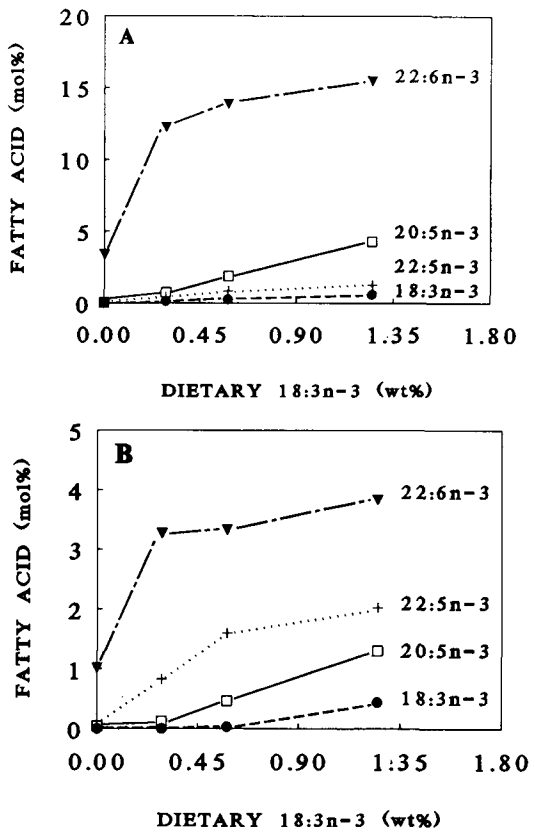


FIG. 7. The effect of increasing dietary α-linolenic acid on select tissue phospholipid n-3 PUFA levels of A) liver and B) peritoneal cell phospholipids.

tetraenyl LT formation, *i.e.*, LTC_4 and LTE_4, declined as the fish oil content of the diet increased, whereas a 48% reduction was observed in animals consuming diet 6 (n-3/n-6 ratio of 0.4) *vs.* the animals on the control diet, with no further reduction observed in animals on diet 7 (Fig. 4). The 20:5n-3 can compete with 20:4n-6 for enzymatic oxidation by the 5-lipoxygenase, thereby decreasing LTA_4 formation, and LTA_5 is a known inhibitor of tetraenyl LT formation (35).

The biological significance of 5-series SP-LT formation has yet to be elucidated. While LTB_5 has received increasing attention because it is biologically less active than LTB_4 (37,38), little attention has been focused on the pentaenyl SP-LT, possibly because the 4- and 5-series SP-LT have been shown to be equipotent physiological mediators in a limited number of bioassays (20,29,31) and only a few studies have reported *in vivo* production of these pentaenyl compounds (27,30,31).

There was no indication that α-LNA inhibited either Δ6, Δ5, or Δ4 desaturase activities, as dietary α-LNA appeared to be preferentially elongated and desaturated to 22:6n-3. It has been reported that n-3 PUFA, as derived from linseed oil and fish oil, can inhibit rat liver microsomal Δ6 desaturase activity (39). Therefore, desaturase inhibition cannot be ruled out. However, if there was inhibition of this activity in the mouse model, it was not reflected in the 18:2n-6 or α-LNA tissue levels, nor by the elongation products of α-LNA. The inclusion of dietary α-LNA resulted in the accumulation of 22:6n-3 in both the liver and peritoneal cell phospholipids (Fig. 7). Similar results have been observed elsewhere (14). Only after tissue phospholipid 22:6n-3 levels approached a maximum did 20:5n-3 levels rise. Alterations of phospholipid fatty acid composition in the liver appear to precede those changes seen in the peritoneal cells, since resident peritoneal cells, such as the macrophage, do not possess Δ4 desaturase activity nor the capacity for retroconversion (40). The increases in 22:5n-3 levels in peritoneal cells are most likely the result of the elongation of 20:5n-3. This is supported by other data demonstrating that 22:6n-3 is not retroconverted to 22:5n-3, but rather 22:5n-3 is derived from 20:5n-3 (36,40).

Though these conclusions appear consistent with our data, they may not strictly apply to all tissues or species. The 22:6n-3 of rat liver phosphatidylcholine is unaffected by dietary α-LNA levels (41). However, 22:6n-3 may be incorporated preferentially into other phospholipid classes, such as phosphatidylethanolamine (13,34). It has been demonstrated that liver phospholipid 22:6n-3 levels increase dramatically, even with low dietary intakes of α-LNA (similar to the present results), while the lung phospholipids may not be as responsive (14). It has been shown that the 22:6n-3 content of rat liver microsomal phospholipids is responsive to dietary α-LNA (39), as it is in those tissues which require 22:6n-3 (42). Therefore, the limited incorporation of 20:5n-3 and the significant elevation of 22:6n-3 into tissue phospholipids may have contributed to the poor 5-series SP-LT production in the peritoneal cells of animals fed α-LNA (36,40).

Fish oil feeding also resulted in a significant reduction in 6-keto-$PGF_{1\alpha}$ formation. The capacity of macrophages (95% of the peritoneal cell population are resident macrophages) or other tissues to produce PGI_2 could be of interest with regard to a number of metabolic processes, including cholesterol homeostasis. It has been observed that PGI_2 and 6-keto-$PGF_{1\alpha}$ can influence cholesterol efflux in cultured smooth muscle cells by elevating cholesteryl ester hydrolase activity (43). Macrophages appear to be intricately involved in scavenging extravascular LDL cholesterol and this uptake has been related to eicosanoid metabolism (44,45). Mouse peritoneal macrophages, incubated with oxidized LDL, produced twice as much 6-keto-$PGF_{1\alpha}$ and three times more SP-LT than controls (46). In addition, PGI_2 has been shown to be very important in thrombosis and hemostasis (47).

Depression of 6-keto-$PGF_{1\alpha}$ production in mice fed the α-LNA diets was observed only with diet 4. This is in contrast to the results reported in rat lung homogenates where α-LNA had an intermediate effect on decreasing 6-keto-$PGF_{1\alpha}$ production when compared with the control and fish oil groups (14). Other *in vitro* studies have shown that when the dietary 18:3n-3/18:2n-6 ratio exceeds 1.0, prostaglandin production is significantly depressed as compared to diets limited in α-LNA (36,48).

The mechanism by which n-3 PUFA depress prostanoids appear to be the result of competitive inhibition of PGH synthase by 20:5n-3 (11) and by decreasing tissue content of 20:4n-6. In addition, 20:5n-3 is a poor substrate for the formation of the 3-series prostanoids (14).

In summary, a limited amount of data exists in the literature which directly compares the effects of progressive increases of dietary n-3 fatty acids in the form of

α-LNA and fish oil on eicosanoid production. The present study revealed that dietary α-LNA can contribute to 5-series SP-LT formation when sufficient quantitites of α-LNA are provided in the diet to significantly alter tissue 20:5n-3 levels. However, α-LNA appears to be preferentially metabolized to 22:6n-3 in this animal model. In addition, the limited incorporation of long-chain n-3 PUFA (Fig. 1) into tissue phopholipids following a dietary regimen of α-LNA appears to influence the efficacy of α-LNA in altering eicosanoid metabolism. The effects observed with fish oil feeding suggest that preformed (dietary) n-3 HUFA appear to be readily incorporated into tissue phospholipids and that eicosanoid formation is related to the extent of this incorporation.

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Effects of Age and Dietary Essential Fatty Acids on Desaturase Activities and on Fatty Acid Composition of Liver Microsomal Phospholipids of Adult Rats

L. Ulmann[a], J.P. Blond[a], C. Manniongui[a], J.P. Poisson[a], G. Durand[b], J. Bézard[a,*] and G. Pascal[b]
[a]Laboratoire de Physiologie Animale et de la Nutrition, Faculté des Sciences Mirandé, 21004 Dijon Cedex and [b]Station de Recherches de Nutrition INRA-CRJ, 78350 Jouy en Josas, France

The combined effects of age and dietary n-6 and n-3 fatty acids were studied in 3-, 6- and 9-month-old rats. At each age, two groups were fed diets containing 5% (w/w) of vegetable oils rich in either 18:3n-6 (borage group) or 18:3n-6 plus 18:4n-3 (black currant group), for a period increasing with age. A control group was fed the essential fatty acids 18:2n-6 and 18:3n-3 only. For each group, Δ6, Δ5 and Δ9 desaturase activities were measured in liver microsomes, and fatty acid composition was determined in microsomal phospholipids. Desaturase activity varied as a function of age and dietary lipids. Δ6 Desaturation of 18:3n-3 was more sensitive to these factors while Δ6 desaturation of 18:2n-6 and Δ9 desaturation were more dependent on season than the other two. Desaturase activity was influenced more by the black currant than by the borage diet, especially at 6 and 9 months of age. A large proportion of arachidonic acid was maintained in the microsomes independent of the diet. Changes in the fatty acid composition did not strictly reflect the differences in desaturase activities. The effects of the two factors (age and diet) on the activities of the desaturases are complex, suggesting that the enzymes are susceptible to other factors as well.
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It is well established that in animals unsaturated fatty acids are synthesized by a series of alternating position-specific desaturation and malonyl-CoA-dependent elongation steps. The enzymatic conversion of fatty acids to their corresponding desaturated products has been particularly well studied in rat liver microsomes. Different desaturases introduce double bonds at Δ9, Δ6, Δ5 or Δ4 position in the fatty acid chain (1). Thus, γ-linolenic acid (18:3n-6) and stearidonic acid (18:4n-3) are synthesized by Δ6 desaturation, a rate-limiting step in the metabolism of linoleic acid (18:2n-6) and α-linolenic acid (18:3n-3), which are the main dietary essential fatty acids (EFA). Another enzyme, the Δ5 desaturase, converts dihomo-γ-linolenic acid (20:3n-6) into arachidonic acid (20:4n-6), whereas a Δ9 desaturase is responsible for the transformation of stearic acid, a non-essential saturated fatty acid (18:0), into oleic acid (18:1n-9). Desaturations of fatty acids by liver microsomes are influenced by numerous hormonal and nutritional factors (2–7) and are sensitive to circadian variations (5,8). Losada and Peluffo (9) observed that a cold environment decreased the Δ9 desaturase activity while Δ6 desaturation was insensitive to temperature variations.

Physiological factors, such as age, may induce modifications of desaturase activities. Strouve-Vallet and Pascaud (10) investigated Δ6 desaturation in liver microsomes and brain during the first month of rat growth. Satomi and Matsuda (11) examined the microsomal Δ6 desaturation of linoleic acid in rat liver fetus. Peluffo and Brenner (12) demonstrated that in rat liver Δ6 desaturation of 18:2n-6 and 18:3n-3 decreased while Δ9 desaturation of 18:0 increased at 12 months of age as compared to 3 months. Recently, Bordoni *et al.* (13) showed that a linear decrease of Δ6 desaturase activity occurred with age (1- to 22-month-old rats) and compared the Δ6 desaturation of 18:2n-6 to that of 18:3n-3 (14). However, the standard diets provided to the animals did not allow the influence of dietary essential fatty acids in enriched diets to be studied. Blond *et al.*(15) studied Δ6 and Δ5 desaturations in Zucker rats with age, but only during a short period of growth, *i.e.*, 6, 9 and 12 weeks.

Evening primrose seed oil is a well-known source of γ-linolenic acid. The use of dietary γ-linolenic acid avoids the Δ6 desaturation step. Other species, such as *Boraginaceae*, are also known to contain significant amounts of 18:3n-6 (16). Thus, borage oil also can be used to supply diets with this fatty acid. Black currant seed oil (17), a Ribes seed oil, is proposed currently as a source of both stearidonic acid and 18:3n-6. Hoy *et al.* (18) demonstrated that liver Δ6 and Δ5 desaturase activities were not modified by dietary 18:3n-6 when rats were fed evening primrose oil or safflower oil for 11 weeks. In contrast, the Δ5 desaturase activity was depressed in young animals fed a diet containing 18:3n-6 (19). Choi and Sugano (20), using a diet containing evening primrose oil, showed a decrease of liver Δ6 desaturase activity in 8-month-old rats but not in 3-week-old animals. These previous studies dealing with dietary fatty acids, age and desaturations usually reported results only for certain ages (often for young rats) and more particularly for Δ9 and Δ6 desaturation. A time-course investigation of Δ9, Δ6 and Δ5 desaturation together with a liver microsome fatty acid compositional study was needed.

Nutritional experiments were therefore carried out on 3-, 6- and 9-month-old rats. The use of borage and black currant seed oils permitted the study of the effect of a partial substitution of 18:3n-6 and 18:4n-3 for 18:2n-6 and 18:3n3, respectively, on the desaturation capacity of rat liver microsomes as well as on the composition of microsomal phospholipids.

*To whom correspondence should be addressed at Laboratoire de Physiologie Animale et de la Nutrition, Faculté des Sciences Mirande, BP 138, 21004 Dijon Cedex, France.

Abbreviations: Bc, black currant; Bg, borage; C, control; EFA, essential fatty acids; HPLC, high performance liquid chromatography; PUFA, polyunsaturated fatty acid; SFA, saturated fatty acid.

MATERIALS AND METHODS

Animals and diets. Male Wistar rats, supplied by INRA (Jouy-en-Josas, France), were randomized into three groups according to age and housed in stainless steel cages in a well-ventilated room maintained at 22 ± 2°C on a 12 hr/12 hr light/dark cycle. Diets and tap water were provided *ad libitum*. After weaning, the rats were fed the basal diet (control diet) for 7, 16 and 26 weeks. It contained (g/kg): casein, 220; D,L-methionine, 1.6; cellulose, 20; starch, 440; saccharose, 218; mineral mixture, 40; vitamin mixture, 10; and oil mixture (50 % peanut oil + 50 % rapeseed oil), 50 (21). Then, within each group, the animals were again divided into three groups (three rats per group). The first group continued to receive the control diet (group C). The two other groups were given a diet in which the oil mixture was replaced by 5% (w/w) fat including borage oil (group Bg) or 5% (w/w) fat containing black currant seed oil (group Bc) for 2, 5 and 7 weeks, respectively, in the three increasing age groups. For 100 g of diet, the n-6 fatty acids represented 945, 973 and 988 mg for C, Bc and Bg groups, respectively; the n-3 fatty acids represented 188 mg for each diet. The three oil mixtures had a n-6/n-3 fatty acid ratio of about 5.2 and similar contents of n-6 and n-3 fatty acids. The fatty acid compositions of these mixtures are reported in Table 1. The three groups of animals were killed at 3, 6 and 9 months. To minimize seasonal variations in desaturase activities, two groups of three rats were used at 4–6 month intervals throughout the year, for a given age and for a given diet. Means were calculated on the 6 values.

Chemicals. [1-^{14}C]Stearic acid (52 mCi/mmol, 97.5% radiochemical purity) and [1-^{14}C]linoleic acid (58 mCi/mmol, 99% radiochemical purity), were purchased from CEA (Gif sur Yvette, France). [1-^{14}C]α-Linolenic acid and [2-^{14}C]dihomo-γ-linolenic acid (56 mCi/mmol, 96% radiochemical purity) were purchased from the Amersham Radiochemical Centre (Amersham, U.K.). The reported radiopurity of each substrate was that indicated by the manufacturer. Each substrate was diluted in ethanol with unlabelled fatty acid to a specific activity of 10 mCi/mmol. Unlabelled fatty acids, coenzymes and biochemicals were provided by Sigma Chemical Co. (St. Louis, MO) and by NuChek Prep (Elysian, MN). All other chemicals purchased from Sigma and Merck (Darmstadt, Germany) were of analytical grade.

Isolation of microsomes. Rats were killed by exsanguination between 07:00 and 08:00 hr to avoid any circadian variation (22) and to insure conditions of high desaturase activity (8). Livers were excised, rinsed with ice-cold physiological saline solution, blotted, weighed and cut into thin slices. Liver (3.5 g) was homogenized at 4°C in a Potter-Elvejhem homogenizer with 6 volumes of 0.05 M phosphate buffer (pH 7.4) and 0.25 M sucrose solution. Homogenates were centrifuged at 13,000 × *g* for 20 min to sediment cell fragments, mitochondria and nuclei. The microsomal pellet was obtained by centrifugation of the supernatant at 105,000 × *g* for 60 min.

Microsomal pellets were resuspended in 0.4 mL supernatant and 0.8 mL of 0.05 M phosphate buffer (pH 7.4) and 0.25 M sucrose solution for determination of desaturase activities. Microsomal proteins were estimated by the method of Layne (23) with fatty acid-free bovine serum albumin as standard.

TABLE 1

Fatty Acid Composition (% moles) of Dietary Lipids

Fatty acids	Control[a]	Black currant[b]	Borage[c]
14:0	0.2	0.8	0.5
16:0	7.7	26.1	18.1
16:1n-7	0.3	—	0.2
18:0	2.9	5.7	4.5
18:1n-9	57.3	39.8	49.6
18:1n-7	2.3	1.1	—
18:2n-6	20.1	16.3	17.3
18:3n-6	—	4.4	3.6
18:3n-3	4.0	3.2	4.0
18:4n-3	—	0.8	—
20:0	1.1	0.6	0.5
20:1n-9	1.5	0.3	1.3
22:0	1.8	0.9	0.4
24:0	0.8	—	—
SFA/PUFA[d]	0.2	0.8	0.5
Σ n-6	20.1	20.7	20.9
Σ n-3	4.0	4.0	4.0
n-6/n-3	5.0	5.2	5.2

[a]Control: (rapeseed + peanut) oils.
[b]Black currant: (black currant seed + peanut + palm) oils.
[c]Borage: (borage + rapeseed + hydrogenated palm) oils.
[d]SFA, saturated fatty acids; PUFA, polyunsaturated fatty acids.

Enzyme assays in vitro. Five mg of microsomal proteins were incubated in an open flask. Two quantities of each substrate in ethanolic solution were used: a nonsaturating level, 50, 60, 30 and 40 nmol and a saturating level, 100, 120, 80 and 120 nmol for 18:0, 18:2n-6, 20:3n-6 and 18:3n-3, respectively. These levels and the effect of microsomal protein concentration and incubation time were established in preliminary experiments (unpublished data). Incubations were performed at 37°C for 15 min in a shaking water bath with a total volume of 2.1 mL incubation medium containing 72 mM phosphate buffer, pH 7.4, 4.8 mM $MgCl_2$, 0.5 mM coenzyme A, 3.6 mM ATP and 1.2 mM NADPH. Incubations were stopped by adding 15 mL chloroform/methanol (1:1, v/v). Lipids were saponified and methylated as reported by Slover and Lanza (24). The distribution of radioactivity between substrate (stearic, linoleic, dihomo-γ-linolenic or α-linolenic acid) and desaturation product (oleic, γ-linolenic, arachidonic or stearidonic acid) was determined by the reversed phase high performance liquid chromatography (HPLC) method described by Narce *et al.* (25), using a Waters Chromatograph and a Lichrocart column (Superspher RP 18, 250 mm × 4 mm i.d. Waters, Milford, MA). After separation by HPLC, each fraction corresponding to a fatty acid methyl ester was collected at the detector outlet (differential refractometer) and its radioactivity was directly measured in the solvent by liquid scintillation counting. The conditions established by the authors permitted a good separation of the different unsaturated fatty acid methyl esters. Moreover, the desaturation product was eluted before its substrate,

avoiding possible radioactive contamination. Enzyme activity was calculated from the desaturation product/substrate ratio and was expressed as nmoles of substrate transformed in 15 min with 5 mg of total microsomal proteins.

Fatty acid analysis. Lipids from aliquots of liver microsomes were extracted according to Delsal (26). Phospholipids were obtained by liquid column chromatography following the procedure described by Hirsch and Ahrens (27). After addition of heptadecanoic acid, fatty acids were methylated in the presence of methanolic BF_3 and extracted as previously described (24). The fatty acid methyl esters were analyzed by capillary gas-liquid chromatography on a model 417 Becker-Packard apparatus (Packard Instrument, Rungis, France) equipped with a laboratory-made 50 m × 0.3 mm i.d. glass capillary column coated with Carbowax 20 M. Gas chromatographic peaks were identified on the basis of their retention time relative to methyl heptadecanoate. Peak area was measured using a Delsi model Enica 21 computing integrator (Delsi Instruments, Suresnes, France).

Statistical analysis. All data are presented as the mean ± SE for n=6 animals. After analysis of variance using a Duncan's multiple range test, means were compared according to the least significant difference ($p<0.05$).

RESULTS

Table 2 shows that, at each age, the composition of dietary lipids had no significant effect on body and liver weights and on microsomal protein content of liver microsomes. In each dietary group, body weight increased between 3 and 6 months, but in the control group it also increased between 6 and 9 months.

Tables 3 and 4 report the desaturation activity, first expressed as quantities in Table 3 (nanomoles of desaturation product) and second, as conversion percentages (Table 4). The Δ9 desaturase showed the highest activity independent of age and diet. This activity clearly increased between 3 and 6 months but then decreased at 9 months to approximately the three month-level. The diet had no significant influence. At a 50-nmol substrate concentration, the conversion percentage was about 1.5 times higher than at a substrate concentration of 100 nmol.

The two other desaturases were less active than the Δ9 desaturase. The Δ5 desaturation of 20:3n-6 was more active than the Δ6 desaturation of 18:2n-6 or 18:3n-3 in any of the experimental situations. As for Δ9 desaturation, Δ5 desaturation progressed at a higher rate in the presence of a lower concentration of substrate (30 nmol). The activity increased from 3 to 6 months, at which time it reached a plateau. The influence of diet appeared only at 6 and 9 months when black currant and borage oils had been ingested over a longer time period. In 3-month-old animals, the desaturation of n-3 fatty acid was much higher than that of n-6 fatty acid for all dietary groups. The difference disappeared by 6 and 9 months. The conversion percentage was the same at the two concentrations of the two substrates in contrast to results obtained with the Δ9 and Δ5 desaturases. The effect of season (Table 5) was investigated in the 6-month-old rats. The Δ9 desaturation activity markedly increased in winter in all dietary groups. Δ5 Desaturation activity was also higher in winter but only at high concentration of substrate and only for the C and Bc groups. At the same saturating concentration of substrate (120 nmol), the Δ6 desaturation of the n-3 fatty acid was not affected by the season, whereas the desaturation of the n-6 fatty acid was greatly increased in winter.

Table 6 presents the percentages of the major fatty acids of liver microsomal phospholipids at the different ages and for the different diets. Palmitic and stearic acids were the main saturated fatty acids. Their cumu-

TABLE 2

Body Weight, Liver Weight and Protein Content of Liver Microsomes of Rats. Effects of Age and Dietary Lipids[a]

Age (months)	Dietary group	Body weight (g)	Liver weight (g)	Liver weight/ body weight (%)	Microsomal proteins (mg/g of liver)
3	C[b]	327 ± 27^{x}	11.5 ± 0.6^{w}	3.51 ± 0.11^{y}	19.12 ± 3.46^{z}
	Bc[c]	330 ± 33^{x}	12.2 ± 1.5^{wx}	3.70 ± 0.13^{z}	16.40 ± 2.14^{yz}
	Bg[d]	331 ± 17^{x}	12.7 ± 0.9^{wxy}	3.85 ± 0.08^{z}	17.19 ± 2.42^{yz}
6	C	411 ± 27^{y}	12.7 ± 1.2^{wxy}	3.08 ± 0.15^{vwx}	15.35 ± 2.17^{y}
	Bc	448 ± 20^{yz}	13.9 ± 1.8^{yz}	3.12 ± 0.11^{wx}	16.22 ± 1.96^{yz}
	Bg	440 ± 52^{y}	14.2 ± 0.9^{yz}	3.22 ± 0.15^{x}	16.82 ± 3.37^{yz}
9	C	479 ± 40^{z}	14.2 ± 1.5^{z}	2.97 ± 0.13^{vw}	17.08 ± 2.79^{yz}
	Bc	442 ± 38^{yz}	13.4 ± 0.8^{xyz}	3.01 ± 0.17^{vw}	18.95 ± 1.07^{z}
	Bg	460 ± 26^{yz}	13.4 ± 1.8^{xyz}	2.92 ± 0.19^{v}	15.97 ± 2.33^{y}

[a]Results are means ± SE for n=6 animals in each group. After analysis of variance (Duncan's multiple range test), means were compared in each column (three groups and three diets) according to the least significant difference, and classified according to increasing order. Means assigned different manuscript letters were significantly different ($p<0.05$).

[b]Control diet.

[c]Black currant seed oil diet.

[d]Borage oil diet.

lated percentages generally decreased with age in the three groups. The influence of diet at each age was not evident. The decrease in saturated fatty acid content was generally compensated (especially in the control group) by an increase of the monounsaturated fatty acids 16:1n-7 and 18:1n-9, the products of Δ9 desaturation. The percentage of linoleic acid did not really change with age and diet. The arachidonic acid content did not increase with age in the C group. With the experimental diets containing γ-linolenic acid, an increase in arachidonic acid was observed, but only at 9 months (corresponding to ingestion of 18:3n-6 for a longer time). This caused a similar variation in the total n-6 fatty acid content. The ratio 20:4n-6/18:2n-6, which reflects the overall conversion of 18:2n-6 to 20:4n-6, did not generally change between 3 and 9 months in each dietary group. However, the ratio was higher in the black currant and borage dietary groups (especially the latter). Docosahexaenoic acid (22:6n-3) was the major n-3 fatty acid in the microsomal phospholipids. The percentage of 22:6n-3 tended to increase with age for each group. At 9 months, the total n-3 fatty acid content was higher than at 3 months, especially in the two groups on the experimental diets, Bc and Bg. The presence of stearidonic acid in the black currant oil did not induce any specific changes.

DISCUSSION

The purpose of this study was to see whether dietary manipulation could overcome a possible age-related decrease in PUFA content of tissue lipids due to a decrease in desaturation activity. A decrease in Δ6 desaturation is of special concern as Δ6 desaturation is the major regulating reaction step in EFA metabolism.

The experimental approach was to bypass the Δ6 desaturation reaction by adding to the diet γ-linolenic acid (n-6 series) or stearidonic acid (n-3 series). Δ9 Desaturase activity was also studied because it broadly reflects lipogenic activity. In studies with guinea pigs (28), humans (29) and rats (30), it was shown that administration of γ-linolenic acid results in the biosynthesis of arachidonic acid by shunting the Δ6 desaturase step. An increase of n-6 PUFAs has been observed in various organs after the addition to the diet of black currant seed oil or borage oil, both containing γ-linolenic acid.

In our experiments rats were fed low quantities of 18:3n-6 (206.8 mg and 169.1 mg per 100 g of Bc and Bg diets, respectively). Black currant and borage oils were mixed with other oils to maintain the same total levels of n-6 and n-3 fatty acids as in the control group. Experimental diets were given at different time points as a function of age, the duration of feeding being increased from 3 to 9 months of age. The protocol was justified by the aim of this study, which was to compensate for, and not to prevent a possible deficiency in desaturation capacity. Because the turnover rate of tissue lipids supposedly decreases with age, the duration of the substituted experimental diet was increased with age.

Results of *in vitro* desaturation by liver microsomes (Tables 3 and 4) showed that the Δ9 desaturase

TABLE 3

Desaturase Activity in Rat Liver Microsomes. Effects of Age and Dietary Lipids[a]

Dietary group	Age (months)	Δ n-3 18:3 → 18:4		Δ6 n-6 18:2 → 18:3		Δ5 n-6 20:3 → 20:4		Δ9 18:0 → 18:1n-9	
		40	120	60	120	30	80	50	100
		nmoles							
C[b]	3	3.3 ± 0.4^{z}	9.7 ± 0.9^{yz}	3.5 ± 0.5^{xy}	5.3 ± 1.3^{wx}	6.3 ± 0.7^{xy}	8.3 ± 1.1^{x}	21.3 ± 6.2^{yz}	27.9 ± 12.0^{xy}
	6	3.5 ± 0.9^{z}	9.0 ± 1.2^{xy}	5.2 ± 1.2^{z}	8.0 ± 2.6^{z}	9.0 ± 1.0^{z}	12.7 ± 2.1^{y}	30.5 ± 5.4^{z}	43.6 ± 11.5^{z}
	9	3.0 ± 0.5^{yz}	8.2 ± 1.0^{wx}	4.7 ± 0.9^{yz}	6.1 ± 1.1^{xy}	8.5 ± 0.8^{yz}	12.6 ± 2.4^{y}	14.6 ± 5.0^{y}	16.6 ± 7.7^{w}
Bc[c]	3	3.4 ± 0.5^{z}	10.6 ± 1.4^{z}	3.1 ± 0.4^{xy}	3.8 ± 0.5^{vw}	5.8 ± 0.7^{x}	8.5 ± 1.4^{x}	25.0 ± 4.9^{yz}	31.2 ± 8.8^{y}
	6	2.3 ± 0.2^{y}	7.9 ± 0.7^{w}	2.3 ± 0.2^{x}	7.1 ± 2.6^{yz}	5.9 ± 0.9^{x}	16.7 ± 3.5^{z}	30.3 ± 5.0^{z}	42.9 ± 13.8^{z}
	9	2.8 ± 0.8^{yz}	8.2 ± 0.6^{wx}	2.3 ± 0.2^{x}	7.1 ± 2.6^{yz}	9.2 ± 1.6^{z}	14.8 ± 3.5^{yz}	22.1 ± 5.8^{yz}	35.0 ± 10.6^{yz}
Bg[d]	3	2.8 ± 0.5^{yz}	9.2 ± 1.3^{y}	2.4 ± 0.2^{x}	3.5 ± 0.5^{v}	5.2 ± 0.3^{x}	7.4 ± 1.2^{x}	20.6 ± 7.3^{yz}	25.9 ± 10.5^{wxy}
	6	3.1 ± 0.8^{yz}	8.2 ± 1.0^{wx}	4.6 ± 0.7^{yz}	7.2 ± 1.8^{yz}	5.6 ± 0.7^{x}	13.3 ± 1.3^{y}	30.6 ± 3.8^{z}	45.2 ± 13.1^{z}
	9	2.3 ± 0.3^{y}	9.2 ± 1.1^{y}	4.6 ± 1.1^{yz}	8.3 ± 2.1^{z}	10.2 ± 2.1^{z}	16.5 ± 3.9^{z}	15.8 ± 9.9^{y}	20.2 ± 13.8^{wx}

[a]Desaturase activities are expressed as nmoles of substrate converted per 15 min per 5 mg microsomal protein. Results are means ± SE for n=6 animals in each group. After analysis of variance (Duncan's multiple range test), means were compared in each column (three groups and three diets) according to the least significant difference, and classified according to increasing order. Means assigned different manuscript letters were significantly different ($p<0.05$).
[b]Control diet.
[c]Black currant seed oil diet.
[d]Borage oil diet.

TABLE 4

Desaturase Activity in Rat Liver Microsomes Expressed as Conversion Percentages. Effects of Age and Dietary Lipids[a]

Desaturation		Δ6 n-3 18:3 → 18:4		Δ6 n-6 18:2 → 18:3		Δ5 n-6 20:3 → 20:4		Δ9 18:0 → 18:1n-9	
Dietary group	Age (months)	40	120	60	120	30	80	50	100
		nmoles of substrates							
C[b]	3	8.3	8.1	5.8	4.4	21.0	10.4	42.6	27.9
	6	8.8	7.5	4.3	6.7	30.0	15.9	61.0	43.6
	9	7.5	6.8	3.9	5.1	28.3	15.8	29.2	16.6
Bc[c]	3	8.5	8.8	5.2	3.2	19.3	10.6	50.0	31.2
	6	5.8	6.6	3.8	5.9	19.7	20.8	60.6	42.9
	9	7.0	6.8	3.8	5.9	30.7	18.5	44.2	35.0
Bg[d]	3	7.0	7.7	4.0	2.9	17.3	9.3	41.2	25.9
	6	7.8	6.8	7.7	6.0	18.7	16.6	61.2	45.2
	9	5.8	7.7	7.7	6.9	34.0	20.6	31.6	20.2

[a]Conversion percentages were calculated from the desaturation product/substrate ratio.
[b]Control diet.
[c]Black currant seed oil diet.
[d]Borage oil diet.

was more active than the Δ6 and Δ5 desaturases. This high activity of Δ9 desaturation, especially at 6 months, may be related to active lipogenesis in the liver. Growth is most active between 3 and 6 months and the need for fatty acids for tissue synthesis is elevated. Saturated fatty acids provided by the dietary oils were relatively low, enhancing lipogenic reactions which give rise to saturated fatty acids that undergo desaturation by Δ9 desaturase.

In comparison, the activities of Δ5 and, more particularly, of Δ6 desaturases were clearly lower than that of the Δ9 desaturase. The Δ5 and Δ6 desaturations do not have the same physiological significance as the Δ9 desaturation, since they are required in the biosynthesis of PUFAs from dietary linoleic and linolenic acids. The low activity of the Δ6 desaturase means that it constitutes a limiting step in the biosynthesis of the essential PUFAs.

The difference observed regarding the activity of the enzymes with different substrates is in agreement with observations reported previously by others. Brenner and Peluffo (31) demonstrated a more rapid desaturation of α-linolenic acid than of linoleic acid by the Δ6 desaturase. This was confirmed by Marcel *et al.* (32) and by Sprecher (33). Data regarding the Δ5 desaturation rate are sparse and sometimes contradictory. Bernert and Sprecher (34) observed a rate lower than the Δ6 desaturation rate, but others (31,32) found it higher.

The activities of Δ6 and Δ5 desaturases toward n-6 fatty acids increased from 3 to 6–9 months. This corresponds to an active period of growth during which PUFA demand is high for membrane phospholipid synthesis in the newly formed tissues. Desaturation activities adapt themselves to this need probably by the synthesis of new enzyme under hormonal control. The Δ6 desaturation of the n-3 fatty acid was more active than that of the n-6 fatty acid only at 3 months. Nervous tissue is very rich in n-3 PUFAs and at three months of age the brain is still rapidly developing (35), increasing the demand for this type of PUFAs.

Our results regarding the influence of age on the desaturation rates (Tables 3 and 4) can be compared to those reported by Peluffo and Brenner (12) and Bordoni *et al.* (13). These authors studied the Δ6 desaturation in rats fed a diet containing two levels of proteins (12) or a commercial standard diet (13). They observed that Δ6 desaturation activity was lower at 12 or 14 months than at 3 months. This is not contradictory to our results since we observed the maximum at 6 and 9 months; the activity is then likely to decrease. The changes in Δ6 desaturation with age may be similar to those we observed with Δ9 desaturation, but at a slower rate. Results of studies at a more advanced age, *i.e.*, at 12, 18 and 24 months, may answer this question.

The presence of γ-linolenic acid in the diet did not induce any change in the Δ6 desaturation of linoleic acid. In contrast, Δ5 desaturation was slightly more active, especially at 9 months. These low effects are likely due to the low level of γ-linolenic acid in the experimental diets (Bc and Bg). When ingested for a longer time (rats of 9 months of age), the stimulating effect on Δ5 desaturation was more significant. The presence of stearidonic acid (18:4n-3) in black currant seed oil did not affect the Δ6 desaturation of α-linolenic acid, probably because the amount of this n-3 fatty acid in the diet was very low. Choi and Sugano (20) observed an inhibitory effect of dietary γ-linolenic acid on the Δ6 desaturation of linoleic acid. In these experiments, a higher amount of dietary evening primrose oil (9.3 % of γ-linolenic acid) was provided (10% weight of oil in the diet) than in our study.

Our studies confirmed the seasonal variations of desaturation previously observed by Peluffo and Brenner (12). The desaturations were generally more active

TABLE 5

Desaturase Activity of Liver Microsomes of 6-month-old Rats. Effect of the Season[a]

Desaturase		Δ6 n-3		Δ6 n-6		Δ5 n-6		Δ9	
Dietary group	Season	40	120	60	120	30	80	50	100
		nmoles							
C[b]	Summer	2.9 ± 0.9^{y}	9.1 ± 1.2^{z}	4.2 ± 0.4^{x}	5.6 ± 0.7^{w}	9.2 ± 1.1^{z}	11.1 ± 0.8^{x}	26.2 ± 4.4^{y}	33.6 ± 5.6^{z}
	Winter	4.1 ± 0.3^{z}	8.9 ± 1.4^{z}	6.3 ± 0.2^{z}	10.4 ± 0.3^{z}	8.6 ± 0.8^{z}	14.2 ± 1.8^{y}	34.7 ± 0.1^{z}	53.6 ± 0.7^{y}
Bc[c]	Summer	2.4 ± 0.2^{y}	7.7 ± 0.8^{y}	2.5 ± 0.2^{y}	4.6 ± 0.1^{v}	6.8 ± 0.4^{yz}	14.7 ± 0.9^{y}	25.9 ± 0.7^{y}	30.9 ± 3.1^{z}
	Winter	2.2 ± 0.2^{y}	8.1 ± 0.6^{z}	2.1 ± 0.1^{w}	9.7 ± 0.4^{y}	5.1 ± 0.1^{y}	18.7 ± 4.3^{z}	34.6 ± 2.4^{z}	54.9 ± 5.5^{y}
Bg[d]	Summer	2.9 ± 0.7^{y}	8.4 ± 0.2^{z}	3.9 ± 0.2^{x}	5.6 ± 0.2^{w}	6.1 ± 0.2^{y}	13.6 ± 1.3^{y}	27.4 ± 2.5^{y}	34.1 ± 6.9^{z}
	Winter	2.5 ± 0.9^{y}	8.0 ± 1.6^{z}	5.2 ± 0.4^{y}	8.9 ± 0.3^{x}	5.1 ± 0.6^{y}	13.1 ± 1.5^{y}	33.7 ± 0.6^{z}	56.4 ± 2.8^{y}

[a]Results are expressed as in Table 3 for n=3 animals. After analysis of variance (Duncan's multiple range test), means were compared in each column (three diets and two seasons) according to the least significant difference, and classified according to increasing order. Means assigned different manuscript letters were significantly different ($P < 0.05$).
[b]Control diet.
[c]Black currant seed oil diet.
[d]Borage oil diet.

TABLE 6

Major Fatty Acids (% moles) of Liver Microsomal Phospholipids. Effects of Age and Dietary Lipids[a]

		Fatty acids							
Dietary group	Age (months)	16:0 + 18:0	16:1n-7 + 18:1n-9	18:2n-6	20:4n-6	22:6n-3	total n-6[e]	total n-3[f]	20:4n-6/ 18:2n-6
C[b]	3	38.5 ± 1.8^{yz}	13.5 ± 2.0^{wx}	7.2 ± 0.6^{xyz}	23.7 ± 3.0^{u}	8.9 ± 1.4^{wxyz}	35.1 ± 3.7^{w}	10.7 ± 2.3^{xy}	3.4 ± 0.4^{x}
	6	39.6 ± 1.5^{z}	14.8 ± 2.2^{xy}	8.0 ± 0.6^{z}	24.8 ± 2.6^{uv}	7.9 ± 1.3^{vw}	35.7 ± 2.6^{wx}	9.6 ± 1.6^{wx}	3.1 ± 0.5^{x}
	9	33.2 ± 1.2^{wx}	17.0 ± 2.8^{z}	7.7 ± 0.5^{yz}	25.7 ± 3.0^{uvw}	9.7 ± 1.1^{xyz}	36.4 ± 1.9^{wx}	12.0 ± 1.0^{zy}	3.3 ± 0.5^{x}
Bc[c]	3	39.8 ± 1.2^{z}	12.5 ± 1.5^{w}	6.1 ± 0.8^{w}	26.8 ± 1.7^{vw}	8.5 ± 0.3^{wx}	36.9 ± 1.6^{wx}	9.8 ± 0.2^{wx}	4.4 ± 0.8^{y}
	6	37.7 ± 2.8^{y}	15.9 ± 1.0^{yz}	6.5 ± 0.3^{w}	26.9 ± 3.1^{vw}	7.1 ± 1.8^{v}	38.8 ± 5.3^{xy}	8.5 ± 1.8^{w}	4.1 ± 0.3^{y}
	9	31.6 ± 2.2^{w}	13.3 ± 1.7^{w}	7.3 ± 0.5^{xy}	31.8 ± 1.7^{yz}	10.0 ± 0.6^{yz}	42.0 ± 1.7^{yz}	12.3 ± 0.6^{z}	4.4 ± 0.3^{y}
Bg[d]	3	38.5 ± 0.8^{yz}	11.0 ± 0.7^{v}	5.3 ± 0.9^{v}	27.9 ± 1.7^{wx}	8.9 ± 0.9^{wxy}	37.1 ± 2.8^{wx}	10.2 ± 0.8^{x}	5.3 ± 0.6^{z}
	6	32.6 ± 0.8^{wx}	13.6 ± 0.3^{wx}	7.4 ± 0.3^{yz}	30.1 ± 0.8^{xy}	9.5 ± 0.2^{xyz}	41.7 ± 1.0^{yz}	10.7 ± 0.2^{xy}	4.1 ± 0.3^{y}
	9	32.3 ± 2.3^{wx}	12.2 ± 0.7^{vw}	6.6 ± 0.3^{wx}	33.5 ± 3.1^{z}	10.1 ± 0.7^{z}	43.6 ± 2.4^{z}	12.1 ± 0.6^{yz}	5.0 ± 0.3^{z}

[a]Results are expressed as molar percentages of total fatty acids. Results are means ± SE for n=6 animals in each group. After analysis of variance (Duncan's multiple range test), means were compared in each column (three groups and three diets) according to the least significant difference, and classified according to increasing order. Means assigned different manuscript letters were significantly different ($p < 0.05$).
[b]Control diet.
[c]Black currant seed oil diet.
[d]Borage oil diet.
[e]Total n-6: include unreported fatty acids: 18:3, 20:2, 20:3, 22:4 and 22:5n-6.
[f]Total n-3: include unreported fatty acids: 18:3, 20:5 and 22:5n-3.

in winter (January) than in summer (June). Surprisingly, the Δ6 desaturation of α-linolenic acid was not affected in contrast to the Δ6 desaturation of linoleic acid. The variations in desaturation activity are likely to follow variations in hormonal secretions. The physiological significance of these modifications is difficult to understand since the rats were submitted to identical environmental conditions throughout the year. The results underline the importance of taking into account seasonal variations when studying the desaturation rates, especially in the course of aging. In our experiments with 3- and 9-month-old rats, two groups of three rats were tested at 4- to 6-month intervals to minimize seasonal variations. Modifications in Δ6 and Δ5 desaturation rates are likely to influence the rate of biosynthesis of essential PUFAs and to modify their pattern in membrane phospholipids. To test this hypothesis, we analyzed the fatty acid composition of microsomal phospholipids at the three different ages and with the three different diets. This choice of membrane appears appropriate for this study because the desaturases are thought to reside in the phospholipid bilayers of the endoplasmic reticulum. The question is often raised whether changes in desaturase activity would change the fatty acid composition of the phospholipids or, conversely, whether modification of phospholipid fatty acids would induce changes in the desaturation rate. Eventually, correlation between the two phenomena could be established. In this work, the fatty acid composition of microsomal phospholipids (Table 6) did not necessarily correlate with changes in desaturase activity. For example, the proportion of monounsaturated fatty acids was not modified between 6 and 9 months, whereas the Δ9 desaturation rate sharply decreased during this period (Tables 3 and 4). Similarly, the proportion of arachidonic acid did not change from 3 to 9 months, whereas the Δ6 and Δ5 desaturation rates increased from 3 to 6 months. However, the type of diet seemed to influence the phospholipid fatty acid composition. The presence of γ-linolenic acid in dietary black currant or borage oil tended to increase the Δ5 desaturation rate and, consequently, the arachidonic acid content of microsomal phospholipids. This property is important since the activity of the rate-limiting Δ6 desaturation step may decrease with age.

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Change in Bile Salt Dependent Lipase in Human Breast Milk During Extended Lactation

Patricia Dupuy[a], J. Frédéric Saunière[a], Henry L. Vis[b], Michèle Leclaire[c] and Dominique Lombardo[d,*]
[a]Service de Gastroenterologie, Hôpital Sainte Marguerite, 13009 Marseille, [b]Hôpital des Enfants Reine Fabiola, 1020 Bruxelles, Belgium, [c]Service de Pédiatrie, Hôpital Michel-Levy, 13006 Marseille, and [d]Centre de Biochimie et de Biologie Moléculaire du Centre National de la Recherche Scientifique, 13402 Marseille, France

Two hundred eighty-one milk samples collected from Zaïrian nonprivileged, undernourished mothers, in series of nine groups from 1 month to 18 months after parturition, and 66 milk samples collected from French privileged mothers in series of four groups from 2 days to 16 months *postpartum*, were analyzed for their lactose, lipid and protein contents. In addition, the activity of bile salt-dependent lipase (esterase), which may play an important role in the newborn infant's lipids digestion, was measured. After the first month *postpartum*, independent of the nutritional state of the mother, sugar and protein concentrations were identical. Lipid content of French mothers' milk was lower in transitional milk, but appeared constant in mature milk with an average value of 29.1 ± 5.8 mg/mL of milk. In Zaïrian mothers' milk, the lipid content of mature milk plateaued at around 50–55 mg/mL independent of the stage of lactation. Bile salt-dependent lipase showed constant esterase activity within the lactation stage in privileged mothers' milk, but decreased by almost 80–90% during the first four months of lactation in undernourished mothers. The data suggest that milk from nonprivileged mothers may lose some of its ability to hydrolyze milk lipid esters, which could also be of consequence to the infant's normal growth in view of its effect on the esters of the lipid-soluble vitamins A, E and D.
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Human milk has been shown to contain two lipases (1). One, referred to as the bile salt-stimulated lipase (or bile salt-dependent lipase), is responsible for milk fat degradation and acts in the newborn's intestinal lumen (2). This enzyme is stable enough to withstand the acidic conditions of the stomach (2) and was shown to be structurally and functionally closely related to the human pancreatic carboxylic ester hydrolase (3–7). Both enzymes share rather wide substrate specificities. They hydrolyze water-insoluble long-chain fatty acid esters of glycerol and cholesterol as well as esters of vitamin A, D and E in the presence of primary bile salts (8). They also hydrolyze water-soluble esters such as short-chain fatty acid esters of glycerol, 4-nitrophenyl acetate, and methyl butyrate (9); however, in this case activation by bile salts is not essential. The wide specificity of the bile salt-dependent lipase suggests that in the presence of bile salt the enzyme may hydrolyze essentially any lipid ester. As a milk protein, the bile salt-dependent lipase appears to be unique to the highest primates and is carnivorous. So far, it has been found only in milk from man (1), gorilla (10), dog and cat (11), but not in milk from several other species including rat, pig, cow, horse and monkey (12,13).

In addition to the bile salt-dependent lipase, the occurrence of traces of lipoprotein lipase (or serum-stimulated lipase) in milk has been reported; it is likely to result from cell damage during the secretion of milk by mammary gland (14). In contrast to bile salt-dependent lipase, lipoprotein lipase is rapidly inactivated in the gastric environment (15). Hence, it serves no useful function in the newborn's gastrointestinal tract. The presence of lipoprotein lipase in the mammary gland is essential for the synthesis of milk lipids.

Previous investigations have established that the bile salt-dependent lipase is present in milk from Caucasian privileged and undernourished Ethiopian mothers (16–21). The aim of the present work was to extend these studies to other African mothers and to establish whether bile salt-dependent lipase is present in the milk of women living in an area where malnutrition is widespread. We also set out to determine whether the esterase activity changes as a function of length of lactation.

EXPERIMENTAL PROCEDURES

Collection of samples. Subjects were breast-feeding primiparous or multiparous women. Milk samples (401) representing 281 subjects were collected from nonprivileged African women living in the urban area of Bukavu (Kivu, Zaïre). In parallel, milk samples collected from 66 privileged Caucasian mothers living in the urban area of Marseilles (France) were also analyzed. Sixty-seven percent of the Zaïrian mothers and 25% of French mothers were less than 24-years-old; 6.5% of the Zaïrian mothers and 20% of French mothers were more than 35-years-old. Mothers were between 2 days and 18 months *postpartum* and nursing full-term infants who were generally fed on demand. Milk aliquots (5–10 g) were taken between nursing (fore-milk) and were expressed indistinctly from either breast by means of a breast-pump in the morning or in early afternoon (9 a.m. to 2 p.m.).

African milk samples were sent frozen and stored at −20°C until thawed slowly before analysis. European milk samples were also frozen and thawed before analysis. Note that freezing-thawing cycles (up to 3 cycles, at least) have no effect on the bile salt-dependent lipase activity (22). Nevertheless, multiple freezing and thawing was avoided.

Sampling. Milk from nonprivileged mothers were put into series of nine groups, from I to IX corresponding to: Group I, one month *postpartum* milk samples (n = 21); group II, two months (n = 36); group III, three months (n = 24); group IV, four months (n = 31); group V, six months (n = 15); group VI, nine months (n = 52); group VII, twelve months (n = 49); groups VIII, fifteen months (n = 44); and group IX, eighteen months (n = 9). Milk samples from privileged mothers were in a series of four

*To whom correspondence should be addressed at Centre de Biochimie et de Biologie Moléculaire-CNRS. 31, chemin Joseph Aiguier, B.P. 71, 13402 Marseille-Cédex 9, France.

groups from A to D, corresponding to: Group A, 2–5 days (transitional milk, n = 26); group B, 1–2 weeks (mature milk, n = 12); group C, 1–4 months (n = 18); and group D, 5–16 months *postpartum* (n = 10).

Chemicals. 4-Nitrophenyl acetate was provided by Ega-Chemie (Steinheim/Albuch, Germany) and sodium taurocholate and deoxycholate were purchased from Calbiochem-Behring (La Jolla, CA). Delipidized bovine serum albumin was a product from Sigma (St. Louis, MO) and α-DL-glucose came from Fluka (Buchs, Switzerland).

Protein determination. Protein concentrations were determined according to Lowry *et al.* (23) using bovine serum albumin as standard. Measurements were done on diluted skim milk samples obtained after centrifugation (6) to avoid turbidity and lipid interference (24). Under our conditions, sugars do not interfere with protein determination.

Lactose determination. Lactose was assayed by the phenol-sulfuric acid method of Dubois *et al.* (25), using glucose as standard.

Lipid determination. Total lipid content of milk was determined by gravimetry after extraction (26). Extracted lipids were dried under vacuum until no further weight loss was observed. Duplicate determinations were done on pools constituted from milk samples of nonprivileged groups (2–9 pools per group) and on each sample of milk from privileged groups.

Bile salt-dependent esterase activity assay. The bile salt-dependent esterase activity was determined at 25 ± 1°C using 4-nitrophenyl acetate as substrate in the presence of 6 mM sodium taurocholate (27). Bile salt is added to the assay to activate the bile salt-dependent esterase (1) and to inhibit, if any, the lipoprotein lipase activity (14). Controls with known enzyme activities were always run in parallel with the unknown samples. In a series of experiments, we determined intra-assay and inter-assay errors which were less than 5% and 10%, respectively.

One enzyme unit was the enzyme quantity able to hydrolyse 1 μmole of substrate per mL and minute (U/mL). The enzyme activity was also expressed as the number of enzyme units per mg of milk total protein (U/mg milk protein).

When milk samples were incubated for 2 hr at 4°C and pH 7.5 in the presence of 1 mM α-toluenesulfonyl fluoride as lipoprotein lipase inhibitor (28), within experimental error, no decrease in 4-nitrophenyl acetate hydrolysis by milk enzymes was observed. This shows that the contribution of lipoprotein lipase activity to this hydrolysis is negligible under our experimental conditions.

Bile salt-dependent lipase activity assay. Bile salt-dependent lipase activity was determined titrimetrically on emulsified tributyrin in the presence of 1 mM sodium deoxycholate as already described (9).

Cholesterol ester hydrolase activity assay. The cholesterol ester hydrolase activity of bile salt-dependent lipase was determined as described by Lombardo and Guy (29).

Bile salt-dependent lipase purification. The bile salt-dependent lipase was purified to homogeneity using the two Heparin-Sepharose columns procedure of Bläckberg and Hernell (6).

Statistical analysis. Data were statistically analyzed by Student's *t*-test. They were considered to be significantly different when $p < 0.01$.

RESULTS

Bile salt-dependent lipase and bile salt-dependent esterase activities of human milk. In an attempt to demonstrate that the bile salt-dependent lipase and bile salt-dependent esterase activities of human milk reside in the same protein molecule, we determined the activity on 4-nitrophenyl acetate and on tributyrin during the purification step of the bile salt-dependent lipase (6). The data presented in Table 1 show that the ratio of esterase *vs* lipase activities is independent of the purification step of the enzyme. Since the bile salt-dependent lipase of human milk also bears cholesterol ester hydrolase activity (3), we determined the milk activity as well as the homogenous enzyme activity on cholesteryl esters. The data are presented in Table 1 and show that the esterase *vs* cholesterol esterase activity ratios are not significantly different in the two fractions.

Incubation of skim milk and bile salt-dependent lipase with *p*-nitrophenyl-*N*-butyl-*N*-methyl carbamate, a specific active site titrator for bile salt-dependent lipases (Fourneron J.D., Abouakil, N., Chaillan, C. and Lombardo, D., unpublished data), also decreases to the same extent the esterase and lipase activities of skim milk and of pure bile salt-dependent lipase. These results indicate that the esterase activity of human milk is due to the bile salt-dependent lipase. The results cannot, of course, rule out the possibility that milk can contain different esterase activities too low to make a significant contribution to the activities recorded under our conditions.

Analysis of milk from Zaïrian nonprivileged mothers. Milk samples were put into a series of nine groups collected from 1 month up to 18 months *postpartum.* Samples were analyzed for the bile salt-dependent esterase activity, protein, lactose and total lipid contents. Lactose content of breast milk from nonprivileged mothers did not vary from 1 month to 18 months; the mean (± standard deviation) value for all samples was 71 ± 10 mg/mL. Lipid (mostly triglyceride) content also remained quite stable at 53.6 ± 13.4 mg/mL, independent of the stage of lactation (Fig. 1).

TABLE 1

Esterase *vs* Lipase and Cholesterol Esterase Activities in Human Milk and During the Purification of Bile Salt-Dependent Lipase[a]

	Esterase/lipase	Esterase/cholesterol esterase
Skim milk	0.21 ± 0.05 (n = 3)	2.11 ± 0.14 (n = 4)
First Heparin-Sepharose	0.20 ± 0.06 (n = 3)	n.d.
Second Heparin-Sepharose	0.19 ± 0.02 (n = 3)	2.56 ± 0.40 (n = 4)

[a]The values given are mean ± standard deviation. Bile salt-dependent lipase was purified from skim milk according to Bläckberg and Hernell (see ref. 6). The bile salt-dependent lipase activity was eluted from a first Heparin-Sepharose column with a linear gradient from 0.05 to 1 M NaCl in 5 mM Tris/HCl, pH 7.4 buffer. The fractions of lipase were then dialyzed and applied to an Affi-Gel blue column to eliminate albumin. Fractions containing lipase activity were then loaded onto a second Heparin-Sepharose column (5 mM Tris/HCl, 50 mM NaCl, pH 7.4 buffer), the column was washed step-wise with sodium chloride in buffer, and the lipase was eluted at 0.5 M NaCl.

Figure 1 shows that protein concentrations were statistically higher ($p < 0.01$) in group I (1 month *postpartum*, 12.0 ± 2.0 mg/mL) than in the other groups, where the average value was 10.0 ± 0.5 mg/mL. Despite the constant concentration of fat, the bile salt-dependent esterase activity decreased (Fig. 1) from 12.9 ± 2.3 U/mL in the first group to 1.6 ± 0.7 U/mL after six months *postpartum*. This value, which represents an 80% decrease, remained constant until the end of breast-feeding. The decrease of the enzyme activity over the period extending to the sixth month of lactation is likely to result from a decrease of the total amount of breast-secreted bile salt-dependent esterase as shown by the parallel decrease of the activity of the enzyme per mg of milk protein (Fig. 1).

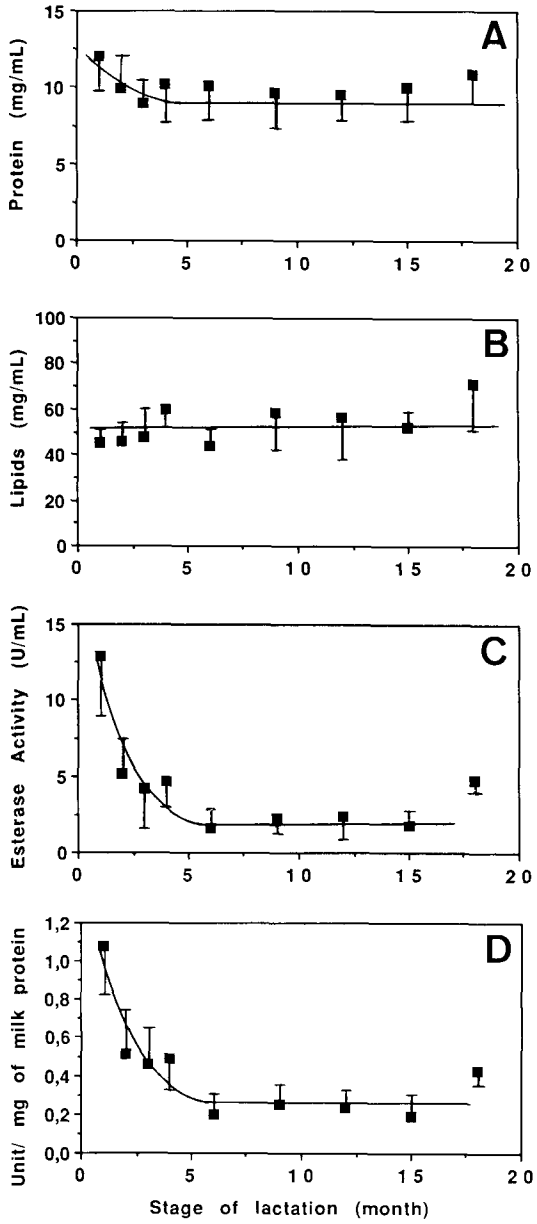


FIG. 1. Composition and enzyme content of milk samples from undernourished mothers. A, concentration of proteins; B, lipid concentration; C, bile salt-dependent esterase activity; D, bile salt-dependent esterase activity per mg of milk protein. Vertical bars represent the standard deviation.

Analysis of milk from French privileged mothers. Milk samples from privileged mothers were analyzed and the results compared to those obtained for undernourished mothers. The values obtained for lactose and lipids are presented in Table 2. In transitional milk samples (group A), the sugar content was significantly lower, then it increased slightly and reached a constant value between the first and the sixteenth month (group C and D). The sugar contents are identical to those found in the corresponding groups of milk samples from undernourished mothers (see Discussion section, Table 3). Total lipid contents of French milk increased slightly from transitional (group A) to mature milk (group B and C). A higher lipid content was then observed in group D (Table 2). This probably reflects the end of exclusive breast-feeding (20,30), since late lactation specimens of French women were taken during weaning. The lipid contents of Zaïrian milks did not increase significantly between the 1-4 months and the 5-16 months groups (Table 3).

Protein concentration (Table 2) decreased during the first month of lactation. Afterward the protein concentration remained constant at about 13 mg/mL, which is not very different from the value obtained for undernourished mothers' milk at the corresponding stage of lactation. All nutrient contents, independent of the milk source, agree with those found in the literature (31-34).

Bile salt-dependent esterase activity of milk from privileged mothers was constant and independent of the stage of lactation. Concomitantly to the protein decrease from transitional to mature milk, the enzyme activity per mg protein increased ($p < 0.01$) and reached its maximal level at one month *postpartum*. These data indicate that the amount of enzyme secreted by the breasts of privileged mothers was constant during the lactation period.

DISCUSSION

In adults, efficient fat digestion is achieved through the concerted action of three lipolytic enzymes, namely pancreatic colipase-dependent lipase, carboxylic ester hydrolase, and preduodenal acid lipase (9,35-37). In the breast-fed newborn infant, pancreatic colipase-dependent lipase secretion is low (38), while the time frame of the secretion of pancreatic carboxylic ester hydrolase is still unknown. The bile salt-dependent milk lipase, which has a very wide substrate specificity, appears to play an important role in fat digestion (2). Nevertheless, the amount of enzyme present in milk would account for no more than 40% hydrolysis in 2 hr, which is the estimated transit time from the duodenum to the ileum (39). Also, fat absorption is reduced by approximately one-third when raw milk is heated (40), but this value is improved when fresh milk is fed (41). Furthermore, the bile salt-dependent lipase seems unable to hydrolyze milk fat globules, without prior gastric lipolysis (42). Intragastric lipolysis depends upon the presence of preduodenal acid lipase in the stomach (43) and accounts for some 20% of formula fat hydrolysis (37). It is evident that the combined activity of the milk bile salt-dependent lipase and of the preduodenal lipase can lead to sufficient hydrolysis of dietary fats and can thus effectively substitute for the low level of pancreatic lipase secretion (37). Pancreatic lipase *in vitro*, even in the presence of bile salts and colipase, cannot initiate the hydrolysis of milk fat globules

TABLE 2

Esterase Activities and Nutrient Contents of Milk Samples from Privileged Mothers[a]

Group	Number of sample	Lactose (mg/mL)	Lipids (mg/mL)	Proteins (mg/mL)	Esterase activity (U/mL)	Esterase activity (U/mg milk protein)
2-5 Days (A)	26	59.3 ± 8.3*	19.2 ± 10.9*	21.2 ± 4.2*	6.6 ± 2.9	0.34 ± 0.14*
1-2 Weeks (B)	12	65.7 ± 9.6	24.3 ± 9.2	18.1 ± 4.5	7.5 ± 2.4	0.47 ± 0.20
1-4 Months (C)	18	78.4 ± 7.9*	25.7 ± 8.3	13.0 ± 3.0*	6.6 ± 2.1	0.52 ± 0.18*
5-16 Months (D)	10	73.7 ± 6.2*	37.2 ± 9.9*	13.5 ± 4.2*	6.6 ± 2.2	0.51 ± 0.16*

[a]The asterisks indicate a significance of $p < 0.01$ as compared to group A. The values given are mean ± standard deviation.

TABLE 3

Esterase Activities and Nutrient Contents of Milk Samples from Undernourished Mothers[a]

Group	Number of sample	Lactose (mg/mL)	Lipids[b] (mg/mL)	Proteins (mg/mL)	Esterase activity (U/mL)	Esterase activity (U/mg milk protein)
1-4 Months	112	72.3 ± 11.2	50.7 ± 9.5	10.2 ± 2.5	6.3 ± 2.2*	0.60 ± 0.34*
5-16 Months	169	70.0 ± 9.9	55.7 ± 1.54	9.0 ± 2.0	2.0 ± 0.8*	0.22 ± 0.09*

[a]The asterisks indicate a significance of $p < 0.01$. The values given are mean ± standard deviation.

[b]Lipids were assayed on pooled samples as described in Experimental Procedures (1-4 months, n = 26; 5-16 months, n = 35).

(44). This is consistent with the fact that rat milk lacks bile salt-dependent lipase, while pancreatic carboxylic ester hydrolase is the dominant lipolytic activity in newborn rat duodenum (45). The acid-stable lipolytic systems (bile salt-dependent and preduodenal lipases) in newborns appears to be preferred over the colipase-dependent lipase route.

No gross differences were noted between the French and Zaïrian groups with respect to the major nutrients, at least as far as protein and lactose are concerned. The main differences between the milks of undernourished and privileged mothers existed in the lipid contents and in the activities of the bile salt-dependent esterase.

The lipid content of the Zaïrian milk samples was significantly higher than that determined in the French milk samples (Table 3). Because an inverse relationship between fat content and milk volume exists (46), the higher lipid amounts could have been related to differences in daily milk volumes produced by Zaïrian *vs* French mothers. However, this does not seem to be the case since daily milk volumes of nonprivileged mothers were comparable to or even higher than those of privileged mothers (31,47,48). The higher amount of lipids in Zaïrian milk as compared to French milk thus may rather be due to the expression of hind-milk instead of fore-milk (34,49). Although it would have been more desirable to obtain milk samples by entire breast expression, this would have been difficult to control especially with Zaïrian mothers. Our collection procedure in France was therefore carefully standardized with respect to that used in Zaïre. Independent of the origin and the stage of lactation, the fat content of milk samples used here is compatible with that of fore-milk (or with mid-milk) but not with that of hind-milk (21,31,32,34,40,49).

In European and North-American milk, esterase and lipase activities decrease (16,18,19,21) or remain constant (17,19, this study) during lactation. These inconsistent observations cannot be due, firstly, to the mode of sampling because the enzyme activity is independent of within-feed variation (34) or the milk volume produced (20); secondly, to the fat content of milk which does not correlate with the lipase activity (21); and, thirdly, to the assay used *i.e.*, the determination of lipase or esterase activity, because we, as well as others (19,50), have demonstrated that both activities reside in the same enzyme. It is also consistent with the fact that the same amino acids are essential for lipase and esterase activities, indicating that the same catalytic site may be involved in both activities displayed by the pure human milk bile salt-dependent lipase (7). Inhibition studies also confirmed these data (51; Fourneron *et al.*, unpublished data). Therefore, within-group variation can explain the conflicting observations.

In Zaïrian milk, bile salt-dependent esterase activity is higher during the early stages of lactation, and then decreases to less than 20% of the initial value. This decrease of bile salt-dependent lipase activity in breast milk from nonprivileged mothers is apparently not in agreement with the study of Hernell *et al.* (17) which was done with breast milk from nonprivileged Ethiopean mothers. This latter study concluded that the activity of the bile salt-dependent lipase did not depend upon the nutritional state of the mother and did not change with time. Therefore, Zaïrian milk samples were divided in two groups (1-4 months and 5-16 months *postpartum*, see Table 3) as described by Hernell *et al.* (17). Table 3 shows that i) the average amount of bile salt-dependent esterase present in the first group (1-4 months) is identical to that of the corresponding French group (Table 2), and that ii) the bile salt-dependent esterase activity as well as its activity per mg of milk protein are still significantly lower ($p < 0.01$) in the second than in the first group and than

in the corresponding milk group of privileged mothers. This shows that our results differ from those mentioned above (17) only when considering late lactation, and that the difference cannot be due to the way the groups have been defined. French and Zaïrian fore-milk samples were used here, and differences between the two studies (17), which are only observed in non-privileged milk samples for longer lactation time (>5 months), cannot be due to sample collection (*i.e.,* fore-milk *vs* mid- or hind-milk study) because no variation of the bile salt-dependent lipase activity as a function of feeding time has been reported (34). However, it is conceivable that the Zaïrian mothers living in the urban area of Bukavu were under more severe nutritional stress than the low socioeconomic Ethiopian mothers. Thus, the 80% decrease of the bile salt-dependent lipase (esterase) activity in Zaïrian milk may reduce the ability of hydrolyzing lipid-soluble vitamin esters and triglycerides in the intestine of infants breast-fed by undernourished mothers.

Despite the large number of studies concerned with breast milk composition, only a few have been devoted to changes in bile salt-dependent lipase (16-21) over time in underprivileged populations (17). The present study extends previous findings and shows that the enzyme activity is present in human breast milk at all stages of lactation, although it is found in much lower amounts in undernourished populations. Interestingly, enzymatic secretion by the pancreas is also decreased in the malnutrition state.

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Unusual Product Ratios Resulting from the Gamma-Irradiation of Cholesterol in Liposomes

G. Maerker* and **K.C. Jones**
U.S. Department of Agriculture, Eastern Regional Research Center, Agriculture Research Service, Philadelphia, Pennsylvania 19118

Cholesterol in aqueous suspensions of multilamellar vesicles (MLV) was exposed to gamma-irradiation (0.5–10 kGy) at 0–4°C. Cholesterol oxidation products resulting from the irradiation were isolated by dry column extraction followed by preparative thin-layer chromatography (TLC) and were quantitated by on-column gas chromatography (GC). The ratio of 7-ketocholesterol/cholesterol 5,6-epoxides generated by irradiation was less than one, much lower than the ratio of ten commonly produced by autoxidation. Irradiation also produced relatively higher amounts of 7-hydroxycholesterol than did autoxidation. These unique product ratios may be suitable indicators of past exposure to irradiation.
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As part of our search for a diagnostic test capable of discriminating meat and poultry that have been irradiated from those that have not, we have studied the effect of gamma-radiation on cholesterol in liposomes. The identification of irradiated foods has been the subject of worldwide interest (1–3), but until now no discriminating method suitable for animal tissue has been perfected. Much of the research effort in the identification of irradiated foods has been on the attempted characterization and measurement of unique radiolytic products, *i.e.* compounds whose formation can be attributed solely to the effect of ionizing radiation. The extent to which this effort has been unsuccessful, despite determined efforts of a worldwide community of talented scientists, is perhaps a measure of the trivial effect that low-dose ionizing radiation has on the chemical structure of food components.

The interaction of ionizing radiation with fatty acids, triglycerides and food fats has been studied intensively for more than twenty years, and much of this work has been summarized (4,5). Among the radiolytic products formed are hydrocarbons, aldehydes, lactones, ketones and other volatile products that are also characteristic of lipid oxidation such as would be experienced when lipids are temperature-abused in the presence of air. In model lipid systems containing polyunsaturated fatty acids, radiation-induced peroxidation has been observed and measured (6–8).

The effect of oxidizing conditions on cholesterol has been studied in detail (9), and current knowledge of cholesterol autoxidation has been reviewed recently (10,11). The principal product of the autoxidation of cholesterol in aqueous sodium stearate dispersions (12), in organic solvents (13) and in liposomes (14,15) is 7-ketocholesterol (3β-hydroxycholest-5-en-7-one). Other prominent products of cholesterol autoxidation include 7α- and 7β-hydroxycholesterol (cholest-5-ene-3β,7α-diol and cholest-5-ene-3β,7β-diol, respectively) and the α- and β-epoxide (cholesterol 5α,6α-epoxide and cholesterol 5β,6β-epoxide, respectively). In aqueous dispersions, the 7-ketone/5,6-epoxides ratio has typically been determined to range from six to ten, while about twice as much 7-ketocholesterol was formed as total 7-hydroxycholesterol (12,16).

The effect of ionizing radiation on cholesterol has also been examined in various systems, and it has been shown that the chemical changes produced are similar in nature to those known to occur during autoxidation (17–19). Recently, work performed in our laboratory revealed that irradiation of cholesterol in aqueous sodium stearate dispersions produced relatively less 7-ketocholesterol and relatively more 5,6-epoxides than did autoxidation of similar dispersions. Ratios of 7-ketocholesterol/5,6-epoxides resulting from irradiation were less than unity (20), while autoxidation, although at higher temperatures, gave ratios between six and ten (21). These results suggested that unique product ratios, rather than unique radiolytic products, might be useful indicators of past exposure to irradiation.

The current work was undertaken to determine whether cholesterol dispersed in the relatively hydrophobic medium of model membranes, *i.e.* liposomes, might also give unusual product ratios when exposed to low-dose ionizing radiation. Our earlier work had provided an instance in which ionizing radiation affected 7-ketocholesterol quite differently in liposomes than it did in aqueous dispersions (22). In that study, 7-ketocholesterol exposed to gamma radiation experienced 90% degradation in aqueous sodium stearate dispersion but only 10% degradation in liposomes.

EXPERIMENTAL

Materials and reagents. Cholesterol (99 + %), 6-ketocholestanol (3β-hydroxycholestan-6-one), 7-ketocholesterol (3β-hydroxycholest-5-en-7-one), cholesterol 5α,6α-epoxide and Sephadex G 50–80 were purchased from Sigma Chemical Co. (St. Louis, MO), cholesterol 5β,6β-epoxide and 7α-hydroxycholesterol (cholest-5-ene-3β,7α-diol) from Research Plus, Inc. (Bayonne, NJ), and 7β-hydroxycholesterol (cholest-5-ene-3β7β-diol) from Steraloids, Inc. (Wilton, NH). Dipalmitoyl phosphatidylcholine (DPPC) and dipalmitoyl phosphatidylglycerol (DPPG) were obtained from Avanti Polar Lipids, Inc. (Pelham, AL). Celite 545 was purchased from Fisher Scientific (Malvern, PA), and thin-layer chromatography (TLC) plates, silica gel GHL (250 μm) from Analtech, Inc. (Newark, DE). All solvents used were "distilled in glass grade," and chemicals were of reagent grade quality. Water was double-deionized, glass-distilled.

*To whom correspondence should be addressed at the U.S. Department of Agriculture, 600 E. Mermaid Lane, Philadelphia, PA 19118.

Abbreviations: DPPC, dipalmitoyl phosphatidylcholine; DPPG, dipalmitoyl phosphatidylglycerol; GC, gas chromatography; MLV, multilamellar vesicles; TLC, thin-layer chromatography.

Liposome preparation. Multilamellar vesicles (MLV) were prepared from 70.5 mg (96.0 μmoles) DPPC, 23.1 mg (32.0 μmoles) DPPG and 24.8 mg (64.0 μmoles) of cholesterol. The phospholipids and cholesterol were dissolved in 3.0 mL chloroform in a 15-mL test tube provided with a Teflon-lined screw cap, and the solution was mixed thoroughly. Solvent was removed under a stream of nitrogen while the tube was rotated at an angle, so that the lower half of the tube was coated evenly with a mixture of solids. The remaining solvent was removed until the odor of chloroform was no longer detectable. The coated lipids were hydrated with 8 mL water and allowed to swell for 3 hr in a water bath held at 55°C with vortexing every 15 min.

The liposome preparation was allowed to cool to room temperature and was filtered through a 0.4 μm pore polycarbonate membrane to insure rather uniform size distribution (23). The suspension was chromatographed on a 26 cm × 1 cm column of Sephadex G 50-80 using double deionized water as eluent. Vesicles were collected, as eluted, in a total volume of 16 mL. The batch was vortexed to insure homogeneity, and 1 mL aliquots under air were placed into 2-mL glass vials provided with Teflon-lined screw caps. Samples were refrigerated (0–4°C) overnight prior to irradiation. Phospholipid content of the vesicles was determined by measuring inorganic phosphorus according to the procedure of Eng and Noble (24).

Irradiation of cholesterol in liposomes. Duplicate 1-mL aliquots of MLV were irradiated in a ^{137}Cs source (0.118 KGy/min) at 0–4°C at dose levels of 0.5, 1.0, 2.5, 5.0, or 10.0 KGy. Controls consisted of unirradiated, refrigerated (0–4°C) samples. Controls and irradiated samples were extracted immediately after irradiation.

Autoxidation of cholesterol in liposomes. Liposome suspension (10 mL) was placed in a 50-mL screw cap test tube equipped with magnetic stirring bar. The tube was sealed with air in the headspace. The tube was suspended in a water bath kept at 45°C and stirred for 48 hr. Unoxidized aliquots were kept 0–4°C. Duplicate 1-mL portions of oxidized and unoxidized controls were analyzed.

Dry column extraction of cholesterol oxides. Anhydrous, granular sodium sulfate (4.0 g) and Celite 545 (3.0 g, previously washed with methylene chloride) were ground in a porcelain mortar, and 1 mL of a liposome preparation was applied to the powder evenly. The mixture was ground thoroughly for three minutes and transferred in small increments to a 10-mL glass syringe containing a thin pad of glass wool that had been previously washed with methylene chloride. The dry mixture was tamped firmly into the syringe with a flattened glass rod to a total volume of 6–7 mL. The mortar was rinsed with 5 mL methylene chloride, and the rinse was added to the solids in the syringe. Additional methylene chloride was added gently to the top of the column until 10 mL eluate had been collected at a flow rate of <1 mL/min. Eluates were blanketed with nitrogen and stored in the refrigerator (0–4°C) pending isolation and analysis.

Isolation of cholesterol oxidation products. Each dry column eluate was evaporated to dryness with nitrogen. The residue was reconstituted in 250 μL chloroform and the solution streaked on the middle 10 cm section of a scored 20 × 20 cm TLC plate. The plate had been prewashed with chloroform/methanol (2:1,v/v), again with ethyl acetate, and activated at 110°C. The residue container was rinsed with 150 μL chloroform and the rinse streaked over the previously applied sample. The end sections of the scored plate were spotted with 2 μL each of 7-ketocholesterol and 7α-hydroxycholesterol solution (1 mg/mL). The plate was developed with benzene/ethyl acetate (60:40,v/v) and development continued for 10 min after the solvent front had reached the top of the plate. The end sections, intended for visualization, were snapped off and sprayed with 50% sulfuric acid and heated. They were used to locate the band containing both 7-ketocholesterol and 5,6-epoxide and the 7-hydroxycholesterol band in the center section of the plate. The corresponding bands of the middle section were scraped and separately extracted with 2 × 5 mL acetone. After each extraction the samples were centrifuged to pellet the silica. The two extracts of each band were combined, filtered through a 2 μm Nylon 66 filter and evaporated to dryness under nitrogen. To each residue was added internal standard 6-ketocholestanol (4 μL, 1 μg/μL in ethyl acetate), and the solvent was removed with nitrogen. The sample was reconstituted in 100 μL ethyl acetate for gas chromatography (GC) analysis.

Determination of cholesterol content of liposomes. The sample of MLV (1 mL) was ground with anhydrous sodium sulfate (4.0 g) and Celite 545 (3.0 g) as described above. Before the ground mixture was transferred to the 10-mL syringe, the latter was provided with 1.0 g of a mixture of Celite 545/$CaHPO_4 \cdot 2H_2O$ (9:1, w/w) which was tamped securely. Addition of the dry powder containing the sample then brought the total volume in the syringe to 8–9 mL. The column was eluted with methylene chloride, and approximately 10 mL of eluate was collected. Solvent was removed under nitrogen and the sample was reconstituted in methylene chloride to 10.0 mL in a volumetric flask. An aliquot (10 μL) was transferred to a vial, internal standard 6-ketocholestanol (4 μL, 1 μg/μL in ethyl acetate) was added, the solvent was removed under nitrogen, and the residue was reconstituted in 100 μL ethyl acetate prior to GC analysis.

Gas chromatography (GC). Samples were analyzed by GC as described previously (25) with the following modifications. The column was a 0.25 mm i.d. × 30 m bonded phase 5% phenylsilicone column with 0.25 μm film thickness (Durabond 5, J & W Scientific, Folsom, CA). A retention gap was not used. For cholesterol oxide determinations, the initial column temperature of 100°C was held 2 min, then programmed at 30°C/min to 265°C, then at 0.5°C/min to 275°C where it was held for 5 min, then programmed at 5°C/min to 300°C and held for 15 min. For cholesterol determinations the program was modified by programming from 275 to 300°C at 30°C/min and holding at 300°C for only 2 min. Sample size injected in all cases was 0.6–0.8 μL.

GS response factors. Response factors of cholesterol and of the five cholesterol oxidation products of interest relative to the internal standard 6-ketocholestanol were determined by the method of Lee *et al.* (26).

RESULTS AND DISCUSSION

Various types of liposomes have been employed as model membrane systems to study the reactions of lipids, including their peroxidation (27), in environments that approach those of biological membranes. We chose to study the effect of ionizing radiation in multilamellar vesicles (MLV), because these types of liposomes are easily prepared in the quantities that we required. In the current series of experiments the MLV were composed of the fully saturated phospholipids dipalmitoyl phosphatidylcholine (DPPC) and dipalmitoyl phosphatidylglycerol (DPPG), and cholesterol. The molar ratio DPPC/DPPG/cholesterol was 3:1:2. The liposomes were formed in double deionized water, because exploratory experiments with *N*-2-hydroxyethyl piperazine-*N'*-2-ethane sulfonic acid (HEPES) buffer and with phosphate buffer had indicated objectionable interactions of the buffer with the radiation. The liposomes were formed and swelled above 41°C, the transition temperature (T_c) for both phospholipids (28).

Swelling of the proper lipid components in aqueous media above the T_c of the lipids leads to spontaneous vesiculation with the formation of liposomes (29). While liposome formation is spontaneous, it is not necessarily complete. One of the techniques used to free liposomes from non-trapped solutes is molecular sieve chromatography (27,30). Table 1 lists six experiments in which liposomes were prepared under supposedly identical conditions, including separation of unincorporated lipids on a Sephadex G 50-80 column. The data indicate that 66–77% of the added cholesterol was found incorporated in the liposomes, while 80–96% of the phospholipids added were present in the vesicles. The actual cholesterol content of the liposomes ranged from 28–32 mol%, slightly different than the intended 33 mol%. The MLV were characterized by analysis of total phosphorus content (24) and by cholesterol determination.

To determine cholesterol and its major oxides it is necessary to extract the compounds from the vesicles. Since the sterols are fairly soluble in ethyl acetate while the phospholipids are not, a simple extraction was thought to be necessary. However, repeated extractions of the liposomes with ethyl acetate revealed that cholesterol was retained by the liposomes tenaciously, so that after five consecutive extractions more than 5% of the cholesterol remained in the vesicles. Jacobsohn *et al.* (31,32) also reported retention of cholesterol in MLV containing phosphatidylcholine and suggested that the phospholipid in the aqueous phase prevents access to cholesterol by nonpolar solvents. The difficulty of quantitative extraction of sterols from foods has also been noted (33).

Extraction of cholesterol from phospholipids should be facilitated if the aqueous phase is removed first. The dry column extraction procedure (34) is designed to remove water from partly aqueous systems such as animal tissue, and seemed suitable for the dehydration of liposomes. For the purposes of the current work the procedure was modified, so that a 10-mL glass syringe

TABLE 1

Cholesterol and Phosphorus Content of Liposomes

	Cholesterol				Phosphorus (P_i)		
Experiment	Amount added μmol/mL	Amount found μmol/mL	Percent incorporated in liposomes	Liposome content mol%	Amount added μmol/mL	Amount found μmol/mL	Percent incorporated in liposomes
1	4.01	2.64	65.8	28.9	8.00	6.51	81.4
2	4.01	3.04	75.8	29.7	8.00	7.20	90.0
3	4.01	2.90	72.3	28.7	8.00	7.20	90.0
4	4.01	2.94	73.3	27.8	8.00	7.64	95.5
5	4.01	3.09	77.1	31.0	8.00	6.88	86.0
6	4.01	3.03	75.5	31.9	8.00	6.47	80.5

TABLE 2

Purities and GC Response Factors of Various Cholesterol Oxidation Products

Compound	Purity	Response factor[a]
Cholesterol (5-cholesten-3β-ol)	95.1	0.87
α-Epoxide (cholesterol 5α,6α-epoxide)	100.0	1.10
β-Epoxide (cholesterol 5β,6β-epoxide)	96.0	1.09
7-Ketocholesterol (3β-hydroxycholest-5-en-7-one)	96.8	1.03
6-Ketocholestanol (3β-hydroxycholestan-6-one)	94.8	1.00
7α-Hydroxycholesterol (cholest-5-ene-3β,7α-diol)	93.2	1.11
7β-Hydroxycholesterol (cholest-5-ene-3β,7β-diol)	97.1	1.11

[a]Relative to 6-ketocholestanol.

could serve as the extraction column. Since cholesterol was the only neutral lipid in the liposomes, methylene chloride was a suitable solvent for its extraction. Elution with 10 mL of this solvent gave complete recovery of cholesterol, as judged by TLC. Unfortunately, however, small but significant amounts of phospholipids co-eluted. The latter compounds are not volatile in our GC procedure and constituted a potential contaminant for our capillary column.

Three procedures were investigated to overcome this difficulty. Reduction of the methylene chloride eluate in volume followed by preparative TLC gave a discrete cholesterol band, well resolved from the phospholipid band which remained at the origin. Extraction of the scraped cholesterol band with acetone led to a clean GC trace of cholesterol suitable for quantitation. This procedure was rather tedious and sometimes gave erratic recoveries. In an alternative procedure, 1-mL aliquots of liposome suspensions were saponified in a modification of the procedure of Slover *et al.* (35), which was effective in destroying phospholipids and hence made extraction of cholesterol feasible. In our hands this technique lacked reproducibility and was time-consuming, and hence was abandoned. Finally, the dry column procedure was modified by insertion of a Celite/$CaHPO_4$ trap (see Experimental) to minimize elution of the phospholipids. The technique gave 86% recoveries when applied to liposome suspensions containing known amounts of cholesterol, *i.e.* vesicles that were allowed to swell but were not filtered or passed through molecular sieves. The cholesterol values listed in Table 1 were determined by this method but were not adjusted to compensate for the 14% loss mentioned above.

Recovery and measurement of cholesterol oxidation products from irradiated liposomes was also carried out with use of the dry column procedure (no trap) followed by preparative TLC. To test the procedure, liposomes were prepared that, in addition to the usual components, also contained known amounts of 7-ketocholesterol (9.7 μg/mL of suspension), the two isomeric 5,6-epoxides (31.6 μg/mL) and the two isomeric 7-hydroxycholesterols (31.3 μg/mL). The vesicles were swelled but not filtered or passed through the Sephadex column to avoid losses of compounds that had failed to be incorporated into the MLV. Recovery of 7-ketocholesterol (92.9%) and 7-hydroxycholesterols (91.8%) validated the procedure. The two 5,6-epoxides, however, were unstable to the vesicle swelling conditions (55°C, pH 6.4) and were largely converted to 5α-cholestane-3β,5,6β-triol, as expected from a previous study (36). The triol was incompletely resolved from the phospholipids by TLC and was recovered only partially. Total recovery of 5,6-epoxides and triol was 63.4%.

Purities of standard compounds as determined by GC and GC response factors relative to the internal standard are shown in Table 2. The response factors were used in calculating the data reported in Table 3. The low response factor of cholesterol may be a function of its lower-than-expected purity.

Table 3 lists the ratios of 7-ketocholesterol/5,6-epoxides generated as a result of exposure of cholesterol in MLV to ionizing radiation at 0–4°C. The ratios of all irradiated samples are less than unity, and actu-

TABLE 3

Cholesterol Oxidation Products Resulting from γ-Irradiation of Cholesterol in Liposomes[a,b]

	μg/mL							
Dose kGy	7-keto-cholesterol	Cholesterol 5β,6β-epoxide	Cholesterol 5α,6α-epoxide	7α-hydroxy-cholesterol	7β-hydroxy-cholesterol	Total oxides measured	Ratio 7-ketone/ 5,6-epoxides	Ratio 7-ketone/ 7-hydroxy-cholesterols
0	0.50 ± 0.27	0.36 ± 0.12	0.20 ± 0.11	trace	trace	1.49 ± 0.75	0.86 ± 0.11	—
0.5	0.65 ± 0.15	3.10 ± 1.21	0.75 ± 0.24	0.38 ± 0.06	0.38 ± 0.11	5.26 ± 1.52	0.20 ± 0.12	0.85 ± 0.10
1.0	1.46 ± 0.48	5.60 ± 2.42	1.30 ± 0.46	0.90 ± 0.34	0.99 ± 0.44	10.25 ± 3.90	0.22 ± 0.05	0.79 ± 0.11
2.5	1.60 ± 0.26	7.46 ± 2.48	1.65 ± 0.50	5.30 ± 1.44	3.84 ± 1.23	19.84 ± 5.49	0.19 ± 0.05	0.19 ± 0.04
5.0	1.82 ± 0.50	8.96 ± 4.27	1.95 ± 0.77	6.07 ± 2.02	3.89 ± 1.48	22.65 ± 9.07	0.18 ± 0.05	0.19 ± 0.02
10.0	2.89 ± 0.93	10.1 ± 2.41	2.32 ± 0.44	8.01 ± 2.61	4.52 ± 1.05	27.84 ± 5.80	0.24 ± 0.07	0.24 ± 0.01

[a]Amounts corrected for GC response factors.
[b]Mean value of 6 samples ± standard deviation.

ally <0.35 in most experiments. The reason for the slight increase in the ratio at higher doses in some experiments requires further investigation. The unirradiated vesicles contained a somewhat higher ratio of 7-ketone/5,6-epoxide as would be expected, since these oxidation products were formed during the liposome swelling process in the presence of air, rather than by means of ionizing radiation. In addition it should be noted that the levels of 7-ketone and 5,6-epoxides in the unirradiated samples are very low, so that a greater amount of uncertainty is involved in their quantification. Irradiation, even at the low dose of 0.5 kGy, increases the amounts of cholesterol oxidation products greatly, so that these compounds can be measured at this dose with good precision.

The ratios of 7-ketone/5,6-epoxides produced by exposure of cholesterol to γ-radiation in the presence of air can best be understood by comparing them to the ratios of the same products, *i.e.* 6–10 (see introduction) generated by the principal alternative free radical reaction, autoxidation. It is apparent that the ratios that are now seen to result from irradiation, *i.e.* <1.0, are quite different from those generated by autoxidation. Hence in the current work concerning cholesterol in saturated liposomes the signature of the irradiation process is unique and easily distinguishable from the principal competing process, autoxidation.

In order to determine whether the higher 7-ketone/5,6-epoxides ratio holds for the autoxidation of cholesterol in saturated liposomes, a suspension of such vesicles was exposed to air oxidation at 45°C for 48 hr and the products were isolated. About 20% of the cholesterol oxidized during that period and the experimental 7-ketone/5,6-epoxide ratio was 10.6 ± 3.0.

It has been reported (37) that γ-irradiation of dispersions of cholesterol gave rise to a distinctly different ratio of 5α,6α-epoxide/5β,6β-epoxide, *i.e.* 3.5, than that obtained by autoxidation (~0.1). If confirmed, the α/β-epoxide ratio might also be a useful marker to distinguish irradiated from autoxidized cholesterol. In our earlier study of the irradiation of cholesterol in aqueous dispersions, we reported α/β-epoxide ratios of less than one, usually 0.3–0.4. In the current work the irradiation of cholesterol in liposomes gave an α/β-epoxide ratio of 0.2–0.3 consistently. It must be concluded that this ratio cannot be used as evidence for past irradiation.

Table 3 contains a list of the ratios of 7-ketocholesterol/7-hydroxycholesterols obtained when cholesterol in MLV was irradiated at 0–4°C with 0.5 to 10.0 kGy of γ-radiation. With the exception of two values at 0.5 kGy, all of the ratios are less than unity and most of them are less than 0.5. In calculating the values of Table 3, the 7α- and 7β-hydroxycholesterols, determined individually, but not always resolved completely, have been added.

Again it is useful to compare the ratios of Table 3 with those reported to have been produced by autoxidation. In aqueous dispersions, autoxidation of cholesterol produced about twice as much 7-ketocholesterol as 7-hydroxycholesterol (11,12,16,21), while uncatalyzed autoxidation of cholesterol in liposomes has been reported to give a ratio of about 1.5 (19). In the current work, autoxidation of cholesterol in liposomes gave a 7-ketone/7-hydroxycholesterol ratio of 3.4 ± 0.6. It is apparent, then, that the difference in this ratio between irradiated and autoxidized cholesterol is not as clear-cut as it is for the 7-ketocholesterol/5,6-epoxide ratio. Nevertheless, the two ratios taken together may serve as strong evidence of past irradiation.

Finally, there is the matter of the total amounts of measured cholesterol oxidation products. These include 7-ketocholesterol, the isomeric 5,6-epoxides, and the isomeric 7-hydroxycholesterols. Their sums are shown in Table 3. The level of oxidation products in the unirradiated samples is quite low. In a previous study (20), an aqueous dispersion of cholesterol was subjected to a flow of air at 0–4°C for two weeks without a detectable increase in cholesterol oxidation.

The amounts of cholesterol oxidation products increase dramatically with radiation dose, but the increase does not appear to be linear. As can be seen from Table 3, we experienced some difficulties in experiment-to-experiment reproducibility of the amounts, but not of the ratios, of cholesterol oxides. This in spite of exhaustive, detailed studies of the variables of each step of the procedure. Our investigations along these lines are continuing.

This research has clearly demonstrated that γ-irradiation of cholesterol, contained in MLV prepared from saturated phospholipids, leads to ratios of oxidation products that are substantially different from those generated by autoxidation. New procedures for isolating and measuring cholesterol and its oxidation products in aqueous liposome suspensions have been developed in the course of these studies and are reported here. Further research is in progress to identify and to define reaction parameters.

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Effects of Hexadecylphosphocholine on Protein Kinase C and TPA-Induced Differentiation of HL60 Cells

Mamoru Shoji[a]*, Robert L. Raynor[b], Edward A.M. Fleer[c], Hansjörg Eibl[c], William R. Vogler[a] and J.F. Kuo[b]

Departments of [a]Medicine (Hematology/Oncology) and [b]Pharmacology, Emory University School of Medicine, Atlanta, Georgia 30322 and [c]Max-Planck Institute for Biophysical Chemistry, D-3400 Göttingen, Federal Republic of Germany

Several structural analogs of alkylphosphocholine (APC) were studied for their effects on protein kinase C (PKC) and 12-*O*-tetradecanoylphorbol-13-acetate (TPA) elicited biochemical and cellular events in HL60 cells. Hexadecylphosphocholine (He-PC_2), the APC prototype, inhibited PKC competitively with respect to phosphatidylserine and noncompetitively with respect to $CaCl_2$, both with an apparent K_i of about 15 μM. Inhibition of PKC by He-PC_2 was selective, since cyclic AMP dependent protein kinase and Ca^{2+}/calmodulin dependent protein kinase II were relatively unaffected. He-PC_2 inhibited TPA-induced depletion of PKC and TPA-stimulated phosphorylation of cellular proteins in HL60 cells. TPA-induced differentiation of HL60 cells was also inhibited by He-PC_2, and this inhibition was synergistic or additive to the effects of 1-(5-isoquinolinesulfonyl)-2-methylpiperazine (H-7), a PKC inhibitor. The present findings are consistent with the hypothesis that inhibition of PKC might be related, in part, to the antineoplastic effect of He-PC_2 and ether lipid analogs such as ET-18-OCH_3 (1-octadecyl-2-methyl-glycero-3-phosphocholine).

Lipids 26, 145–149 (1991).

Alkyllysophospholipids (ALP) are analogs of the cell membrane component 2-lysophosphatidylcholine, and represent a new class of antitumor agents (1). ET-18-OCH_3 (*rac*-1-*O*-octadecyl-2-*O*-methyl-glycero-3-phosphocholine), the ALP prototype, has been found to be cytotoxic to human leukemic cells (2,3) and solid tumors (4,5) *in vitro*, and is effective in purging murine leukemic cells from remission simulated bone marrow for autologous bone marrow transplantation (6) and treatment of experimental rat tumors (7,8). Recently, a TLP (thioether analog of ALP) derivative BM 41.440 (3-hexadecylmercapto-2-methoxymethyl-propylphosphocholine) has been shown to possess antineoplastic activity against a variety of leukemias and solid tumors (9,10). Both ET-18-OCH_3 and BM 41.440 are currently undergoing clinical trials (11,12), and the former is being used to purge leukemic cells from remission marrows (13). These compounds have different mechanisms of action than the conventional chemotherapeutic drugs. Several modes of action have been postulated to account for their biologic effects. These include enhancement of the cytotoxic properties of macrophages (1,7,14), alterations of phospholipid metabolism (15), and inhibition of protein kinase C (PKC) (16) and Na,K-adenosinetriphosphatase (ATPase) (sodium pump) (17,18). More recently, another subclass of antineoplastic agents, APC (alkylphosphocholine), has been developed (19). One of the agents, hexadecylphosphocholine (He-PC_2), has been shown in a recent clinical trial to be active topically against locally recurring breast cancer skin metastases without serious side effects on patients (20). In the present study, we investigated the effects of various APC analogs on the PKC system and 12-*O*-tetradecanoylphorbol-13-acetate (TPA)-induced differentiation of HL60 cells. We found that He-PC_2 inhibited PKC and TPA-induced differentiation of HL60 cells and protein phosphorylation in the leukemia cells.

MATERIALS AND METHODS

Materials. RPMI-1640 medium, fetal bovine serum, penicillin/streptomycin solution (× 100), L-glutamine (200 mM), trypsin/ethylenediaminetetraacetic acid (EDTA) solution (× 10) were obtained from GIBCO (Grand Island, NY); TPA was from L.C. Services Corp. (Woburn, MA); dimethylsulfoxide, phosphatidylserine (PS)—(bovine brain), histone H1 (type III-S), PMSF and H-7 were from Sigma Chemicals (St. Louis, MO); ET-18-OCH_3 was from Medmark Chemicals (Grünwald bei München, FRG); APC analogs were synthesized as described (21); ampholytes (pH ranges of 4–6, 4–7 and 3–10) were from LKB (Gaithersburg, MD) and Serva Biochemicals (Westburg, NY).

Cell culture and cell differentiation. HL60 and K562 leukemic cells were cultured in RPMI-1640 medium supplemented with 10% fetal bovine serum (heat-inactivated), L-glutamine (2 mM), and penicillin G (100 units/mL), and streptomycin (100 μg/mL) in a humidified incubator at 37°C in 95% air/5% CO_2. The cells at mid-log phase were harvested and used in all experiments reported herein. The TPA-induced differentiation toward the macrophage pathway was determined by cell adherence after treatment of cells with 10 nM TPA for 48 hr in the presence or absence of various PKC inhibitors. The adherent cells were washed once

*To whom correspondence should be addressed.

Abbreviations: ALP, alkyllysophospholipid; APC, alkylphosphocholine; A-PK, cAMP dependent protein kinase; BM 41.440, 3-hexadecylmercapto-2-methoxymethyl-propylphosphocholine; CaM-PK-II, Ca^{2+}/calmoduline dependent protein kinase II; EDTA, ethylenediaminetetraacetic acid; EGTA, ethyleneglycol-*bis*(β-aminoethyl ether)*N,N,N',N'*-tetraacetic acid; ET-18-OCH_3, *rac*-1-*O*-octadecyl-2-*O*-methyl-glycero-3-phosphocholine; H-7, 1-(5-isoquinolinesulfonyl)-2-methylpiperazine; He-PC_2, hexadecylphosphocholine, hexadecyl (*N,N,N*-trimethylamino)ethyl phosphate; He-PC_3, hexadecyl (*N,N,N*-trimethylamino)propyl phosphate; He-PC_4, hexadecyl (*N,N,N*-trimethylamino)butyl phosphate; He-PC_6, hexadecyl (*N,N,N*-trimethylamino)hexyl phosphate; He-*O*-P-C_2, *O*-hexadecyl (*N,N,N*-trimethylamino)-ethylphosphonate; He-P-*O*-C_2, hexadecyl-phosphorylcholine; He-PS, hexadecylphosphoserine; Oc-PC_2, octadecylphosphocholine; PKC, protein kinase C; PMSF, phenylmethylsulfonyl fluoride; PS, phosphatidylserine; TLP, thioether lysophospholipid; TPA, 12-*O*-tetradecanoylphorbol-13-acetate.

with RPMI-1640 medium and resuspended by incubating with trypsin-EDTA solution (0.5 mL) for about 20 min. An equal volume (0.5 mL/well) of 20% fetal bovine serum in the RPMI-1640 medium was then added to each well in order to block the activity of trypsin, and cells were transferred to tubes. Each well was washed once with 1 mL of 10% fetal bovine serum in RPMI-1640 medium. Aliquots (200 μL) of cell suspension were counted for cell number using a Coulter counter, and viability was determined by trypan blue dye exclusion.

PKC preparation and assay. PKC was purified from pig brain extracts through the phenyl sepharose step (22). PKC was assayed under the standard conditions as described elsewhere (23). Briefly, the reaction mixture (0.2 mL) contained 5 μmol of piperazine-*N,N'*-*bis*(2-ethanesulfonic acid) (pH 6.5), 2 μmol of $MgCl_2$, 2 μg of PS, 40 μg of histone HI, 0.06 μmol of ethyleneglycol-*bis*-(β-aminoethyl ether) *N,N,N',N'*-tetraacetic acid (EGTA), with or without 0.1 μmol of $CaCl_2$, 1.0 nmol of [γ-^{32}P]ATP (containing about 1×10^6 cpm), and various kinds and concentrations of agents, as indicated. When present, the final concentration of $CaCl_2$ was 200 μM (in excess of 300 μM EGTA). The reaction was carried out at 30°C for 5 min.

Endogenous protein phosphorylation. Phosphorylation of endogenous proteins in intact HL60 cells and the subsequent two-dimensional gel electrophoresis and autoradiography of phosphoproteins were carried out as described (24), with a few modifications. Ampholyte mixtures used for isoelectric focusing gels consisted of 67% of pH 4-6, 16% of pH 5-7 and 17% of pH 3-10. Separation gels contained 8% acrylamide and 0.1% sodium dodecyl sulfate.

Other methods. A-PK (25) and CaM-PK-II (26) were purified and assayed as described. [γ-^{32}P]ATP was prepared by the method of Post and Sen (27).

RESULTS

The structures of APC analogs are shown in Figure 1. All compounds inhibited PKC, with IC_{50} values of about 15-20 μM (Fig. 2). By contrast, hexadecylphosphoserine (He-PS) was inactive, indicating that the phosphoserine group cannot substitute for phosphocholine. The data on the structure-activity relationship of the APC analogs further illustrated that neither the distance (2 to 6 carbons) between the negatively charged phosphate group and the positively charged ammonium group (*e.g.* He-PC_2 and He-PC_6), nor the deletion of the oxygen at either side of the phosphoric acid moiety (*i.e.* in He-P-O-C_2 and He-O-P-C_2) was critically important for activity. The IC_{50} of 10 μM observed for the ALP prototype ET-18-OCH_3 (Fig. 2) was the same as previously reported (16). Kinetic analysis indicated that He-PC_2 inhibited PKC competitively with respect to PS, with an apparent K_i value of 15 μM (Fig. 3), but it inhibited the enzyme noncompetitively with respect to $CaCl_2$, with a similar K_i value (data not shown). These findings on the inhibition mechanism of He-PC_2 were in good agreement with those reported previously for the glycerophospholipid analogs ET-18-OCH_3 (16) and BM 41.440 (23), consistent with the notion that He-PC_2 also likely inter-

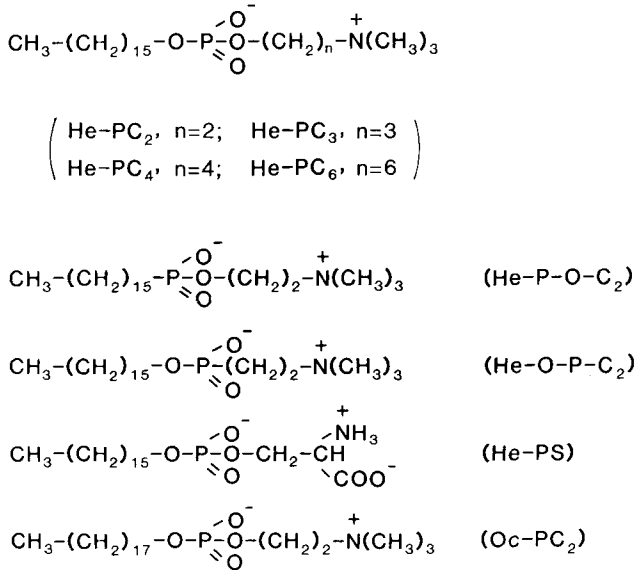


FIG. 1. Structure of alkylphosphocholines and analogues.

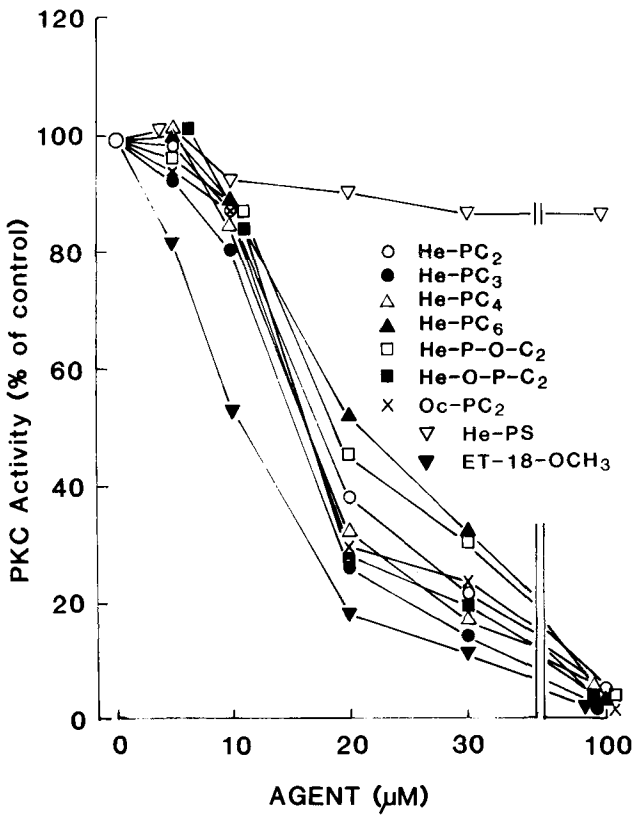


FIG. 2. Inhibition of PKC by alkylphosphocholine analogues compared with ET-18-OCH_3. The enzyme was assayed essentially as described previously (23) but in the presence or absence of the agents, as indicated. The agents were dissolved in water and added directly to the incubation mixture without presonicating with PS. The control enzyme activity obtained in the absence of the agent (18.4 pmol P transferred/min) was taken as 100%.

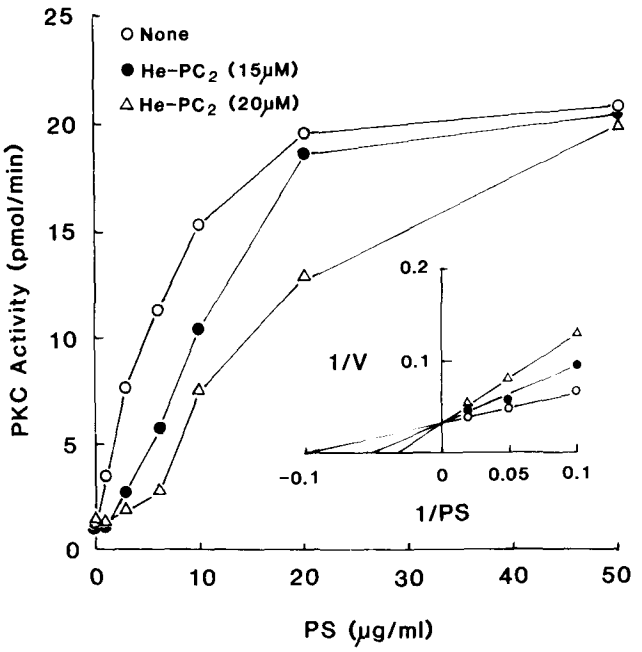


FIG. 3. Inhibition of PKC by He-PC_2 as a function of PS concentration. The enzyme was assayed under the standard condition except for various concentrations of PS and the agent, as indicated. The double-reciprocal plots of the data are shown in the inset.

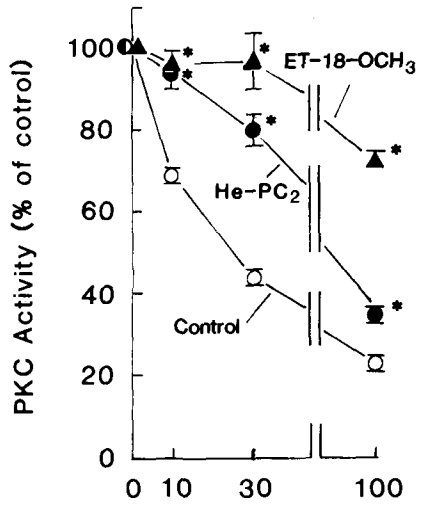


FIG. 4. Comparative effects of He-PC_2 and ET-18-OCH_3 on the TPA-induced depletion of PKC in HL60 cells. Cells were incubated with or without 40 μM of He-PC_2 or ET-18-OCH_3 for 1 hr, followed by incubation with various concentrations of TPA for 20 min, as indicated. Enzyme activity in the soluble fraction (1 μg of protein) was assayed under standard conditions. The enzyme activity levels seen in cells treated with 0.1% of DMSO (solvent control) were taken as 100% in all cases. The asterisk signifies a difference from the respective control ($p<0.05$). Similar results were obtained in three other experiments.

TABLE 1

Selective Inhibition of PKC by APC Analogs and ET-18-OCH_3[a]

Inhibitor	Inhibition (%) of PKC	CaM-PK-II	A-PK
APC (30 μM)	65–85[b]	12[c]	0–10[b]
ET-18-OCH_3 (20 μM)	80	10	8

[a]The data presented are means of triplicate incubations, with assay errors being less than 5%.
[b]Values obtained for He-PC_2, He-PC_3, He-PC_4, He-PC_6, He-P-O-C_2, He-O-P-C_2 and Oc-PC_2.
[c]Value obtained only for He-PC_2.

acted with site(s) on PKC shared by PS but not by Ca^{2+}.

Selective inhibition of PKC by APC analogs and ET-18-OCH_3 was substantiated by the findings that these agents, at concentrations that inhibited PKC up to 85%, had little or no effect on other classes of protein kinases, such as cAMP dependent protein kinase (A-PK) and Ca^{2+}/calmoduline dependent protein kinase II (CaM-PK-II) (Table 1).

We previously reported that TPA caused PKC translocation from the cytoplasm to the plasma membrane and the subsequent depletion of the cytoplasmic enzyme in HL60 (28) and KG-1 cells (29). The present study showed that He-PC_2 partially blocked the TPA-induced depletion of the cytoplasmic enzyme in HL60 cells (Fig. 4). ET-18-OCH_3 was more effective (Fig. 4) whereas He-PC_6 was less effective than He-PC_2 (data not shown) in this respect, in agreement with their relative potency in inhibiting PKC as shown in Figure 2 above. Exposure of HL60 cells to TPA resulted in an enhanced phosphorylation of a number of cellular proteins, notably those in block I (pI of 5.45–5.65; M_r of 60,000–67,000) (Fig. 5). He-PC_2 at 40 and 60 μM inhibited, to different degrees, the TPA-stimulated phosphorylation of these proteins, similar to the effects reported previously for ET-18-OCH_3 (24).

The TPA-induced differentiation of HL60 (28,30) and KG-1 cells (29,31) has been shown to be PKC-dependent, consistent with the findings that PKC inhibitors ET-18-OCH_3 (24), BM 41.440 (23) and H-7 (32,33) blocked this process. Because H-7 inhibited PKC by interacting with the ATP-binding sites (34) whereas the lipid analogs did so by interacting with PS-binding sites on PKC (16,23), it was of interest to examine the effects of these agents, having different mechanisms of action on HL60 cell differentiation, when they were used in various combinations. We found that the combined effects of the agents were either synergistic or additive, depending upon the types of the inhibitors and their concentrations used. Thus, 10 or 20 μM H-7 and 3 μM He-PC_2, while without significant effect on the TPA-induced differentiation when present alone, acted synergistically to decrease the differentiating cell population from a control value of

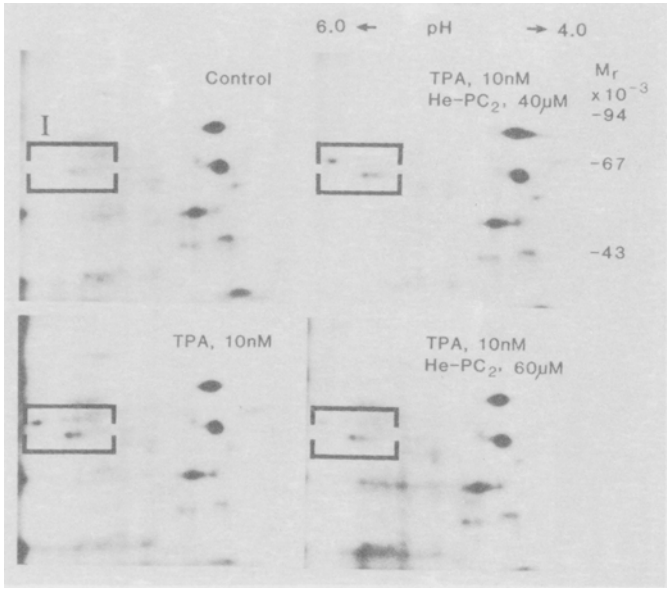


FIG. 5. Effect of He-PC_2 on protein phosphorylation in HL60 cells stimulated by 10 nM TPA. Cells were labeled with $^{32}P_i$ in the phosphate-free medium for the total of 4 hr. He-PC_2 was added to the cells in the final 1 hr, whereas TPA was added in the final 15 min of the labeling period. Other experimental details were as described under Materials and Methods and in Reference 24. Similar results were obtained in two other experiments.

78–86% to 42 and 32%, respectively (Table 2). Only a simple additive effect, however, was observed when a higher concentration (10 μM) of He-PC_2 (showing a marked inhibition by itself) was used in combination with 10 or 20 μM H-7. It was also observed that 1 μM ET-18-OCH_3, which decreased cell differentiation from 88 to 52%, decreased it further to 42% in the presence of 10 μM H-7 (Table 2). In separate experiments, He-PC_2 concentration up to 50 μM did not cause rounding up or detachment of the TPA-induced adherent HL60 cells in 1 hr (data not shown), indicating that its effects on HL60 cells were not likely due to a non-specific detergent-like activity of the agent.

DISCUSSION

In the present study the APC prototype He-PC_2 was found to be nearly as active as ALP (*e.g.* ET-18-O'CH_3) in all parameters studied. These include inhibition of PKC and various TPA-mediated events in HL60 cells (*i.e.* PKC down-regulation, cellular protein phosphorylation and differentiation to become macrophage-like cells). He-PC_2, again as ET-18-OCH_3, inhibited PKC by a similar mechanism and preferentially inhibited PKC over other classes of protein kinases. In addition, He-PC_2, like ET-18-OCH_3 and the TLP analog BM 41.440, has been shown to possess antineoplastic activity (19,20,35), displayed a greater cytotoxic effect toward

TABLE 2

Comparative Inhibition of TPA-Induced Differentiation of HL60 Cells by H-7, ET-18-OCH_3 or He-PC_2, Present Singly or in Combination[a]

Incubation number	Treatment				Cell adhesion (%)
	TPA (nM)	H-7 (μM)	ET-18-OCH_3 (μM)	He-PC_2 (μM)	
1	0	0	0	0	<2
2	10	0	0	0	88 ± 2[b]
3	0	10	0	0	<2
4	0	0	1 or 3	0	<2
5	0	0	0	3 or 10	<2
6	10	10	0	0	80 ± 3
7	10	20	0	0	78 ± 1
8	10	0	1	0	52 ± 4[c]
9	10	0	3	0	18 ± 1[c]
10	10	0	0	3	86 ± 2
11	10	0	0	10	24 ± 2[c]
12	10	10	1	0	42 ± 1[d]
13	10	10	3	0	12 ± 1[d]
14	10	10	0	3	42 ± 3[d]
15	10	10	0	10	14 ± 1[d]
16	10	20	1	0	36 ± 1[e]
17	10	20	3	0	12 ± 1[e]
18	10	20	0	3	32 ± 4[e]
19	10	20	0	10	16 ± 1[e]

[a]The cells (5×10^5/2 mL/well) were incubated for 2 hr with the agents prior to the addition of TPA or dimethylsulfoxide (DMSO), as solvent control) followed by culturing for an additional 48 hr. The adherent (differentiating) cells were removed by using trypsin/EDTA solution, and the cell number and viability were determined as described under Materials and Methods. The values presented are means ± SE of triplicate incubations. Similar findings were confirmed in two other experiments. Viability of the adherent cells in all groups was > 90%.
[b]Significantly different from 1) ($p<0.01$).
[c]Significantly different from 2) ($p<0.01$).
[d]Significantly different from 6) ($p<0.01$).
[e]Significantly different from 7) ($p<0.01$).

leukemia HL60 line compared to K562 line (35,36), and similarly inhibited synaptosomal Na,K-ATPase (17,18) and sodium pump in HL60 cells (18). This strongly indicated that He-PC_2, a simple alkylphosphocholine lacking the usual glycerol backbone present in other ALP analogs, might have the minimal structural requirement essential for the general biological activities demonstrated for this group of membrane-active agents.

The selective cytotoxicity of He-PC_2 for HL60 and K562 cell lines (35,36) probably was not due to differences in uptake and intracellular distribution of the agent because we observed that the rate and extent of uptake of [^{3}H]He-PC_2 and its distribution in certain subcellular fractions (cytosol and crude membranes) of the two cell lines were practically the same (data not shown). Similar results on He-PC_2 uptake by the two cell lines have been recently reported (37). Because PKC inhibition is a common effect for He-PC_2 and ether lipid analogs, a basis for their cellular effects would likely reside on specific substrate proteins for PKC shown to be present, for example, in HL60 and K562 cells (24). Identification of functional properties of these cellular proteins would yield new insights into the mechanisms underlying the selective toxicity (or lack of it) of the drugs on various neoplastic cells lines.

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Acyl-CoA:Cholesterol Acyltransferase Activity in the Rat Mammary Gland: Variation During Pregnancy and Lactation

J.H. Shand and D.W. West*
Hannah Research Institute, Ayr, KA6 5HL, Scotland

Cholesterol esterification by acyl-CoA:cholesterol acyltransferase (ACAT; EC 2.3.1.26) has been studied in microsomes isolated from the mammary glands of rats in late pregnancy, in early and mid-lactation, and following premature weaning. The mammary glands were freeze-clamped and the microsomes prepared in the presence of phosphatase inhibitors to preserve the phosphorylation status of the enzyme. Optimal conditions were established for the assay of enzyme activity in the presence of endogenous cholesterol. Supplementation of the microsomes with exogenous cholesterol as a dispersion in Triton WR-1339 was shown to lead to an increase in enzyme activity. Incubation of microsomes with MgATP led to an increase in ACAT activity which could be reversed by treatment of the microsomes with a phosphoprotein phosphatase preparation from rat liver. The results suggested that ACAT activity in the mammary gland was activated by phosphorylation in a similar way to that observed for the hepatic enzyme. The mammary glands from pregnant animals contained a higher level of ACAT activity than did the glands of the lactating animals and this correlated with the higher cholesteryl ester content of the pregnant glands. The highest level of ACAT activity was found in the weaned animals but the cholesteryl ester content of the microsomes was lower than expected. The influence of progesterone levels and changes in feeding patterns during gestation were considered as factors in these variations in ACAT activity.
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Many cells respond to an increased uptake of cholesterol by esterifying it with fatty acids, a reaction catalyzed by the microsomal enzyme acyl-CoA:cholesterol acyltransferase (ACAT; EC 2.3.1.26) (1,2). Storage of the sterol in this form is an important part of the overall regulation of cholesterol metabolism. However, in recent years experimental work on the cellular metabolism of cholesterol has emphasized the importance of cholesterol esters in steroid hormone and lipoprotein synthesis, and in the regulation of cholesterol synthesis, rather than merely as a storage depot (3–5). ACAT activity has been shown to exist in the lactating rat mammary gland (6) and since a considerable amount of cholesterol is taken up by the gland during lactation (7) cholesteryl esters may be involved in cholesterol metabolism within the gland. However, ACAT activity in the lactating gland is low when compared with that present in the liver and adrenal cortex, two tissues with very active cholesterol metabolism. This low level of ACAT activity has been assumed to reflect the generally low level of cholesteryl ester found in the milk of most species. Nevertheless, recent studies (8,9) have indicated that milk obtained from the pregnant gland has an elevated cholesteryl ester content when compared with post-partum milk. This finding prompted us to investigate the activity of ACAT in the rat mammary gland before and during lactation to ascertain whether the ester content of the gland correlated with the activity of the cholesterol esterifying enzyme during these developmental changes.

In addition, since studies of ACAT activity in other tissues have suggested (4,10,11) that the activity of this enzyme could be regulated by a phosphorylation-dephosphorylation mechanism we have investigated the effect of such modifications on ACAT activity in the mammary gland.

MATERIALS AND METHODS

Materials. Cholesteryl [1-^{14}C]oleate (1.85–2.22 GBq/mmol) and [9,10-^{3}H]oleic acid (74–370 GBq/mmol) were purchased from Amersham International (Amersham, U.K.). [9,10-^{3}H]Oleoyl-CoA was synthesized from [9,10-^{3}H]oleic acid and coenzyme-A by the method of Goldman and Vagelos (12), yielding a product with specific activity of between 3.5 and 3.7 GBq/mmol. Intestinal alkaline phosphatase (EC 3.1.3.1., 1095 units/mg protein), cholesterol, cholesteryl oleate, coenzyme-A, dithiothreitol, oleoyl-CoA and Triton WR-1339 (tyloxapol) were obtained from Sigma Chemical Co. (Poole, Dorset, U.K.). Fatty acid-free bovine serum albumin was from Miles Scientific (Stoke Poges, U.K.). and was dialyzed before use. Protein phosphatase was partially purified from rat liver by the method described for sheep liver (13) and had 38 units/mL of activity when assayed by inactivation of phosphorylase a.

Preparation of microsomes. Female Wistar rats were kept on a constant light cycle (lights on 08:00–20:00) and were sacrificed at 14:30. The animals used were 18–19 days pregnant (late pregnant), 2 days post-partum (early-lactating) and 10–14 days post-partum (mid-lactating) and prematurely weaned (pups removed at day 10–12 post-partum and animals used 2 days later). They were anaesthetized (pentobarbital; 80 mg/Kg) and the abdominal mammary glands dissected free from the skin and abdominal wall before being rapidly freeze-clamped with tongs pre-cooled in liquid nitrogen. The glands were crushed at liquid nitrogen temperature and approximately 2 g were homogenized (Polytron, PMA20N probe, 17000 rpm, 30 sec) in 5 volumes of an ice-cold medium consisting of 250 mM sucrose, 100 mM KF, 1 mM EDTA and 50 mM KH_2PO_4 buffer, pH 7.1. The homogenate was centrifuged (10,000 × *g*,

*To whom correspondence should be addressed.

Abbreviations: ACAT, acyl-CoA:cholesterol acyltransferase; TLC, thin-layer chromatography; VLDL, very low density lipoproteins.

15 min), the pellet discarded and the supernatant split into two equal portions before being re-centrifuged for 30 min at 100,000 × *g*. One of the resulting pellets was resuspended in a medium containing 50 mM Tris, 50 mM MES, pH 7.1, 100 mM KF, 50 mM KCl and 7.5 mM glutathione, while the other was resuspended in a similar medium in which the KF had been replaced by an equimolar concentration of KCl. Both suspensions were centrifuged at 100,000 × *g* for 30 min and the resultant microsomal pellets resuspended at a concentration of 7 mg/mL in the appropriate resuspension medium containing either KF ("fluoride" microsomes) or KCl ("fluoride-free" microsomes). A portion of freeze-clamped gland was also homogenized in medium containing 50 mM Tris, 50 mM MES and 100 mM KCl before centrifugation (100,000 × *g* 30 min) to provide a "fluoride-free" cytosolic fraction. The cholesterol and cholesteryl ester content of the microsomes was determined by the procedure given by Gamble *et al.* (14). DNA content was determined according to the method of Labarca and Paigen (15) and protein as described by Bradford (16) with bovine serum albumin as standard.

Assay of ACAT activity. ACAT activity was measured both utilizing the cholesterol endogenous to the microsomes and in the presence of exogenous cholesterol (250 μg/mL) added as a dispersion in Triton WR-1339 (17). The reaction mixture contained 2 mM dithiothreitol, 50 mM KH_2PO_4 buffer (pH 7.1), 60 mM KCl, 175 μg microsomal protein, 100 μM [9,10-^{3}H]oleoyl-CoA (20 nmol; 2 × 10^6 dpm), 110 μM bovine serum albumin and a mixture of phosphatase inhibitors (10 mM KF, 10 mM Na pyrophosphate, and 0.2 mM Na orthovanadate), in a total volume of 200 μL. After pre-incubation (20 min at 37°C) the reaction was initiated by the addition of radiolabeled oleoyl-CoA (10 μL) and terminated after 20 min (3 min for microsomes from weaned animals) by the addition of methanol/water (3:2, v/v; 1.8 mL) followed by cholesteryl [1-^{14}C]oleate (500 dpm, 0.25 mg) as internal standard. The mixture was extracted by standard methods (18) and the labelled cholesteryl oleate separated by thin-layer chromatography (TLC). Incubations were performed in triplicate and loses during extraction and TLC were estimated from the recovery of the internal standard. Blank incubations without microsomes were incubated concurrently to assess non-enzymatic breakdown of the substrate.

Modulation of microsomal ACAT activity. Microsomes (fluoride-free, 75–150 μg) were preincubated at 37°C for 30 min with ATP (4 mM) with or without $MgCl_2$ (1 mM), 100,000 × *g* supernatant (fluoride-free) and KF (40 mM). Alternatively, the microsomes were preincubated for 10 min with a partially-purified preparation of rat liver phosphoprotein phosphatase or purified intestinal alkaline phosphatase. ACAT activity in the microsomes was then assayed in the presence of exogenous cholesterol.

RESULTS

Assay conditions. Initial experiments indicated that with microsomes prepared from mammary glands at mid-lactation the formation of cholesteryl ester correlated directly with increasing substrate concentration up to 100 μM provided that the molar ratio of bovine serum albumin to oleoyl-CoA was 1 or more (Fig. 1). At 100 μM oleoyl-CoA with a slight molar excess of albumin the rate of incorporation of substrate was linear with respect to both microsomal protein (up to 200 μg) and time of incubation (up to 40 min). Addition of cholesterol as a dispersion in Triton WR-1339 markedly increased ACAT activity although the detergent alone inhibited the enzyme (Fig. 2). Inclusion of high levels of KF in the assay inhibited ACAT activity but inhibition was not observed in the presence of 10 mM KF, 10 mM Na pyrophosphate, 0.2 mM Na orthovanadate or a com-

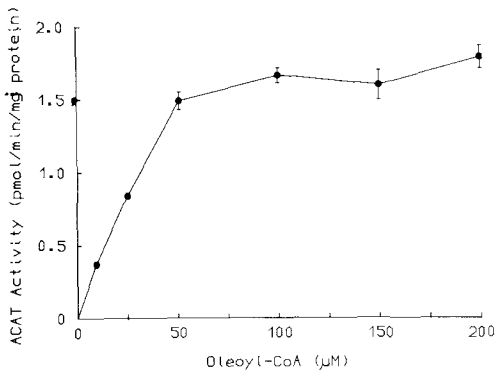


FIG. 1. The effect of the concentration of oleoyl-CoA on the yield of esterified cholesterol. Results were obtained by the appropriate variations of the oleoyl-CoA concentration in the presence of 110 μM bovine serum albumin and 175 μg microsomal protein (fluoride-free). Incubation time, 20 min. Other incubation details are listed in Materials and Methods. Data are the mean ± SEM of triplicate incubations corrected in each case for a blank value obtained in the absence of microsomes.

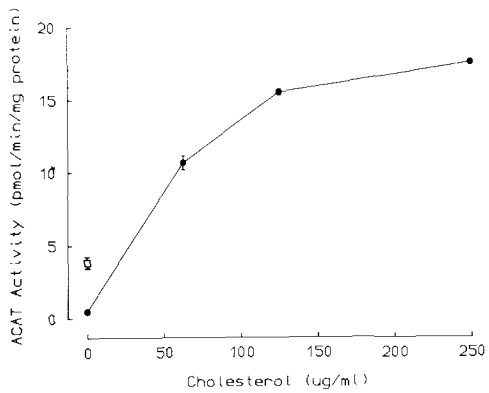


FIG. 2. The variation in microsomal ACAT activity with exogenous cholesterol. The cholesterol was added as a dispersion in Triton WR-1339 (0.3% w/v, final concentration). Microsomes (fluoride-free, 175 μg) were pre-incubated with the cholesterol-detergent suspension for 30 min under standard assay conditions. Reaction was initiated by the addition of labeled oleoyl-CoA and was terminated after 20 min. The results are the means ± SEM of triplicate incubations. The values obtained in the absence of Triton are indicated (□).

TABLE 1

Influence of MgATP on Mammary ACAT Activity[a]

Additions	Cholesteryl oleate formed (pmol/min/mg protein)
1. None	62.45 ± 2.35
2. ATP (4 mM)	85.02 ± 3.29
3. 2 + 100,000 × *g* supernatant (300 μg protein)	87.77 ± 4.65
4. 3 + KF (40 mM)	92.81 ± 0.57
5. 4 + $MgCl_2$ (1 mM)	117.19 ± 2.35
6. 5 + Phenylboronic acid (0.4 mM)	116.19

[a]Microsomes (fluoride-free, 120 μg) from weaned mammary glands were preincubated for 30 min at 37°C with the additions shown. The ACAT activity of the treated microsomes was then determined over 3 min in the presence of exogenous cholesterol and a mixture of phosphatase inhibitors (10 mM KF, 10 mM Na pyrophosphate, 0.2 mM Na orthovanadate and 1 mM EDTA). The results are the mean ± SEM of three different microsome preparations.

TABLE 2

Activation and Reversal of ACAT in Mammary Microsomes[a]

Treatment[b]	Cholesteryl oleate formed (pmol/min/mg protein)	
	Mid-lactation	Weaned
1	9.31 ± 0.49	78.54 ± 2.85
2	12.43 ± 0.25	108.49 ± 1.83
3	7.56 ± 0.43	82.65 ± 3.39
4	8.89 ± 0.17	78.20
5	Not determined	77.20

[a]Microsomes (fluoride-free) from weaned mammary glands were incubated at 37°C under a number of conditions[b] (shown below) before being assayed for ACAT. Enzyme activity was measured over 20 min (mid-lactators) or 3 min (weaned) in the presence of exogenous cholesterol in assays equalized with regard to the concentration of phosphatase inhibitors. Where indicated the results shown are the mean ± SEM for three microsomal preparations.

[b]Treatments: 1. Control microsomes were incubated for 30 min in buffer mixture without additions, recovered by centrifugation (100,000 × *g*, 30 min) and then incubated for an additional 30 min. 2. Microsomes (1.13 mg) were incubated with ATP (4 mM), $MgCl_2$ (1 mM), KF (50 mM) and 100,000 × *g* supernatant (fluoride-free, 2.7 mg protein) for 30 min before recovery and further incubated as in 1-3. As in 2, but the recovered microsomes were incubated with partially-purified rat liver phosphoprotein phosphatase (4.4 units/mg microsomal protein) for 30 min. 4. Microsomes were treated with the rat liver phosphatase (12 units/mg microsomal protein) for 30 min before the addition of KF (40 mM) and incubated for an additional 30 min. 5. As in 4, but using intestinal alkaline phosphatase (753 *p*-nitrophenyl phosphate units/mg microsomal protein).

bination of these phosphatase inhibitors (results not shown).

Modulation of ACAT activity. When microsomes obtained from weaned animals were preincubated with ATP a small increase was observed in the amount of cholesteryl ester formed (Table 1). The presence of 100,000 × *g* supernatant (2.5 mg protein per mg microsomal protein) caused no further increase nor did the inclusion of KF. However the inclusion of $MgCl_2$ provoked a larger elevation in enzyme activity. Assays were performed in the presence of phosphatase inhibitors and with exogenous cholesterol to ensure that endogenous phosphatases were inactive and that cholesterol substrate was not limiting. The inclusion of phenylboronic acid (0.4 mM), which inhibits cholesteryl ester hydrolase (19), did not modify the effects of Mg-ATP, which indicates that the esterase activity known to be present in these microsomes (20) did not interfere with the ACAT assay. A similar result was obtained when microsomes from mid-lactating animals were employed. Pre-incubation of microsomes with rat liver phosphoprotein phosphatase or high levels of intestinal alkaline phosphatase had no effect on their ACAT activity. Nevertheless, the elevated ACAT activity that resulted from treatment of microsomes with Mg-ATP and 100,000 × *g* supernatant containing KF (40 mM) could be reduced back to the control levels by incubation of the recovered microsomes with the rat liver phosphoprotein phosphatase (Table 2). This was observed in microsomes from both weaned and mid-lactating animals which represented the extremes of ACAT activity measured. In contrast, the intestinal alkaline phosphatase was not able to reverse the phosphorylation of ACAT which resulted from the treatment of the microsomes with ATP and $MgCl_2$ (Table 2). Microsomes prepared either in the presence or absence of phosphatase inhibitors exhibited the same ACAT activity (Table 3) when assayed under the same conditions.

Effects of pregnancy and lactation. ACAT activity and the free and esterified cholesterol content of microsomes prepared from mammary glands at different lactational stages are shown in Tables 3 and 4, respectively. ACAT activity, measured in the presence of endogenous cholesterol, was higher in the pregnant glands than in glands taken immediately post-partum, although there was no significant difference in the cholesterol contents of the two sets of microsomes. Enzyme activity was even lower at mid-lactation and this correlated with a reduction in the cholesterol content of the microsomes. There was a good correlation ($r=0.93$) between ACAT activity and the levels of cholesteryl ester in these three sets of microsomes (Fig. 3). The highest enzyme activities were observed in the microsomes from animals weaned for two days where the cholesterol contents of the microsomes were again elevated to the levels seen in the pregnant animals. The cholesteryl ester contents of these microsomes were much lower than would have been predicted on the basis of the correlation between enzyme activity and cholesterol ester levels observed previously (Fig. 3). The addition of exogenous cholesterol caused a greater than three-fold increase in ACAT activity in early and mid-lactators, but with the pregnant and weaned animals this increase was much more muted.

DISCUSSION

Our results demonstrate that the ACAT activity of the mammary gland late in pregnancy is elevated relative to the activity either immediately post-partum or at

TABLE 3

ACAT Activity in Microsomes Isolated from the Mammary Glands of Pregnant, Lactating and Weaned Rats: Influence of Exogenous Cholesterol and Fluoride[a]

	Cholesteryl oleate formed (pmol/min/mg DNA)		
	Fluoride-containing	Fluoride-free	
Animals	Endogenous cholesterol	Endogenous cholesterol	Exogenous cholesterol
Pregnant	8.81 ± 0.67 (5)	9.15 ± 0.45 (9)	16.00 ± 1.11 (9)
Early-lactators	5.03 ± 0.54 (5)[b]	5.49 ± 0.55 (8)[c]	17.06 ± 1.35 (8)
Mid-lactators	2.48 ± 0.42 (6)[c]	2.84 ± 0.41 (11)[c]	11.50 ± 0.89 (11)[b]
Weaned	26.24 ± 1.96 (4)[c]	22.35 ± 1.54 (6)[c]	35.89 ± 2.09 (6)[c]

[a]Fluoride-free and fluoride-containing microsomes were isolated from freeze-clamped mammary glands and ACAT activity determined in triplicate as detailed in Materials and Methods. Cholesterol was added as a dispersion in Triton WR-1339. The data are the means ± SEM for the number of animals given in parenthesis. Statistical differences (Student's *t*-test) are indicated between pregnant glands and the other glands in each column.
[b]$p<0.01$.
[c]$p<0.001$.

TABLE 4

Cholesterol and Cholesterol Ester Content of Mammary Gland Microsomes[a]

Animals	Cholesterol (μg/mg microsomal protein)	Cholesteryl ester (μg/mg microsomal protein)
Pregnant	109.9 ± 8.5 (9)	90.3 ± 10.9 (9)
Early-lactators	127.0 ± 3.3 (4)	42.0 ± 3.5 (4)[b]
Mid-lactators	59.1 ± 10.2 (11)[b]	15.4 ± 1.3 (11)[c]
Weaned	109.1 ± 5.0 (6)	57.3 ± 1.1 (6)[b]

[a]Cholesterol and cholesterol ester content of microsomes were determined as indicated in Materials and Methods. The results are means ± SEM for the number of animals given in parenthesis. Statistical differences (Student's *t*-test) are shown; [b]$p<0.01$, [c]$p<0.001$ compared with the appropriate pregnant glands.

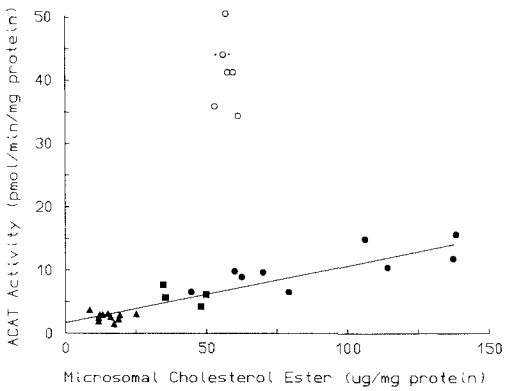


FIG. 3. Scatter diagram relating ACAT activities, assayed with endogenous cholesterol, to microsomal cholesteryl esters. Linear regression analysis was performed on the ACAT activities in "fluoride-free" mammary microsomes from pregnant (●), early-lactating (■) and mid-lactating (▲) rats and their corresponding cholesteryl ester contents. The relationship was linear with regression line $y = 1.67 + 0.096\ x$ and correlation coefficient $r = 0.93$. ACAT activity in microsomes from prematurely weaned animals are shown (○) for comparison.

mid-lactation. It is even further increased when milk is allowed to accumulate by premature weaning of the gland. In the latter situation there would be a large build-up of cholesterol in the gland and a consequent need to remove it by esterification for storage. Interestingly, the activity of the enzyme did not correlate with the ester content of these microsomes in the same way that was seen for the other three sets of microsomes (Fig. 3), suggesting that other mechanisms for removal of cholesterol were also operative in the weaned glands.

In the pregnant glands the enhanced level of ACAT activity may have been a compensatory response to the increasing concentrations of progesterone in the animals (21,22) since this hormone has been shown to inhibit the enzyme in a number of tissues (6). However, other factors may be important. A high cholesterol diet leading to an increased cholesterol level in the rat liver has been reported to give rise to elevated hepatic ACAT activity (23,24). The pregnant animal has an increased food intake and becomes hypercholesterolemic in late pregnancy (25–27), and this may cause a similar elevation in ACAT activity in the mammary gland. This effect would be accentuated in the preg-

nant gland and probably also in the weaned animal since neither has the high flux of cholesterol into milk characteristic of the fully lactating animal.

The activity of the enzyme in all of these microsomal samples could be stimulated by the addition of cholesterol, indicating that enzyme activity was at least partially regulated by substrate supply. However, there was no clear correlation between cholesterol content of the microsomes and ACAT activity at the different developmental stages, suggesting that other factors were also controlling enzyme activity. This was also indicated by the results observed when exogenous cholesterol was added since a similar stimulation (>three-fold) was obtained with microsomes from the early and mid-lactators, whereas only about half this increase was elicited with the microsomes from the pregnant and weaned glands.

In other experiments in which anti-hormone agents were used to arrest lactation the mammary gland ACAT activity was similar to that of the weaned animal, but was increased three-fold when assayed under the same conditions with exogenous cholesterol (28). Thus the smaller increase provoked by additional cholesterol seen with the weaned and pregnant microsomes was unlikely to be due either to the initially elevated ACAT or to insufficient equilibration of the exogenous cholesterol with the membranes. The cause of the relatively poor response to exogenous substrate in these particular microsomes is unknown, but clearly indicates that other mechanisms must act to control ACAT in the gland. Previous reports have suggested that ACAT could be regulated by a phosphorylation-dephosphorylation mechanism, although whether phosphorylation was associated with an increase or a decrease in enzyme activity was dependent on the tissue studied (4,8). In microsomes from both rat liver and rat intestinal cells ACAT activity was increased by incubation of the microsomes with the 100,000 $\times$ *g* supernatant fraction in the presence of MgATP and NaF (8), whereas a similar incubation of microsomes from the bovine adrenal cortex resulted in an inhibition of ACAT activity (4). The results presented here would suggest that the activity of ACAT in the mammary gland is increased on phosphorylation. Thus the enzyme in the mammary gland responds to this covalent modification in the same way as the hepatic and intestinal enzymes. The responses observed with the mammary enzyme were much smaller than those seen with the hepatic enzyme, and particularly the addition of the cytosolic fraction and KF had little effect. Hepatic microsomes prepared in the presence of fluoride to inhibit phosphatases did not show increased ACAT activity when compared with appropriate controls except in the situation where cholesterol-rich VLDL was first administered to the animals *in vivo* (29). In the present work tissue was obtained by freeze-clamping at liquid nitrogen temperatures, a technique extensively used in previous studies (see *e.g.*, ref. 30) to preserve the intracellular activity status of enzymes, and subsequently homogenized in media containing both phosphatase and kinase inhibitors. Nevertheless microsomes isolated in this manner did not differ significantly in ACAT activity from microsomes prepared without any of these precautions suggesting that the enzyme was not phosphorylated *in vivo*. This correlated with the failure of the partially-purified rat liver phosphoprotein phosphatase to alter the activity of the microsomal enzyme *in vitro* unless it had first been increased by treatment with Mg-ATP. Thus, although ACAT was responsive to phosphorylation *in vitro*, the function of this enzyme modification in controlling the activity of ACAT in the mammary gland requires further investigation.

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METHODS

Analysis of Aldehydic Lipid Peroxidation Products by TLC/Densitometry

Jeffrey K. Beckman*, Samuel A. Morley, Jr. and **Harry L. Greene**
Department of Pediatrics, Division of Gastroenterology, Vanderbilt University Medical Center, Nashville, Tennessee

We have explored the use of thin-layer chromatography (TLC)/densitometry in both the reflectance and fluorescence mode for quantitation of specific products of lipid peroxidation. Aldehydic peroxidation products were generated by exposure of arachidonic acid to iron and ascorbic acid for 24 hr. Several methods for the quantitative analysis of peroxidation products by TLC/densitometry were compared using two different aldehyde-specific derivatizing reagents, namely dinitrophenylhydrazine (DNPH) and cyclohexanedione (CHD). DNPH hydrazones of the arachidonic acid-peroxidation products, upon TLC separation on silica gel, revealed prominent alkanal and hydroxyalkenal bands. Reverse phase high performance liquid chromatography confirmed that the primary alkanal component was hexanal, while the primary hydroxyalkenal was 4-hydroxynonenal. Semiquantitative methods for the direct analysis of these products by TLC/densitometry were worked out based on the use of external hydrazone standards. TLC/densitometry (fluorescence mode) was used to measure CHD adducts of aldehydes by forming the derivatives in the presence of decanal (used as an internal standard) and separating the derivatives by reverse phase TLC. Hexanal-CHD was detectable upon application of 0.5 nanomoles while 4-hydroxynonenal showed a lower response and was detectable with 10 nanomoles. Using appropriate response factors, hexanal and 4-hydroxynonenal were measured in the aldehyde sample from arachidonic acid and results were similar to those obtained by the DNPH method.

Similar approaches were used to analyze the peroxidation products of docosahexaenoic acid (24-hr exposure) and of rat liver microsomes exposed to iron for 30 min. The DHA peroxidation products contained extremely low levels of alkanals, while polar aldehydes and hydroxyalkenals were prominent. Formation of alkanals, osazones, hydroxyalkenals and phospholipid aldehydes from iron-exposed microsomes was also demonstrated. Uses and limitations of these methods of aldehyde measurement are discussed.
Lipids 26, **155–161 (1991).**

As an indicator of free radical injury, lipid peroxidation is usually assessed by the thiobarbituric acid (TBA) method (1,2) in which a complex of TBA with malondialdehyde is produced and measured by either absorbance or fluorescence monitoring. Although the TBA method is useful due to its rapidity and ease, the method is not specific and fraught with potential difficulties (3,4). The development of methods to detect and measure specific products of lipid peroxidation in recent years has increased the sensitivity of peroxidation analysis and has overcome many of the earlier problems. Various studies have shown that specific aldehydic peroxidation products, including the hydroxyalkenals, are of biological relevances, due, in part, to their high reactivity (5–7). Assays of specific products of lipid peroxidation are usually based on high performance liquid chromatography (HPLC) (8) or gas chromatography (GC)/mass spectrometry (MS) (9,10) and typically require preliminary fractionation of the products to be analyzed.

The purpose of the present investigation was to develop methods for the analysis of aldehydic lipid peroxidation products directly on thin-layer chromagraphy (TLC) plates using densitometric techniques. TLC/densitometry appears particularly useful in obtaining rapid estimates of the extent of formation of particular classes of lipid peroxidation products, which does not preclude more detailed subsequent analyses. Although certain aldehydes, including the hydroxyalkenals, may be detected directly by TLC/densitometry by monitoring at their absorbance maximum, such measurements are not applicable to complex samples where neutral lipids are likely to interfere. Derivatization allows the specific detection of aldehydes and increases the sensitivity of measurements, although it can also complicate assays. This study compares the use of two aldehyde-specific reagents for the densitometric detection of aldehydic peroxidation products. Polyenoic fatty acids (arachidonic acid and docasahexaenoic acid) and rat liver microsomes were used to study lipid peroxidation.

EXPERIMENTAL PROCEDURES

Arachidonic acid and docosahexaenoic acid (each approx. 99%) were purchased from Sigma Chemical Company (St. Louis, MO) and stored at −20°C under nitrogen prior to use. 1,3-Cyclohexanedione (CHD) was obtained from Aldrich Chemical Company (Milwaukee, WI) and recrystallized from methanol. Dinitrophenylhydrazine (DNPH) was obtained from Sigma and recrystallized from methanol.

Oxidation and extraction of fatty acids. Fatty acids (20–100 mg) were oxidized as a dry film by exposure to air for 20–24 hr. Fatty acids were dissolved in chloroform and the progress of oxidation was monitored by TLC (*n*-heptane/ethyl acetate/glacial acetic acid, 75:25:1, by vol) and densitometric assay at 205 and 235 nm. Fatty acid hydroperoxides, along with any residual unoxidized fatty acids (usually less than 25% of the total) were placed in Erlenmeyer flasks, chloroform was evaporated, and the lipids redissolved (1 mg/mL) with vortexing in 0.1 M Tris buffer, pH 7.4. Ferric chloride (20 μM) and ascorbic acid (500 μM) were added, and the solutions were incubated at 37°C for 24 hr. The solutions were acidified to pH 3 and extracted three times with equal portions of chloroform. The pooled chloroform extracts were evaporated under nitrogen (without heating) and redissolved in 2–4 mL of chloroform. The progress of secondary product

*To whom correspondence should be addressed.

Abbreviations: AA, arachidonic acid; CHD, cyclohexanedione; DHA, docosahexaenoic acid (22:6n-3); DNPH, dinitrophenylhydrazine; GC/MS, gas chromatography/mass spectrometry; HPLC, high performance liquid chromatography; HNE, 4-hydroxynonenal; TBA, thiobarbituric acid; TLC, thin-layer chromatography.

formation was monitored by TLC using the system described above and by scanning at 220, 235 and 270 nm.

TLC densitometry. A Shimadzu CS 9000 dual wavelength TLC scanner (Shimadzu, Columbia, MD) equipped with a xenon lamp fluorometry attachment was used for densitometric monitoring of either absorbance or fluorescence. Absorbance was monitored using the reflectance photo mode and the zig zag scan mode (swing width 7.0 mm) with single wavelength monitoring. Precise prescribing of lanes on TLC plates [either by the manufacturer for the Whatman LK5D plates or using an Analtech (Newark, DE) plate scriber for the unscribed LKC18 plates] enabled automatic scanning of multiple lanes. Fluorescence of CHD-adducts was monitored at a 360 nm excitation wavelength using emission filter 2 (wavelength cutoff at 400 nm). Scanning was done in the linear mode for fluorescence with a beam of 0.4 × 0.4 mm.

Dinitrophenylhydrazine derivatization, thin-layer chromatography and standardization of hydrazone thin-layer densitometry. Aliquots of extracts of the secondary products of fatty acid peroxidation (a portion equivalent to 5 mg of the original fatty acid) were evaporated to near dryness, redissolved in one mL of DNPH reagent (0.34 mg/mL in 1 M HCl), and allowed to react for 2 hr at room temperature in the dark. Dinitrophenylhydrazones were extracted with methylene chloride (2 × equal volume), the extractions concentrated to 0.5 mL by evaporating under nitrogen, and the residue was redissolved in methylene chloride. The samples were applied to silicic acid TLC plates along with hydrazone standards of hexanal and HNE, and plates were developed in methylene chloride (8). TLC densitometry was done at 360 nm using an external standard of hexanal-DNPH and an extinction coefficient of 25,500 (8). Various concentrations of hexanal-DNPH were run in separate lanes of the TLC plate, and calibration curves were prepared using the data processing unit of the densitometer. Calibration curves were stored on floppy disks and recalled using the disk drive accessory and the data processing unit for automatic integration of hydrazone bands in respect to concentration.

Phospholipid hydrazones (from microsomal mixtures) were separated by thin-layer chromatography using ethyl acetate/n-propanol/chloroform/methanol/0.25% KCl (25:25:25:13:9, by vol) as solvent system. The plates were initially developed about 15 cm, then dried, and then further developed in methylene chloride to separate the neutral lipids. Hydrazones were analyzed using the densitometric methods and the calibration curves described above.

HPLC. Hydrazones of alkanals and of hydroxyalkenals were separated using a Beckman Gold HPLC system (Beckman Instruments, Fullerton, CA) and an analytical Ultrasphere ODS column (4.6 mm × 25 cm) equipped with a Model 166 UV detector. Samples were injected as 20-μL aliquots and separated using the gradient solvent system (11) initially consisting of methanol/water (3:1, v/v) and changing linearly to methanol/water (4:1, v/v) over the first ten min, and from 4:1 to 100% methanol over 10–25 min. Hydrazones were detected at 360 nm, and peaks were identified by comparison with those obtained for hydrazones prepared from commercially available alkanals or from HNE purified from AA-secondary products and identified by negative ion GC/MS of pentafluorobenzyl oximes as described (10).

CHD adducts of aldehydes were separated by reverse phase HPLC using a semipreparative Ultrasphere ODS column (10 mm × 25 cm) and detected using a McPherson Model FL-750 BX variable wavelength fluorescent detector (McPherson Instruments, Boston, MA) equipped with a Xenon excitation lamp and a 400 nm cut-off emmission filter. CHD adducts were monitored at an emission wavelength of 380 nm.

Cyclohexanedione derivatization and fluorodensitometry. Fluorescent decahydroacridine derivatives of aldehydes were analyzed by fluorescent TLC densitometry using derivatization methods developed by Yoshino *et al.* (12) for fluorometric HPLC assay. The CHD reagent (12) was first boiled for 1 hr and then extracted with three portions of methylene chloride (20 mL/100 mL of reagent). The CHD reagent was used to derivatize standard aldehydes or samples by heating for 90 min at 60°C. Instead of using C18 extraction cartridges as described by Yoshino *et al.* (12), we added methanol, water and chloroform in proportions to arrive at a Bligh and Dyer extraction (13). The chloroform layer was washed once with an equal volume of theoretical upper phase, evaporated to dryness under nitrogen, and redissolved in chloroform (500 or 100 μL as noted). Ten microliters were plated onto prescribed lanes of reverse phase TLC plates. The plates were developed initially about 5 cm in 80% methanol and then completely in 70% methanol. Plates were scanned in the fluorescent mode using an excitation wavelength of 360 nm and an emmission filter which excluded light of wavelengths less than 400 nm.

Microsomal peroxidation. Rat liver microsomes were prepared from animals on standard Chow diets as previously described (14) using a homogenization buffer of 0.1M KCl, 40 mM Tris, pH 7.4, containing 1 mM EDTA, and by resuspending the final pellet in the same buffer at 5–10 mg/mL protein. Microsomes (1 mg/mL final concentration) were incubated in 40 mM Tris buffer, pH 7.4, containing 1mM $CaCl_2$. Ferric chloride (20 μM) and ascorbic acid (500 μM) were used to induce nonenzymatic lipid peroxidation. Peroxidation reactions were stopped by the addition of 0.1% phosphoric acid for TBA-reactivity (1), by addition of methanol (containing decanal internal standard) for the CHD derivatization (12) or DNPH reagent (as described above) as appropriate.

RESULTS

Exposure of arachidonic acid as a dried film to air oxidation resulted in substantial (75%) conversion to fatty acid hydroperoxide as monitored by TLC densitometry at 235 and 205 nm (data not shown). Subsequent exposure to iron (20 μM) and ascorbic acid (500 μM) for up to 24 hr resulted in the conversion of the hydroperoxide and any residual unoxidized fatty acid to various secondary peroxidation products (termed AA-secondary products). TLC densitometry (with scanning) confirmed the formation of significant quantities of hydroxyalkenals (Abs max, 222 nm) and of bands exhibiting ketodiene chromophores (Abs max, 270–275 nm, 235 nm secondary). The formation of alkanals cannot be determined in this manner (without derivatization) due to the lack of a suitable chromophore. Direct densitometry of hydroxyalkenals is useful for TLC preparative work and will be reported separately.

The lipids of more complex systems include compounds that are likely to interfere with the direct assay of underivatized peroxidation products. Assays were therefore developed using aldehyde-specific derivatization reagents.

Derivatization of AA-secondary products with DNPH followed by thin-layer chromatography of extracts on silica plates separated the resulting DNPH hydrazones which were detected by their absorbance at 360 nm. TLC densitometry at 360 nm showed prominent bands of hydrazones of alkanals, hydroxyalkenals and polar aldehydes, the identity of which was confirmed by comparison with DNPH-alkanal and DNPH-hydroxyalkenal standards (Fig. 1). Direct (on-plate) spectra in the visible range confirmed that the bands all contained the hydrazone chromophore (Abs. max, 370 nm). A semiquantitative external standardization method for measuring hydrazone bands was developed as described in the methods section. A calibration curve for hexanal-DNPH was established (Fig. 2) which was found to be essentially the same for other hydrazone classes (hydroxyalkenals and phospholipid aldehydes). Alkanal and hydroxyalkenal TLC bands of the AA-secondary products were eluted and fractionated by reverse phase HPLC for further characterization. The alkanals included predominately (58%) hexanal (Fig. 3a), while the hydroxyalkenals included a major (57%) compound identified (by comparison with HPLC separation of standards) as 4-hydroxynonenal (Fig. 3b). Based on the percentage composition of the HPLC chromatograms and the TLC densitometric analysis of the classes we calculated the yield of hexanal and hydroxyalkenal hydrazones obtained from arachidonic acid (Table 1).

A fluorescent method involving the use of cyclohexanedione reagent (12) was also adapted to TLC densitometry and used to analyze the AA-secondary products. CHD adducts of varying concentrations of standard alkanals and hydroxyalkenals were prepared and separated by reverse phase TLC. Propanal, 4-hydroxynonenal, a diene analog of HNE (HNDE), hexanal and decanal CHD adducts gave separate peaks (Fig. 4a). The CHD reagent always yielded fluorescent peaks corresponding to acetaldehyde and propanal as well as a smaller peak comigrating with butanol [despite recrystallization of the CHD and extraction of the reagent (Fig. 4b)]. Standard curves for alkanals were generally linear up to about 100 μM (r=0.998 for hexanal) which corresponded to applying 25 nmole onto the plate. Response factors of individual alkanals, alkenals and hydroxyalkenals differed. Relative to a 1.0 value for hexanal, we observed the following response factors: Propanal, 0.8; pentanal, 1.0; decanal, 1.1; *trans*-2-hexenal, 0.3; *trans*-2-nonenal, 0.3; and HNE, 0.1. HNE-CHD was virtually undetectable after a 1 hr derivatization reaction (which is sufficient for hexanal and other alkanals). Higher temperature (75°C) did not enhance the response for HNE significantly and increased formation of reagent blank peaks. Malondialdehyde (prepared by hydrolysis of malondialdehyde dimethylacetal) did not react to form a detectable product.

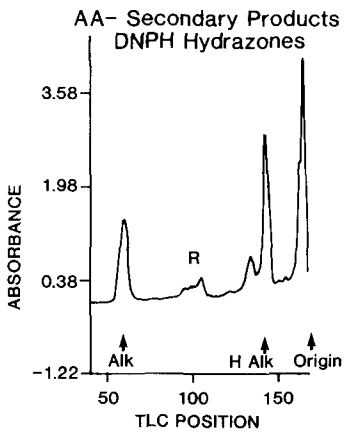


FIG. 1. TLC densitometry of DNPH hydrazones of peroxidation products of arachidonic acid. AA-secondary products of lipid peroxidation were generated and extracted as described in the Experimental section. Thin-layer chromatography on silicic acid plates (using methylene chloride as developing solvent) separated hydrazone classes which were identified using alkanal and hydroxyalkenal hydrazone standards (designated as Alk and H Alk, respectively). Densitometry was performed at 360 nm. TLC position is measured from the top of the plate with the border of the loading zone (origin) at position 168. "R" designates a reagent peak.

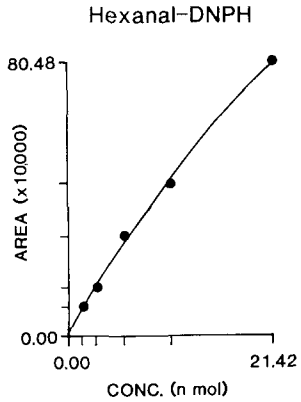


FIG. 2. Calibration curve for DNPH hydrazones. Hexanal-DNPH was isolated and quantitated by UV spectrometry. Various concentrations were plated on individual lanes in duplicate. TLC and TLC/densitometry were performed as in Figure 1. Odd numbers for concentrations reflect the use of even numbered volumes plated.

Aliquots of AA-secondary products were derivatized with CHD in the presence or absence of decanal which was added as an internal standard. Following reverse phase TLC, fluorescent densitometry revealed a substantial hexanal-CHD peak and a small peak of slightly greater R_f (Fig. 4c and Table 1) in addition to the reagent associated peaks. The latter product was subsequently identified by fluorescent HPLC as HNE and was quantified using its 0.1 relative response factor (Table 1).

The n-3 polyenic acid, docosahexaenoic acid, was used as a source for peroxidation products (DHA-secondary products) by the method described for AA-secondary products. DHA-secondary products wer analyzed by both the DNPH and the CHD method and compared with AA-secondary products. DNPH-hydrazones corresponding in R_f value to DNPH-hydroxyalkenal and to polar aldehydes were prominent (Fig. 5). The much lower yield of alkanals as compared with that seen for the AA-secondary products was noteworthy (Table 1). A DNPH hydrazone of R_f equivalent to the hydroxyalkenal (HNE) TLC band was observed which contained a lower concentration than the hydroxyalkenals of AA-secondary

TABLE 1

Comparison of DNPH and CHD TLC Densitometry Measurements of Aldehydic Lipid Peroxidation Products of Arachidonic Acid, Docosahexaenoic Acid and of Rat Liver Microsomes

Product[a] source	Aldehyde class	Method	
		DNPH	CHD
		(nmol/mg fatty acid or mg protein)	
Arachidonic acid	Alkanal[b]	32.4 ± 6.6[c]	57.6 ± 2.0
	Hydroxyalkenal	44.2 ± 8.1	50.4 ± 8.0
Docosahexaenoic acid	Alkanal	3.6 ± 2.4	nd[d]
	Hydroxyalkenal	13.2 ± 4.0	nd
Microsomes	Alkanal	25.0 ± 1.7	24.9 ± 2.9
	Hydroxyalkenal	1.4 ± 0.5	2.0 ± 0.7
	Polar aldehydes	53.0 ± 6.2	na

[a]The fatty acids were exposed to air (24 hr) followed by exposure of the resulting hydroperoxides and unoxidized fatty acid to iron/ascorbic acid (24 hr). Rat liver microsomes were exposed to iron/ascorbic acid for 30 min. Values for control microsomes are given in the text. Details of the methods are described under Experimental Procedures.

[b]The DNPH assay measured total alkanals while the CHD assay measured hexanal only.

[c]Values are expressed as mean ± SD for four analyses of a single representative sample of lipid peroxidation reaction mixtures from each source.

[d]Not determined.

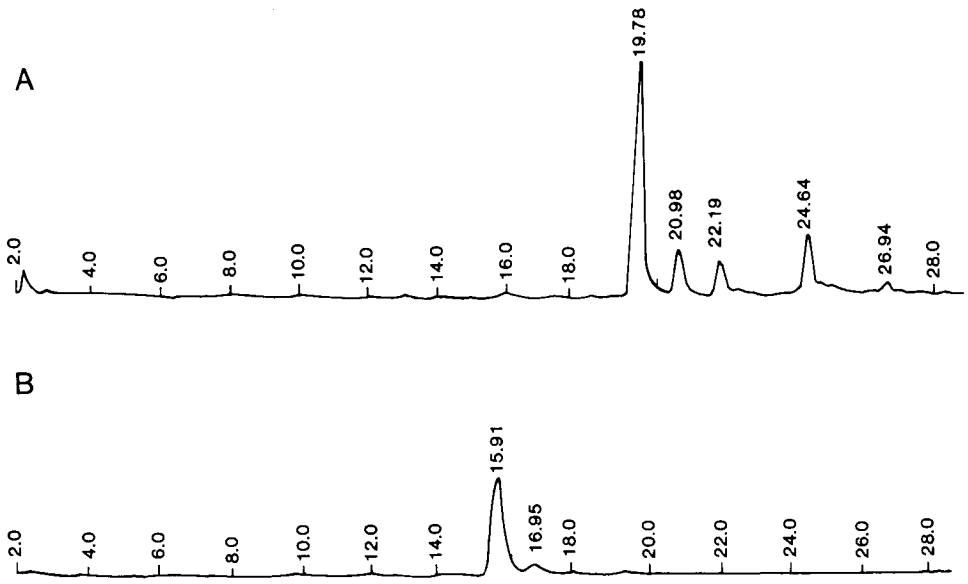


FIG. 3. *Panel A.* HPLC of DNPH hydrazones of alkanals formed from arachidonic acid. The peak at 19.78 min corresponds to hexanal-DNPH. *Panel B.* HPLC of DNPH hydrazones of hydroxyalkenals formed from arachidonic acid. The peak at 15.9 min corresponds to 4-hydroxynonenal-DNPH.

products. Most of the DHA hydrazones were "polar aldehydes" (more polar than the hydroxyalkenals), including a distinct peak at position 135 (Fig. 5), which was absent from the AA-secondary products. Three other preparations of iron-induced peroxidation products of DHA and of AA have been generated and have confirmed the relative lack of alkanal formation from DHA with DHA-hydroxyalkenal formation ranging from 55–82% of the hydroxyalkenals from AA.

Derivatization of DHA-secondary products with CHD followed by reverse phase TLC and fluorescent densitometry gave the chromatogram shown in Figure 4d. The profile was devoid of any peaks other than those found in the reagent blank. In addition, there was no evidence of an augmentation of the propanal peak (which is contained in the blank).

Rat liver microsomes were exposed to iron and ascorbic acid for 30 min and analyzed following derivatization

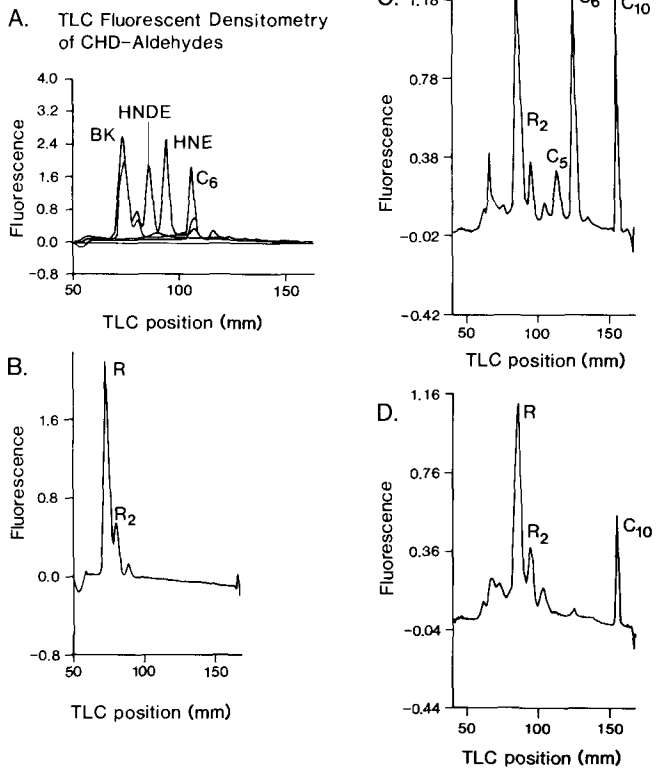


FIG. 4. Fluorescence TLC densitometry of alkanals and hydroxyalkenals of AA and DHA. Reverse phase TLC was used to separate fluorescent CHD acridine derivatives of aldehydes as described in the text. Panel A exhibits an overlay of several lanes of a TLC plate which includes various concentrations of hexanal (C_6) as well as HNE and a diene analog of HNE (HNDE). A separate TLC plate (which also included standards; not shown) was scanned for fluorescence and chromatograms of selected lanes containing either CHD-reagent blank (panel B), AA-secondary products (panel C) or DHA-secondary products (panel D). Decanal (C_{10}) was added to the fatty acid peroxidation products to serve as an internal standard. The propanal standard (not shown) coincides with the second blank peak (R2).

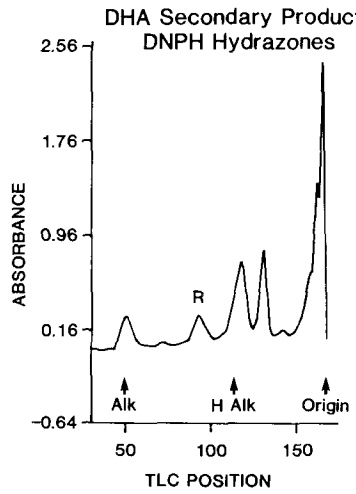


FIG. 5. TLC densitometry of DNPH-hydrazones formed from DHA. Methods wre those in Figure 1. The hydroxyalkenal band ran somewhat higher than in Figure 1, but was identified using the same HNE-DNPH standard.

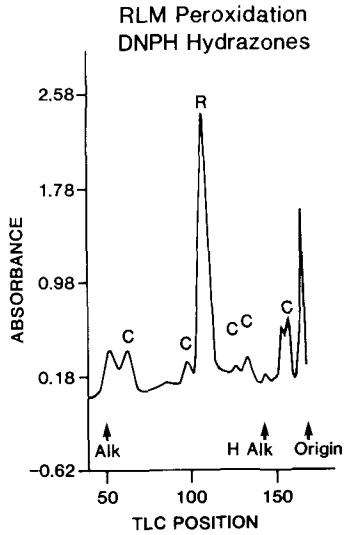


FIG. 6. TLC densitometry of DNPH-hydrazones formed from iron-exposed microsomes. The reagent peak is designated by an R, while peaks present and unchanged from control microsomes are designated with a C. A control alkanal peak was observed, but greatly augmented as described in the text. Alkanal and hydroxyalkenal hydrazones wre located using standards.

with either DNPH or CHD. A parallel sample was assayed for TBA-reactivity which established that at least 30 nmol/mg protein of MDA equivalents were formed. The TLC densitometic chromatogram of DNPH products (Fig. 6) showed the overwhelming reagent peak as well as other peaks (designated with a C) that were present in control microsomal incubations and not augmented by exposure to iron. The alkanal band increased from the control level of 9.2 nmol per mg protein to 25.6 nmol/mg protein while the hydroxyalkenal band increased from 0 to 1.42 nmol/mg protein. The polar aldehyde peak increased from 10 to 53 nmol/mg protein. The hydroxyalkenal band (identified using HNE-DNPH) was much smaller than observed previously using fatty acids as product source. Visible scans of the various bands demonstrated that while most of the bands were hydrazones, the band at position 153.8 gave a spectrum (Abs max, 430-440 nm) characteristic of osazones (8). HPLC fractionation of the alkanals demonstrated that hexanal was the predominant product (53% of the total), while no other peak exceeded 15%. Thin-layer chromatography of hydrazones of the same microsomal reaction mixtures was run in a solvent system designed to separate phospholipid classes. TLC densitometry (absorbance 360 nm) demonstrated that 55% of the polar aldehydes migrated in a band consisting of 4–5 peaks (not resolved) that migrated in the region of standard egg phosphatidylcholine (monitored at 205 nm) with the remainder about equally divided between a group of peaks that migrated with standard phosphatidylethanolamine and a peak that migrated at the polar solvent front (beneath the hydroxyalkenal band). The "phospholipid aldehydes" were absent in the control microsome sample whole the peak migrating at the polar solvent front was present at a level similar to that of the oxidized microsome sample.

Analysis of aldehydes (alkanals and hydroxyalkenals) of the microsome reaction mixtures by the CHD

densitometric method (confirming peak identification by fluorescent HPLC) similarly demonstrated the formation of hexanal as well as HNE (Table 1). Neither hexanal nor HNE were detected in control microsome samples by TLC densitometry.

DISCUSSION

Lipid peroxidation is most often assessed in general terms by use of the TBA assay which measures malondialdehyde and peroxidation products (such as hydroperoxides) which then degrade to form malondialdehyde under the acidic, high-temperature assay conditions (2). However, in an effort to enhance sensitivity of analysis and/or to explore the significance of lipid peroxidative processes in more detail, investigators have, in recent years, developed methods for the analysis of specific products of lipid peroxidation. The aldehydic lipid peroxidation products formed by degradation of lipid hydroperoxides are the major products that have been characterized in a number of biological lipid peroxidation systems both *in vitro* (8) and *in vivo* (15,16). One aldehyde that is particularly interesting is 4-hydroxynonenal (HNE), which has been shown by Esterbauer and co-workers to exhibit reactivity and toxicity in biological systems (5–7) and to be measurable *in vivo*. The method usually applied to measure aldehydic peroxidation products is tedious, involving reaction with DNPH, preparative TLC (multiple lanes per sample), elution of bands, quantitation of classes by spectrometry, and analysis of the individual molecular components by HPLC. In order to study the formation of aldehydes in multiple samples, it appeared advantageous to quantitate the compounds by automated TLC densitometry. In this study, we have explored the use of TLC densitometry for the measurement of two classes of aldehyde derivatives, DNPH hydrazones and CHD acridine adducts.

The applicability of TLC densitometry to the measurement of DNPH hydrazones of alkanals and hydroxyalkenal is hampered by the presence of unreacted reagent in the samples. DNPH reagent (the peak of which is seen prominently in the microsomal peroxidation sample) limits the concentration of extract that can be applied to the plate and hinders the analysis of samples containing low levels of peroxidation product. Reagent blanks also contain alkanal and other hydrazone bands which are insignificant for the highly peroxidized fatty acid extracts or even the iron-exposed microsomes, but which will lessen sensitivity for many peroxidation assays (such as for *in vivo* experiments). Polar lipid hydrazone fractions "phospholipid aldehydes" were more readily separated from interfering reagent bands, but consisted of multiple unresolved peaks that are difficult to accurately quantitate by densitometry. Another disadvantage of measuring hydrazones (in general) is the lack of a suitable internal standard. Reproducible spotting methods are therefore required for the method to be quantitative. We typically spot 20 μL onto the loading zone of prechanneled, precoated TLC plates. Mechanized spotting devices may prove useful as an alternative. Despite the limitations described above, DNPH derivatization with TLC densitometry is, however, a useful method for indentifying isolated peroxidation products by aldehyde class or for semiquantitative measurement of aldehydes in chemical or highly peroxidized biological samples. The detection limits were about 10 nmol of alkanal, 1 nmol of hydroxyalkenal and 1 nmol of "phospholipid aldehyde".

An alternative method for analyzing the aldehydes of lipid peroxidation uses CHD reagent to form fluorescent acridine adducts with peroxidation products. Reverse phase TLC effectively separated alkanals according to their chain length and permitted the use of decanal as an internal standard. We determined that the various alkanals and hydroxyalkenals differed in the extent to which they reacted with CHD and/or were detected by fluorescent TLC densitometry. The CHD-TLC densitometric method detected alkanals with fair sensitivity but was less sensitive for the measurement of hydroxyalkenals. The reported response factor of HNE relative to hexanal for the HPLC assay was 0.25 while our measured value was 0.1 for a 90-min derivatization reaction and 0.15 following a 3-hr reaction. In the presence of comparable levels of hexanal (AA-secondary products), HNE was detected as a much smaller peak. One would anticipate that it would be difficult to detect HNE by this method in most biological samples. Major fluorescent peaks were always observed with the CHD blank and samples that corresponded to two, three and four carbon aldehydes. Similar peaks were observed in the HPLC chromatograms reported by Yoshino *et al.* (12). These reagent peaks thus limit the sensitivity of the assay for these short-chain aldehydes even when using more sensitive detection methods, such as fluorescent HPLC. Despite this drawback and the difficulty of measuring HNE, the CHD-TLC densitometric method may prove useful for assessing hexanal content in multiple samples as a general index of peroxidation, and perhaps prior to HPLC analysis. The reproducibility of the method is excellent for hexanal measurement in iron-exposed microsomes and appeared to be specific for lipid peroxidation as hexanal was not detected in control microsomes. The CHD method for analyzing hexanal by TLC densitometry may detect less than 1 nmol of hexanal but requires about 10 nmol to measure HNE.

The methods described here were used to compare the AA-secondary products with those formed from DHA and from *in vitro* peroxidation of rat liver microsomes. A major finding confirmed by both assays was that considerably more alkanal/alkenal product was formed from arachidonic acid than from the n-3 fatty acid, DHA. It is possible that propanal and/or hexenal are formed from DHA but not detected due to their volatility. We have, for example, not detected substantially higher propanal contents in n-3 enriched microsomes (from rats on fish oil diets) following iron-induced peroxidation and assay by the DNPH and CHD methods (data not shown). Since the assays were performed by derivatization of the microsomal mixtures there was less of a chance for volatile compounds to be lost as would be during evaporation in a nitrogen stream, suggesting that volatility may not be the only cause for the lesser level of n-3 alkanals.

In agreement with previous studies, microsomal peroxidation mixtures contained hydrazones of all of the classes found in the fatty acid peroxidation samples as well as another TLC band exhibiting osazone characteristics (8). The levels of alkanal product were comparable to those obtained by Poli *et al.* (8) using ADP-iron and an NADPH

generating system for the study of enzymatic microsomal lipid peroxidation (8). In our study, the microsomal peroxidation products contained much lower levels of hydroxyalkenals than reported by Poli *et al.* (8). Our system induced lipid peroxidation by non-enzymatic means which may form less hydroxyalkenal than the enzymatic system. The osazone peak is likely underestimated by our current external standard method as the osazone absorbance at 360 nm is about 25% less than its peak value at 430 nm. The identity or significance of osazones has not been explored. Substantial levels of polar aldehydes were generated during microsomal peroxidation. Most migrated on TLC with either phosphatidylcholine or phosphatidylethanolamine standards but could not be accurately quantitated for the reasons described above. These products have been described previously (17), although they have not been fully characterized.

The CHD densitometric method confirmed the formation of hexanal from microsomes exposed to iron. The concentration so estimated was about 30% higher than that measured by DNPH assay (TLC densitometry and HPLC). Values for the AA-secondary product hexanal content were similarly higher, according to the CHD method. Perhaps the CHD derivatization reaction is more complete than the DNPH reaction. Reagent blanks and control microsomes (incubated without iron) did not show any hexanal formation by the CHD method while the DNPH method always exhibited significant levels of DNPH-alkanal including DNPH-hexanal.

Our results demonstrate the utility, as well as the limitations, of TLC densitometry for assessing the generation of aldehydic peroxidation products. TLC densitometry of hydrazones (DNPH method) may be used as a semiquantitative measure for a variety of alkanals, hydroxyalkenals and perhaps phospholipid aldehydes for *in vitro* samples. However, the limitations of the DNPH densitometric method (described above) will likely prevent its general use for *in vivo* peroxidation investigations. The fluorescent TLC densitometric assay of CHD adducts offers advantages as it uses milder conditions (12), is sensitive for measuring hexanal (usually a prominent peroxidation product), and uses an internal standard. Limitations include a low sensitivity to hydroxyalkenals and interference of reagent peaks with measurement of n-3 fatty acid peroxidation products, such as propanal. The CHD method may be particularly suited for the preliminary assessment of aldehyde formation which can be followed then by fluorescence HPLC in appropriate samples.

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COMMUNICATIONS

A New Eicosapentaenoic Acid Formed from Arachidonic Acid in the Coralline Red Algae *Bossiella orbigniana*

John R. Burgess[a,*], Roger I. de la Rosa[b], Robert S. Jacobs[a] and Alison Butler[b]
Departments of Biological Sciences[a] and Chemistry[b], University of California, Santa Barbara, California 93106

Aqueous extracts of the coralline red algae *Bossiella orbigniana* have been shown to catalyze the enzymatic oxidation of arachidonic acid to a previously unreported ω6 eicosapentaenoic acid product. This unique fatty acid contains a conjugated tetraene with absorption maxima at 293, 306, and 321 nm, and was identified by spectral methods as 5(Z),8(Z),10(E),12(E),14(Z)-eicosapentaenoic acid. The compound was given the trivial name bosseopentaenoic acid. Under experimental conditions, production of bosseopentaenoic acid was linear up to 2 hr and dioxygen was consumed. Bosseopentaenoic acid, along with several other conjugated tetraenes, was also present in the algae endogenously as revealed by a comparison of the ultraviolet (UV) spectra and the high-performance liquid chromatographic (HPLC) pattern of the purified product and the organic extract of *Bossiella*.
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Oxidation of polyunsaturated fatty acids (PUFA) to biologically active localized hormones occurs in the majority of living systems analyzed (1). A large variety of PUFA metabolites have been identified and the number continues to grow. Some of the most unique metabolites of arachidonic acid (AA) and other PUFA have been isolated from marine invertebrates (1,2). Recently, attention has centered on the presence of eicosanoid and eicosanoid-like compounds as natural products in marine algae (3). Many of these compounds appear to be produced biosynthetically through an initial step involving a lipoxygenase (LO) enzyme (4) as evidenced by several reports on the presence of 12(S)-hydroxyeicosatetraenoic acid (12-HETE), 12(S)-hydroxyeicosapentaenoic acid, hepoxilins, and prostaglandins E_2 and $F_{2\alpha}$ in the organic extracts of several red algae species (5–8). We have recently examined the eicosanoid compounds and lipoxygenase activity present in coralline red algae (Burgess and Jacobs, manuscript in preparation). We report herein that aqueous extracts of *Bossiella orbigniana* spp. *orbigniana* Silva catalyze the oxidation of arachidonic acid, forming a previously unreported ω6 eicosapentaenoic acid product containing a conjugated tetraene. This enzyme catalyzed product, which is also present endogenously, is identified as 5(Z),8(Z),10(E),12(E),14(Z)-eicosapentaenoic acid.

*To whom correspondence should be addressed at current address: Department of Foods and Nutrition, Stone Hall, Purdue University, West Lafayette, IN 47907.

Abbreviations: AA, arachidonic acid; BPA, bosseopentaenoic acid; HPLC, high-pressure liquid chromatography; 12-HETE, 12(S)-hydroxyeicosatetraenoic acid; LO, lipoxygenase; NMR, nuclear magnetic resonance spectroscopy; PUFA, polyunsaturated fatty acid; Tris-HCl, hydroxymethylaminomethane hydrochloric acid.

MATERIALS AND METHODS

Material. Bossiella orbigniana was collected off the coast of Goleta, California, between September 1989 and March 1990. The alga was kept in flowing seawater tanks until used. Arachidonic acid (99%, porcine liver), deuterated-benzene, and Tris-hydrochloride were obtained from Sigma Chemical Co. (St. Louis, MO). Organic solvents—high-performance liquid chromatography (HPLC)-grade or better—were obtained from Fisher Scientific (Pittsburgh, PA).

Algae and product preparation. Aqueous extracts (33%) of freshly collected *B. orbigniana* were prepared by homogenization in 20 mM Tris-HCl buffer pH 8.0 followed by centrifugation at 500 × *g* to remove particulate matter. The supernatant from this centrifugation was incubated in 3 vol of 0.1 M Tris-HCl buffer pH 7.1 in the presence or absence of AA (100 μM) for up to 2 hr at 37°C. The reaction was terminated by the addition of 1N HCl to pH 3.5 and extracted with 3 vol of solvent A (heptane/2-propanol, 4:1, v/v). After separation, the organic layer was scanned in the ultraviolet (UV) range and evaporated to dryness under a stream of N_2. The dried sample was resuspended in solvent B (heptane/hexane/2-propanol/acetic acid, 499:499:1:1, by vol) and the components separated by HPLC. The HPLC separation was conducted isocratically in solvent B at a flow rate of 4 mL/min using an LC5000 liquid chromatograph (Varian Instrument Group, Sugarland, TX) on a semi-preparative silica column (Supelco, Inc., Bellefonte, PA). Purification of the enzymatically produced product was conducted by first processing the crude extract by open-column silica chromatography (2.5 × 30 cm column) in solvent C (hexane/2-propanol/acetic acid, 994:5:1, v/v/v), followed by HPLC separation and collection of the fractions.

Spectroscopic techniques. UV-visible spectroscopy was conducted with an IBM 9430 UV-VIS scanning spectrophotometer. Mass spectral analysis by FAB was conducted with a *m*-nitrobenzylalcohol matrix on a VG analytical mass spectrometer. Proton, ^{13}C and, 2D correlation spectroscopy (COSY) nuclear magnetic resonance (NMR) spectroscopy were performed on a General Electric 500 MHz NMR spectrometer.

RESULTS

Bossiella was homogenized in Tris-buffer and, following centrifugation, the UV-spectrum of the organic extract of the homogenate showed absorption maxima at 293, 306, and 321 nm (Fig. 1b) indicating the presence of a conjugated tetraene (9). When AA was incubated with the aqueous algal homogenate prior to the organic extraction, a 5-fold absorbance increase in the total conjugated tetraene was observed (Fig. 1a), with no other observable

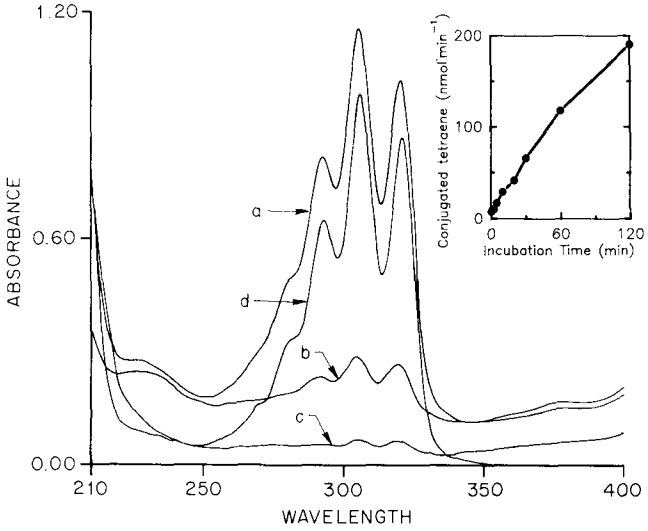


FIG. 1. UV spectrophotometric scans of organic extracts of *Bossiella orbigniana* incubations. Aqueous algal homogenates were incubated in the presence (a) or absence (b) of arachidonic acid (100 μM) and analyzed as described under Materials and Methods. (c) Heat-treatment of aqueous algal homogenate at 50°C for 30 min prior to incubation with AA eliminates conjugated tetraene formation. (d) HPLC Fraction II, produced from incubation with AA after purification by open-column silica and straight-phase HPLC chromatographies. Insert. Time course of conjugated tetraene formation in incubation of aqueous algal homogenate with arachidonic acid.

change in the UV spectrum. Product formation was roughly linear over 2 hr (Fig. 1 insert). Heat treatment of the aqueous homogenate prior to addition of arachidonic acid did not produce the conjugated tetraene (Fig. 1c), thus indicating that the conjugated tetraene is formed enzymatically from AA. When AA was incubated with the algal homogenate, oxygen was consumed as measured polarographically, which is consistent with the oxidation of AA to conjugated olefins. O_2 consumption was not observed upon prior heat-treatment of the homogenate.

Separation of the organic extract of the algal homogenate by HPLC yielded four fractions (Fig. 2A) all having the same UV-spectral pattern. Retention times were as follows: peak I, 18.0 min; peak II, 19.0 min; peak III, 20.5 min; peak IV, 22.0 min. Four fractions were also obtained when the AA incubated sample was resolved on HPLC, with an enhancement of the peak area for Fraction II (data not shown). To determine whether AA oxidation resulted in the formation of a single or multiple conjugated tetraene products, the algal homogenate was processed by repeated centrifugation and extracted with acetone (90%) to remove endogenous fatty acids. The resuspended protein pellet retained 100% of its original activity when incubated with AA. The organic extract of this incubation when injected on the HPLC resolved one major peak which eluted with the same retention time as Fraction II of the endogenous tetraene chromatogram (Fig. 2B). The two peaks co-eluted on HPLC when extracts from each sample were injected simultaneously (Fig. 2C).

Large scale preparation of the enzymatic product involved using the acetone-precipitated protein from 2 kg of algae, and 40 mg of AA. Fraction II was purified as

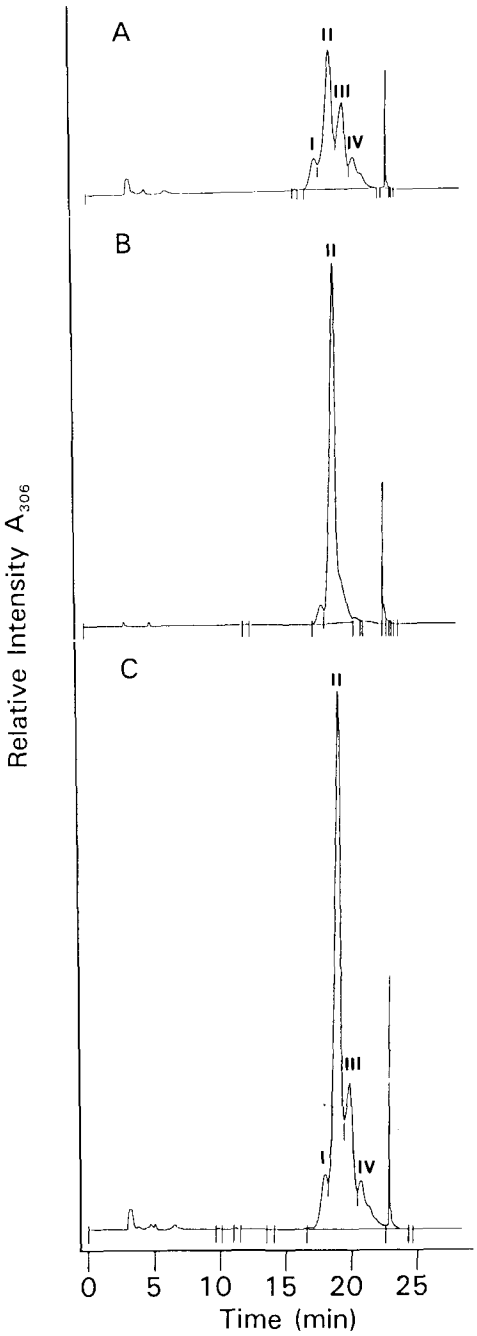


FIG. 2. HPLC chromatograms of the organic extracts of *Bossiella orbigniana* incubations. (A) Organic extract of aqueous algal homogenate prior to incubation. (B) Organic extract of incubation of AA with acetone-precipitated algal protein fraction. (C) Coinjection of extracts A and B.

a colorless oil (5.2 mg) and showed the following UV spectral pattern (solvent A) λ_{max} 293, 306, 321 (ε=50,794; 71,958; 62,698 $M^{-1}cm^{-1}$, respectively). These results were very similar to those obtained for other naturally occurring fatty acids possessing a conjugated tetraene function (9-11). FAB-Mass spectrometry of the underivatized compound gave the following (rel. intensity): 303 $[M+1]^+$ (24), 302 $[M]^+$ (51), 119 (42), 105 (48), 91 (100), 79 (72), 67 (97), 55 (95). The ^{13}C NMR spectrum showed

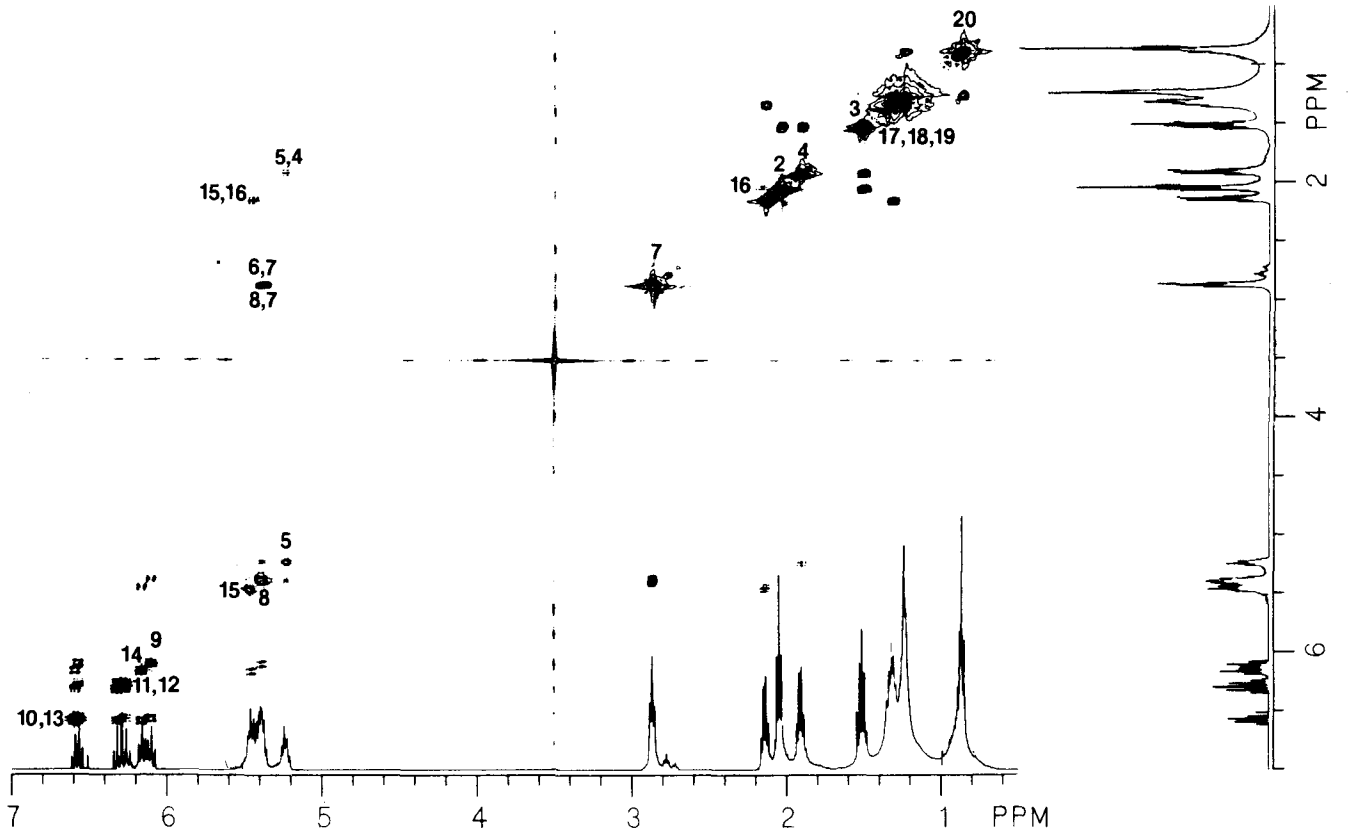


FIG. 3. ^1H-^1H correlation spectroscopy of 5(Z),8(Z),10(E),12(E),14(Z)-eicosapentaenoic acid showing key correlations between coupled protons (*ca.* 5 mg of BPA in 0.5 mL C_6D_6, 500 MHz).

10 olefinic carbon atoms, one aliphatic methyl carbon, and eight methylene carbons, indicating conversion of AA to a pentaene. The ^1H and ^{13}C NMR spectra are consistent with 10 vinylic, 2 allylic, 14 secondary, and 3 primary protons. These results yielded a molecular formula of $C_{20}H_{30}O_2$, and the compound was named bosseopentaenoic acid (BPA). The linear array of the methylene and olefinic groups of BPA and the stereochemical configuration were analyzed by ^1H-^1H 2D shift correlation spectroscopy (COSY) (Fig. 3). The chemical shift parameters and the coupling constants were assigned based on these results and are summarized in Table 1. Assignment of the *bis*-allylic methylene proton signal centered at δ 2.87 to C-7 was made based on coupling between this signal and the olefinic multiplet at δ 5.4, as well as, coupling of this multiplet with both the olefinic signal at δ 5.25 and the triplet peak centered at δ 6.1. These results confirmed the linear array of double bonds residing at positions 5,8, 10,12 and 14 of the carbon backbone. The symmetry of the COSY pattern suggested that the configuration within the conjugated tetraene was *cis,trans,trans,cis* which was confirmed by the large coupling constants (>13) obtained for carbons 10 through 13 (12).

DISCUSSION

Bosseopentaenoic acid is a unique and previously unreported polyunsaturated fatty acid. It is endogenous to *Bossiella orbigniana*, and can be enzymatically formed through the oxidation of arachidonic acid. Based on the

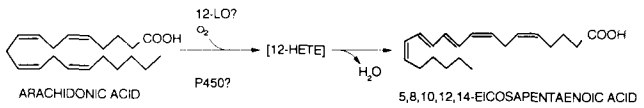
TABLE 1

NMR Data for the Eicosapentaenoic Acid Formed from Arachidonic Acid by the Aqueous Extract of *Bossiella orbigniana*[a]

C No.	^1H			^{13}C[b]
	δ	m	J(Hz)	δ
1	—	—	—	—
2	2.04	t	7.3	32.85
3	1.51	p	7.4	24.28
4	1.91	q	7.1	27.91
5	5.25	m	—	128.63
6	5.44	m	—	129.90
7	2.87	t	7.4	29.09
8	5.40	m	—	129.21
9	6.10	t	10.7	128.41
10	6.58	q	13.4,10.8	126.56
11	6.30	p	13.8,11.5	133.02
12	6.28	p	13.8,11.5	133.67
13	6.58	q	13.4,10.8	127.03
14	6.16	t	11.0	129.06
15	5.46	m	—	132.13
16	2.14	q	7.5	31.36
17	1.32	m	—	26.26
18	1.23	m	—	26.20
19	1.23	m	—	22.55
20	0.86	t	6.9	13.88

[a] Spectra of purified conjugated tetraene (Fraction II, 5 mg) obtained in C_6D_6 on a General Electric 500 MHz NMR spectrometer. Chemical shift values in ppm expressed relative to tetramethylsilane (TMS) as an internal standard.

[b] Assigned by comparison to conjugated olefin natural products, as well as, several model compounds (18-21).



SCHEME 1. Possible biosynthetic pathway for BPA formation.

observed O_2 incorporation associated with the formation of BPA, we propose a possible biosynthetic pathway from AA through a more polar intermediate such as 12-HETE involving a lipoxygenase or cytochrome P450 (13,14) enzyme in the initial step (Scheme 1). A requirement for molecular oxygen has also been observed in the biosynthesis of the naturally occurring conjugated tetraene, parinaric acid, found in seed oils of *Impatiens balsamina* (15). Furthermore, the presence of 12-LO enzymatic activity has recently been documented in a marine red alga *Gracilariopsis lemaneiformis* (16). A 12-HETE intermediate could then undergo enzymatic dehydration to form bosseopentaenoic acid. A similar dehydration of hydroxyenes has been proposed as a biosynthetic step in the formation of parinaric acid and other naturally occurring conjugated polyenoates (17). Studies are currently underway to isolate and characterize the enzyme(s) involved in the biosynthesis of BPA, and to analyze the stoichiometry of O_2 consumption.

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Dietary Supplementation with Ether-Linked Lipids and Tissue Lipid Composition

Merle L. Blank, Edgar A. Cress, Zigrida L. Smith and **Fred Snyder***
Medical Sciences Division, Oak Ridge Associated Universities, Oak Ridge, Tennessee 37831-0117

The goal of this investigation was to determine the effect of an alkylglycerol dietary supplement on the lipid composition of several major organs. Lipids from kidney, liver, and lung tissues of rats on a laboratory chow diet (controls) were compared to lipids from the same tissues of rats that had received oral supplements (300–600 mg/day) of 1-*O*-alkyl-2,3-diacetyl-*sn*-glycerol (alkyl groups were 65% 18:1 and 17% 16:1) for six days. Incorporation of the alkylglycerol into tissue lipids was indicated by both the presence of a neutral lipid in liver that had the same chromatographic migration as alkyldiacylglycerols and by a substantial increase (~150% of controls) in the octadecenyl group of the alk-1-enyl- and alkyl-glycerol side chains derived from total phospholipids of all three tissues. Compared to controls, there was a significant increase in the amount of alkylacylglycerophosphocholine in all three tissues of the alkylglycerol supplemented group. Total lipids, total phospholipid phosphorus, or the distribution of phospholipid classes (except for small differences in lung tissue) were not affected by the dietary supplement. The increase in ether lipids was offset by a corresponding decrease in the diacyl subclass in tissues from animals on the alkyldiacetylglycerol supplement. Our results indicate that the amount of ether-linked glycerolipids in rat tissues can be easily increased with dietary supplements of alkylglycerols.
***Lipids 26,* 166–169 (1991).**

Virtually all animal cells contain ether-linked aliphatic moieties as membrane lipid constituents. With few exceptions the *O*-alkyl moieties are most prominent in choline glycerolipids, whereas the *O*-alk-1-enyl chains are found primarily as ethanolamine plasmalogens. An exhaustive listing of available reports about the tissue distribution of ether-linked phospholipids in comparison to the diacyl types of lipids for tissues of various species has been compiled by Sugiura and Waku (1) and Horrocks (2). Although ether lipids were once thought to serve strictly as structural components of membranes, it is now apparent that some types of ether lipids possess biological activities. The most potent lipid mediator ever discovered is a novel ether-linked phospholipid subclass possessing the chemical structure 1-alkyl-2-acetyl-*sn*-glycero-3-phosphocholine (3–5). This phospholipid is commonly referred to as platelet-activating factor (PAF), although it elicits a wide variety of biological responses that involve many different types of cells (6–8).

Earlier studies have shown that orally administered fatty alcohols (9,10) or glycerol ethers (10–14) can be incorporated into the glycerol ether lipids of mammalian tissues. Incorporation into tissue glycerol ethers was based on either qualitative changes in composition of tissue alkyl/alk-1-enyl groups or the use of radioactive tracers. L-M fibroblasts grown in culture media supplemented with alkylglycerols were shown to contain increased amounts of glycerol ethers in their total phospholipids (15). However, except for the reported increase in the plasmalogen content of erythrocytes in one human subject (16) and no change in the plasmalogen content of rat tissues (12) after feeding a supplement of alkylglycerols, none of the earlier reports attempted to determine the quantitative levels of ether lipids found in tissues after feeding the glycerol ethers (or alcohols) to intact mammals. Because PAF can be biosynthesized from the precursor pool of 1-alkyl-2-acyl-*sn*-glycero-3-phosphocholine (alkylacyl-GPC) *via* a remodeling pathway (8), we felt that it was important to see if this precursor pool size could be increased in mammalian tissues by the oral administration of an alkylglycerol diacetate. An increase in the PAF precursor pool size could have important biological ramifications because of the multiple cellular responses produced by this autacoid.

MATERIALS AND METHODS

The dietary supplement of 1-*O*-alkyl-2,3-diacetyl-*sn*-glycerol (ADAG) used in these studies was a gift from Western Chemicals (Vancouver, Canada). Diacetyl derivatives of alkylglycerols were used as the dietary supplement in order to mimic the esterified forms of alkylglycerols that would occur in a normal diet. Analysis of the ADAG supplement by thin-layer chromatography (TLC) and high-performance liquid chromatography (HPLC) (17) revealed the supplement was >95% alkyldiacetylglycerols with the two major alkyl chains being 16:1 (17%) and 18:1 (67%); no other single species accounted for >5% of the total alkyl chains. Male CDF rats, seven weeks of age, were given 300-mg doses of carrier-free ADAG by stomach tube. The rats received one dose of ADAG on the first day of the experiment followed by two equivalent doses per day, 7 hr apart, on days two through five and a final single dose on day six. Rats were sacrificed six hours after the last dose of ADAG. Livers, lungs, and kidneys were removed and immediately frozen in liquid nitrogen until the time of lipid extraction. Both controls and the ADAG-fed rats (five animals per group) had access *(ad libitum)* to regular laboratory chow and water during the entire experimental period.

Lipids were extracted from the tissues by the method of Bligh and Dyer (18). TLC of neutral lipids was carried out on plates coated with 250 μm layers of Silica Gel G developed in a solvent system of hexane/diethyl ether/acetic acid (85:15:1, v/v/v). Alkyldiacylglycerols are easily separated from cholesterol esters, triacylglycerols, and fatty acids in this neutral lipid TLC system. Phospholipids were separated by TLC on 250 μm layers of silica gel H using a solvent system of chloroform/methanol/acetic acid/water (50:25:8:2, by vol). For preparative

*To whom correspondence should be addressed: Medical Sciences Division, Oak Ridge Associated Universities, P.O. Box 117, Oak Ridge, TN 37831-0117.

Abbreviations: ADAG, 1-*O*-alkyl-2,3-diacetyl-*sn*-glycerol; GPC, *sn*-glycero-3-phosphocholine; GPE, *sn*-glycero-3-phosphoethanolamine; HPLC, high-performance liquid chromatography; PAF, platelet-activating factor; TLC, thin-layer chromatography.

TABLE 1

Distribution of Phospholipid Classes in Selected Tissues of Controls and Rats Fed Alkyldiacetylglycerol (ADAG)[a]

Tissue	Diet	Sphingomyelin + LysoGPC	Diradyl-GPC	Phosphatidylinositol/serine	Diradyl-GPE
Kidney	control	14.5 ± 0.4	44.5 ± 0.8	9.1 ± 0.7	32.1 ± 0.6
	ADAG-fed	15.5 ± 0.4	43.0 ± 0.8	10.7 ± 0.6	30.8 ± 0.4
Liver	control	6.2 ± 0.7	61.5 ± 2.3	4.2 ± 0.8	25.7 ± 0.9
	ADAG-fed	4.9 ± 0.7	62.8 ± 0.5	4.3 ± 0.5	28.0 ± 0.8
Lung	control	11.9 ± 0.2	52.9 ± 0.6	11.6 ± 0.2	23.5 ± 0.4
	ADAG-fed	13.2 ± 0.1[b]	49.5 ± 0.4[b]	13.4 ± 0.3[b]	23.9 ± 0.6

[a] All values represent mean mole percent ± S.E. from tissues of five rats in each group, except for one lung extract sample that was lost in the control group.

[b] Values are significantly different from corresponding controls ($P < 0.01$).

purposes, the developed TLC plates were exposed to NH_3 fumes (3–4 min), then sprayed with a solution of 0.1% 2, 7-dichlorofluorescein in ethanol and viewed under ultraviolet (UV) light to locate the separated phospholipid bands. The phospholipids were extracted from the silica gel (18) for subsequent analysis. For quantitative analysis of phospholipid classes the developed TLC plates were charred with H_2SO_4 and the amount of phosphorus in the separated phospholipids was determined (19). The percentage of octadecenyl (18:1) groups in the alkyl- and alk-1-enyl-glycerol ethers derived by Vitride reduction, benzoylation, and HPLC of the total phospholipid fractions was determined by earlier methodology (17). Quantitation of the subclasses in diradyl-*sn*-glycero-3-phosphoethanolamine (GPE) and diradyl-GPC was performed *via* phospholipase C hydrolysis, benzoylation of the diradylglycerols, and HPLC of the benzoate derivatives, as previously described (20).

All data are expressed as means ± S.E. and statistical significance between groups was based on Student's t-test (only P values of <0.05 were considered significant).

RESULTS AND DISCUSSION

Except for an unexplained lower percent weight gain by rats fed the ADAG supplement (5.3 ± 1.7% *vs* 18.5 ± 0.8% for the controls), the glycerol ether supplement was well tolerated by the animals with no other visible indications of problems or morbidity. There were no significant differences in the amounts (as percent of tissue wet weight) of total lipids extracted from the tissues of the control *vs* the ADAG-fed rats (liver, 4.61 ± 0.08 *vs* 4.85 ± 0.17; kidney, 4.41 ± 0.13 *vs* 4.56 ± 0.23; and lung, 3.43 ± 0.17 *vs* 3.66 ± 0.15; for control *vs* ADAG-fed, respectively). Total tissue phospholipids (as mg P per g wet weight) were also not statistically different between the control and ADAG-supplemented animals (liver, 1.08 ± 0.06 *vs* 1.21 ± 0.04; kidney, 0.95 ± 0.05 *vs* 0.99 ± 0.01; and lung, 0.74 ± 0.03 *vs* 0.80 ± 0.03; for control *vs* ADAG-fed, respectively).

TLC analysis of the neutral lipids from the three tissues revealed livers from the ADAG-fed group contained significant amounts (estimated at 2–3% of the total lipid mass) of a lipid component having the same chromatographic mobility as alkyldiacylglycerols. This lipid component was also detected (in lesser amounts) in the lung lipids of the ADAG-fed rats but was present in no more than trace amounts in the lipids of these three tissues from control animals. These results indicate the alkylglycerol portion of the ADAG supplement was incorporated into the liver and lung lipids. Only trace amounts of this neutral lipid could be detected in the kidney lipids from both groups of rats. There was no evidence for the presence of alkyldiacetylglycerols in the TLC analysis of tissue neutral lipids from ADAG-fed rats.

The dietary supplement of ADAG had little effect on the distribution of phospholipid classes in the three rat tissues (Table 1). There were small, but statistically significant, changes noted in three of the phospholipid classes from lungs of the ADAG-fed group; sphingomyelin/lysoGPC and phosphatidylinositol/serine were increased and diradyl-GPC was decreased when compared to controls. The biological significance, if any, of these small changes is unknown. HPLC analysis of alkyl- and alk-1-enyl-glycerol dibenzoates, derived from total

TABLE 2

Effect of Dietary Supplements of Alkyldiacetylglycerol (ADAG) on the 18:1 Content of Ether Groups in the Total Phospholipid Fraction of Selected Rat Tissues[a]

Tissues and glycerol ether subclasses	% of glycerol ether chains as 18:1	
	Control	ADAG-fed
Kidney		
alk-1-enylacyl	19.2 ± 0.4	32.6 ± 0.4
alkylacyl	44.2 ± 1.2	61.1 ± 1.0
Liver		
alk-1-enylacyl	18.8 ± 2.0	38.9 ± 1.0
alkylacyl	45.2 ± 1.9	78.7 ± 3.4
Lung		
alk-1-enylacyl	14.1 ± 0.6	26.0 ± 0.1
alkylacyl	42.2 ± 1.9	64.5 ± 0.8

[a] The total phospholipid fractions from the same tissues as in Table 1 were analyzed for the composition of the aliphatic ether chains as described in Materials and Methods. All values represent the mean percent ± S.E. (n=3) and were significantly higher ($P < 0.001$) in 18:1 for all tissues from rats that were fed alkyldiacetylglycerol.

TABLE 3

Effect of Dietary Alkyldiacetylglycerol (ADAG) on the Distribution of Ether Subclasses in the Diradyl-GPE and Diradyl-GPC Phospholipids of Selected Rat Tissues[a]

Tissues and glycerol ether subclasses	% of phospholipid class			
	Control		ADAG-fed	
	GPE	GPC	GPE	GPC
Kidney				
alk-1-enylacyl	20.0 ± 1.0	2.3 ± 0.1	21.3 ± 0.9	3.9 ± 0.2[b]
alkylacyl	0.9 ± 0.1	1.7 ± 0.1	2.2 ± 0.2[b]	6.3 ± 0.2[b]
Liver				
alk-1-enylacyl	3.3 ± 0.2	0.4 ± 0.0	4.9 ± 0.2[b]	0.6 ± 0.1
alkylacyl	0.1 ± 0.0	0.3 ± 0.0	3.4 ± 0.6[b]	3.5 ± 0.5[b]
Lung				
alk-1-enylacyl	41.3 ± 1.0	1.6 ± 0.1	47.1 ± 1.1[c]	2.6 ± 0.2[c]
alkylacyl	1.1 ± 0.1	2.6 ± 0.1	2.9 ± 0.2[b]	10.1 ± 0.4[b]

[a]Ether-containing subclasses of diradyl-GPE and diradyl-GPC were determined as described in Materials and Methods from the same tissues as in Table 1. All values are means ± S.E. (n=5, except as noted in Table 1). Values for the diacyl subclasses (not shown) are the difference between 100 percent and the sum of the two ether subclasses. Values that are significantly higher than corresponding data for controls are indicated by (b) = $P < 0.001$ and (c) = $P < 0.01$. Diacyl subclasses, except for diacyl-GPE of the kidneys, were significantly lower ($P < 0.01$) in tissues of the animals fed ADAG.

phospholipids of the tissues, showed that the ether chains were highly enriched in the 18:1 moiety in rats that were fed ADAG as compared to controls (Table 2). The increased content of 18:1 in the ether-linked moieties was so dramatic that we felt it was necessary to analyze the total phospholipid fractions of tissues from only three rats of each group. Also, others have fed unlabeled alkylglycerols to rats and reported a shift in the qualitative makeup of the tissue glycerol ether groups toward the composition of the dietary alkylglycerol (10,12). In one study, only the level and composition of the alk-1-enyl group of ethanolamine plasmalogens from several different rat tissues was analyzed (12) and in the other instance the chain length composition of both *O*-alkyl and *O*-alk-1-enyl groups were analyzed but only from liver (10). Our results (Table 2) support the findings of both of these papers.

We next examined the amounts of ether-containing subclasses present in the diradyl-GPE and diradyl-GPC phospholipid classes. Except for the diradyl-GPC of lung, the amounts of diradyl-GPC and diradyl-GPE were not significantly different between the tissues of controls and those of the rats on the ADAG-supplemented diet (mg P/g wet weight times the percent of the phospholipid class, Table 1); therefore, the percent distribution of subclasses shown in Table 3 is representative of actual changes in their relative mass (Table 3). In agreement with earlier results (1,2), these three tissues from both groups of rats showed a general pattern of higher concentrations of plasmenyl (alk-1-enylacyl-) ether lipid in the GPE class, whereas the GPC class was enriched in the plasmanyl (alkylacyl-) type. Except for the alk-1-enylacyl-GPE of kidney and the alk-1-enylacyl-GPC of liver, the plasmenyl subclasses were slightly increased in the tissues as a result of dietary supplementation with ADAG. In every instance, the concentration of the alkylacyl- subclasses was significantly increased, compared to controls, in both GPC and GPE phospholipid fractions from tissues of rats that received the ADAG supplement (Table 3). The precursor of PAF in the remodeling pathway, alkylacyl-GPC, was increased by a factor of almost four-fold (kidney and lung) to ten-fold (liver) in the three tissues of the ADAG-fed rats. The increase in alkylacyl and alk-1-enylacyl subclasses of the diradyl-GPE and diradyl-GPC fractions was compensated for by decreases in the corresponding diacyl subclasses (see legend of Table 3). We are unaware of any other experiments in which attempts were made to quantitate the levels of alkylacyl glycerolipids in tissues after feeding alkylglycerols to intact mammals. The exact mechanism for incorporation of the alkylglycerol portion of the dietary ADAG into rat tissues is presently unknown. However, one possible explanation is *via* hydrolysis of the acetate groups from ADAG by intestinal and/or other tissue lipases followed by phosphorylation of the resulting alkylglycerol (21,22) to produce alkylglycerophosphate. This compound serves an intermediate in the *de novo* biosynthesis of alkylacyl-GPE, and alkylacyl-GPC (23). The increased levels of alkylacyl-GPC (the precursor of PAF) found as a result of oral administration of alkyldiacetylglycerols in the current study could have a very pronounced influence on biological responses elicited by PAF and eicosanoid mediators. Future experimentation in this project is designed to explore this possibility.

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Gas Chromatographic Analysis of Malonaldehyde and 4-Hydroxy-2-(E)-nonenal Produced from Arachidonic Acid and Linoleic Acid in a Lipid Peroxidation Model System

H. Tamura[1] and **T. Shibamoto***
Department of Environmental Toxicology, University of California, Davis, California 95616

Malonaldehyde (MA) and 4-hydroxynonenal (4-HN) formed upon oxidation with Fe^{2+}/H_2O_2 from arachidonic acid and linoleic acid, and their ethyl esters were analyzed by gas chromatography (GC). The MA and 4-HN produced were reacted with *N*-methylhydrazine (NMH) to give 1-methylpyrazole and 5(1′-hydroxyhexyl)-1-methyl-2-pyrazoline, respectively. The derivatives were analyzed by GC on a fused silica capillary column using a nitrogen-phosphorus detector. With arachidonic acid, more MA and 4-HN were formed from the ester (88 nmol/mg and 23 nmol/mg, respectively) than from the free acid (25 nmol/mg and 9 nmol/mg, respectively). In contrast, with linoleic acid, more MA and 4-HN were produced from the free acid (53 nmol/mg and 13 nmol/mg, respectively) than from the ester (39 nm/mg and 8 nmol/mg, respectively).
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It is well known that peroxidation of unsaturated fatty acids produces various carbonyl compounds. Some of these, in particular the low molecular weight aldehydes such as malonaldehyde (MA), were reported to be genotoxic (1). As a bifunctional reagent, MA can crosslink proteins or nucleotides (2), entailing the potential for carcinogenic activity (3). MA levels have also been used as diagnostic indicators of the freshness of lipid-rich foods because unsaturated fatty acids are decomposed by free radicals formed by sunlight (4), by metal ions such as Fe and Cu (5), or by lipoxygenases (6), resulting in MA formation. MA can also serve as biomarker of the hepatotoxicity of CCl_4 (7).

Recently, 4-hydroxy-2-(E)-nonenal (4-HN), which is one of the major aldehydes formed during the peroxidation of linoleic, γ-linolenic and arachidonic acids (8), has attracted attention as a cytotoxic agent (1). 4-HN inhibits various biological functions, including microsomal glucose-6-phosphatase and cytochrome P-450 activities (9), and it has been used as an indicator of lipid peroxidation in biological systems, such as rat liver microsomes (10).

Analysis of the complex mixture of carbonyl compounds formed by lipid peroxidation is rather difficult (1). The most common method of analyzing carbonyl compounds involves derivatization with 2,4-dinitrophenyl hydrazine. The hydrazones produced are then analyzed by either gas chromatography (GC) or high-performance liquid chromatography (HPLC) (8,11). However, MA does not yield a satisfactory derivative with 2,4-dinitrophenyl hydrazine (12). Recently, MA was analyzed by GC as *N*-methylhydrazine (NMH) derivative (13,14).

4-HN in biological samples, such as rat tissue or lipoprotein, was analyzed as pentafluorobenzyl oxime by gas chromatography-mass spectrometry (15,16). This method requires trimethylsilylation of the hydroxyls. In the present study, MA and 4-HN formed from arachidonic acid and linoleic acid, and their ethyl esters, in a model system were analyzed as the NMH derivatives 1-methylpyrazole (1-MP) and 5(1′-hydroxylhexyl)-1-methyl-2-pryazoline (HMP), respectively.

MATERIALS AND METHODS

Synthesis of 4-HN. 4-HN was synthesized by modification of a method reported previously (17). Magnesium (0.6 g) was added to 6 mL tetrahydrofuran (THF) in a 50-mL Erlenmeyer flask and then cooled to −5°C. Ethyl bromide (22 mmol) in 6 mL THF was added to the above solution. The viscous solution was left for 30 min at −10°C, then propargyl aldehyde diethyl acetal (21 mmol) in 6 mL THF was added. The reaction mixture was stirred with a magnetic stirrer for 2 hr at 0°C and then cooled to −10°C. Hexanal (22 mmol) in 6 mL THF was added and the reaction mixture was stirred for 4 hr at −10°C. Approximately 10 mL saturated ammonium sulfate solution was added, followed by 200 mL of distilled water to remove excess magnesium. The reaction solution was extracted with 20 mL of diethyl ether, and the extract was dried over anhydrous potassium carbonate for 12 hr.

After removal of the solvent, the reaction product 4-hydroxy-2-nonenal diethyl acetal (3.4 g) was obtained by fractional distillation *in vacuo* (*ca.* 0.1 mm Hg) at 107–112°C. 4-Hydroxy-2-nonenal diethyl acetal (2.5 g) was dissolved into 20 mL of diethyl ether, and $LiAlH_4$ (18.2 mmol) was added at −70°C over 2 hr under a nitrogen stream. The reaction solution was kept below −50°C with dry ice/acetone/acetonitrile for 5 hr to prevent formation of cyclic furanoid by-products (17). The temperature of the solution was then raised to between −20 and −30°C. After 2.5 hr, the reaction was terminated by adding ammonium sulfate at −70°C.

The reaction mixture was precipitated at room temperature, the white precipitate was washed with 20 mL of diethyl ether, and the filtrate was dried over anhydrous potassium carbonate for 12 hr. After removal of the solvent, 4-hydroxy-2-nonenal diethyl ace-

[1]Present address: Department of Bioresource Science, Kagawa University, Kagawa 761-07, Japan.
*To whom correspondence should be addressed.

Abbreviations: BHT, butylated hydroxytoluene; GC, gas chromatography; HMP, 5(1′-hydroxyhexyl)-1-methyl-2-pyrazoline; 4-HN, 4-hydroxynonenal; HPLC, high-performance liquid chromatography; MA, malonaldehyde; 1-MP, 1-methylpyrazole; NMH, *N*-methylhydrazine; THF, tetrahydrofuran, TLC, thin-layer chromatography.

TABLE 1

Spectra of 4-Hydroxynonenal (4-HN) and 5(1′-Hydroxyhexyl)-1-methyl-2-pyrazolines (HMP)

Compound	MS, *m/z* (%)	IR, cm^{-1}	NMR, δ (ppm)[a]
4-HN	M^+ = 156, 57 (100), 71 (56), 43 (45), 86 (35), 85 (32)	3650 (O-H), 2939 (C-H), 1715 (C=O), 1637 (C=C)	9.56 (1H, *d*, *J* = 8.0 Hz), 6.80 (1H, *dd*, *J* = 4.0, 15.5 Hz), 6.26 (1H, *ddd*, *J* = 1.0, 7.0, 15.5 Hz), 4.40 (1H, *m*), 2.58 (1H, *brs*), 1.4 (8H, *m*), 0.88 (3H, *brt*)
HMP (I)	M^+ = 184, 83 (100), 42 (86), 84 (52), 56 (42), 41 (41)	3647, 3534, (O-H), 3070, 2940 (C-H), 1592 (C=N)	6.75 (1H, *brs*), 3.84 (1H, *brs*), 2.93 (1H, *ddd*, *J* = 2.5, 10.0, 13.0 Hz), 2.83 (3H, *s*), 2.80 (1H, *ddd*, *J* = 1.5, 13.0, 16.5 Hz), 2.58 (1H, *ddd*, *J* = 1.8, 10.0, 16.5 Hz), 2.33 (1H, *brs*), 1.5 (2H, *m*), 1.3 (6H, *m*), 0.90 (3H, *t*, *J* = 7.5 Hz)
HMP (II)	M^+ = 184, 83 (100), 42 (85), 84 (44), 41 (41), 56 (41)	3556 (O-H), 3070, 2940 (C-H), 1590 (C=N)	6.71 (1H, *brs*), 3.53 (1H, *brs*), 3.0 (1H, *ddd*, *J* = 5.0, 10.5, 11.0 Hz), 2.90 (1H, *ddd*, *J* = 2.0, 10.5, 15.5 Hz), 2.87 (3H *s*), 2.63 (1H, *ddd*, *J* = 2.0, 10.5, 15.5 Hz), 2.21 (1H, *brd*), 1.5 (2H, *m*), 1.3 (6H, *m*), 0.90 (3H, *s*)

[a]Broadened peaks are identified by the affix *br*; *e.g.*, *brs* = broadened singlet.

tal (1.7 g) was obtained by fractional distillation *in vacuo* at 105°C. The acetal (32 mg) was added to 2 mL of 1% citric acid solution at room temperature. After 15 hr, 10 mL of saturated sodium chloride solution was added to the reaction mixture, and the mixture was extracted with three 10-mL portions of dichloromethane. Removal of the solvent yielded 18 mg of 4-HN. Spectral data are shown in Table 1.

Synthesis of standard 5(1′-hydroxyhexyl)-1-methyl-2-pyrazoline (HMP). 4-HN (100 mg) was dissolved in 2 mL of diethyl ether, and then 80 μL NMH was added. The reaction mixture was stored in a refrigerator at 4°C for 4 hr. GC analysis of the mixture indicated the presence of two products. After the solution was cooled to 4°C, a white precipitate appeared, and the solution was filtered. The filtrate contained compound I (Fig. 1) as the major component. Compound I was purified by preparative thin-layer chromatography (TLC) using hexane/ethyl acetate (1:1, v/v) as developing solvent. The precipitate was washed with cold diethyl ether and dissolved in 2 mL ethanol, which was subsequently evaporated off, yielding 20 mg of compound II (Fig. 1). Because 4-HN forms two isomers (I and II), it is necessary to use the total amount of I and II for the quantitative analysis of 4-HN. Spectral data of I and II are shown in Table 1.

Oxidation of arachidonic acid, linoleic acid, and their ethyl esters in a Fe^{2+}/H_2O_2 model system. An aqueous solution (5 mL) containing a fatty acid or its ethyl ester (1.5 mg/mL), 0.25 mmol trizma buffer (pH 7.4), 0.75 mmol potassium chloride, and sodium dodecyl sulfate (0.2%), was stirred with or without 200 μmol ferrous chloride and 200 μmol hydrogen peroxide at 37°C for 16 hr. The reaction was stopped by adding 4 ppm butylated hydroxytoluene (BHT), 40 μL NMH was added, and stirring was continued for 1 hr. The aqueous reaction mixture was extracted with 20 mL of dichloromethane using a liquid-liquid continuous extractor for 3 hr. The solution was saturated with NaCl prior to extraction to mixture emulsion formation. The extract was analyzed for MA and 4-HN by GC.

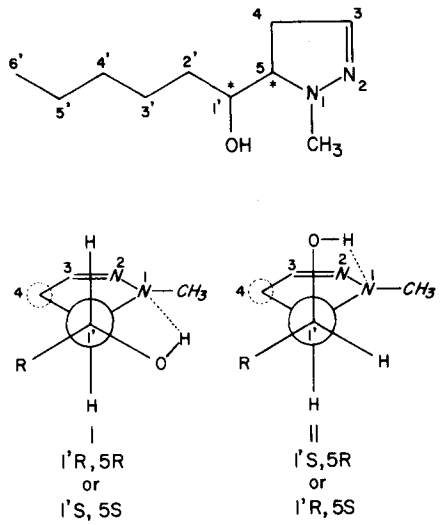


FIG. 1. Structure of 5(1′-hydroxyhexyl)-1-methyl-2-pyrazoline and the proposed configurations of diastereomers I and II.

Recovery of 1-MP and HMP. The test sample was prepared by spiking 36 nmol/mL of 1-MP and 30 nmol/mL of HMP in 5 mL aqueous solution containing the ingredients as mentioned above except for ferrous chloride and hydrogen peroxide. The spiked sample was stirred for 1 hr and then analyzed.

RESULTS AND DISCUSSION

With NMH, 4-HN yielded the isomeric pyrazoline derivatives I (55%) and II (45%). Figure 1 shows the proposed configurations for the two diastereomers. Isomer I should be more stable than isomer II. In isomer I, intramolecular hydrogen bonding between the hy-

TABLE 2

Amount of MA and 4-HN Formed from Fatty Acids and Their Ethyl Esters in a Fe^{2+}/H_2O_2 Lipid Peroxidation Model System

		Arachidonic acid				Linoleic acid			
		Free acid		Ethyl ester		Free acid		Ethyl ester	
Time (hr)	Fe^{2+}/H_2O_2	MA	4-HN	MA	4-HN	MA	4-HN	MA	4-HN
0	−	4.8 ± 2.2[a]	0.6[b]	13.0 ± 6.7	0	0.7 ± 0.2	0	0.1[b]	0
16	−	14.6 ± 9.0	3.7 ± 1.8	26.4 ± 25.1	4.9 ± 4.5	8.9 ± 1.6	2.5 ± 1.0	0.7 ± 0.1	0
16	+	27.5 ± 3.8	10.3 ± 2.2	97.2 ± 15.6	26.1 ± 12.6	57.9 ± 6.9	15.1 ± 1.5	43.1 ± 6.1	9.1 ± 2.8

[a]All values are mean ± standard deviation (n=3).
[b]n=2.

droxy hydrogen and ring nitrogen-1 would tend to rotate the bulky R group away from the carbon-4 CH_2 group of the pyrazoline ring. In contrast, in isomer II the R group would rotate toward the carbon-4 methylene causing steric hindrance. It also could be expected that isomer I has a shorter GC retention time (Kovats Index, 2009) than does isomer II (Kovats Index, 2133) on a polar GC liquid phase such as DB-WAX due to intramolecular hydrogen bonding.

The lowest detection levels of 1-MP and HMP by nitrogen phosphorus detector (NPD) were 8.9 pg and 8.4 pg, respectively; these values are equivalent to 7.8 pg of MA and 7.1 pg of 4-HN, respectively. GC detection limits were the amounts of analyte that produced peak heights more than three times the noise level. The percent recoveries of the spiked 1-MP (36 nm/mL) and HMP (30 nm/mL) standards from the test mixture were 91.0 ± 0.6 (7) and 87.5 ± 0.2, respectively. The values are the means of three replicates ± standard deviation.

The results of MA and 4-HN analyses are shown in Table 2. Figure 2 shows a typical GC of NMH derivatives of the lipid peroxidation products formed from arachidonic acid in the Fe^{2+}/H_2O_2 model system. Some MA (4–10 nmol/mg) was already present in both the fatty acids and their ethyl esters. Thus, it was previously reported that 1 g of nontreated linolenic and linoleic acids contained 57 μg and 14 μg of MA, respectively (12). A trace amount of 4-HN was also found in nonoxidized arachidonic acid but not in its ethyl ester, nor in linoleic acid or its ethyl ester. The addition of an oxidizing agent increased MA and 4-HN formation significantly.

In the present study, more MA and 4-HN were formed from ethyl arachidonate than from arachidonic acid. In contrast, more MA and 4-HN were produced from linoleic acid than from ethyl linoleate.

MA is thought to be formed from polyunsaturated fatty acids *via* a cyclic peroxide intermediate (18) produced by $^1O_2$ (19). This mechanism is consistent with the observation that a more unsaturated fatty acid produces more MA than does a less unsaturated fatty acid. The oxidation of fatty acids initiated by a hydroxy radical also produced MA in the present study. However, linoleic acid produced more MA than did arachidonic acid. The results suggest that alternate pathways of MA formation do exist.

There has been no prior report on the simultaneous analysis of MA and 4-HN. The gas chromatographic method developed in the present study gives higher resolution and sensitivity than does the HPLC method, and it may be applied to multiple analyses of reactive carbonyl compounds formed in various biological systems.

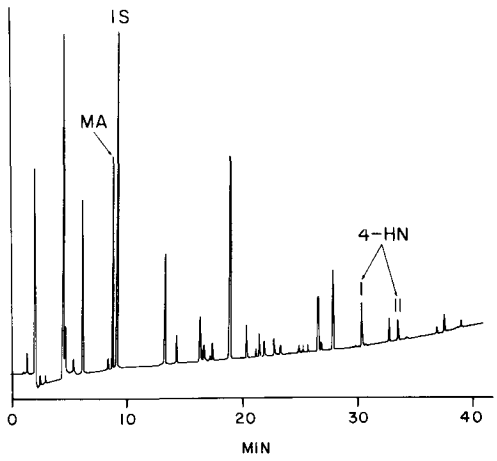


FIG. 2. Typical gas chromatogram of a mixture of *N*-methylhydrazine derivatives obtained from the products formed by arachidonic acid oxidation with Fe^{2+}/H_2O_2. MA, malonaldehyde; IS, internal standard (2-methylpyrazine); 4-HN, 4-hydroxynonenal.

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ERRATUM

"Squalene in Grapefruit Wax as a Possible Natural Protectant Against Chilling Injury" by Harold E. Nordby and Roy E. McDonald, *Lipids 25*, 807-809, 1990. The correct figure captions to appear on page 809 are FIG. 1. Cumulative profiles of squalene and of percent sound fruit from six temperature conditioning studies throughout the grapefruit season. FIG. 2. Squalene in epicuticular wax of October 1988 grapefruit temperature conditioned at 15° for 1 to 21 days and for grapefruit "conditioned" 7 days at 1, 5, 10, and 21°C. FIG. 3. Squalene in epicuticular wax of February 1989 grapefruit stored for 1 to 21 days at 5°C (nonconditioned) and for fruit stored 1 to 21 days at 15°C (conditioned).

Effect of Essential Fatty Acid Deficiency on Lipid Composition of Basolateral Plasma Membrane of Pig Intestinal Mucosal Cells

Véronique Duranthon, Lucie Frémont* and **Claude L. Léger**
Laboratoire de Nutrition et Securité Alimentaire, CRJ-INRA, Domaine de Vilvert, 78352 Jouy-en-Josas Cedex, France

The effect of essential fatty acid (EFA) deficiency on the lipid composition of basolateral plasma membranes (BPM) from intestinal mucosal cells was investigated in weaning pigs fed control or EFA-deficient diets for 12 weeks. The phospholipid and cholesterol contents relative to protein were similar in both groups, showing a cholesterol/phospholipid molar ratio of 0.6. The distribution of phospholipid classes was also unaffected by the diet. In contrast, fatty acid profiles of the two phospholipid main classes, phosphatidylcholine and phosphatidylethanolamine were altered by EFA deficiency. Linoleic acid (18:2n-6) was largely reduced, whereas arachidonic acid (20:4n-6) only slightly decreased in EFA-deficient pigs. The unsaturation index was essentially maintained by high levels of oleic acid (18:1n-9) and by conversion of oleic acid to 5,8,11-eicosatrienoic acid (20:3n-9). Finally, during the period of EFA deficiency, the lipid composition of BPM of the intestinal mucosal cells was little affected, suggesting a preferential uptake of 20:4n-6 and (or) precursor mobilized from other tissues. However, an effect of dietary treatment on the function of membrane-associated proteins cannot be ruled out.
Lipids 26, 175–181 (1991).

Evidence exists that the lipid matrix structure of a cell membrane influences the biological activities of intrinsic membrane proteins and that dietary lipids can change the fatty acid composition and modulate membrane function (1–3). The effect depends on the cell type, the subcellular membrane fraction and possibly the membrane domain. In the intestinal mucosal cell plasma membrane, the basolateral and brush-border regions differ in morphology, composition and function (4). The phospholipid and cholesterol patterns of the two regions have been described for rats (5–7) and other animals (6), and it is being recognized that large species differences do exist. Several reports have shown that changes in the lipid composition of the intestinal mucosal cell plasma membrane induced by dietary fats alter the physicochemical properties and biological functions in both plasma membrane regions (8–11).

The difficulty of carrying out such studies in humans implies the necessity of finding appropriate experimental animal models. As assessed by numerous reports (12,13), the pig is considered a well fitted model because its gastrointestinal system and pathways of lipid metabolism resemble those of humans. Moreover, the piglet exhibits biochemical and physiological similarities to man during the perinatal period (14).

Recent studies in our laboratory have focused on the effect of an essential fatty acid (EFA)-deficient diet on lipid composition and lipid-protein interactions in pig brush-border membrane vesicles (15,16). In pigs, the basolateral region has been investigated mainly for proteins and peptides (17), but little is known about the lipid composition. Therefore, the present study was undertaken to characterize the basolateral plasma membrane (BPM) and to determine the influence of EFA deficiency on lipid distribution and fatty acid composition of phospholipid classes.

*To whom correspondence should be addressed.

Abbreviations: BPM, basolateral plasma membrane; EFA, essential fatty acids; PC, phosphatidylcholine; PE, phosphatidylethanolamine; PUFA, polyunsaturated fatty acids; SDS, sodium dodecylsulfate; SDS-PAGE, SDS-polyacrylamide gel electrophoresis; UNID, unsaturation index.

METHODS

Animals and diets. Eight male piglets of the Large-White breed were divided into two groups just after weaning (10-days-old). They were fed a semi-purified diet (INRA Institute, Jouy-en-Josas, France) *ad libitum,* containing 7% (w/w) lipids, in the form of either corn oil plus soybean oil mixture (control diet) or hydrogenated coconut oil (EFA-deficient diet). The composition of diets is given in Table 1.

Piglets were maintained on the experimental diets for 12 weeks and sacrificed after an 18-hr fast. The small intestine was rapidly removed, everted, washed with cold saline and immediately used for membrane preparation.

Membrane preparation. The isolation procedures were similar for piglets of both groups. Jejunal segments, free of mucus, were scraped and mucosal scrapings suspended

TABLE 1

Composition of Experimental Diets[a]

Fatty acids % total	Control diet	EFA-deficient diet
8:0		1.2
10:0		4.2
12:0		46.5
14:0	2.1	20.2
16:0	11.3	11.5
18:0	2.8	14.7
18:1n-9	20.4	0.8
18:2n-6	59.0	0.7
18:3n-3	4.4	0.2
Saturated	16.2	98.3
Monounsaturated	20.4	0.8
Polyunsaturated	63.4	0.9

[a]Fats were added to the basal diet in the following proportions: 35 g corn oil plus 35 g soybean oil (control diet); 70 g hydrogenated coconut oil (EFA-deficient diet) per kg of diet. The basal diet contained the following components (g/kg diet): potato starch, 300; cassava meal, 250; casein, 200; cellulose, 100; molasses, 36.5; dicalcium phosphate, 17; calcium carbonate, 17; sodium chloride, 4.5; and trace mineral and vitamin mixture, 5. Mineral and vitamin mixture (in mg/kg diet): $ZnSO_4 \cdot 7\ H_2O$, 44; $MnSO_4 \cdot H_2O$, 6; $CuSO_4 \cdot 5\ H_2O$, 3; KI, 0.1; $CoSO_4$, 0.1; Na_2Se, 0.1; vitamin A (500.000 I.U./g), 1.3; vitamin D_3 (100.000 I.U./g), 1.3; vitamin E (500 I.U./g), 2.5; vitamin K_3, 0.3; thiamin, 0.1; riboflavin, 0.5; pyridoxine hydrochloride, 0.1; nicotinic acid, 1.9; folic acid, 0.1; vitamin B_{12}, 5.10^{-3}; biotin, 25.10^{-3}; calcium pantothenate, 1.3; and choline, 63.

in 4 vol (w/v) of an ice-cold buffer consisting of 250 mM sucrose, 3 mM $MgCl_2$, 10 mM Tris-HCl (pH 7.4). All buffers contained 0.1 mM phenylmethylsulfonyl fluoride. The pooled suspension was homogenized using a motor-driven glass-Teflon homogenizer with twelve down and up strokes at 1500 rpm. The homogenate was then filtered through nylon gauze (150 μm) and diluted 1:1 with the cold 250 mM sucrose buffer. The procedure of purification was a modification to that reported by Scalera *et al.* (18) and del Castillo and Robinson (19) involving a Percoll (Pharmacia, Uppsala, Sweden) gradient. The crude homogenate was centrifuged at 300 × *g* for 10 min (MSE "high speed" 25 centrifuge, Crawley Sussex, U.K., 59582 angle rotor). The pellet, P_1, was discarded and the supernatant, S_1, centrifuged at 900 × *g* for 5 min to eliminate nuclei (pellet P_2). The supernatant fraction, S_2, was centrifuged at 18000 × *g* for 25 min to give a double pellet consisting of a lower layer (P_3) and a white, fluffy upper layer. The upper layer was gently collected, resuspended in 250 mM sucrose, 10 mM Tris-HCl (pH 7.4) and homogenized with six strokes of the glass-Teflon pestle. The fraction was mixed with Percoll and 500 mM sucrose, 10 mM Tris-HCl (pH 7.4) to a final concentration of Percoll 11%, 250 mM sucrose (initial density 1.046). Fractions of 25 mL of the mixture were centrifuged at 48000 × *g* for 40 min (Beckman ultracentrifuge, 50.2 Ti fixed angle rotor) to form a self-orientating continuous gradient. It was separated into 1-mL fractions numbered 1 to 25, from the bottom to the top, with a slow-rate peristaltic pump. The material-rich fractions 3–5 and 15–17 were named Layer 2 (L_2) and Layer 1 (L_1), respectively. They were diluted with 10 mM Tris-HCl buffer (pH 7.4) and centrifuged three times for 40 min at 50000 × *g* to remove the Percoll particles. Membranes were finally resuspended in 10 mM Tris-HCl buffer (pH 7.4) and immediately tested for yield and contamination. They were stored at −80°C until analysis.

The purity of fractions L_1 and L_2 was assayed using $(Na^+ + K^+)$-ATPase activity as a specific marker of BPM (20–23). Contamination with brush-border membrane and mitochondrial membrane was tested, estimating the marker enzymes of these organelles, namely sucrase (24) and succinic dehydrogenase (25).

Enzyme assays. Sucrase activity was measured by the method of Dahlquist (26). Succinic dehydrogenase activity was determined as described by Green *et al.* (25). The $(Na^+ + K^+)$-ATPase activity was considered as the ouabain-sensitive ATPase activity measured in the presence of Mg^{2+}, Na^+, K^+ and 1 mM ouabain (20). Phosphorus liberated from Na_2-ATP was measured by the method of Baginski *et al.* (27). Just before incubation, the samples were pretreated with 0.06% sodium deoxycholate, 1 mM EDTA (final concentrations) for 30 min at 20°C in order to open closed vesicles (19). Protein was determined by the method of Lowry *et al.* (28), using bovine serum albumin as a standard.

SDS-polyacrylamide gel electrophoresis. Membrane proteins were separated by slab gel electrophoresis on a linear gradient (3–15%) of polyacrylamide in 375 mM TRIS-HCl (pH 8.8) containing 0.1% SDS according to the method of Laemmli (29). Aliquots of membrane preparations (fraction L_1) were solubilized in the gel buffer containing 2% SDS, 10% glycerol, 80 mM dithiothreitol and traces of bromophenol blue. The proteins were dissociated by immersing the samples for 3 min in boiling water. The gels were run at 40 mA for 3–4 hr and the proteins stained with Coomassie blue R 250. Molecular weight markers (MW-SDS-200 Kit, Sigma Chemical Co., St. Louis, MO) were run as well.

Lipid analysis. Total lipids were extracted from BPM using $CHCl_3$/MeOH (2:1, v/v) (30). Phospholipids were separated from neutral lipids and glycolipids on a silica cartridge (SEP-PACK, Waters Associates, Milford, MA) by successive elutions with chloroform, acetone and methanol (31,32). Phospholipids were estimated by the amount of lipid phosphorus (33). Cholesterol was measured in the neutral lipids by an enzymatic method (Boehringer Mannheim, Meylan, France). Phospholipid classes were separated by high-performance liquid chromatography (34). The equipment used was a gradient liquid chromatograph (Beckman, Model 332) with a detector (UVICORD 2158 5D, LKB) set at 206 nm. The separation was done on a column (250 × 10 mm) packed with silica (5 μm Ultrasphere-Si). The mobile phases were: Solvent A, n-hexane/2-propanol/water (6:8:0.75, v/v/v), and Solvent B, n-hexane/2-propanol/water (6:8:1.4, v/v/v), at a flow rate of 2.5 mL/min. The column was eluted for 10 min with Solvent A. This was followed by a linear gradient of Solvent B in Solvent A developed from 0 to 100% over 10 min. In these conditions phospholipid classes were separated within 75 min. The percentages of phospholipid classes were estimated by the phosphorus content (33).

The fatty acid distribution in phospholipid classes was determined by gas-liquid chromatography of the fatty acid methyl esters obtained after transmethylation (35). A Packard Model 427 (Packard, Rungis, France) gas chromatograph was equipped with a flame ionization detector and a CP Wax 52 CB bonded fused silica capillary column (50 m × 0.2 mm internal diameter). Hydrogen was the carrier gas, at a flow rate of 1 mL/min. The injector and detector were set at 260 and 300°C, respectively, and the oven temperature was scheduled from 140 to 225°C at a rate of 4°C/min. Peaks were identified by comparing their equivalent chain lengths with those of authentic fatty acid methyl esters. Peak areas and automatic expression of data were obtained by an integrator (Enica 10, Delsi Instr., Surenes, France) coupled with a microcomputer.

Statistics. Results are presented as means ± S.D. Comparisons between groups were made by the Student's *t*-test. Values of $p<0.05$ were considered statistically different.

RESULTS AND DISCUSSION

Purity of BPM-preparation. The distribution of markers in the membrane fractions of control pigs isolated on the Percoll gradient showed that the two layers, L_1 and L_2, were enriched in BPM (Table 2). Fraction L_1 (mean density 1.03) appeared as the most enriched in $(Na^+ + K^+)$-ATPase relative to the initial homogenate. A twelve-fold increase in specific activity was found, whereas the sucrase specific activity remained close to that of the homogenate, revealing a minor contamination by the brush-border. Moreover, L_1 was free of any significant contamination by mitochondrial membranes as assessed by the enrichment factor of 0.6 for succinic

TABLE 2

Marker Enzyme Activities in Crude Homogenate and BPM-enriched Fractions (L_1 and L_2) of Intestinal Mucosal Cells from Pigs Fed Either a Control or an EFA-Deficient Diet

		Ouabaine-sensitive $(Na^+ + K^+)$-ATPase		Sucrase		Succinic dehydrogenase	
		Control	EFA-deficient	Control	EFA-deficient	Control	EFA-deficient
Homogenate	Specific activity[a]	22 ± 2	17 ± 2*	25 ± 3	23 ± 2	24 ± 5	16 ± 3
	Enrichment factor[b]	1	1	1	1	1	1
Membrane fraction L_1	Specific activity	257 ± 29	181 ± 21**	33 ± 4	28 ± 3	14 ± 1.5	13 ± 1.5
	Enrichment factor	11.7	10.6	1.3	1.2	0.6	0.8
	Recovery[c]	1.7	1.5	0.19	0.17	0.09	0.14
Membrane fraction L_2	Specific activity	145 ± 13	107 ± 12**	115 ± 13	101 ± 14	N.D.[d]	N.D.
	Enrichment factor	6.6	6.3	4.6	4.4	N.D.	N.D.
	Recovery	2.9	2.5	2.2	2	N.D.	N.D.

[a] Specific activities are expressed as nmol per mg protein per min (mean ± S.D. for four animals). Values significantly different from controls at *, $p<0.05$; **, $p<0.01$.

[b] Enrichment factor is the ratio of the specific activity of the membrane fraction to that of the homogenate.

[c] Recovery is the percentage of total activity of the membrane fraction relative to the homogenate.

[d] Not determined.

dehydrogenase. Fraction L_2, enriched in sucrase activity by a factor of 4.6, was discarded.

The $(Na^+ + K^+)$-ATPase specific activities measured in fractions isolated from EFA-deficient pigs were slightly lower than those measured in control pigs. This finding agrees with a previous study showing that $(Na^+ + K^+)$-ATPase specific activity was lower in BPM from rats on a saturated as compared to an unsaturated diet (8). The fact that homogenates from EFA-deficient pigs also exhibited a reduced specific activity indicates that the deficient material had not been damaged during the process of isolation as reported in the case of mitochondria (36,37). Hence, the difference in the enzyme activity might be related to changes in the membrane function induced by the diet. Accordingly, we considered that a similar degree of purification was achieved with membranes from both EFA-deficient and control pigs. Thus, fraction L_1 of either group was satisfactorily enriched in BPM, practically free of mitochondria and minimally contaminated by brush-border membranes.

SDS-PAGE of BPM proteins. The protein populations of BPM are presented in Figure 1. They show that the protein patterns were similar in preparations obtained from EFA-deficient pigs and from control pigs.

Several bands were observed with apparent molecular weights ranging from 100 to 29 kDa. The major band had a molecular weight of 46 kDa. Four secondary bands with higher molecular weights were found (56, 70, 78, 89 kDa). The fastest components consisted of two closed bands (35 and 37 kDa).

When compared to profiles previously described for the same region of the plasma membrane, the present protein pattern differed from that of mouse small intestine cells (38), but it resembled that of rat colonic epithelial cells (39).

Lipid composition of BPM. The lipid composition in fraction L_1 is given in Table 3. It reveals that phospholipid and cholesterol contents relative to protein were not significantly influenced by the dietary regimen. The cholesterol/phospholipid molar ratio of about 0.6 was

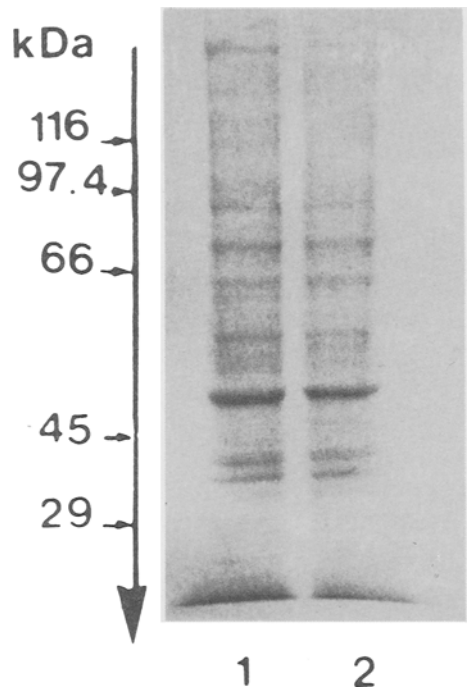


FIG. 1. SDS-PAGE of proteins from pig intestinal mucosal cell BPM. Lane 1 represents BPM from control pigs. Lane 2 represents BPM from EFA-deficient pigs. See text for experimental details. The position of molecular weight standards are shown to the left. The standards were: carbonic anhydrase, 29 kDa; albumin, egg 45 kDa; bovine albumin, 60 kDa; phosphorylase b, 97.4 kDa; and β galactosidase, 116 kDa.

in the same range as that found in rat BPM (5,40) although a higher ratio had been reported by others (7). The absence of any significant change in the lipid distribution due to the dietary fats was also observed by Brasitus *et al.* (8) in rat intestinal BPM.

The distribution of phospholipid classes did not differ in the two groups of pigs (Table 4). Phosphatidylcholine

TABLE 3

Lipid Composition of Intestinal Mucosal Cell BPM from Pigs Fed Either a Control or an EFA-Deficient Diet[a]

	Control	EFA-deficient
Phospholipid phosphorus (μg/mg protein)	15.0 ± 2.5	12.9 ± 0.8
Cholesterol (mol/mg protein)	0.28 ± 0.04	0.24 ± 0.03
Cholesterol/phospholipid (molar ratio)	0.59	0.60

[a]Values are means ± S.D. (four animals per group with two or three membrane preparations for each pig). There were no significant differences due to diet.

TABLE 4

Phospholipid Class Distribution of Intestinal Mucosal Cell BPM from Pigs Fed Either a Control or an EFA-Deficient Diet[a]

Phospholipid classes	Control	EFA-deficient
Phosphatidylcholine	45.8 ± 2.1	44.2 ± 3.3
Phosphatidylethanolamine	30.5 ± 1.9	31.1 ± 1.2
Phosphatidylserine	8.6 ± 0.9	9.1 ± 1.6
Sphingomyelin	6.9 ± 1.2	7.5 ± 0.9
Phosphatidylinositol	5.8 ± 1.6	5.8 ± 1.6
Lysophosphatidylcholine	1.1 ± 0.5	1.4 ± 0.9
Diphosphatidylglycerol	1.1 ± 0.4	0.8 ± 0.1

[a]Individual phospholipids are expressed as percentage (w/w) of total phospholipid phosphorus. Values are means ± S.D. (four animals per group) with two or three membrane preparations for each pig. There were no significant differences due to diet.

(PC) and phosphatidylethanolamine (PE) accounted for 45 and 31%, respectively, of total phospholipid phosphorus. They were also found as major classes in rat BPM (7), but according to another study on the same species, sphingomyelin was preponderant (8). In the pig intestinal mucosal cell, only the brush-border membrane has been investigated. In this membrane, a recent study (16) showed that the cholesterol/phospholipid ratio, as well as the phospholipid class distribution, were unchanged by dietary fats. Diets were similar to those we used but the experimental period only lasted six weeks after weaning.

The fatty acid distribution in the two main classes of BPM phospholipids is shown in Table 5. The major saturated fatty acids were 16:0 and 18:0, the sum of which

TABLE 5

Fatty Acid Composition of the Major Phospholipid Classes of Intestinal Mucosal Cell BPM from Pigs Fed Either a Control or an EFA-Deficient Diet for 12 Weeks Post-weaning[a]

	Phosphatidylcholine		Phosphatidylethanolamine	
Fatty acids	Control	EFA-deficient	Control	EFA-deficient
14:0	0.6 ± 0.48	2.7 ± 0.53**	1.0 ± 0.84	2.2 ± 1.57
16:0	34.1 ± 3.40	31.8 ± 1.61	10.8 ± 1.40	11.8 ± 3.19
16:1	1.8 ± 0.35	4.0 ± 0.79*	2.1 ± 0.84	5.4 ± 1.24*
18:0	14.1 ± 1.13	12.9 ± 0.94	17.5 ± 1.97	15.8 ± 0.85
18:1n-9	13.6 ± 0.68	28.5 ± 1.40***	12.8 ± 0.18	21.3 ± 0.92***
18:1n-7	1.6 ± 0.08	2.8 ± 0.15***	1.9 ± 0.10	1.4 ± 0.17*
18:2n-6	21.5 ± 2.82	4.0 ± 0.53**	21.6 ± 0.91	6.7 ± 0.18***
18:4n-3	—	—	1.4 ± 0.40	1.4 ± 0.28
20:0	1.1 ± 0.25	1.0 ± 0.08	0.9 ± 0.12	0.9 ± 0.23
20:1n-9	0.3 ± 0.06	0.3 ± 0.01	0.5 ± 0.06	0.4 ± 0.04
20:2n-6	1.1 ± 0.17	—	1.0 ± 0.15	—
20:3n-9	0.4 ± 0.05	2.9 ± 0.36***	0.7 ± 0.17	10.1 ± 2.24**
20:3n-6	0.4 ± 0.02	0.4 ± 0.02	0.7 ± 0.08	0.6 ± 0.08
20:4n-6	3.9 ± 0.57	2.7 ± 0.19*	16.1 ± 2.38	12.9 ± 1.90
20:5n-3	0.2 ± 0.05	0.4 ± 0.06*	0.4 ± 0.04	1.3 ± 0.17**
22:0	1.2 ± 0.13	1.4 ± 0.12	0.8 ± 0.09	0.8 ± 0.19
22:1n-9	0.3 ± 0.16	0.2 ± 0.03	0.3 ± 0.07	0.2 ± 0.07
22:4n-6	0.5 ± 0.20	0.2 ± 0.02	4.3 ± 0.70	1.6 ± 0.25**
22:5n-3	0.4 ± 0.09	0.4 ± 0.09	2.6 ± 0.35	2.1 ± 0.34
22:6n-3	0.3 ± 0.26	0.3 ± 0.05	0.9 ± 0.17	1.4 ± 0.19*
24:0	1.6 ± 0.2	1.7 ± 0.24	1.0 ± 0.13	1.0 ± 0.19
24:1n-9	1.3 ± 0.23	1.4 ± 0.23	0.6 ± 0.15	0.7 ± 0.14
Saturated	52.7 ± 3.5	51.5 ± 1.1	32.0 ± 3.4	32.6 ± 4.9
Monounsaturated	18.9 ± 0.4	37.2 ± 2.2***	18.2 ± 0.6	29.4 ± 0.9***
Polyunsaturated	28.4 ± 3.1	11.0 ± 0.6**	49.8 ± 3.9	38.0 ± 5.3*
20:3n-9/20:4n-6	0.09 ± 0.01	1.09 ± 0.20**	0.05 ± 0.01	0.79 ± 0.17**
UNID	88 ± 7.9	71 ± 1.7*	175 ± 15.4	164 ± 17.5

[a]Values are expressed as a percentage (w/w) of the total fatty acids and are means ± S.D. for four piglets. Values significantly different from controls at *, $p<0.05$, **, $p<0.01$; ***, $p<0.001$.

Unsaturation Index (UNID) = Σ (relative percentage of each unsaturated fatty acid times the number of double bonds for that particular fatty acid).

represented over 45% of total fatty acids in PC and 25% in PE regardless of the diet.

With the control diet, PC and PE contained similar levels of 18:1n-9 (about 13%) and 18:2n-6 (about 21%). By contrast, 20:4 only represented 3.9% in PC, whereas it accounted for 16.1% in PE. The proportions of 22:4 were 0.5% in PC and 4.3% in PE. A higher content of 20:4 in PE than in PC was also found in other fractions of intestinal mucosal cells, namely rat microsomal (41) and pig brush-border (16) membranes.

EFA deficiency altered the polyunsaturated fatty acid (PUFA) levels in BPM phospholipids. However, all fatty acids were not equally affected. The percentage of 18:2n-6 decreased by 82% in PC ($p<0.01$) and 69% in PE ($P<0.001$), whereas 20:4 was minimally reduced in both classes. It decreased 1.4 times ($p<0.05$) in PC and 1.2 times (non-significant) in PE.

Preliminary data revealed that after a six-week-post-weaning regimen, EFA deficiency already reduced the BPM levels of 18:2, but not 20:4 (Frémont, L., and Duranthon, V., unpublished data). The influence of dietary fats on phospholipids of the intestinal mucosal cells plasma membrane without changing the 20:4 levels has already been reported. In both PC and PE, the 20:4 percentages were also unaffected in pig brush-border membranes (16) and in rat plasma membranes (1) with a saturated diet. Nevertheless, Brasitus *et al.* (8) found a 50% decrease of 20:4 in the intestinal BPM of rats fed a saturated as compared to an unsaturated regimen. This finding could be due to the high lipid content of the diets (37%).

In both classes of phospholipids, EFA deficiency caused an approximately two-fold increase in 18:1n-9. Moreover, 20:3n-9 present in very small amounts in BPM phospholipids of control pigs accounted for 10.1% in PE and 2.9% in PC of EFA-deficient pigs.

The 20:3n-9/20:4n-6 ratio which expresses EFA deficiency (42) reached 1.1 in PC and 0.8 in PE, whereas it remained lower than 0.1 in control pigs. A similar but smaller effect was also observed in the pig brush-border membrane of intestinal mucosal cells after a six-week feeding of an EFA-deficient diet (16).

The essential fatty acids of the n-3 series were 20:5, 22:5 and 22:6. With the control diet, the overall content represented about 1% of total fatty acids in PC and 4% in PE. The EFA-deficient diet did not reduce the level of these fatty acids. Thus, the percentages were similar or even slightly higher (20:5 in PC and PE, 22:6 in PE). These findings show that PUFA with 20 and 22 carbons of both series, n-6 and n-3, were more incorporated in PE than in PC and that PE was also less affected by EFA deficiency than PC.

The unsaturation index (UNID) was higher in PE than in PC and with the EFA-deficient diet the value remained practically unchanged in PE and was minimally reduced in PC. The higher unsaturation of PE as compared to PC has been found in other tissues (3). The maintenance of n-3 PUFA and the partial replacement of n-6 PUFA by 18:1n-9 and 20:3n-9 levels might contribute to preserving the fluidity of the BPM. However, the position of the double bond largely influences the membrane properties (3). Hence, changes similar to those observed in our experiment in fatty acid profile of pig brush-border membranes by EFA deficiency were found to be associated with a low but significant decrease in lipid fluidity (16). Moreover, a harmful effect of 20:3n-9 cannot be ruled out (43).

The influence of dietary lipids on the fatty acid composition of BPM and serum phospholipids is shown in Figure 2. It is clear that with the EFA-deficient diet, the 20:4 levels remained unchanged in BPM, whereas it decreased by 65% ($p<0.01$) in the serum. On the contrary, the precursor 18:2 decreased in the same proportions in both BPM and serum (70%, $p<0.001$ *vs.* 73%, $p<0.01$).

It is well known that the conversion of 18:2 into 20:4 takes place mainly in the liver; however, the existence of a Δ6 desaturase activity has been described in rat intestinal mucosa (44). Assuming that such an activity also exists in pig intestinal mucosa, a fraction of 18:2 previously incorporated into the tissue could be converted into 20:4 *in situ*.

Endogenous PUFA are provided to the intestinal mucosal cell by reabsorbed bile and by circulating lipids mainly incorporated in lipoproteins. Serum lipoproteins might play a functional role in the regulation of certain cellular processes in maintaining membrane lipid composition (45,46). The authors suggest the existence of intact phospholipid molecule exchanges between lipoproteins and the cell plasma membrane. Moreover, lipoprotein binding sites have been described in rat (47,48) and human

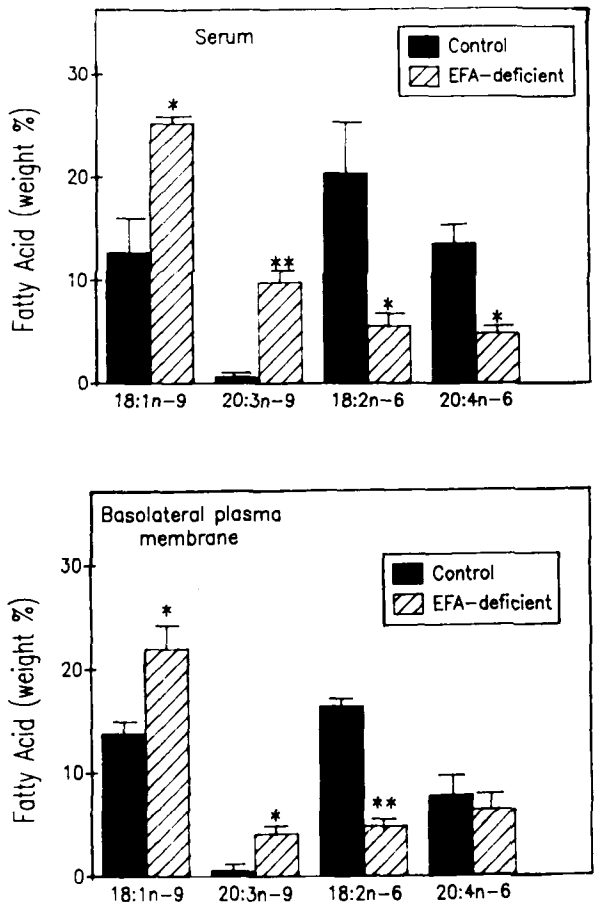


FIG. 2. Incorporation of fatty acids of the series n-9 and n-6 into phospholipids isolated from serum and intestinal mucosal cell BPM from pigs fed either a control or an EFA-deficient diet for 12 weeks post-weaning. Values significantly different from controls at *, $p<0.01$; **, $p<0.001$.

(49) intestinal plasma membrane. Binding has only been observed on BPM.

The intestinal mucosal cell has a rapid rate of turnover and in the present experiment, piglets were in a phase of intensive growth. Therefore, the demand of PUFA for the formation and replacement of structural phospholipids was very high. In the EFA-deficient group, these fatty acids were of maternal origin since pigs began to receive experimental diets just at weaning.

The placental transfer of 18:2 and 20:4 from the maternal blood to the fetus has been described in many species (50–52). In pigs, only trace amounts of free fatty acids cross the placental tissues (52,53), but the fetal piglet is capable of elongating and desaturating 18:2 into 20:4 (54). The newborn pig has very little body fat (1–2%), which contains 5–8% 18:2 and 5–6% 20:4 (55,56). For one or two days after birth, suckling pigs ingest colostrum containing 20–25% lipids (dry matter), in which 18:2 and 20:4 account for 12–18 and 1%, respectively, of total fatty acids (55,57). Afterwards, the sow's milk supplies very high fat levels (40% of dry matter) with 16% 18:2 and 1% 20:4 (56). It has been reported (58) that the lipid percentage of the total body increased eight-fold within a week after birth. At seven days, the backfat contained 11% 18:2 and traces of 20:4, whereas muscle and liver contained 12% 18:2 and 2–9% 20:4. Accordingly, one may assume that the PUFA reserves were still low when the diet treatment started.

The present findings show that the intestinal mucosal cell BPM of piglets is relatively resistant to a 12-week EFA deficiency. It therefore seems likely that PUFA previously incorporated into other tissues are preferentially mobilized for the synthesis of intestinal BPM. It has been suggested that the intestine might have a higher priority than certain organs, because of its role in the transport of nutrients between the environment and the body (59). However, EFA deficiency alters the structure and function of the jejunal epithelial cells impairing fat absorption (59,60).

During the period of dietary EFA deficiency, the uptake by BPM of endogenous PUFA vehicled by plasma lipoproteins depends on the efficiency of their mutual interactions. EFA deficiency modifies the lipoprotein profile and lipid supply to the cells (61). Thus, changes in the conformation of the particles might influence the transfer of PUFA to the intestinal mucosal cell. Moreover, it has been suggested that lipoprotein binding sites are protected against any enzyme attack by the membrane phospholipids in which they are embedded (62). It may thus be hypothesized that changes of fatty acid distribution in BPM phospholipids induced by EFA deficiency influence the binding activity of BPM specific sites for plasma lipoproteins. A reduced activity might impair the supply of endogenous PUFA for the synthesis of the other intestinal mucosal cell membranes.

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Effect of Magnesium Deficiency on Post-Heparin Lipase Activity and Tissue Lipoprotein Lipase in the Rat

Y. Rayssiguier[a,*], L. Noé[b], J. Etienne[b], E. Gueux[a], P. Cardot[a] and A. Mazur[a]

[a]Laboratoire des Maladies Métaboliques, Institut National de la Recherche Agronomique, INRA, Theix F-63122 Ceyrat and [b]Laboratoire de Biochimie, CHU Saint-Antoine F-75571 Paris, France

Previous studies have provided evidence that Mg deficiency affects lipid metabolism. The present experiments were designed to assess whether the hypertriglyceridemia associated with Mg deficiency was related to alterations in post-heparin lipase activity (PHLA). Mg-deficient and control diets were pair-fed to weanling Wistar rats for eight days and plasma lipoproteins were separated into various density classes by sequential preparative ultracentrifugation. Triglycerides were significantly increased in chylomicrons and in the very low density lipoprotein, low density lipoprotein and high density lipoprotein (HDL) fractions. Cholesterol and phospholipid levels were significantly lower in the HDL fraction. PHLA in deficient rat was substantially lower than in control rats. The inverse correlation between plasma triglyceride concentration and PHLA strongly suggests that hypertriglyceridemia is the result of defective lipolysis of plasma triglycerides in Mg-deficient rats. Further examination of the PHLA was carried out by salt-mediated inhibition of lipoprotein lipase (LPL) and by heparin sepharose affinity chromatography and purified rat LPL antiserum. The results indicate that hepatic lipase is significantly decreased in Mg-deficient rats but the low PHLA is due mainly to a decline in LPL. However, total LPL activity, that is, both the intracellular and the extracellular pools of LPL in adipose tissue, heart and diaphragm, were unaffected by Mg deficiency. The results suggest that the decrease of LPL activity in the plasma of Mg-deficient rats may be due to a selective decrease in the heparin-releasable pool of enzyme.

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Several studies have provided evidence that magnesium deficiency affects lipid metabolism (1–5). Magnesium deficiency can have pronounced effects on the physical state of membrane bilayer lipids, and defective membrane function could be the primary lesion underlying the cellular disturbances that occur in magnesium deficient animals (6).

The mechanisms behind the hypertriglyceridemia of Mg-deficient rats have been addressed in several studies. Hyperlipemia can be produced either by excessive production and release of lipids into the circulation, by their impaired removal from the circulation, or by a combination of both. Previous experiments indicate that decreased clearance of circulating triglycerides is a major mechanism contributing to hyperlipemia in Mg-deficient rats (7). Because lipoprotein lipase is the rate-limiting enzyme in the removal process, reduction of its activity can lead to hypertriglyceridemia (8). The present experiment was designed to test the hypothesis that hypertriglyceridemia associated with Mg deficiency can be causally related to alterations in LPL activity.

*To whom correspondence should be addressed.

Abbreviations: HDL, high density lipoprotein; HL, hepatic lipase; IHD, ischemic heart disease; LDL, low density lipoprotein; LPL, lipoprotein lipase; PHLA, post-heparin lipolytic activity; VLDL, very low density lipoprotein.

MATERIALS AND METHODS

Weanling male Wistar rats weighing about 60 g were randomly divided into magnesium-deficient and control groups. They were housed four to a cage and pair-fed with the appropriate diets for eight days using an automatic feeding apparatus. The initial food intake of 6 g/day rose to 8 g/day per rat. Distilled water was provided *ad libitum.* The synthetic diets contained (g/kg) casein 200, sucrose 705, corn oil 50, mineral mixture 35, and vitamin mixture 10, as described previously (1). Their magnesium contents determined by analysis were 30 mg/kg (deficient) and 960 mg/kg (control).

The experiments were performed between 9–10 a.m. on non-fasting rats. In some studies, the rats were food deprived 12 hr before experimentation. Plasma or other tissue samples were obtained on rats anesthetized with sodium pentobarbital (40 mg/kg of body weight).

Lipoprotein isolation and analysis. Lipoprotein isolation and analysis were carried out on unfrozen plasma from non-fasting rats. NaN_3 (0.02%), merthiolate (0.005%) and Na_2-EDTA (0.04%) were added. Samples were overlayered with 0.15 M NaCl/0.01% EDTA (d=1.006 g/mL) and chylomicrons were recovered following two centrifugations for 30 min at 12000 × *g*. Lipoproteins were separated into various density classes by sequential preparative ultracentrifugation. Very low density lipoproteins (VLDL) (d < 1.006 g/mL), low density lipoproteins (LDL) (d=1.006–1.050 g/mL) and high density lipoproteins (HDL) (d=1.050–1.21 g/mL) were isolated after the density had been adjusted with solid KBr. Ultracentrifugation was performed at 20°C in a Beckman model L5-50B ultracentrifuge (Beckman Instruments Inc., Palo Alto, CA) with a Ti-50 rotor.

Post-heparin plasma. Blood samples were withdrawn from the subclavian venous plexus of fed or fasted rats into tubes containing heparin. The plasma was separated in a refrigerated centrifuge and was used for the determination of Mg and triglycerides. Blood was taken again 10 min after intravenous administration of heparin (Produits Roche, Neuilly, France) at 25 U per 100 g of body weight.

Tissue lipoprotein lipase activity. Epididymal fat pads in fed rats, and the diaphragm and heart in fasted rats were excised immediately. Tissues were rinsed in 0.15 M NaCl solution at 4°C, blotted on filter paper, weighed and frozen on dry ice.

Measurement of post-heparin lipase activity in plasma. Lipolytic activity was measured with an emulsion of glycerol tri[1-^{14}C]oleate as substrate (9,10). The stock substrate contained 25 μCi (926.10^3 Bq) glycerol tri-[1-^{14}C]oleate, 69 mg unlabelled triolein, 3.33 mg soybean phosphatidylcholine and 5 mL glycerol. The mixture was

emulsified on ice for 5 min using a polytron (IKA, Staufen, Germany). The emulsion was stable for six weeks. The assay mixture contained 150 μL of the enzyme solution and 100 μL of diluted substrate (40 μL of emulsified stock substrate, 40 μL of 5% bovine serum albumin solubilized in 0.62 M Tris/0.16 M NaCl and adjusted to pH 8.6, and 20 μL of fresh human serum). After incubation at 37°C for 20 min, the labelled fatty acid products released were extracted and radioactivity was counted. Enzyme activity (1 mU) was defined as the amount of enzyme required to catalyze the release of 1 nmol of fatty acid per min at 37°C.

Measurement of tissue lipoprotein lipase (LPL) activity. Acetone/diethyl ether powder was prepared, and the crude lipoprotein lipase was extracted with 25 mM NH_4OH/NH_4Cl buffer (pH 8.5). The enzyme preparation was then homogenized with a polytron, centrifuged for 30 min at 4000 × *g* at 4°C and the supernatant collected. Measurements were made on 50–150 μL of supernatant.

Characterization of post-heparin lipase activity in plasma. In some assays to measure the effect of 1 M NaCl and the serum dependence of the lipolytic activity, serum was omitted from the incubation medium and was replaced by 0.16 M NaCl. The incubation was carried out with 100 μL of enzyme plus 50 μL of 5 M NaCl and 100 μL of substrate.

Heparin-Sepharose affinity chromatography was done on CNBr Sepharose 4B containing covalently linked crude heparin. The 1 × 5 cm column was equilibrated with 0.005 M barbital buffer containing 0.4 M NaCl (pH 7.4), and chromatography was carried out at 4°C. Two mL of diluted post-heparin plasma 0.5 M NaCl (v/v) was applied to the column, and the column was washed with 10 mL 0.25 M NaCl buffer and 30 mL 0.5 M NaCl. Successive 2-mL fractions were eluted with NaCl solutions of increasing molarity [10 mL of 0.7 and 1.5 M NaCl in 0.005 M barbital buffer (pH 7.4)]. Measurements were made on 150 μL enzyme fractions.

Lipase activity was also measured by use of an antiserum against purified LPL from rat adipose tissue, raised in goats as previously described (11). This antiserum has been shown to partially inhibit whole post-heparin plasma and it did not inhibit hepatic triacylglycerol lipase separated from rat post-heparin plasma by affinity chromatography. Post-heparin plasma (75 μL) diluted 1:10 was preincubated for 5 min at 37°C with 75 μL antiserum. The mixture was then centrifuged and incubated with substrate. Lipolytic activity was measured before and after incubation with the antiserum.

Other analytical methods. Magnesium was determined with a Perkin-Elmer series 400 atomic absorption spectrophotometer. Plasma was analyzed directly after dilution in lanthanum chloride solution containing 1 g La/L. Plasma triglycerides (Biotrol, Paris, France), cholesterol (BioMérieux, Charbonnières-les-Bains, France) and phospholipids (BioMérieux, Charbonnières-les-Bains, France) in plasma were determined by enzymatic procedures (12–14).

Calculations and statistical analysis. Values are given as means ± SEM. Data were analyzed by Student's *t*-test or Mann-Whitney U test and regression analysis.

RESULTS

The mean final body weights of the magnesium-deficient and control rats were 85 and 91 g, respectively, at the end of the experimental period and clinical symptoms of Mg deficiency were observed. An obvious turbidity and hyperlipemia was visible in the plasma from deficient animals when rats were not fasted before sacrifice; this was due mainly to hypertriglyceridemia. Triglyceride, cholesterol and phospholipid distribution in lipoprotein fractions are shown in Table 1. With Mg deficiency, triglycerides were significantly increased in chylomicrons and in the VLDL, LDL and HDL fractions. Both phospholipids and cholesterol were significantly increased in the VLDL and LDL fractions, but significantly lower in the HDL fraction.

Plasma Mg levels were significantly lower in the Mg-depleted group. PHLA in non-fasting deficient rats was significantly lower than in control rats (Table 2) and was inversely correlated with plasma triglyceride concentration (Fig. 1). The plasma post-heparin activity of Mg-deficient rats was also lower than that of the control rats

TABLE 1

Effect of Dietary Mg on Lipid Composition of Plasma Lipoproteins on Non Fasted Rats[a]

	Triglycerides (mg/100 mL)	Cholesterol (mg/100 mL)	Phospholipids (mg/100 mL)
Chylomicrons			
Control	17 ± 3	—	—
Mg deficient	102 ± 25**	—	—
VLDL			
Control	43.8 ± 5.9	7.2 ± 1	11.6 ± 1.8
Mg deficient	151.7 ± 15.8***	19.1 ± 3.3**	40.1 ± 7.5**
LDL			
Control	9.5 ± 1.1	7.4 ± 0.6	7.9 ± 0.4
Mg deficient	26.5 ± 1.7***	10.8 ± 0.5***	15.7 ± 0.9***
HDL			
Control	4.7 ± 0.4	29.0 ± 1.3	34.4 ± 2.4
Mg deficient	6.5 ± 0.4**	14.7 ± 0.7***	27.5 ± 2.7*

[a]Means ± S.E.M. of eight determinations per group. Statistical analysis was by Student *t*-test. ***, $P < 0.001$; **, $P < 0.01$; *, $P < 0.05$.

TABLE 2

Effect of Dietary Mg on Post-Heparin Plasma Lipolytic Activity in Non Fasted Rats[a]

	Mg (mM)	PHLA total (nmol/min/mL)	+ NaCl[b] (nmol/min/mL)
Control	0.77 ± 0.02 (7)	803 ± 24 (6)	82 ± 2.3 (5)
Mg-deficient	0.14 ± 0.01 (7)	278 ± 42 (7)	55 ± 3.0 (6)
	$P < 0.001$	$P < 0.001$	$P < 0.001$

[a]Numbers in parentheses indicate number of determinations for each assay. Values are given as means ± SEM. Statistical analysis was by Student *t*-test.

[b]Determination of enzyme activity under standard conditions described in Material and Methods, + NaCl, assay in the presence of 1 M NaCl without addition of serum.

TABLE 3

Characterization of Post-Heparin Plasma Lipolytic activity in Control and Mg-Deficient Rats[a]

	Total affinity (nmol/min/mL)	+ antiserum to LPL (nmol/min/mL)	LPL after affinity chromatography (nmol/min/mL)
Fed control (3)	679.7 ± 50.6	66 ± 6	143 ± 15
Fed Mg-deficient (3)	362.0 ± 23.6	38 ± 3	58 ± 13
	P < 0.05	P < 0.05	P < 0.05

[a]Numbers in parentheses indicate number of determinations for each assay. Values are given as means ± SEM. Statistical analysis was by Mann-Whitney U test.

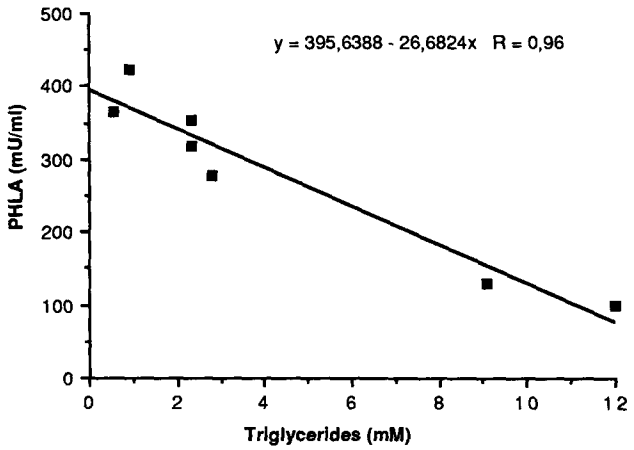


FIG. 1. Relationship between plasma triglyceride concentrations and post-heparin lipolytic activity in Mg-deficient animals.

when lipolytic activity was determined at 1.0 mol NaCl in the absence of human serum (Table 2).

For further characterization of PHLA (Table 3), the LPL fraction was separated on a heparin Sepharose affinity column. The fraction, which was inhibited by antilipoprotein lipase serum, was significantly reduced in Mg-deficient rats. When post-heparin plasma was incubated with antiserum, the lipolytic fraction of Mg-deficient rats was also lower than that of control rats. Thus, low PHLA was shown to be mainly due to a decline in LPL, but hepatic lipase (HL) was also significantly decreased. When rats were food deprived before sacrifice, the effect of Mg deficiency on plasma triglyceride concentration was also noticeable and PHLA was significantly lower than in control rats (Table 4). The adipose tissue LPL activity was the same in non-fasting control and Mg-deficient rats, and the heart and diaphram enzyme activity was not different in the two experimental fasted groups (Table 5).

DISCUSSION

The hyperlipemic effect of Mg deficiency was observed in post prandial conditions or following fasting. The post prandial phase is a non-static phase where activation of the processes of formation, catabolism and interchange of the different lipoprotein fractions takes place. The results are often variable since they depend, at least

TABLE 4

Effect of Dietary Mg on Post-Heparin Plasma Lipolytic Activity in Fasted Rats[a]

	Mg (mM)	TG (mM)	PHLA (nmol/min/mL)
Control (8)	0.80 ± 0.03	0.24 ± 0.02	575.5 ± 17.2
Mg-deficient (7)	0.27 ± 0.02	3.41 ± 0.86	312.4 ± 20.4
	P < 0.001	P < 0.01	P < 0.001

[a]Numbers in parentheses indicate number of determinations for each assay. Values are given as means ± S.E.M. Statistical analysis was by Student *t*-test.

TABLE 5

Effect of Dietary Mg on Lipoprotein Lipase Activity in Rat Organs[a]

	Control (nmol/min/g)	Mg-deficient (nmol/min/g)	
Epididymal fat (fed)	227.6 ± 30.6 (10)	252.0 ± 28.6 (11)	NS
Heart (fasted)	20.9 ± 0.7 (6)	23.2 ± 3.0 (6)	NS
Diaphragm (fasted)	206.0 ± 19.2 (6)	230.2 ± 28.8 (6)	NS

[a]Numbers in parentheses indicate number of determinations for each assay. Values are given as means ± S.E.M. Statistical analysis was by Student *t*-test. NS, not significant.

partially, on the stage of intestinal absorption. An automatic feeding apparatus was therefore used in the present experiment in order to obtain the same pattern of feeding in both control and Mg-deficient rats. This was not the case in the previous experiment (1). Moreover, as the hyperlipemic effect of Mg deficiency was still observed when rats were food deprived 12 hr before sacrifice, this effect was not the consequence of a modification in intestinal absorption.

Chylomicrons, VLDL and LDL levels were higher in Mg-deficient rats, but HDL levels were lower than in controls. Triglycerides were elevated and this was primarily due to an increase in chylomicron and VLDL concentrations. Cholesterol levels increased in the VLDL and LDL fractions. Since plasma cholesterol levels tend to decrease as a result of the decrease in HDL levels, total cholesterol

levels were not modified. In fact, the contribution of VLDL and LDL cholesterol to the total plasma cholesterol is very small in the rat (15). In contrast to the slight modification in total cholesterol in severe magnesium deficiency of short duration, other experiments indicate a significant increase in total cholesterol during moderate magnesium deficiency of long duration (16).

Several defects can produce hyperlipemia. These defects can consist of either an overproduction of a lipoprotein or a decrease in catabolism. By studying intralipid clearance, we were able to show that hyperlipemia developed because triglyceride removal was impaired (7). Moreover Mg-deficient rats exhibited delayed clearance of ^{14}C-labelled VLDL compared to control rats, whereas the catabolism of [^{125}I]LDL was not affected (17).

The present experiment provides what is believed to be the first direct evidence that Mg deficiency decreases PHLA. After an intravenous injection of heparin, triglyceride hydrolase activity appears in plasma (8,18). LPL is not the only lipase released by heparin into the bloodstream, HL is also released. Both enzymes are located in the capillary bed where lipolytic activity is expressed. LPL is stimulated by apolipoprotein CII and inhibited by NaCl 1.0 M, whereas HL seems not to require any specific serum factor and is activated by high salt concentrations. The lipolytic degradation of circulating triglyceride-rich lipoproteins involves loss of triglyceride and phospholipid from the particles. The two enzymes are believed to participate in this process. LPL is associated primarily with adipose tissue, and skeletal muscle shows a particular affinity for chylomicrons and VLDL. The function of the other enzyme, HL, is less well documented (19) and has a higher affinity for smaller particles. Several studies have suggested that its actions may be directed primarily at the metabolism of small VLDL, LDL and HDL. The separation of enzymes was efficiently obtained by heparin-Sepharose chromatography, a useful procedure when it is imperative to exclude the possible effects on enzymatic activity of other components in the post-heparin plasma samples (20). Inhibition of LPL by specific antibodies was another way to assay for HL and to quantitate LPL by subtracting the resistant lipolytic activity from total post-heparin lipolytic activity. Suppression of LPL by NaCl was another approach.

Two factors may be responsible for defective lipolysis of plasma triglycerides in Mg-deficient rats—a reduction in the availability of LPL or an abnormality in the lipoproteins themselves, rendering them a poor substrate for the enzyme. The apolipoproteins of VLDL isolated from rats fed deficient diets differ significantly from those of rats fed adequate diets. The VLDL in Mg-deficient rats appear to have reduced proportions of apo E but increased apo C. The role of this altered VLDL composition on defective clearance has previously been discussed (10). In these studies, it could not be determined how the changes in apolipoprotein patterns were related to hyperlipoproteinemia. The question remained whether the alterations in pattern cause hyperlipoproteinemia or whether these alterations were the results of the metabolic changes that produces hypertriglyceridemia (17).

In the present study, the assays for post-heparin lipolytic activity were carried out in the presence of human serum, that is providing any lipoprotein lipase cofactor protein requirement. Plasma triglyceride concentration correlates inversely with post-heparin plasma lipolytic activity. The experiment suggests that the alterations in the lipoprotein profile are the result of a reduction in the availability of LPL and not of an abnormality in the lipoproteins themselves.

The low lipolytic activity provides an appropriate explanation for both elevated levels of VLDL fraction and chylomicrons and for the reduction of HDL, because LPL activity is one determinant of plasma HDL levels. The accumulation of lipids in the blood occurs when their rate of entry into the blood exceeds their rate of removal. Sucrose, as compared to starch, markedly increases the hyperlipidemic effect of magnesium deficiency (1). The hypertriglyceridemic effect of sucrose is associated with a greater conversion of fructose to fatty acids in liver into plasma relative to dietary glucose or starch. Impairment of triglyceride clearance following a reduction in PHLA in Mg-deficient rats may explain the synergistic hypertriglyceridemia in Mg deficiency and sucrose feeding.

Since magnesium deficiency was associated with a dramatic decrease in LPL, it seemed worthwhile to determine the LPL activity in adipose tissue and muscle. After a carbohydrate-rich meal, enzyme activity increases rapidly in adipose tissue and declines in muscle. During a fast, the lipolysis of triglycerides stored in adipose tissue accelerates. This change is accompanied by decreasing levels of LPL in adipose tissue, but by stable or even increasing levels of lipase in muscle (cardiac, skeletal) (21,22). Therefore, LPL activity of adipose tissue was measured in post prandial conditions, whereas LPL activity of muscles was measured following food deprivation. Assuming that LPL activity in epididymal adipose tissue reflects that in other localizations (23), and activity in the heart and diaphragm is representative of that of muscles (24), an interesting aspect of the decrease in plasma LPL in Mg-deficient rats is that it occurs without decreases in muscle and adipose tissue LPL.

LPL is synthesized as an inactive precursor in the endoplasmic reticulum. This inactive form is transported to the Golgi apparatus where terminal processing and activation occur (18). Active LPL is released from the cells and moves to the vascular lumen where it is bound at the endothelial surface by glycosaminoglycans. In tissues such as adipose tissue and muscle, LPL exists in both functional and non-functional forms. The functional form appears to be largely associated with the endothelial surface of tissue capillaries and is readily released by heparin. The non-functional form of LPL, which is not readily releasable by heparin, appears to be associated with the tissue parenchymal cells (20). Two techniques, acetone/diethyl ether powder preparations and heparin eluates, have been described for assay of enzyme activity in adipose tissue and muscle. It is generally considered that the acetone/diethylether preparation represents the total tissue activity, *i.e.*, both the intracellular and the extracellular pools, including the physiologically active fraction at the endothelial surfaces. Heparin eluates, however, largely reflect extracellularly located enzyme (20). It would be of interest to determine if there is a defect within the tissues from Mg-deficient rats in maintaining the heparin-releasable pool of enzyme activity. With the acetone/diethylether preparation in whole tissue only, this selective decrease may not be discernable.

The mechanism responsible for the effect of Mg deficiency on LPL activity is unclear. The role of Mg in secretion and mechanisms of endothelial binding of LPL is unknown, and there is still only a limited knowledge of the heparin releasable pool of enzyme activity and its important relationship to the *in vivo* function of the enzyme. There are several explanations for the low post PHLA in Mg-deficient rats. One possibility is that Mg deficiency could impair secretion and that Mg is necessary for the transport of the active enzyme, and relocation to the endothelium. Other possibilities could be that the enzyme is degraded by the endothelial cells or that Mg deficiency increases the removal of the enzyme from the circulation, thus explaining why low activity is found in post-heparin plasma. Severe acute Mg deficiency induces hypertriglyceridemia and a decrease in PHLA. The time course development and the reversibility of these modifications by Mg refeeding should be examined.

Since the effect of marginal Mg deficiency of long duration is more relevant to human nutrition (25), the influence of moderate Mg deficiency and whether long term adaptation may occur which would affect the response remain also to be determined. While acute Mg deficiency has been shown to induce hyperlipemia and vascular lesions in experimental animals (2), the possibility that chronic Mg deficiency may be one among several factors that favor vascular disease in humans, is often disregarded (2). However, several observations indicate that patients with ischemic heart disease (IHD) are often Mg-deficient and that Mg deficiency may, together with several other factors, be involved in the development of IHD. Recently, Rasmussen *et al.* (26) found that oral Mg supplementation reduced plasma concentrations of triglycerides, VLDL and apo B in patients with IHD, suggesting that LPL activity might be reduced by Mg deficiency and increased by oral supplementation. Future investigations should clarify the influence of magnesium on LPL activity and lipid metabolism.

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Antioxidant Activity of Oxidation Products of α-Tocopherol and of its Model Compound 2,2,5,7,8-Pentamethyl-6-chromanol

Cacang Suarna and **Peter T. Southwell-Keely***
School of Chemistry, University of New South Wales, Kensington N.S.W., 2033 Australia

A variety of oxidation products (4-29) of α-tocopherol, 1 and of its model compound, 2,2,5,7,8-pentamethyl-6-chromanol (2) has been tested for antioxidant activity against autoxidizing safflower oil (ASO) and autoxidizing methyl linoleate (AML). The following compounds showed good antioxidant activity against both substrates: 5-hydroxymethyl-2,2,7,8-tetramethyl-6-chromanol (4), 5-(2,2,5,7,8-pentamethyl-6-chromanoxy)methyl-2,2,7,8-tetramethyl-6-chromanol (15), 1,2-*bis*(2,2,7,8-tetramethyl-6-chromanol-5-)ethane (16), 5-ethoxymethyl-7,8-dimethyltocol (19), 5-methoxymethyl-2,2,7,8-tetramethyl-6-chromanol (21), 5-ethoxymethyl-2,2,7,8-tetramethyl-6-chromanol (20), 5-propoxymethyl-2,2,7,8-tetramethyl-6-chromanol (22), 5-butoxymethyl-2,2,7,8-tetramethyl-6-chromanol (23), 5-(2-methyl-1-propoxy)methyl-2,2,7,8-tetramethyl-6-chromanol (24), 5-(2-methyl-2-propoxy)methyl-2,2,7,8-tetramethyl-6-chromanol (25), 5-heptoxymethyl-2,2,7,8-tetramethyl-6-chromanol (26), 5-undecoxymethyl-2,2,7,8-tetramethyl-6-chromanol (27), 5-phytoxymethyl-2,2,7,8-tetramethyl-6-chromanol (28) and 5-cholesteroxymethyl-2,2,7,8-tetramethyl-6-chromanol (29). 2,2,7,8-Tetramethylchroman-5,6-dione (17) and 1,2-*bis*(2,2,7-trimethylchroman-5,6-dione-8-)ethane (18) showed significant antioxidant activity against ASO but not against AML. If the corresponding oxidation products of 1 are formed *in vivo* it means that the antioxidant activity of 1 is not lost on oxidation. This may help to explain the outstanding capacity of 1 to protect cell membranes.
Lipids 26, **187-190 (1991).**

It is believed that α-tocopherol (1) functions *in vivo* as an antioxidant by being oxidized to the tocopheroxyl radical and then regenerated by a reducing agent such as ascorbic acid (1-8). This one-electron redox cycle offers an explanation for the remarkable efficiency of 1. The tocopheroxyl radical is easily formed due to its stabilization by the pyran ring oxygen (9).

Recent studies on the model compound of 1, 2,2,5,7,8-pentamethyl-6-chromanol (2), have suggested another reason for the extraordinary effectiveness of 1 as an antioxidant. Many of the oxidation products of 2 are themselves phenolic compounds and therefore potentially capable of acting as antioxidants (10-13). The present work was undertaken to determine the effectiveness of a variety of oxidation products of 2 as antioxidants.

MATERIALS AND METHODS

Compound 1 (Roche Products, Australia) and butylated hydroxyanisole (BHA, Embanox, May and Baker, Dagenham, England) were obtained commercially and used without further purification. Compound 2, 2,2,7,8-tetramethyl-6-chromanol (3) and 5-hydroxymethyl-2,2,7,8-tetramethyl-6-chromanol (4) were synthesized to satisfactory purity by established methods (10,11).

The following compounds were isolated from oxidation reactions of 1 and 2 as described previously (12-16): α-tocoquinone (5), 2-(3-hydroxy-3-methylbutyl)-3,5,6-trimethyl-1,4-benzoquinone (6), 1H-2,3-dihydro-3-(4,8,12-trimethyltridecyl)-7a-(3hydroxy-3,7,11,15-tetramethylhexadecyl)-3,5,6,9,10,11a-hexamethyl-pyrano[2,3-a]xanthene 8(7aH), 11(11aH) dione (7), 1H,2,3-dihydro-3,3,5,6,9,10,11a(R)-heptamethyl-7a(S)-(3-hydroxy-3-methylbutyl)-pyrano[2,3-a]xanthene 8(7aH), 11(11aH) dione (8), 5-ethoxycarbonyl-7,8-dimethyltocol (9), 5-formyl-7,8-dimethyltocol (10), 5-formyl-2,2,7,8-tetramethyl-6-chromanol (11), the spirodimer of 1 (12), the spirodimer of 2 (13), the spirotrimer of 2 (14), 5-(2,2,5,7,8-pentamethyl-6-chromanoxy)methyl-2,2,7,8-tetramethyl-6-chromanol (15), 1,2-*bis*(2,2,7,8-tetramethyl-6-chromanol-5)ethane (16), 2,2,7,8-tetramethyl-chroman-5,6-dione (17) (Suarna, C. and Southwell-Keely, P.T., unpublished data), 1,2-*bis*(2,2,7-trimethylchroman-5,6-dione-8)ethane (18) (Suarna, C., and Southwell-Keely, P.T., unpublished data), 5-ethoxymethyl-7,8-dimethyltocol (19), 5-ethoxymethyl-2,2,7,8-tetramethyl-6-chromanol (20), 5-methoxymethyl-2,2,7,8-tetramethyl-6-chromanol (21), 5-propoxymethyl-2,2,7,8-tetramethyl-6-chromanol (22), 5-butoxymethyl-2,2,7,8-tetramethyl-6-chromanol (23), 5-(2-methyl-1-propoxy)-methyl-2,2,7,8-tetramethyl-6-chromanol (24), 5-(2-methyl-2-propoxy)methyl-2,2,7,8-tetramethyl-6-chromanol (25), 5-heptoxymethyl-2,2,7,8-tetramethyl-6-chromanol (26), 5-undecoxymethyl-2,2,7,8-tetramethyl-6-chromanol (27), 5-phytoxymethyl-2,2,7,8-tetramethyl-6-chromanol (28), and 5-cholesteroxymethyl-2,2,7,8-tetramethyl-6-chromanol (29).

Safflower oil (400 g; Becel Pty. Ltd, Marrickville, N.S.W., Australia) was purified by passage over alumina (200 g) which had been activated at 600°C for 6 hr immediately before use. The alumina column (4.5 × 35 cm) and collection vessels were wrapped in aluminum foil and the oil pushed through the column using a slight nitrogen pressure. The peroxide value of the purified oil was 1.24 meq/kg and gas liquid chromatography (GLC) analysis gave the following fatty acid composition: palmitic acid, 7.5%; stearic acid, 3.3%; oleic acid, 14.7%; and linoleic acid, 74.5%.

Methyl linoleate was prepared from safflower oil by transesterification (17) followed by removal of the saturated methyl esters by urea clathration and, finally, fractional distillation (18,19). The fraction boiling at 150-152°C/0.3-0.6 mm Hg was shown by GLC analysis to be >99% pure.

Glass tubes (50 × 25 mm) for antioxidant testing were washed with detergent, tap water, immersed in 50% nitric acid overnight, washed again with tap water, distilled water and dried in an oven before use.

The substrates were analyzed for trace metals as follows: To the substrate (2 g) was added 50% nitric acid

*To whom correspondence should be addressed at School of Chemistry, University of New South Wales, P.O. Box 1, Kensington, N.S.W., 2033, Australia.

Abbreviations: AML, autoxidizing methyl linoleate; ASO, antioxidizing safflower; BHA, butylated hydroxyanisole; GLC, gas-liquid chromatography.

(2 mL) and the mixture shaken at 50°C for 1 hr. The nitric acid layer was withdrawn, the substrate washed with nitric acid (2 × 2.5 mL), and the washings added to the original nitric acid layer. The combined nitric acid solution was taken to dryness, made up to 10 mL with 10% nitric acid and analyzed for trace elements by an Inductively Coupled Plasma Atomic Emission Spectrometer (Labtam, Melbourne, Australia). The experiment was performed in duplicate and compared with duplicate reagent blanks.

Antioxidants were dissolved in substrates to give solutions equimolar with 0.25% BHA and were diluted with substrates to a test concentration equimolar with 0.01% BHA as required.

Antioxidant test procedure. Duplicate test samples (1 g) in glass tubes (50 × 25 mm) in a two-tiered aluminum rack designed to accommodate 60 samples were placed in a convection oven at 40 ± 1°C for methyl linoleate or 60 ± 1°C for safflower oil. At daily intervals the tubes were cooled in a desiccator and weighed accurately. The procedure was continued until a significant weight gain was noted. Antioxidant activity was expressed as an induction period (in days; mean ± standard deviation) which was determined by the point of intersection of tangents drawn to the oxidation curve early in the run and late in the run after the oxidation rate had assumed its uninhibited value (20).

RESULTS AND DISCUSSION

The variety of methods used to test for antioxidant activity is testimony to the fact that no one method is absolutely satisfactory in all situations. The oxygen uptake method is probably the most accurate, but can only handle limited numbers of samples at any one time (21-23).

The weight gain method was chosen in this instance as being the only method available which would allow simultaneous comparison of a large number of compounds under identical conditions (24). Variation in antioxidant performance in different substrates is noted often, and therefore two substrates were chosen, safflower oil and methyl linoleate.

Both substrates were tested initially at 60°C, but it was found that no compounds protected methyl linoleate successfully at this temperature and subsequent experiments on methyl linoleate were performed at 40°C. Analysis of both substrates for trace elements revealed that only iron was present at a higher level than that of the reagent blank and that methyl linoleate contained 1.6 ppm of iron, whereas safflower oil contained 0.3 ppm. This was surprising since the isolation and purification (including distillation) of methyl linoleate had been carried out in glass vessels. However, the higher level of iron in methyl linoleate may help explain its greater ease of oxidation.

Compound 3 (the γ-tocopherol model compound) was more active than all of the products of 1 and 2 against both substrates and was only surpassed by BHA against methyl linoleate (Table 1). Compounds 1 and 2 were almost as active as 3 against safflower oil but, surprisingly, were much less active against methyl linoleate and, in fact, were inferior to many of the oxidation products of 2 against this substrate. The lack of agreement between different methods of antioxidant assay is shown clearly by a comparison of the activities of 1 and 2 using three methods—i) inhibition of weight gain (present results), ii) inhibition of the destruction of β-carotene by autoxidizing hydrogenated corn oil (25), and iii) inhibition of the initiated oxidation of styrene (9). In method i) the order of activities was 2 > 1, in method ii) 1 > 2 and in method iii) 2 > 1. Comparison of 1,2 and 3 using methods i) and ii) gave in method i) 3 > 2 > 1 and in method ii) 1 > 3 > 2. The relative activities of compounds 2 and 3 were as predicted from earlier literature on α- and γ-tocopherol (26,27) but were contrary to more recent results (9,28). The reason for the difference between the two sets of results has been related to the use of an uninitiated (present results) or initiated autoxidation test system (9). However, the recent results (9,28) were concerned only with the reaction of 1 and γ-tocopherol with peroxyl or phenoxyl radicals and did not take into account the influence of oxidation products of 1 and of γ-tocopherol on the overall autoxidation process. It may be that the main reason for the difference in the activities of 2 and 3 lies in the fact that a major product from the oxidation of 3 is the ortho quinone 17, which retains significant activity (see below) while the major products of 2 are the spirodimer 13, the spirotrimer 14 and the para quinone 6 (Suarna, C., and Southwell-Keely, P.T., unpublished data) all of which have little activity (see below).

If the higher level of iron in the methyl linoleate is the main reason for its more rapid oxidation than safflower oil, an alternative explanation for the greater efficacy of 3 is possible. The intermediate in the oxidation of 3 to 17 is 2,2,7,8-tetramethyl-5,6-dihydroxy-chroman (30), which would be an excellent chelator of iron, and many other metals, and could function both by prevention of propagation and of initiation. Sufficient 30 to chelate all of the iron would have accumulated well before all of 3 had been oxidized.

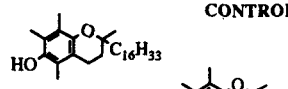
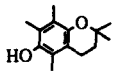
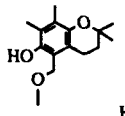
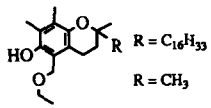
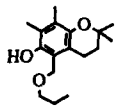
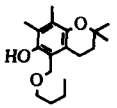
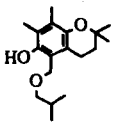
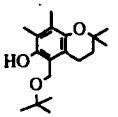
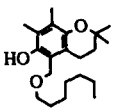
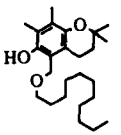
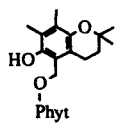
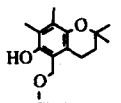
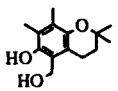
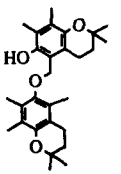
Compounds 5–8 showed no activity against either substrate while compounds 9–14 showed slight activity against both substrates. Compounds 4, 15, and 16 all showed good antioxidant activity against both substrates. This would have been predicted, since all of these compounds, although at a higher oxidation state than 2, are still phenols and, therefore, capable of further oxidation.

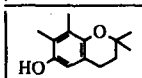
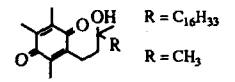
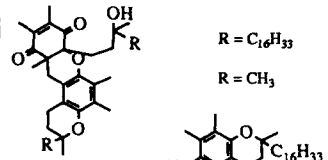
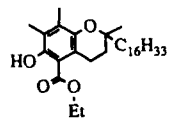
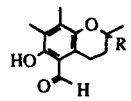
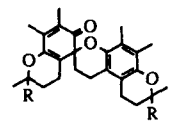
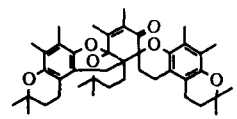
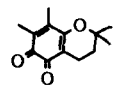
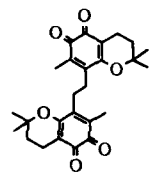
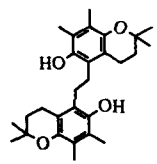
Compounds 17 and 18 showed no activity against methyl linoleate but significant activity against safflower oil. This is an interesting result since 17 and 18 are at a higher oxidation state than 5 and 6, which have no activity. The result with 17 confirms earlier observations on the ability of tocored, the equivalent compound from α-tocopherol, to inhibit the autoxidation of lard (29,30). The ability of 17 and 18 to protect safflower oil (0.3 ppm iron), but not methyl linoleate (1.6 ppm iron), may mean that 17 and 18 function predominantly by hydrogen transfer (prevention of propagation) rather than metal chelation (prevention of initiation). There is no doubt that 17 can function by hydrogen transfer since its conversion into 18 or into 2,2,7-trimethyl-6-hydroxy-chroman-5,8-dione are both oxidations which proceed by hydrogen abstraction (Suarna, C., and Southwell-Keely, P.T., unpublished data). Similarly, oxidation of 18 could occur readily with the loss of two hydrogen atoms from the ethane bridge between the two monomers to join the two ortho-quinone rings in a completely conjugated system.

All 5-alkoxymethyl derivatives of 2 showed good antioxidant activity against both substrates. The effect

TABLE 1

Antioxidant Activity of α-Tocopherol, 2,2,5,7,8-Pentamethyl-6-Chromanol and Some of their Oxidation Products

COMPOUND	No	INHIBITION (INDUCTION PERIOD), DAYS	
		Me. Linoleate (40°C)	Safflower Oil (60°C)
CONTROL	—	2.78 ± 0.04 *	1.11 ± 0.01
	1	3.68 ± 0.04	6.55 ± 0.14
	2	4.53 ± 0.04	7.00 ± 0.14
	21	4.55 ± 0.21	4.48 ± 0.32
 R = $C_{16}H_{33}$, R = CH_3	19	6.10 ± 0.14	4.08 ± 0.11
	20	5.55 ± 0.21	4.30 ± 0.14
	22	5.90 ± 0.14	5.53 ± 0.24
	23	5.38 ± 0.04	4.33 ± 0.11
	24	6.60 ± 0.28	4.35 ± 0.21
	25	8.18 ± 0.04	4.80 ± 0.64
	26	5.75 ± 0.49	4.35 ± 0.21
	27	6.60 ± 0.07	4.05 ± 0.07
	28	5.60 ± 0.14	3.55 ± 0.07
	29	6.98 ± 0.39	3.95 ± 0.07
	4	4.40 ± 0.14	5.85 ± 0.92
	15	6.10 ± 0.14	4.15 +

COMPOUND	No	INHIBITION (INDUCTION PERIOD), DAYS	
		Me. Linoleate (40°C)	Safflower Oil (60°C)
	3	8.70 ± 0.84 *	7.55 ± 0.07
 R = $C_{16}H_{33}$, R = CH_3	5	2.46 ± 0.08	1.25 ± 0.07
	6	2.90 ± 0.07	1.15 ± 0.14
 R = $C_{16}H_{33}$, R = CH_3	7	2.69 ± 0.09	1.13 ± 0.04
	8	2.73 ± 0.18	1.20 ± 0.14
	9	3.48 ± 0.04	1.60 +
 R = $C_{16}H_{33}$, R = CH_3	10	3.08 ± 0.11	1.45 ± 0.07
	11	3.68 ± 0.10	1.30 ± 0.07
 R = $C_{16}H_{33}$, R = CH_3	12	3.00 ± 0.14	1.23 ± 0.04
	13	3.53 ± 0.11	1.35 ± 0.07
	14	3.08 ± 0.25	1.28 ± 0.04
	17	2.80 ± 0.21	4.60 ± 1.13
	18	2.55 ± 0.21	3.80 ± 0.28
	16	3.90 ± 0.14	7.55 ± 0.64
BHA	—	> 12	6.10 ± 0.14

of replacing the 5-methyl group of 2 by an alkoxymethyl group was to decrease its antioxidant activity against safflower oil but to increase its activity against methyl linoleate. The explanation for this may be that the 5-alkoxymethyl derivatives can function not only by hydrogen transfer (prevention of propagation) but also by metal chelation (prevention of initiation). The low level of iron in safflower oil may mean that, in this substrate, the 5-alkoxymethyl compounds function predominantly as propagation inhibitors rather than as initiation inhibitors. If this is so, internal chelation of the 6-hydroxy group by the 5-alkoxymethyl may make it more difficult to remove the hydrogen and therefore lead to a slower reaction with peroxyl radicals (and therefore less activity) than that of 1 and 2. By contrast, the higher level of iron in methyl linoleate may mean that prevention of initiation (by metal chelation) in that substrate is more important than prevention of propagation and therefore the 5-alkoxymethyl compounds, with their much greater metal chelation capacity, are superior to 1 and 2. It appears that an alkoxy group of almost any length attached to position 5 of a chromanol ring is more beneficial to antioxidant activity against methyl linoleate than the phytol residue at position 2 of compound 1. The activity of 29 against methyl linoleate was particularly noteworthy in view of the presence of cholesterol in all cell membranes.

Thus it is clear that many of the oxidation products of 2 and, by inference, of 1 possess substantial antioxidant activity and that, if formed *in vivo,* could help explain the outstanding capacity of 1 to protect cell membranes. Nonetheless one must be cautious in drawing too many conclusions since many derivatives of 1 and 2 have shown the ability to act as antioxidants but none has shown the capacity of 1 to protect against liver necrosis or resorption sterility in rats, encephalomalacia in chicks or muscular dystrophy in rabbits (25,31,32).

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Implication of Steroid Saponins and Sapogenins in the Hypocholesterolemic Effect of Fenugreek

Yves Sauvaire[a,*], Gérard Ribes[b], Jean-Claude Baccou[a] and Marie-Madeleine Loubatieres-Mariani[b]

[a]Laboratoire de Physiologie Vegetale, Universite Montpellier II, 34095 Montpellier, Cedex 5 and [b]Faculte dé Médecine, Laboratorie de Pharmacologie, URA 599 du CNRS, Institut de Biologie, 34060 Montpellier, France

The transformation of fenugreek subfractions, rich in steroid saponins, was studied upon their passage through the digestive tract to determine the contribution of saponins and/or diosgenin and other steroid sapogenins to the hypocholesterolemic effect of fenugreek seeds. Feces of alloxan diabetic dogs fed fenugreek subfractions were analyzed, and diosgenin, smilagenin and gitogenin were identified and measured using capillary gas chromatography/mass spectrometry. Our results show that saponins are, in part (about 57%), hydrolyzed into sapogenins in the digestive tract. It appears that saponins may be implicated, alone or together with diosgenin, in the observed hypocholesterolemic effect of fenugreek seeds in diabetic dogs.

Lipids 26, 191–197 (1991).

Fenugreek (*Trigonella foenum graecum L., Leguminosae*) is an annual herb, cultivated in Southern Europe, Northern Africa and India. The seeds of fenugreek are known to have hypocholesterolemic activity (1,2). We have previously shown that this hypocholesterolemic effect was due to the defatted part (3) and especially to the saponin-rich subfraction (4). This subfraction, which contains most of the saponins present in the seed, reduced hypercholesterolemia and hypertriglyceridemia of alloxan-diabetic dogs. The hypocholesterolemic effect was not observed with the saponin-free subfraction (4).

Saponins are plant glycosides whose aglycone structure is triterpenoid or steroidal. Studies on the effects of saponins on cholesterol homeostasis concern mainly the triterpenoid saponin from alfalfa (5) and from soybean (6). It has also been reported that a steroidal saponin, digitonin, prevents hypercholesterolemia in monkeys (7).

Fenugreek seed saponins are of steroidal nature (type furostanol saponins) with diosgenin [(25 R)-spirost-5-en-3β-ol] as main sapogenin (Fig. 1) (8). Diosgenin has various effects on cholesterol metabolism, one of the most important being the capacity to lower plasma cholesterol concentration (9). This hypocholesterolemic effect appears to be dependent on the capacity of diosgenin to inhibit cholesterol absorption, to decrease liver cholesterol concentration and to increase biliary cholesterol secretion and fecal excretion of neutral sterols (10–12).

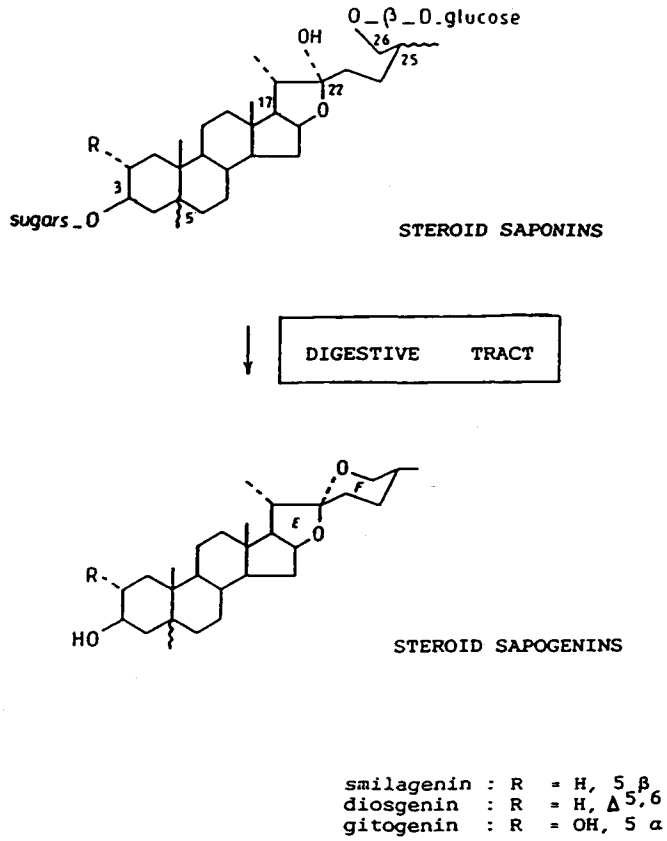


FIG. 1. Transformation of steroid saponins from fenugreek in the digestive tract.

Furthermore, Malinow (13) has shown that diosgenin glucoside was more efficient in reducing intestinal absorption of cholesterol than diosgenin. At comparable small doses, diosgenin glucoside inhibited cholesterol absorption, whereas diosgenin did not (13,14). Saponins and/or diosgenin could hence be involved in bringing about the hypocholesterolemic effect of fenugreek seeds. It was thus important to study the transformation of fenugreek subfractions upon their passage through the digestive tract. Toward this aim, we have carried out a study in alloxan-diabetic dogs.

MATERIALS AND METHODS

Plant material. Fenugreek seeds (cultivar Gouka) were obtained from our experimental field in Montpellier, France (Université des Sciences et Techniques du Languedoc).

The defatted fenugreek seeds were obtained as previously described (15) and were further fractionated to obtain subfractions enriched in saponins and pro-

*To whom correspondence should be addressed.

Abbreviations: FAE, feces alcoholic extract; FLE, feces lipid extract; GC, gas chromatography; GC/MS, gas chromatography/mass spectrometry; HPLC, high-performance liquid chromatography; NMR, nuclear magnetic resonance; TLC, thin-layer chromatography.

tein. The defatted seeds were fractionated into two parts—a fraction containing testa plus endosperm (Subfraction A) and a fraction containing cotyledons plus axis (Subfraction B). The latter was extracted with isopropanol/water (70:30, v/v). The lyophylized extract contained practically all the saponins (Subfraction S), but not the sapogenins (aglycone part of saponins). The insoluble portion contained protein without saponin (Subfraction P).

The techniques used to determine the composition of the subfractions of defatted fenugreek seeds have been previously described (16). Subfraction B includes 7.2% saponins and 52.8% protein; Subfraction S contains 22.2% saponins without protein; Subfraction P contains 70.5% protein without saponins.

Alloxan-diabetic dogs. Conscious mongrel male dogs, weighing 18–21 kg, were made diabetic by an intravenous injection of alloxan (50 mg/kg) at least 45 days before the experiment. The animals were housed in individual cages. They were fed twice daily with a standard diet (30 g/kg at each meal, in the form of patties containing 9% protein, 5% lipid and 78% moisture; UAR, Epinay, France).

Each animal received two daily subcutaneous injections (14–24 IU) of soluble insulin (Endopancrine, Organon, France) so that they were clearly glycosuric (about 50 g/24 hr) and had high plasma levels of cholesterol, but they were not ketotic. The dose of insulin injected was kept constant throughout the experiment.

Experimental protocols. Fenugreek seed subfractions were administered with the animals diet for 21 days. Four sets of experiments were performed: i) Four dogs received Subfraction B at 1.13 g/kg corresponding to 0.08 g/kg per day of saponins and 0.59 g/kg per day of protein; ii) four dogs received Subfraction S at 0.37 g/kg corresponding to the same dose of saponins—0.08 g/kg per day; iii) four dogs received Subfraction P at 0.73 g/kg corresponding to 0.51 g/kg per day of protein; and iv) four dogs were fed the standard diet (control experiments). The doses were chosen so that the animals received an amount of saponins (Subfractions B and S) or protein (Subfraction P) corresponding to those of the total defatted fraction previously used (3).

Feces from each dog were collected before and during the treatments and the composition of fecal saponins or sapogenins was determined. Simultaneously, the effects on plasma cholesterol levels were evaluated, according to the colorimetric method of Watson (17), using blood samples collected from jugular vein.

Fecal saponin analysis. Feces were immediately sampled and freeze-dried; 5–15 g were ground in a mortar and extracted in a Soxhlet apparatus overnight with hexane under reflux. The hexane extract was called feces lipid extract (FLE). The defatted fecal samples were reextracted in a Soxhlet apparatus overnight with isopropanol/water (70:30, v/v). The alcoholic extract was called feces alcoholic extract (FAE).

The steroid sapogenins in FLE were characterized by thin-layer chromatography (TLC) on silica gel (precoated silica gel TLC plates, Merck, Darmstadt, Germany) developed with hexane/ethyl acetate (60:40, v/v). Fractions were made visible with $SbCl_3$/*6 N* HCL (1:1, w/v) or with methanol/H_2SO_4 (1:1, v/v) followed by heating.

To isolate the steroid sapogenins, the FLE was applied as band onto TLC plates and the plates were developed in hexane/ethyl acetate (40:60, v/v). The sapogenins were separated into an upper zone (R_f, 0.7–0.8), consisting of monohydroxylated sapogenin that co-developed with standard diosgenin, and a bottom zone (R_f, 0.2–0.3), consisting of dihydroxylated sapogenins that co-developed with standard gitogenin [(25 R)-5α-spirostan-2,3β-diol]. Silica gel from the two zones was scraped from the plates and eluted with a series of solvents, namely hexane, dichloromethane, and then ethanol. Five successive preparative TLC fractionations were necessary to obtain relatively pure fractions, as judged by GC.

The monohydroxy sapogenins recovered from the last TLC separation were further purified by reversed-phase high performance liquid chromatography (HPLC) (C18 column Ultrasphere ODS, 5 μdp, 4.6 mm i.d. × 250 mm) eluted with methanol/water (96:4, v/v). The flow rate was set at 1.6 mL/min, and the detector at 205 nm. Diosgenin was eluted in 7.5 min, cholesterol in 24 min, and sitosterol was eluted in 32.5 min. Monohydroxy sapogenins (like diosgenin) were collected after repeated injection onto the column. The identity of each isolated mono or dihydroxy sapogenin was established by a comparison of retention times in GC and of mass spectra (GC/MS) with those of standards.

Capillary gas chromatography (GC). A Delsi gas chromatograph (model D 1700) equipped with a FID and a Shimadzu integrator (model C-R4A, Shimadzu, Kyoto, Japan) were used. The chromatograph was fitted with a RSL 150 (Alltech, Deerfield, IL) 30-m fused silica capillary column of 0.25 mm i.d. bound and cross-linked with a polydimethylsiloxane film 0.2 μm thick. The analyses were run isothermally at 270°C; the injector and detector temperatures were 300°C. Hydrogen was used as carrier gas with a head pressure of 0.7 bar (10 psi).

Capillary gas chromatography/mass spectrometry (GC/MS). GC/MS was carried out with an LKB Bromma 209 equipped with Vg Digital pdp 8a computer. The gas chromatograph was fitted with a capillary column OV 1701 (25 m, 0.32 mm i.d., 0.20 μm film). Analyses were carried out isothermally at 260°C for monohydroxy sapogenins and at 290°C for dihydroxy sapogenins. Helium was used as carrier gas with a head pressure of 0.5 bar (7 psi). The jet separator was maintained at 230°C. Ionizer temperature was set at 250°C and electron energy and electron multiple voltage were 70 eV and 3.5 kV, respectively. Smilagenin [(25 R)-5β-spirostan-3β-ol] was from Merck, and diosgenin was from K and K Laboratories Inc. (Plainview, CA). Gitogenin was isolated from *Trigonella foenum graecum* and characterized by GC/MS and ^{1}H nuclear magnetic resonance (NMR).

RESULTS

Total food intake was kept the same in the different experimental groups. The data in Table 1 show that subfractions B and S induced a significant reduction

TABLE 1

Effect of Chronic Intake of Fenugreek Seed Subfractions Combined with Insulin Treatment on Plasma Cholesterol Levels (mg/100 mL) in Alloxan Diabetic Dogs[a]

	Days/Insulin treatment[b]										
				Fenugreek intake							
	Day −42	−40	−2	−1	1	6	9	14	17	21	28	33
Fenugreek Subfraction B (4)	80 ± 15		238 ± 7	246 ± 6	215[c] ± 14	189[c] ± 14	199[c] ± 20	200[c] ± 3	193[c] ± 9	202 ± 11	230 ± 20	241 ± 10
Fenugreek Subfraction S (4)	89 ± 12		257 ± 16	256 ± 19	233 ± 17	229 ± 9	226[d] ± 12	206[d] ± 11	192[d] ± 18	203 ± 13	229 ± 14	239 ± 15
Fenugreek Subfraction P (4)	92 ± 13		259 ± 5	268 ± 8	276 ± 8	277 ± 7	274 ± 8	270 ± 10	279 ± 12	286 ± 13	296 ± 21	292 ± 20
Controls (4)	68 ± 10		248 ± 21	263 ± 13	266 ± 18	268 ± 20	267 ± 20	283 ± 17	274 ± 21	272 ± 22	277 ± 19	281 ± 21

[a]Results are expressed as mean ± SEM. The number of animals is indicated in parentheses. Subfraction B (saponins, 7.2%; proteins, 52.8%). Subfraction S (saponins, 22.2%). Subfraction P (proteins, 70.5%).
[b]Alloxan was injected on day −41.
[c]$p<0.01$.
[d]$p<0.05$.

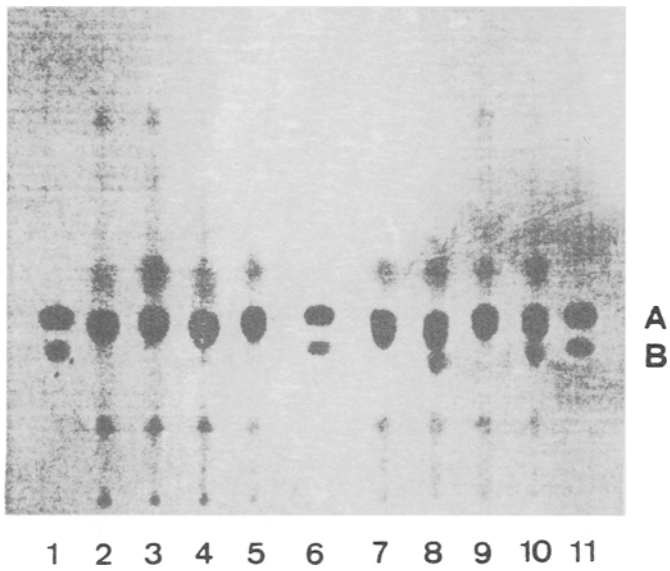


FIG. 2. Thin-layer chromatogram of feces lipid extracts (FLE) from dogs receiving Subfractions P and S of fenugreek. Lanes 1, 6, 11: Cholesterol (R_f, 0.37) plus diosgenin (R_f, 0.30). Lanes 2, 3, 4, 5: FLE from dogs receiving Subfraction P. Lanes 2, 4: Before treatment. Lanes 3, 5: After treatment. Lanes 7, 8, 9, 10: FLE from dogs receiving Subfraction S. Lanes 7, 9: Before treatment Lanes 8, 10: After treatment. A indicates the presence of substances with an R_f equal to that of cholesterol. B indicates the presence of substances with an R_f equal to that of diosgenin.

in plasma cholesterol in diabetic dogs; in contrast, Subfraction P had no effect.

Figure 2 shows a thin-layer chromatogram obtained with FLE from feces of dogs receiving either Subfraction S or Subfraction P. TLC clearly reveals the appearance of a new substance, but only in the feces from dogs given Subfraction S. The same substance was also detected in FLE from dogs given Subfraction B (saponins 7.2%) (results not shown). These chromatographic properties (R_f, chromophore) of the substance suggested that it belonged to the class of monohydroxylated sapogenins, such as diosgenin. Nevertheless, the results obtained by TLC needed to be confirmed by complementary methods.

After purification by preparative TLC and HPLC, several samples were analyzed by GC and GC/MS. The chromatogram of monohydroxy steroid sapogenins upon capillary GC shows three principal peaks, as well as several minor ones (Fig. 3). The retention times of peaks No. 1 and No. 2 correspond to smilagenin and diosgenin, respectively. For further confirmation, we analyzed the samples by capillary GC/MS. The chromatogram exhibits the same pattern as the two principal peaks 1 and 2 (Fig. 3A). The ionogram obtained by ion selective monitoring at *m/z* 139 is practically identical to the ion chromatogram in Figure 3B. It seems likely that the peak at *m/z* 139 $[C_9H_{15}O]^+$ arises from ring E fragmentation by a 1,2 shift of the C-20 hydrogen to C-17 (Fig. 1). The fragment is characteristic of a steroid sapogenin with a spiroketal side chain (18,19).

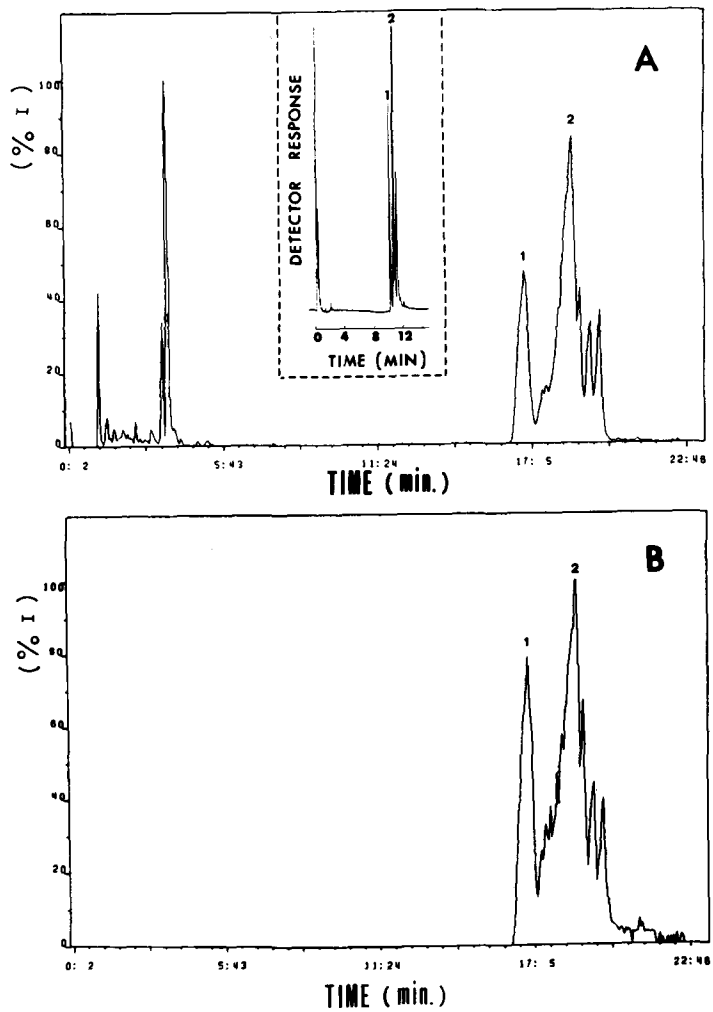


FIG. 3. Capillary GC/MS of isolated monohydroxy steroid sapogenins from feces of dogs receiving steroid saponins. A: Ion electron impact chromatogram (capillary GC). B: Ion current profile of selected ion *m/z* 139.

The mass spectrum of the substance corresponding to peak No. 1 reveals a molecular peak at *m/z* 416 which corresponds to the mass of smilagenin (Fig. 4A). Likewise, the mass spectrum of the substance corresponding to peak No. 2 shows a molecular peak at *m/z* 414 which corresponds to the mass of diosgenin (Fig. 4B).

The other fragmentations observed allowed us to confirm our assignments (Figs. 4A and 4B). Fission of the F ring produces ions $[M\text{-}C_4H_8O]^+$ at *m/z* 344 or 342. Corresponding to $[C_{23}H_{36}O_2]^+$ or $[C_{23}H_{34}O_2]^+$, and ions $[M-C_3H_7O]^+$ at *m/z* 357 or 355. Corresponding to $[C_{24}H_{37}O_2]^+$ or $[C_{24}H_{35}O_2]^+$, in the smilagenin or diosgenin spectra, respectively. Another group of characteristic ions arises by fragmentation in ring E, producing ions at *m/z* 302 or 300 corresponding to $[C_{21}H_{34}O]^+$ or $[C_{21}H_{32}O]^+$ in the smilagenin or diosgenin spectra, respectively. Other characteristic ion peaks occurred at *m/z* 255 (253), 269 (267), and 284 (282) due to hydrocarbon fragments. Standards of smilagenin and diosgenin showed spectra and fragmentation patterns very similar to those of peaks No. 1 and 2, respectively.

The chromatogram of dihydroxy steroid sapogenins obtained upon capillary GC shows several peaks and two principal ones, Nos. 3 and 4 (Fig. 5). The retention time of peak No. 3 corresponds to that of gitogenin. The assignment was confirmed by capillary GC/MS (Fig. 5A). The ionogram obtained by ion selective monitoring at *m/z* 139 is practically identical to the ion chromatogram in Figure 5B. The mass spectrum (Fig. 4C) exhibits a molecular peak at *m/z* 432 corresponding to the mass of gitogenin. The spectrum also shows prominent ions at *m/z* 360 $[C_{23}H_{36}O_3]^+$, *m/z* 363 $[C_{22}H_{35}O_4]^+$, and *m/z* 373 $[C_{24}H_{37}O_3]^+$. Another group of characteristic ions arises from cleavage at the spiroketal site. Ions *m/z* 318 indicates that the additional hydroxy group in gitogenin is nuclear. A standard of gitogenin showed a spectrum and fragmentation pattern very similar to those of

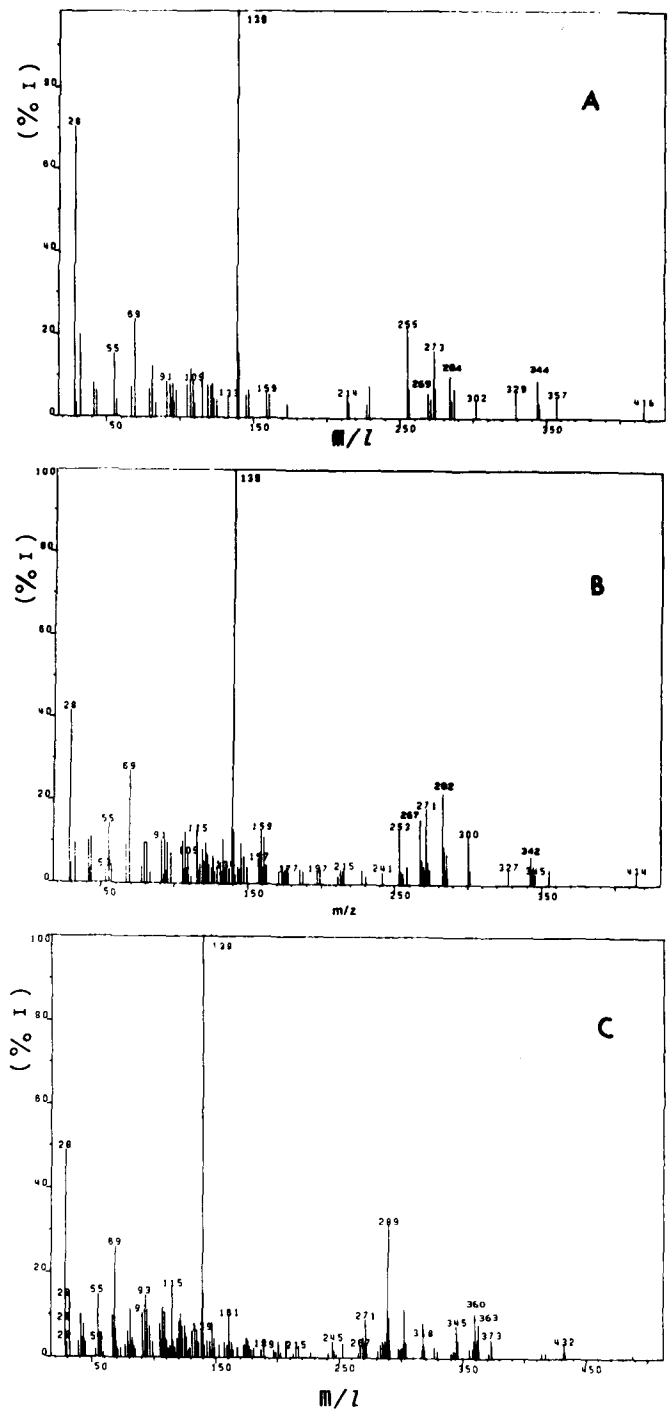


FIG. 4. Mass spectra of isolated steroid sapogenins from feces of dogs receiving steroid saponins. A: Mass spectrum of peak No. 1. B: Mass spectrum of peak No. 2. C: Mass spectrum of peak No. 3.

peak No. 3. Peak No. 4 has not been identified so far.

Assays of sapogenins either in the free form (in FLE) or in the glycosylated form (in FAE) were carried out to determine the extent of hydrolysis of steroid saponins in the digestive tract. The results obtained from six different feces samples indicated that 57.4 ± 5.3% (SD) of the saponins were hydrolyzed to sapogenins.

We also investigated *in vitro* whether β-glucosidase could hydrolyze a sample of fenugreek saponins to the sapogenins. For this purpose, the purified S subfraction was subjected to β-glucosidase digestion for 24 hr. The results showed that no sapogenin was released by this treatment, indicating that β-glucosidase is ineffective in cleaving the glycoside bond at C-3 (Fig. 1). However, the enzyme acted on the saponins releasing the glucose residue from C-26. The native saponins of the furostanic type were then converted to saponins of the spirostanic type. We also calculated the diosgenin/smilagenin ratio based on chromatographic analysis (GC). The ratio was 19 for the S subfraction before ingestion, and 2 for FLE.

DISCUSSION

Our study showed that fenugreek subfractions rich in steroid saponins are, in part, transformed in the gastrointestinal tract. Thus, smilagenin, diosgenin and gitogenin were detected in feces of dogs fed fenugreek (Fig. 1). Hydrolysis averaged 57%. The 25 S epimers corresponding to smilagenin, diosgenin and gitogenin, as well as their saturated or unsaturated derivatives, may also be present in the feces.

The location of fenugreek saponin hydrolysis in the digestive tract has not been determined. If sapogenins are able to partially prevent cholesterol absorption, saponin hydrolysis presumably occurs in the stomach or/and in the proximal small intestine. However, Gestetner *et al.* (20) had previously reported that ingested soybean saponins are hydrolyzed by cecal microflora in chicks, rats and mice. The apparent discrepancy may be due to differences in species or other experimental conditions.

Calculation of the diosgenin/smilagenin ratio revealed a clear difference between the composition of Subfraction S and the lipid extract obtained from the feces (19 *vs* 2). This greater amount of smilagenin in the feces seems to be due to transformation of the sapogenins from Subfraction S during their passage through the gastrointestinal tract.

Previous reports have shown that other plant steroids with a double bond in 5,6-position are reduced and transformed into 5β-hydroxy steroid analogues by gastrointestinal microorganisms in various animals (21,22). It is possible that in our experiments diosgenin was reduced and transformed to smilagenin in the digestive tract of the dogs. However, it is important to recall that differences in the biotransformation of diosgenin have been observed depending on the species studied and the conditions of administration employed (23).

As to the hypocholesterolemic effect of fenugreek seeds, the presence of steroid saponins seems to play an essential role (4). Other saponins have also been reported to modify cholesterol metabolism. Thus, alfalfa saponins, as well as digitonin, inhibited cholesterol intestinal absorption in the monkey (7,24). Soybean saponins reduced intestinal uptake of cholesterol

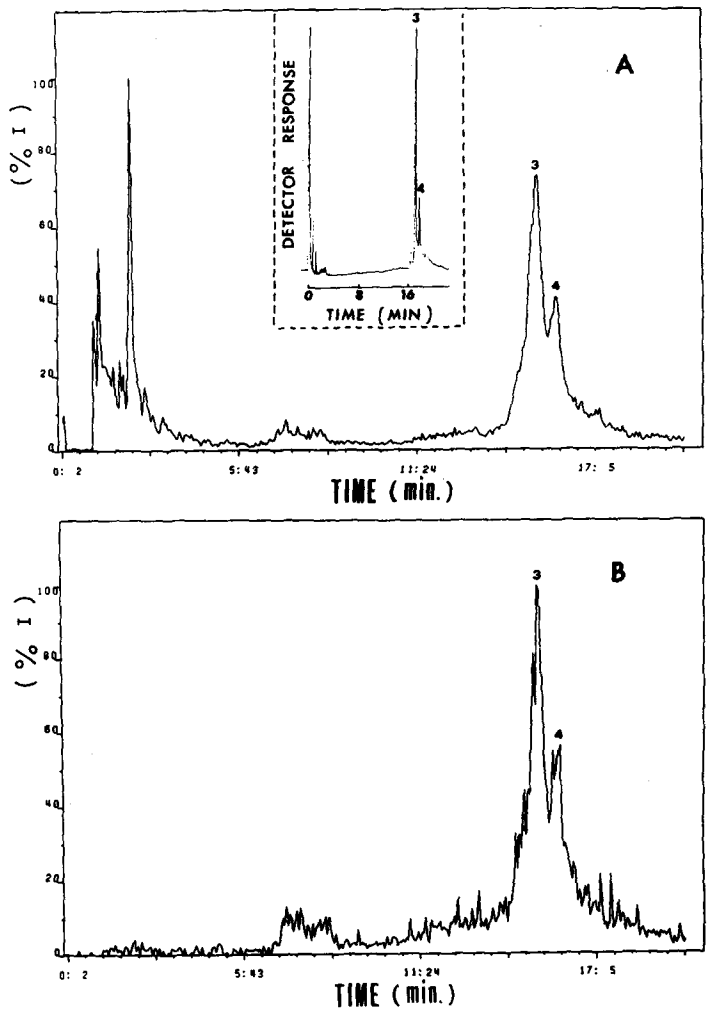


FIG. 5. Capillary GC/MS of isolated dihydroxy steroid sapogenins from feces of dogs receiving steroid saponins. A: Ion electron impact chromatogram (capillary GC). B: Ion current profile of selected ion *m/z* 139.

bean saponins reduced intestinal uptake of cholesterol in the rat (6). It appears likely that saponins and cholesterol form insoluble complexes (25–27) which may retard cholesterol absorption in the intestine. On the other hand, certain saponins from soapwort (*Saponaria officinalis L*), soybeans, and quillaya (*Quillaya saponaria*) also form large mixed micelles with bile acids (28). The increase in fecal excretion of bile acids and neutral steroids could thus be compensated for by an increased conversion of cholesterol to bile acids by the liver. Furthermore, in the rat fenugreek induced an increase in bile acid output (2,29), which suggests increased transformation of cholesterol into bile acids by the liver. This could contribute to the hypocholesterolemic activity of fenugreek.

Our results show that the ingested fenugreek saponins are partially hydrolyzed to diosgenin in the gastrointestinal tract. Other steroid sapogenins (smilagenin, gitogenin) were also found in the feces of the dogs. It is known that diosgenin interferes with the absorption of cholesterol (10). The decreased cholesterol absorption associated with enhanced secretion of cholesterol into bile resulted in increased excretion of neutral sterols (10,12).

Our results suggest that the hypocholesterolemic effect of fenugreek seeds might be due to saponins and that diosgenin produced by gastrointestinal hydrolysis may contribute to this effect.

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Influence of Genotype on Diet-Induced Changes in Membrane Phosphatidylcholine and Phosphatidylethanolamine Composition of Splenocytes, Liver Nuclear Envelope and Liver Mitochondria

J.T. Venkatraman, R.K. Tiwari[1], B. Cinader[2], J. Flory, T. Wierzbicki and M.T. Clandinin*
Nutrition and Metabolism Research Group, Departments of Foods & Nutrition and Medicine, Division of Gastroenterology, University of Alberta, Edmonton, Alberta, Canada T6G 2C2

Inbred congenic mice of strains MRL/Mp-lpr/lpr (lpr/lpr) and MRL/Mp-+/+ (+/+) were fed nutritionally adequate semipurified diets containing 20% (w/w) fat and differing in linoleic acid content. Levels of linoleic acid (18:2n-6) and arachidonic acid (20:4n-6) in phospholipids of splenocytes, liver mitochondria and liver nuclear envelopes were determined. Membranes of lpr/lpr mice exhibited significantly lower levels of 18:2n-6 and 20:4n-6 in phospholipids compared with the +/+ strain. The high linoleic acid diet increased incorporation of 18:2n-6 and 20:4n-6 in most phospholipid fractions of these membranes. These observations indicate that genotype as well as dietary 18:2n-6 content significantly influenced incorporation of 18:2n-6 and 20:4n-6 into membrane phospholipids. The results also suggest that membrane compositional abnormalities found in the lpr/lpr mice, which develop lymphoma and age faster than +/+ mice, are not restricted to the immune system but also extend to other organs. Differences observed in phospholipid fatty acid composition in splenocytes and liver subcellular membranes for mice fed diets differing in linoleic acid content suggest that the early expression of the lpr gene resulting in progression of autoimmunity may be delayed through dietary manipulation.
Lipids 26, 198–202 (1991).

MRL/Mp-lpr/lpr (lpr/lpr) mice have a genetic predisposition for a particular age-related change in H1 Histone subtypes (1) and lipid composition (2). Age-related changes of this mouse represent extremes in the polymorphism of various immunologic features, involving proliferative disorder, autoimmunity and accumulation of lymphocytes bearing the immature phenotype Thy-1$^+$Lyt2$^-$L3t4$^-$ (3–6). The congenic partner, MRL/Mp-+/+ (+/+), does not manifest these abnormalities at an early age. Some of the abnormalities are inherent in the MRL/Mp-genome and are accelerated by the lpr/lpr but not by the +/+ gene (7).

Nutrition profoundly influences development of autoimmune disease, immune functions and life span (8,9). Dietary lipids may influence incidence of autoimmunity by altering the composition of the membrane lipid bilayer and thereby activate or inhibit several key enzymes (10). Through dietary lipid manipulation (11), molecular studies have revealed diminished ribonucleic acid (mRNA) levels for several oncogenes in lpr/lpr mice. The lipid composition of the membrane also influences the conformation of some receptors (12,13). The lpr/lpr mice exhibit impairment of membrane functions (14), altered membrane fluidity (15) and failure to produce regulatory factors which interrupt autoimmunity (16).

In the present investigation, lpr/lpr and +/+ mice were fed nutritionally adequate diets which were high or low in linoleic acid to examine the influence of genetic background and the level of dietary linoleic acid on the phospholipid fatty acid composition of splenocyte and hepatocyte subcellular membranes.

MATERIALS AND METHODS

Animals. Six-week-old female lpr/lpr and +/+ mice purchased from Jackson Laboratories (Bar Harbor, ME) were fed nutritionally adequate semipurified diets (17) *ad libitum*, providing 20% (w/w) fat containing a low (4% of calories) or high (22% of calories) level of linoleic acid (Table 1). The high linoleic acid fat was provided

TABLE 1

Composition of the Diets

Ingredients	g/kg of total diet
Fat	200
Starch	200
Casein	270
Glucose	212.65
Non-nutritive fiber	50
Vitamin mix[a]	10
Mineral mix[b]	50.85
L-Methionine	2.5
Choline	2.75
Inositol	1.25

[a]A.O.A.C. vitamin mix (Teklad Test Diets, Madison, WI) provided the following per kg of complete diet: 20,000 IU vitamin A; 2000 IU vitamin D; 100 mg vitamin E; 5 mg menadione; 5 mg thiamin-HCl; 8 mg riboflavin; 40 mg pyridoxine-HCl; 40 mg niacin; 40 mg pantothenic acid; 2000 mg choline; 100 mg myoinositol; 100 mg *p*-aminobenzoic acid; 0.4 mg biotin; 2 mg folic acid; and 30 mg vitamin B-12.

[b]Bernhart Tomarelli mineral mix (General Biochemicals, Chagrin Falls, OH) was modified to provide 77.8 mg of Mn^{2+} and 0.06 mg Se^{2+}/kg complete diet.

[1]Sloan Kettering Institute for Cancer Research, New York, New York.
[2]Department of Immunology, University of Toronto, Ontario, Canada.
*To whom correspondence should be addressed at the Department of Foods & Nutrition, 318f Home Economics Building, University of Alberta, Edmonton, Alberta, Canada T6G 2M8.

Abbreviations: DNase-1, deoxyribonuclease; IL-2, Interleukin-2; mRNA, ribonucleic acid; TNF_α, tumor necrosis factor α; UV, ultraviolet.

TABLE 2

Effect of Strain, Diet and Duration of Feeding on Fatty Acid Content in Phospholipids of Splenocytes from lpr/lpr and +/+ Mice

	Strain (lpr/lpr)				Strain (+/+)					Level of significance/main effects		
	High[a]		Low		High		Low		Pooled			Duration of
	1[b]	6	1	6	1	6	1	6	SEM	Strain	Diet	feeding
Phosphatidylcholine					(values expressed as percent w/w)							
16:0	20.0	37.4	24.9	33.2	18.5	36.6	26.2	37.0	1.2	***	***	***
18:0	14.2	12.6	11.2	11.0	15.0	13.1	18.5	12.6	1.6	***	NS	NS
18:1n-9	5.9	8.6	10.8	13.0	7.3	9.5	17.2	11.8	1.1	*	***	NS
18:2n-6	4.3	13.6	5.6	5.5	3.8	13.9	4.4	4.0	1.5	NS	***	*
20:4n-6	12.5	13.8	13.8	14.4	9.3	15.2	17.0	23.0	1.5	**	**	NS
20:5n-3	1.4	0.2	0.3	0.2	0.4	0.3	0.4	0.2	0.1	*	**	*
22:6n-3	3.9	2.3	2.7	2.6	4.1	2.2	4.7	9.6	0.8	***	**	NS
Phosphatidylethanolamine					(values expressed as percent w/w)							
16:0	9.4	10.7	4.9	3.3	20.3	18.7	17.7	14.8	2.6	***	*	NS
18:0	26.4	32.0	15.4	17.4	17.1	23.0	21.3	17.1	1.2	***	***	*
18:1n-9	5.3	7.2	7.3	7.2	7.4	7.2	7.2	12.4	0.7	***	***	**
18:2n-6	5.9	6.3	2.0	2.2	3.7	7.5	3.3	2.1	0.6	NS	***	NS
20:4n-6	23.2	26.7	21.8	21.9	8.5	15.4	17.6	6.9	1.5	***	NS	NS
20:5n-3	0.3	0.1	0.8	0.3	1.0	0.7	1.2	0.5	0.2	**	NS	**
22:6n-3	13.1	10.2	8.8	8.3	5.9	6.0	9.7	2.5	0.8	***	*	***

[a]Dietary linoleic acid.
[b]Weeks of feeding.

Statistical analysis as 2 × 3 factorial design *p<0.05; **p<0.01; ***p<0.001; NS — Not significant. Values are mean of 3 independent determinations. Significant interactions were not found.

by soybean oil and the low linoleic acid fat was a blend of beef tallow and sunflower oil. The fatty acid composition of these diets is as follows: the high linoleic acid diet contained 14:0, 0.3%; 16:0, 15.2%; 16:1, 0.2%; 18:0, 5.6%; 18:1, 29.3%; 18:2ω6, 42.3%; 18:3ω3, 3.4%; and the low linoleic acid diet contained 14:0, 3.6%; 16:0, 27.5%; 16:1, 2.1%; 18:0, 22.0%; 18:1, 34.4%; 18:2ω6, 7.5%; 18:3ω3, 0.6%. Mice were fed these diets for a period of one to six weeks in mouse boxes containing feed cups. All animals were housed in a light, temperature and humidity controlled room on a 12/12 hr light-dark cycle. Animals were sacrificed by cervical dislocation and livers and spleens were collected on ice.

Splenocytes. Spleens were minced and passed over a steel mesh (No. 110-150) with a spatula. The resulting suspension in CMRL 1066 medium was further purified (18), passed through cotton gauze and centrifuged at 900 × *g* for 10 min. Cells were washed with CMRL 1066 medium to free them of red blood cells by treatment with Tris NH_4Cl (pH 7.2) three times. Cells were suspended in 1 mL of medium, diluted appropriately and counted. Viability of splenocytes was measured by trypan blue dye exclusion test and cells with 95% viability were used.

Liver mitochondria. Liver mitochondria were prepared by the method of Dow (19). Liver (1 g) was homogenized in a Teflon homogenizer with 10 mL of 0.25 M sucrose buffer and centrifuged at 2500 × *g* for 15 min. The supernatants were pooled and centrifuged (10,000 × *g* for 30 min) to obtain the mitochondrial pellet. The mitochondria were washed again by resuspension with 0.25 M sucrose and centrifuged at 10,000 × *g* for 30 min. Mitochondrial purity was checked by assaying for the presence of succinic dehydrogenase activity and the absence of 5′-nucleotidase and glucose-6-phosphatase.

Nuclear envelopes. Liver nuclear membranes were prepared by extensive digestion of purified nuclei with deoxyribonuclease (DNase-1). Nuclei were isolated by modification of the procedure of Widnell and Tata (20). The resulting pellet of purified nuclei was used to prepare nuclear envelopes by modifications of the procedure of Kay *et al.* (21). The nuclei were resuspended in 0.25 M sucrose containing 1 mM $MgCl_2$ (pH 7.4) and centrifuged at 750 × *g* for 5 min. The pellet was resuspended in digestion buffer (10 mM Tris HCl, 0.30 M sucrose, 0.1 mM $MgCl_2$ and 5 mM 2-mercaptoethanol, pH 8.5) to which freshly dissolved DNase-1 was added to a concentration of 10 μg/mL. The suspension was incubated at room temperature for 20 min. Following centrifugation (12,000 × *g* for 5 min) the crude nuclear envelopes were resuspended in 10 mM Tris HCl (pH 7.4) and 2 mL of this suspension was layered onto a discontinuous sucrose gradient of 5 mL of 0.25 M sucrose and 10 mL each of 1.5 M, 1.8 M, and 2.0 M sucrose (sucrose solutions made up in 10 mM Tris HCl, pH 7.4). The sucrose gradients were centrifuged (100,000 × *g* for 90 min), and the major band of puri-

TABLE 3

Effect of Strain, Diet and Duration of Feeding on the Fatty Acid Composition of Liver Mitochondria from lpr/lpr and +/+ Mice

	Strain (lpr/lpr)				Strain (+/+)					Level of significance/main effects		
	High[a]		Low		High		Low		Pooled			Duration of
	1[b]	6	1	6	1	6	1	6	SEM	Strain	Diet	feeding
Phosphatidylcholine					(values expressed as percent w/w)							
16:0	22.5	19.8	22.8	25.5	25.7	27.4	26.6	25.8	1.1	***	NS	NS
18:0	18.0	22.4	20.5	18.8	17.8	14.9	16.6	16.1	1.1	***	NS	NS
18:1n-9	5.4	4.7	10.0	16.3	5.9	6.0	14.8	15.8	0.9	*	***	*
18:1n-7	1.4	1.4	1.9	4.3	1.6	0.8	1.3	1.3	0.2	***	***	***
18:2n-6	15.3	17.8	12.8	15.2	17.4	16.4	15.0	13.4	0.7	NS	***	NS
20:4n-6	16.7	14.5	11.9	9.3	20.0	20.2	11.1	12.6	1.3	***	***	NS
20:5n-3	0.2	0.7	0.2	0.1	0.1	0.1	0.1	0.1	0.042	***	***	***
22:6n-3	7.7	6.8	5.5	5.2	8.0	8.8	8.7	7.0	0.7	***	**	NS
Phosphatidylethanolamine					(values expressed as percent w/w)							
16:0	16.8	17.1	16.5	13.2	19.2	20.8	12.1	18.9	0.7	***	NS	NS
18:0	24.4	27.7	21.9	27.6	26.6	20.1	23.4	19.7	1.3	**	NS	NS
18:1n-9	6.3	6.3	5.4	11.0	11.6	11.2	11.8	12.9	0.9	***	*	*
18:2n-6	4.3	6.0	2.6	7.4	5.5	4.5	5.2	4.8	0.5	NS	NS	***
20:4n-6	23.2	23.9	23.6	24.9	19.7	23.3	22.3	23.5	1.1	*	NS	*
20:5n-3	0.1	0.1	0.1	0.8	0.1	0.1	0.1	0.1	0.03	**	NS	*
22:6n-3	13.0	11.9	20.4	10.2	14.2	16.7	20.0	15.1	1.4	**	*	**

[a]Dietary linoleic acid.
[b]Weeks of feeding.

Statistical analysis as 2 × 3 factorial design *p<0.05; **p<0.01; ***p<0.001; NS — Not significant. Values are mean of 3 independent determinations.

fied nuclear envelopes was removed from the gradient and washed twice in 10 mM Tris HCl (pH 7.4) at 33,000 × *g* for 10 min. Membrane purity was assessed as described earlier (22,23). Nuclear envelopes prepared in this manner are free of other subcellular fractions (23).

Extraction of lipid and purification of membrane phospholipids. Lipids were extracted from membranes (24) in the presence of 2-ethoxyquin as an antioxidant, and extracts were dried under nitrogen. Lipid samples were subjected to thin-layer chromatography. High performance thin-layer plates, LHPK (HP T2C) (10 × 1.09 cm), were used for separating phospholipids. The solvent system chloroform/methanol/2-propanol-0.25% (w/v) potassium chloride/triethylamine (30:9:25:6:18, by vol) was used (25). Individual phospholipids were visualized by spraying the plates with 1% (w/v) 8-anilino-1-naphthalenesulfonic acid and viewing under ultraviolet (UV) light. Individual phospholipids were scraped directly into tubes to which hexane was added. Phospholipid fatty acids were methylated with BF_3 in methanol (26). Methyl esters were analyzed by automated capillary gas-liquid chromatography (27). Fatty acids analyzed included all saturated and unsaturated fatty acids of 14 to 24 carbons in chain length. Only data for major saturated, monounsaturated and polyunsaturated fatty acids are illustrated where significant effects were observed. Main effects (*i.e.* those due to strain, diet and duration) are presented where no significant interactions were observed.

Statistical analysis. The effect of strain, diet and duration of treatment was determined by analysis of variance procedures (28).

RESULTS

Fatty acid composition of splenocytes. Strain exhibited a significant effect on saturated fatty acid as well as on the 20:4n-6 content of phosphatidylcholine and phosphatidylethanolamine fractions of splenocytes (Table 2). In the phosphatidylcholine fraction, the +/+ strain exhibited higher levels of saturated fatty acids and 20:4n-6, compared to the lpr/lpr strain. The lpr/lpr strain exhibited higher 20:4n-6 and 18:0 levels in the phosphatidylethanolamine fraction. Change in linoleic acid to saturated fat level in the diet also influenced the fatty acid composition of the phosphatidylcholine and phosphatidylethanolamine fractions of splenocytes. Linoleic acid increased in the phosphatidylcholine fraction of the splenocytes for animals fed the high linoleic acid to saturated fat diet while an increase in 16:0, 18:1n-9 and 20:4 was observed in mice fed the low linoleic acid diet. In contrast, in the phosphatidylethanolamine fraction there was an increase in 16:0, 18:0 and 18:2, and a decrease in 18:1n-9 in splenocytes of animals fed the high linoleic acid diet.

TABLE 4

Effect of Strain, Diet and Duration of Feeding on the Fatty Acid Composition of Liver Nuclear Envelopes from lpr/lpr and +/+ Mice

	Strain (lpr/lpr)				Strain (+/+)					Level of significance/main effects		
	High[a]		Low		High		Low		Pooled			Duration of
	1[b]	6	1	6	1	6	1	6	SEM	Strain	Diet	feeding
Phosphatidylcholine	(values expressed as percent w/w)											
16:0	28.4	26.9	30.5	26.6	27.8	26.0	25.9	28.1	0.4	***	NS	***
18:0	18.0	18.7	18.9	18.8	18.1	20.1	19.8	17.9	0.3	NS	NS	NS
18:1n-9	6.2	5.7	14.9	14.2	6.1	5.7	15.6	16.6	0.3	***	***	NS
18:2n-6	18.2	20.3	12.5	12.4	20.6	21.5	14.1	14.2	2.0	**	***	NS
20:4n-6	17.4	17.9	13.2	17.6	17.3	18.0	14.5	13.3	0.7	**	***	***
20:6n-3	5.9	5.8	4.5	5.0	5.5	5.8	4.9	4.6	0.2	NS	**	NS
Phosphatidylethanolamine	(values expressed as percent w/w)											
16:0	19.5	14.6	27.3	16.8	19.4	16.7	22.5	19.1	1.0	NS	***	***
18:0	23.0	26.2	23.7	26.0	22.7	24.0	21.5	29.4	1.5	NS	NS	**
18:1n-9	11.0	9.2	13.5	11.9	11.7	12.5	14.2	13.7	0.9	*	**	NS
18:2n-6	8.6	8.6	5.9	5.2	8.9	11.0	6.5	7.7	0.7	**	***	NS
20:4n-6	18.4	21.1	14.5	22.8	18.4	17.6	19.7	20.7	1.0	NS	NS	***
22:6n-3	14.1	13.6	8.5	11.7	12.9	12.8	11.2	10.1	1.2	NS	**	NS

[a]Dietary linoleic acid.
[b]Weeks of feeding.

Statistical analysis as 2 × 3 factorial design *p<0.05; **p<0.01; ***p<0.001; NS — Not significant. Values are mean of 3 independent determinations.

Fatty acid composition of liver mitochondria. Significantly higher levels of 18:1n-9 and 22:6n-3 and lower levels of 18:0 and 20:5n-3 were observed in phosphatidylethanolamine and phosphatidylcholine fractions from mitochondria of +/+ mice suggesting that strain had considerable influence (Table 3). The phospholipid content of 18:2n-5 was similar for both strains but the level of 20:4n-6 was significantly different in the mitochondrial phosphatidylcholine fraction. Dietary linoleic acid exhibited a significant influence on the composition of fatty acids in mitochondrial phosphatidylcholine while the fatty acid profile of the phosphatidylethanolamine fraction did not exhibit alterations due to dietary linoleic acid level.

Fatty acid composition of nuclear envelopes. The nuclear envelopes isolated were free of cytoplasmic tags and other subcellular membranes. Genetic strain was found to have a significant but small influence on 18:1n-9 and 18:2n-6 content of nuclear envelope phospholipids (Table 4). The concentration of 16:0 was higher in the phosphatidylcholine fraction of nuclear envelopes prepared from the lpr/lpr animals with the exception of animals fed the low linoleic acid diet for 6 wk. Dietary linoleic acid significantly increased levels of 18:2n-6 and 20:4n-6 in the phosphatidylcholine fraction as well as 18:2n-6 and 22:6n-3 content of the phosphatidylethanolamine fraction of the liver nuclear envelope.

DISCUSSION

Age-related changes in MRL/Mp-lpr/lpr mice occur relatively early in life due to the expression of the lpr/lpr gene (7). These mice develop a defect in the regulatory balance of the immune system and autoimmunity relatively early in life (15). Nutritional approaches to treatment of autoimmune disorders have shown that dietary components may prevent, delay or reverse expression of genetically determined autoimmune defects. The present study demonstrates that mice with the lpr/lpr gene exhibited altered membrane fatty acid composition in a variety of membrane types. It is possible to alter significantly the phospholipid fatty acid composition of membrane types by altering the level of dietary linoleic acid. Thus, the present results suggest that it is conceivable that we may be able to control early expression of the lpr gene or the effect of the gene on membrane phenotype through dietary manipulation.

The spleen is a central site for production of immunologically active prostaglandins (29). Arachidonic (20:4n-6) is an important precursor for prostaglandins involved in promoting lymphokine production (30). For example, Interleukin-2 (IL-2) is essential for maturation of specific T cell populations (31). Mice with the lpr/lpr gene develop generalized massive lymphadenopathy resulting from accumulation of immature Thy-1+Lyt2-L3T4- cells (4) due to the lack of secretion of adequate regulatory factors like IL-2 or biological modifiers like tumor necrosis factor α(TNFα) which modulate autoimmunity (32). Our earlier experiments on splenic T and B cells of lpr/lpr and +/+ mice suggested that the fatty acid composition of membranes was genetically determined and could be altered by ma-

nipulation of dietary lipid (2). Immune responses, such as cell-mediated cytolysis and binding of immunoregulatory hormone IL-2 to T-lymphocytes, are sensitive to changes in composition and physical properties of cell membranes suggesting that several molecular events involving receptors may also be involved in these processes.

Liver nuclear envelope phospholipid fatty acid composition between the two congenic strains of mice was markedly different. Moreover, the dietary linoleic acid level significantly altered this difference. Our studies on functions of the liver nuclear envelope of lpr/lpr mice demonstrate that these mice also possess subcellular abnormalities in mechanisms controlling nuclear activity. For example, lpr/lpr mice exhibit significantly higher nuclear envelope nucleoside triphosphatase activity and nuclear ribonucleic acid release in liver compared to +/+ mice that could be related to differences in the nuclear envelope lipid composition (33).

Perhaps it is reasonable to speculate that dietary lipid may influence the incidence of autoimmunity by altering the composition of membrane, and activate or inhibit several key enzymes or gene expression (10). In this regard, it is possible to alter the levels of mRNAs for several oncogenes in lpr/lpr mice through dietary lipid manipulation (11). Dietary lipid also alters the level of expression of oncogenes in mice (34). Age-related change in expression of the α2-globulin gene exists at the transcriptional level in response to diet (35). In the lpr/lpr autoimmune mouse, the lpr gene is expressed at very early stages of life. Whether or not membrane fatty acid composition is one factor contributing to early expression of the lpr gene or results from expression of the gene remains to be defined.

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Effect of Low Intake of n-3 Fatty Acids During Development on Brain Phospholipid Fatty Acid Composition and Exploratory Behavior in Rats

Marc Enslen, Hubert Milon and **Armand Malnoë***
Nestec Ltd., Research Centre, Vers-chez-les-Blanc, CH-1000 Lausanne 26, Switzerland

The effect of dietary restriction of n-3 fatty acids during development on brain phospholipid fatty acid composition and exploratory behavior has been studied in male Sprague Dawley rats. Female rats were fed semi-purified diets containing either 5.5% safflower oil or 6% soybean oil for 6 wk prior to mating and throughout gestation and lactation. Control rats were maintained on laboratory chow. The male pups were weaned to the diets of the dams except for one group which was switched from safflower to soybean oil at weaning. Behavioral studies and brain phospholipid analyses were conducted at 16–18 wk of age. Rats fed safflower oil showed significantly lower levels of 22:6n-3 in phospholipids of synaptic membranes and myelin than rats fed soybean oil or chow. The decrease in 22:6n-3 was compensated for by an increase in 22:5n-6, the total content of polyunsaturated fatty acids remaining approximately constant. The brain phospholipid fatty acid composition of rats switched from safflower to soybean oil at weaning was similar to that of rats fed soybean oil throughout the experiment. There was no difference in spontaneous locomotor activity among the different dietary groups. However, rats raised on safflower oil displayed a significantly lower exploratory activity (horizontal movements and rearings) in a novel environment than rats fed soybean oil or chow. In contrast to the brain phospholipid fatty acid composition, there was no recovery of exploratory behavior in rats raised on safflower oil and switched to soybean oil at weaning suggesting a specific requirement of n-3 fatty acids during development.
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Docosahexaenoic acid (22:6n-3) is one of the major polyunsaturated fatty acids (PUFA) in the phospholipids of the central nervous system (CNS) (1). Although the brain is relatively resistant to diet-induced alterations in n-3 fatty acid levels (2), dietary deprivation of essential fatty acids during brain development, when requirements are particularly high (3,4), produces significant modifications in the fatty acid profile of brain phospholipids (5–9).

A low intake of dietary n-3 fatty acids has been associated with learning deficits in rats (5,10,11) as well as visual abnormalities in both rats and monkeys (11–14). This may be due to abnormal patterns of development as suggested by studies demonstrating the essentiality of these fatty acids for normal cell growth (15). Furthermore, modifications of the membrane phospholipid fatty acid composition may induce changes in the physical properties of membranes (*i.e.*, fluidity) and in the activity of membrane-bound enzymes (16,17) which may ultimately affect animal behavior, although evidence for such changes in the CNS is rather limited (for a review see Neuringer *et al.*, ref. 18). Alternatively, n-3 fatty acids may influence cellular processes in the CNS by interfering with the metabolism of arachidonic acid and the synthesis of eicosanoids (19). This may account for the behavioral effects observed after short dietary treatments with different fats when brain phospholipid fatty acid composition is not expected to be significantly affected (20–22).

In this study, we have investigated the effect of a reduced intake of n-3 fatty acids during development on locomotor activity and exploratory behavior in adult rats. The effect of diets on the phospholipid fatty acid composition of brain synaptic membranes and myelin is also reported. Finally, we have studied the reversibility of these effects upon feeding n-3 fatty acids to weanling n-3 deficient rats.

MATERIALS AND METHODS

Animal studies. Sprague Dawley rats (Iffa Credo, L'Arbresle, France) were used throughout these studies. Animals were housed individually in polypropylene cages (22 × 36 × 15 cm) and maintained in a room with controlled temperature (22°C), humidity (55%) and cyclic light (12 hr light/12 hr dark, light on at 7 a.m.). They were allowed food and water *ad libitum.* Female rats (3 mo old) were randomly assigned into three groups. Two groups were fed semi-purified diets containing either 6% soybean oil or 5.5% safflower oil (Table 1). A third group was maintained on a laboratory chow containing 5% (w/w) fat (Kliba AG, Kaiseraugst, Switzerland). The fatty acid composition of the diets is presented in Table 2. Rats from all groups were mated after 6 wk on the respective diets with male rats of the same age maintained on laboratory chow except during the mating period. The dams were continued on the respective diets during gestation and lactation. The litters were adjusted to 8 pups after parturition. The male pups were weaned on the same diet as the dams except for one group (labelled "safflower/soybean oil" in tables) in which safflower oil was replaced by soybean oil at weaning (21 days). Litter size and weight gains were not significantly different among the different dietary groups.

Exploratory behavior and locomotor activity. Recordings were carried out between 16 and 18 wk of age. Exploratory behavior was measured as the num-

*To whom correspondence should be addressed at Nestec S.A., Labior, CH-1350, Orbe, Switzerland.

Abbreviations: PUFA, polyunsaturated fatty acids; CNS, central nervous system.

TABLE 1

Composition of Diets

Ingredients (g/100 g)	Diets	
	Safflower oil	Soybean oil
Casein	20	20
L-Methionine	0.3	0.3
Vitamin mix. Ain76[a]	1	1
Salt mix. Ain76[b]	3.5	3.5
Choline bitartrate	0.2	0.2
Inositol	0.025	0.025
Cellulose	5	5
Sucrose	25	25
Cornstarch	39.5	39.0
Safflower oil	5.5	—
Soybean oil	—	6

[a]Vitamin mix in g/kg: thiamine hydrochloride, 0.6; riboflavin, 0.6; pyridoxine hydrochloride, 0.7; nicotinic acid, 3; D-calcium pantothenate, 1.6; folic acid, 0.2; D-biotin, 0.02; cyanocobalamin, 0.001; retinyl palmitate, 1.6; DL-α-tocopherol acetate, 20; cholecalciferol, 0.25; menaquinone, 0.050 and sucrose, 972.9.

[b]Salt mix composition in g/kg: calcium phosphate dibasic, 500; sodium chloride, 74; potassium citrate monohydrate, 220; potassium sulfate, 52; magnesium oxide, 24; manganous carbonate, 3.5; ferric citrate, 6; zinc carbonate, 1.60; cupric carbonate, 0.3; potassium iodate, 0.01; sodium selenite, 0.01; chromium potassium sulfate, 0.55 and sucrose, 118.

TABLE 2

Fatty Acid Composition of Diets (%, w/w)

Fatty acid	Diets		
	Safflower oil	Soybean oil	Lab chow
14:0	0.19	0.13	0.65
16:0	6.71	9.55	15.69
16:1n-7	0.09	0.10	0.80
18:0	2.53	3.49	4.05
18:1n-9	13.04	28.10	21.79
18:2n-6	73.70	47.68	45.41
18:3n-6	—	0.47	—
18:3n-3	0.10	6.53	4.37
18:4n-3	0.05	0.06	0.18
20:0	0.30	0.34	0.28
20:1n-9	0.21	0.37	0.58
20:5n-3	—	—	0.77
22:0	0.22	0.51	0.32
22:5n-3	—	—	0.17
22:6n-3	—	—	0.84
22:1n-9	0.18	—	0.35
24:0	0.12	0.17	—
24:1n-9	0.16	0.04	0.12
Unsaturated	87.60	83.50	75.40
n-3 + n-6	73.90	54.80	51.74
n-6/n-3	491	7.30	7.20

ber of horizontal movements (crossings) and rearings of the animal exposed to a novel environment for 60 min. The novel environment consisted of Macrolon cages (48 × 38 × 28 cm) equipped with four infrared photocells (Datalogic) placed 4 cm above the floor for measurements of horizontal movements and three cells placed 10 cm above the floor for detection of rearing activity. The cages were located in a soundproof chamber with a 10 lux illumination. Measurements were started immediately after introduction of the animal into the cage and counts were recorded during 60 min using a SODECO-SAIA recorder (SODECO, Geneva, Switzerland). Activity was measured between 2 and 3 p.m.

The spontaneous locomotor activity was measured as the number of crossings and rearings accumulated in periods of 12 hr during a light/dark cycle. The recording was performed in the same cages and with the same rats as above after allowing the animals to adapt to their new environment for a period of 17 hr (from 2 p.m. to 7 a.m.). During behavioral testing, the animals had free access to their respective diets and water.

Brain membrane preparation. Rats were sacrificed at 16 wk of age by decapitation. The brain was rapidly removed and subcellular fractions consisting of synaptic membranes and myelin were prepared according to the method of Jones and Matus (23).

Fatty acid analyses. Membrane lipids were extracted with chloroform/methanol (2:1, v/v) according to the method of Folch *et al.* (24). The phospholipids were separated from neutral lipids and free fatty acids on Supelclean-NH2 (Supelco, Bellefonte, PA) resin as previously described (25). A phospholipid fraction containing both phosphatidylcholine and phosphatidylethanolamine was isolated by elution with methanol. The fatty acids of this fraction were methylated by alkaline methanolysis (26). The fatty acid methyl esters were analyzed by gas-liquid chromatography on a Carlo-Erba model 5160 Mega series gas chromatograph equipped with a flame ionization detector. The gas chromatographic conditions were: a capillary column of 30 m × 0.32 mm i.d. coated with Carbowax 20 M (Restek Corporation, Bellefonte, PA). The oven temperature program was: 100°C, 1 min isothermal; 15°C/min to 145°C, 1 min isothermal; 4°C/min to 195°C, 6°C/min to 220°C, 11 min isothermal. The detector was set at 320°C. Chromatograms were recorded with a Hewlett-Packard HP3354 Data System integrator. Identification of peaks was made by comparison of retention times with those of known standards run under the same conditions.

Statistics. Results of behavioral recordings and fatty acid compositions were analyzed using one- or two-way analyses of variance (ANOVA) followed by Tukey's test for comparison between groups. Differences between groups were considered statistically significant when p was <0.05.

RESULTS

Brain phospholipid fatty acid composition. In rats fed a diet low in n-3 fatty acids (safflower oil), the major change in brain phospholipid fatty acid composition was a significant reduction in the content of 22:6n-3 in both synaptic membranes and myelin as compared to rats fed soybean oil or chow (Tables 3 and 4). The decrease in 22:6n-3 was compensated for by an increase in the n-6 fatty acids, 22:5n-6 and to a lesser extent 22:4n-6. The total content of PUFA in synaptic membrane phospholipids was similar for all dietary groups. However, in myelin, there was a slight but

TABLE 3

Effect of Dietary Fats on the Fatty Acid Composition of Synaptic Membrane Phospholipids[a]

	Diets			
Fatty acid	Safflower oil	Soybean oil	Safflower/ soybean oil	Lab chow
12:0	0.69 ± 0.04	0.77 ± 0.05	0.70 ± 0.08	0.91 ± 0.08
14:0	0.29 ± 0.03	0.29 ± 0.03	0.33 ± 0.03	0.31 ± 0.04
16:0	30.30 ± 0.42	29.18 ± 0.74	29.51 ± 0.23	30.10 ± 0.17
16:1n-7	0.30 ± 0.01	0.34 ± 0.01	0.35 ± 0.01	0.35 ± 0.02
17:0	0.23 ± 0.02	0.32 ± 0.01	0.27 ± 0.03	0.29 ± 0.01
18:0	17.13 ± 0.20	16.81 ± 0.07	16.80 ± 0.07	16.68 ± 0.15
18:1n-9	16.39 ± 0.09	17.01 ± 0.15	17.81 ± 0.21	17.76 ± 0.11
18:2n-6	0.56 ± 0.07	0.52 ± 0.06	0.55 ± 0.02	0.59 ± 0.06
20:1n-9	0.72 ± 0.04	0.76 ± 0.08	0.71 ± 0.03	0.74 ± 0.12
20:2n-6	0.54 ± 0.09	0.43 ± 0.17	0.50 ± 0.06	0.48 ± 0.09
20:3n-6	0.23*	0.32 ± 0.05	0.32 ± 0.08	0.35 ± 0.04
20:4n-6	10.76 ± 0.35	12.00 ± 0.98	10.03 ± 0.18	10.18 ± 0.07
22:4n-6	4.60 ± 0.13[a]	3.63 ± 0.07[b]	3.72 ± 0.12[b]	3.59 ± 0.06[b]
22:5n-6	12.06 ± 0.31[a]	—	0.76 ± 0.06[b]	—
22:6n-3	5.47 ± 0.40[a]	18.12 ± 0.17[b]	17.96 ± 0.19[b]	17.82 ± 0.16[b]
Unsaturated	51.47 ± 0.50	52.85 ± 0.75	52.51 ± 0.36	51.92 ± 0.32
n-3 + n-6	34.05 ± 0.51	34.73 ± 0.89	33.64 ± 0.21	33.06 ± 0.26
n-6/n-3	5.31 ± 0.49[a]	0.92 ± 0.07[b]	0.87 ± 0.03[b]	0.86 ± 0.01[b]

[a]Groups of rats were fed diets containing safflower or soybean oil throughout development and up to the age of 16 wk except for one group which was switched from safflower to soybean oil at 21 days (safflower/soybean oil). Control rats were raised on lab chow. Brain synaptic membranes were isolated and the fatty acid composition of a phospholipid fraction consisting of phosphatidylethanolamine and phosphatidylcholine was analyzed as described in Materials and Methods. Values are means ± S.E.M. (n=4) expressed as % (w/w) of identified fatty acids. Values with different superscripts in the same row are significantly different ($p<0.05$) from each other (one-way ANOVA followed by Tukey's test for comparison between groups).

*n=1.

significant increase (+15%) in total PUFA (n-3 + n-6) in rats fed safflower oil (Table 4). The levels of arachidonic acid in synaptic membranes (Table 3) were not significantly affected by the dietary fats. In myelin (Table 4), the levels were slightly lower in chow-fed rats than in the other groups but there was no difference between the safflower and the soybean oil group. The phospholipid fatty acid composition of both synaptic membranes and myelin was otherwise similar in rats fed chow or the soybean oil-based diet. Furthermore, rats raised on safflower oil and switched to soybean oil at weaning displayed the same phospholipid fatty acid profile as rats fed soybean oil throughout the experiment (Tables 3 and 4). Thus, the changes in phospholipid fatty acid composition induced by the diet low in n-3 fatty acids appear to be reversible since previous studies in this laboratory (27) have shown that in rats raised on safflower oil, low levels of 22:6 n-3 are already present at birth and throughout the lactation period.

Exploratory behavior. As shown in Table 5 the exploratory activity (crossings and rearings) following exposure to a novel environment was significantly lower in rats raised on safflower oil than in animals raised on soybean oil. The soybean oil- and chow-fed groups displayed similar crossing activities whereas rearing activity was slightly higher in the soybean oil group but the difference was not statistically significant. Rats raised on safflower oil and switched to soybean oil at weaning also displayed a decreased number of crossings and rearings, the latter being significantly lower ($p<0.05$) than that of rats fed soybean oil.

Spontaneous locomotor activity. The results of the spontaneous locomotor activity recorded over two consecutive 12-hr periods of a light/dark cycle are presented in Table 6. In all dietary groups there was a highly significant increase in spontaneous motor activity (crossings and rearings) during the night but no significant effect of diet over the two activity periods.

DISCUSSION

The results of the present study show that a dietary restriction of n-3 fatty acids during development causes profound modifications of the fatty acid composition of brain phospholipids and alterations of the exploratory behavior in rats. The changes in phospholipid fatty acid composition reported here are consistent with previous reports using similar although not identical dietary regimens (5,8,10,11). The phospholipids analyzed (phosphatidylcholine and phosphatidylethanolamine) are the major phospholipid species in brain (1). Thus the decreased levels of 22:6n-3 in these phospholipids are likely to reflect a decrease in the total brain

TABLE 4

Effect of Dietary Fats on the Fatty Acid Composition of Myelin Phospholipids[a]

	Diets			
Fatty acid	Safflower oil	Soybean oil	Safflower/ soybean oil	Lab chow
12:0	0.89 ± 0.07	0.99 ± 0.10	0.86 ± 0.03	0.98 ± 0.11
14:0	0.30 ± 0.01	0.29 ± 0.02	0.30 ± 0.02	0.30 ± 0.01
16:0	13.98 ± 0.17	13.61 ± 0.17	13.55 ± 0.15	13.77 ± 0.11
16:1n-7	0.43 ± 0.02^{a}	0.53 ± 0.03^{b}	0.45 ± 0.01^{a,b}	0.51 ± 0.01^{a,b}
17:0	0.25 ± 0.02	0.29 ± 0.04	0.27 ± 0.01	0.27 ± 0.02
18:0	15.17 ± 0.38	14.99 ± 0.55	14.36 ± 0.17	14.21 ± 0.33
18:1n-9	37.09 ± 0.72^{a}	40.61 ± 0.22^{b}	39.49 ± 0.38^{b}	41.76 ± 0.49^{b}
18:2n-6	0.84 ± 0.00^{a}	0.94 ± 0.04^{a,b}	0.97 ± 0.06^{a,b}	1.04 ± 0.03^{b}
20:0	0.60 ± 0.15	0.68 ± 0.02	0.76 ± 0.07	0.79 ± 0.11
20:1n-9	6.57 ± 0.21	6.36 ± 0.20	7.17 ± 0.30	7.03 ± 0.31
20:2n-6	0.75 ± 0.07	0.81 ± 0.10	0.70 ± 0.02	0.69 ± 0.00
20:3n-6	0.84 ± 0.06	0.85 ± 0.09	0.79 ± 0.01	0.73 ± 0.04
20:4n-6	8.43 ± 0.18^{b}	7.88 ± 0.20^{a,b}	8.27 ± 0.11^{b}	7.35 ± 0.25^{a}
22:0	0.46 ± 0.03	0.49 ± 0.03	0.49 ± 0.04	0.46 ± 0.03
22:1n-9	0.62 ± 0.04^{a,b}	0.61 ± 0.02^{a}	0.76 ± 0.04^{b}	0.61 ± 0.02^{a}
22:4n-6	6.93 ± 0.10^{a}	4.83 ± 0.06^{c}	5.58 ± 0.16^{b}	4.42 ± 0.04^{c}
22:5n-6	3.77 ± 0.40	—	—	—
22:6n-3	0.87 ± 0.06^{a}	4.13 ± 0.11^{b}	3.73 ± 0.17^{b}	3.93 ± 0.23^{b}
24:0	0.50 ± 0.05	0.52 ± 0.04	0.43 ± 0.05	0.58 ± 0.05
24:1n-9	0.69 ± 0.08	0.65 ± 0.05	0.66 ± 0.06	0.62 ± 0.04
Unsaturated	67.85 ± 0.30	68.22 ± 0.43	68.99 ± 0.27	68.70 ± 0.40
n-3 + n-6	22.43 ± 0.51^{c}	19.44 ± 0.16^{a,b}	20.04 ± 0.23^{b}	18.17 ± 0.45^{a}
n-6/n-3	25.11 ± 1.61^{a}	3.71 ± 0.16^{b}	4.39 ± 0.19^{b}	3.65 ± 0.18^{b}

[a]Values are means ± S.E.M. (n=4) expressed as % (w/w) of identified fatty acids. Values with different superscripts in the same row are significantly different ($p<0.05$) from each other (one-way ANOVA followed by Turkey's test for comparison between groups). See legend to Table 3 for experimental conditions.

TABLE 5

Effect of Dietary Fats on Exploratory Behavior in Rats[a]

Diets	Crossings	Rearings
Safflower oil	321 ± 16^{a}	204 ± 17^{a}
Soybean oil	438 ± 26^{b,c}	297 ± 16^{b}
Safflower/soybean oil	341 ± 17^{a,b}	192 ± 15^{a}
Lab chow	445 ± 39^{c}	252 ± 24^{a,b}

[a]The exploratory activity was measured at the age of 16–18 wk as described in Materials and Methods. Values are means ± S.E.M. (n=12) of the number of crossings and rearings during a period of one hour after introduction in the novel environment. One-way ANOVA indicates a significant effect of diets on crossings ($p<0.002$) and rearings ($p<0.001$). Values with different superscripts within a column are significantly different ($p<0.05$) from each other (Tukey's test).

content of 22:6n-3 in rats raised on a safflower oil-based diet.

The levels of arachidonic acid were not significantly affected by the dietary restriction indicating that the brain content of this fatty acid is relatively resistant to large changes in the ratio of n-3 to n-6 fatty acids in the diets. However, as shown recently (28), brain arachidonic acid levels may be decreased by feeding large amounts of fish oil indicating that the type and amount of n-3 fatty acids in the diets, in addition to the n-3/n-6 ratio, may be important factors in determining the interactions of these fatty acids with arachidonic acid metabolism. Moreover, in this study, we found slightly lower levels of arachidonic acid in the myelin fraction of rats fed lab chow (which contains 20:5n-3 and 22:6n-3) than in rats fed safflower oil. There was no difference in the levels of 22:6n-3 between soybean oil- (which contains only 18:3n-3) and chow-fed rats indicating that, in the rat, 18:3n-3 is readily converted into docosahexaenoic acid.

In rats raised on safflower oil, a complete recovery of phospholipid fatty acid composition was observed on switching animals to a soybean oil-based diet from weaning to 16 wk of age. However, previous studies have shown that even if recovery of normal fatty acid composition eventually occurs in brain cells of n-3 deficient rats, it is much slower than in other tissues (29–31). Restriction of n-3 fatty acids during development significantly decreased the exploratory behavior in adult rats. This was not due to a depressing effect of the safflower oil-based diet on spontaneous locomotor activity which was similar in all dietary groups. The changes affected both the horizontal movements and the rearings. These results are consistent with those of Lamptey and Walker (5) who also observed a de-

TABLE 6

Effect of Dietary Fats on Spontaneous Locomotor Activity in Rats[a]

Diets	Period	Crossings	Rearings
Safflower oil	Day	328 ± 13	283 ± 12
Soybean oil	(7a.m.-7p.m.)	369 ± 16	272 ± 23
Safflower/soybean oil		325 ± 21	246 ± 13
Lab chow		470 ± 29	314 ± 22
Safflower oil	Night	1690 ± 150	1166 ± 70
Soybean oil	(7p.m.-7a.m.)	1652 ± 118	1128 ± 71
Safflower/soybean oil		1550 ± 68	1127 ± 38
Lab chow		1583 ± 101	1038 ± 64

[a]The spontaneous locomotor activity was measured in 16-18 week-old rats after an adaptation period of 17 hr in the measuring chamber as described in Materials and Methods. Values are means ± S.E.M. (n=12) for the number of crossings and rearings accumulated during periods of 12 hr. Two-way ANOVA (diet × period) with repeated measures (diet) indicates a main effect of period on both crossings ($p<0.001$) and rearings ($p<0.001$) but no main effect of diet on any parameters.

creased rearing activity in weanling rats raised on safflower oil. Interestingly, protein malnutrition during development in rats has also been shown to be associated with a reduced exploratory activity without changes in spontaneous motor activity (32).

Exploratory behavior has been proposed to reflect the emotional reactivity of the animal to a novel environment (33,34). This is supported by the observation that anxiolytic drugs increase exploratory activity in an open field (35). Moreover, lesions of the mesolimbic dopaminergic system (36) or dopaminergic drugs (37) which influence emotional states also affect the exploratory activity in rats.

Thus, dietary restriction of n-3 fatty acids appears to influence the animal's reactivity to a novel environment. This may be due to changes in sensory perception or to modifications of neural systems regulating emotional behavior. Visual perception may be involved since exploratory activity is known to be modulated by light (38). Moreover, previous studies have shown that dietary restriction of n-3 fatty acids during development was associated with visual abnormalities in rats (11-13) and monkeys (18) which in the latter species are not easily reversible upon supplementation with n-3 fatty acids. Alternatively, dietary n-3 fatty acids may influence neurotransmitter systems (receptors and second messengers) involved in the control of exploratory behavior including serotonin (39) prostaglandin (40) and opiate systems (41,42), some of which have previously been shown to be influenced by dietary fats (19,43).

The finding that the behavioral deficit of rats raised on safflower oil could not be reversed by feeding soybean oil after weaning points to the essentiality of n-3 fatty acids during development. Moreover, the behavioral deficit could be clearly dissociated from the biochemical effects on membrane phospholipid fatty acid composition which were found to be fully reversible. In a previous study (44) we had observed that if n-3 fatty acids were fed to n-3 deficient dams during the lactation period a reversal of the behavioral deficit (decreased exploratory activity) of the pups could be obtained. Thus, the lack of n-3 fatty acids during the early post-natal days may have a detrimental effect on normal brain development. This may be the consequence of an inadequate supply of 22:6n-3 during synaptogenesis and myelination which may ultimately affect brain function and behavior in the adult. Further studies will be necessary to delineate the biochemical mechanisms underlying these behavioral effects in the rat.

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The Effects of Polyoxyethylated Cholesterol on Fecal Bile Acids and Nitrogen and on Cholesterol Balance in Rats

L.F. Amorosa[a,*], C.P. Martucci[a], N.R. Stevenson[b] and A.K. Khachadurian[a]
[a]Department of Medicine and [b]Department of Physiology, University of Medicine and Dentistry of New Jersey, Robert Wood Johnson Medical School, New Brunswick, New Jersey 08903

Polyoxyethylated cholesterol (POEC) is a water soluble derivative of cholesterol which decreases cholesterol absorption in rats without affecting body weight, fatty acid excretion, or intestinal histology. In the present study rat feces were analyzed for cholic, deoxycholic, chenodeoxycholic, muricholic and lithocholic acid following 3 months of feeding a standard or a 2% enriched cholesterol diet with or without 1.5% POEC. In rats maintained on the cholesterol free diet, POEC increased total bile acids (mg/day) by 50% from 14 ± 3 to 21 ± 3 (mean ± SEM) but only the increase in chenodeoxycholic acid was significant ($P<0.05$). The corresponding POEC effect in the 2% cholesterol diet was 31% (70 ± 8 to 93 ± 3, $P<0.01$). Fecal nitrogen and serum cholesterol did not vary among groups. Comparing these data with neutral steroid excretion previously determined showed that POEC in the cholesterol-free diet increased the negative cholesterol balance more than three-fold (34 ± 7 *vs* 118 ± 13 $P<0.01$). In rats fed 2% cholesterol, POEC caused a negative cholesterol balance of 222 ± 8 compared to the control of 27 ± 52 ($P<0.01$). The data indicate that POEC exerts complex effects in the intestinal tract which increase both bile acid and cholesterol excretion.
Lipids 26, 209–212 (1991).

Polyoxyethylated cholesterol (POEC) (Scheme 1) is a water soluble derivative of cholesterol which inhibits cholesterol synthesis in cultured human skin fibroblasts. In contrast to cholesterol and other cholesterol derivatives, POEC increases low density lipoprotein receptor activity (1,2). In addition, POEC decreases oleate incorporation into cholesteryl esters of cultured cells and has a direct inhibitory effect on acyl-CoA:cholesterol acyltransferase (ACAT), an enzyme which mediates intracellular cholesterol esterification and is implicated in cholesterol absorption. When fed to rats, POEC reduces cholesterol absorption by 66%, increases fecal cholesterol excretion two-three-fold, inhibits cholesterol synthesis in intestinal mucosal cells, and increases hepatic cholesterol synthesis. POEC does not significantly alter fatty acid absorption or intestinal histology after three weeks of feeding. Food intake and body weights were not significantly affected following three months of POEC administration (3).

The intestinal effects of POEC may be due to its physicochemical properties as a non-ionic surfactant. The ethoxy chain of the POEC molecule is quite hydrophilic, making the compound surface active at lipid/water interfaces. This property may also allow POEC to alter micellar structures or otherwise disturb the interaction of micelles and cholesterol at the surface of the unstirred water layer, thus limiting the passage of cholesterol into intestinal cells. Bile acids are reabsorbed by different mechanisms—passive diffusion in the upper intestine and sodium dependent active transport in the ileum. Because of structural and physicochemical similarities between POEC and bile acids, we postulated that POEC may also interfere with bile acid reabsorption.

Therefore, we studied the effects of POEC on fecal bile acids in rats fed a standard diet containing corn oil with and without cholesterol supplementation. We also measured fecal nitrogen content to determine if POEC decreases protein absorption, and measured serum cholesterol serially during the three-month feeding experiment. By combining the present results with previously published data on cholesterol excretion, we determined the effect of POEC on cholesterol balance.

MATERIALS AND METHODS

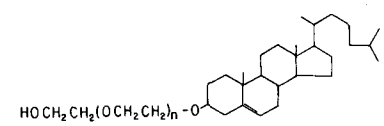
Animals and diets. Male Sprague-Dawley rats, which were previously utilized to determine the effects of POEC on cholesterol excretion, were used in these experiments (3). Animals were fed one of the following four diets: The standard diet contained 90% Purina rat chow (5001) and 10% corn oil (w/w). On this diet the rats consumed less than 1 mg/day of cholesterol. The standard diet was modified by the addition of 1.5% POEC (w/w), or 2% cholesterol (w/w) or both (combined diet). Animals were fed these diets for three months prior to collection of fecal samples over a four-day period.

Reagents. Bile acid standards for gas-liquid chromatography (GLC) were obtained from Steraloid, Inc. (Wilton, NH). 5α-Cholestane was from Sigma Chemical Co. (St. Louis, MO). Solvents used were high performance liquid chromatography (HPLC) grade, and other reagents were analytical reagent grade.

Fecal bile acid analysis. Feces were collected and

*To whom correspondence should be addressed at: Department of Medicine, UMDNJ-RWJMS, CN19, New Brunswick, NJ 08903-0019.

Abbreviations: ACAT, acyl-CoA:cholesterol acyltransferase; ANOVA, analysis of variance; GLC, gas-liquid chromatography; HPLC, high performance liquid chromatography; POEC, polyoxyethylated cholesterol; RT, retention time.



SCHEME 1. Polyoxyethylated cholesterol (POEC) with an average ethoxy chain length (n) of 20 was used in these experiments.

dried in a vacuum oven at 110°C overnight. The dried feces were ground in a mill and the ground material was stored at 4°C. Prior to extraction of bile acids, 10% (w/w) of the ground material from each rat for each of four consecutive days was pooled and further ground to a powder using mortar and pestle. Bile acid extraction was done following the procedure described by Tint *et al.* (4). One gram of powder was extracted for 18 hr with 250 mL of 95% ethanol in a Soxhlet extractor and later saponified for 2.5 hr with 0.5 N KOH and 50% ethanol and water. The volume of the aqueous phase and petroleum ether water washes was adjusted to 12 mL, and the concentration of KOH was brought to 3N. The samples were treated in a pressure cooker at 110°C for 3 hr and then acidified to pH 1-2 with HCl and extracted with 3 mL portions of dimethyl ether. The combined ether layers were dried over anhydrous Na_2SO_4 and evaporated to dryness with nitrogen gas. The residues were reacted with 0.2 mL of Sil-Prep (Alltech-Applied Science Laboratory, Deerfield, IL) for 30 min at 45°C, evaporated and taken up in heptane. Known amounts of 5α-cholestane were added as internal standard prior to duplicate analysis by GLC.

The trimethylsilyl ether derivatives of bile acid methyl esters were chromatographed on a 4 mm I.D. × 80 cm silanized glass column packed with 1% HIEFF-8BP on Gaschrom Q 100/120 mesh (Alltech-Applied Science Labs) at 240°C with helium/air/hydrogen at a flow rate of 40 mL/min. The instrument used was a Varian (Walnut Creek, CA) Model No. 3400 with flame ionization detector and injector temperatures set at 250°C.

The retention times of standards were compared to 5α-cholestane and the major bile acids were identified except for two late running peaks, 9 and 10, with retention times of 30.5 and 33.7 min, respectively. The ratios of these retention times to that of 5α-cholestane were 5.1 and 5.6. POEC in the feces did not chromatograph with the bile acids. Bile acid recovery was estimated to be greater than 90% as determined based on [^{14}C]deoxycholate.

Nitrogen determination. Fecal nitrogen was determined by standard methods based on the oxidation of organic nitrogen to ammonium ions and quantitation of the latter by reaction with Nessler's reagent (5). Nitrogen was determined in an aliquot obtained from the same four-day pooled fecal collection from each rat described above.

Serum cholesterol and triglycerides. Blood was obtained from the rat tail vein every two weeks. Serum cholesterol was determined using the microenzymatic method by Allain *et al.* (6).

Cholesterol balance. Neutral steroid excretion in these rats was previously reported (3). In the present paper, data on plant sterols and their degradation products have been excluded from the neutral steroid result. The addition of POEC to the standard and 2% cholesterol diets did not significantly alter plant sterol intake or β-[4-^{14}C]sitosterol recovery as previously noted. Thus, the cholesterol balance equals [cholesterol intake] minus [excretion of neutral steroids plus bile acids] (7).

Statistical analysis. All data are expressed as mean ± standard error of mean determined by summating the results of fecal or serum analysis for each individual rat. Data were analyzed by one-way analysis of variance (ANOVA) and Student-Newman-Keuls test for group differences (8).

RESULTS

We previously noted (3) that 1.5% dietary POEC did not significantly affect food intake or weight gain over three months. The latter is shown in Table 1 and compared with fecal weights and bile acid excretion. Both POEC and cholesterol supplementation of the standard diet significantly increased dried fecal weight over four days, but POEC did not significantly increase the

TABLE 1

Effects of POEC on Rat and Fecal Weights and on Bile Acid Excretion[a]

Dietary group (n)	Standard (5)	+ 1.5% POEC (6)	+ 2% Cholesterol (5)	Combined (6)
Weight (g) onset	257 ± 10	259 ± 10	258 ± 11	256 ± 19
final[b]	394 ± 14	358 ± 13	387 ± 17	406 ± 8
Fecal wt (g/4 day)	13.96 ± 0.70	19.36 ± 0.69[c]	19.40 ± 1.36[c]	20.78 ± 0.71
Bile acids (mg/day)				
Cholic	2.02 ± 0.44	4.38 ± 0.69	21.63 ± 8.13[c]	23.47 ± 1.25[d]
Deoxycholic	3.09 ± 0.79	4.41 ± 0.93	10.81 ± 2.61[f]	16.01 ± 2.35[d]
Chenodeoxycholic	1.34 ± 0.34	2.57 ± 0.29[f]	3.95 ± 0.54[c]	4.56 ± 0.41[d]
Lithocholic	1.11 ± 0.25	0.92 ± 0.38	3.89 ± 0.99[f]	4.28 ± 0.55[d]
β-Muricholic	1.12 ± 0.38	2.81 ± 0.72	6.08 ± 0.98[c]	9.51 ± 1.13[d]
P9 (RT 30.5 min)	1.25 ± 0.33	1.42 ± 0.35	4.30 ± 1.06[c]	6.27 ± 0.23[d]
P10 (RT 33.7 min)	4.09 ± 0.98	4.85 ± 0.78	19.87 ± 2.83[c]	28.99 ± 2.46[d,e]
Total	14.03 ± 2.87	21.38 ± 3.19	70.01 ± 8.45[c]	93.09 ± 3.39[d,e]

[a]Following three months of POEC feeding. (Data expressed as mean ± SEM).
[b]Amorosa *et al.* (3).
[c]P<0.01 compared to standard group.
[d]P<0.01 compared to 1.5% POEC group.
[e]P<0.01 compared to 2% cholesterol group.
[f]P<0.05 compared to standard group.

fecal weight of cholesterol fed rats. The increase in fecal weight between standard and 1.5% POEC diet is associated with a non-significant increase in bile acid excretion (14.03 ± 2.87 *vs* 21.38 ± 3.19 mg/day). Only the 92% increase in chenodeoxycholic acid excretion was significantly different (P<.05) on the 1.5% POEC diet. A corresponding increase in lithocholic acid, the secondary bile acid derived from bacterial conversion of chenodeoxycholic acid in the intestine, was not found. To determine whether POEC interfered with intestinal conversion of primary to secondary bile acids, we calculated ratios of the secondary to primary bile acids on both the standard and POEC diets. No change in the ratio of deoxycholic acid to cholic acid was found on either diet. In contrast, the ratio of lithocholic to chenodeoxycholic acid varied from 1.02 ± 0.20 on the standard diet to 0.33 ± 0.15, (P<0.05) on the POEC diet, as determined by summating the ratios from individual animals. Cholesterol added to the standard diet induced a five-fold increase in total bile acid excretion (P<0.01). The increase in the excretion of cholic acid and P10 (RT 33.7 min) accounted for almost 60% of the change associated with cholesterol feeding. Feeding of cholesterol plus POEC resulted in a greater than four-fold increase in total bile acid excretion as compared to the POEC diet (P<0.01). When compared to the cholesterol diet, POEC in the combined diet further increased total bile acid excretion by 31% (P<0.01).

Serum cholesterol was determined every two weeks. At no time point during the three-month study did cholesterol or POEC supplementation significantly alter serum cholesterol in these animals. The final serum cholesterol concentrations are shown in Table 2.

Nitrogen in feces was determind to detect protein malabsorption, which might suggest a non-specific toxic effect of POEC on gastrointestinal mucosa. The results (Table 2) show that POEC did not significantly alter nitrogen excretion.

Cholesterol balance data displayed in Table 3 show that POEC increased negative cholesterol balance in the standard diet from 34 ± 7 to 118 ± 13 mg/day (P<0.01). Rats fed a 2% cholesterol diet for three months were in cholesterol balance since −27 ± 52 mg/day does not significantly vary from zero. Addition of POEC to this diet caused a negative cholesterol balance of 222 ± 8 mg/day, P<0.01 from the reference diet.

DISCUSSION

We previously reported that oral administration of POEC to rats for three months inhibited cholesterol absorption and increased hepatic cholesterol synthesis (3). To determine whether the action of POEC was selective to cholesterol absorption, we measured bile acids and total nitrogen in the fecal specimens from the same experiments. These studies were delayed by technical difficulties with the extraction procedures for determining individual bile acids which prompted us to report the other data earlier. The total fecal bile acid excretion of 14.03 mg/day obtained in this study on rats ingesting 10% (w/w) corn oil is similar to the value of approximately 13 mg/day reported by Uchida *et al.* (9) in Sprague-Dawley rats of the same age in-

TABLE 2

Effects of Three Months of POEC on Final Serum Cholesterol Concentrations, and Total Fecal Nitrogen[a]

Dietary group	Fecal nitrogen (mg/day)	Serum cholesterol	
		mmol/L	(mg/dL)
Standard	168 ± 6	3.28 ± 0.28	(127 ± 11)
+ 1.5% POEC	146 ± 11	3.05 ± 0.41	(118 ± 16)
+ 2% Cholesterol	175 ± 19	3.67 ± 0.64	(142 ± 25)
Combined	142 ± 6	3.67 ± 0.25	(142 ± 10)

[a]Data expressed as mean ± SEM.

TABLE 3

Effects of Three Months of POEC Feeding on Cholesterol Balance[a]

Dietary group	Standard	+ 1.5% POEC	+ 2% Cholesterol	Combined
Cholesterol intake	<1	<1	492 ± 21	483 ± 11
Total fecal neutral steroid[b]	20 ± 4	96 ± 10[c]	446 ± 19	612 ± 13[c]
Total fecal acidic steroids	14 ± 3	21 ± 3	70 ± 8	93 ± 3[c]
Total fecal steroids	35 ± 7	118 ± 13[c]	533 ± 29	705 ± 13[c]
Cholesterol balance	−34 ± 7	−118 ± 13[c]	−27 ± 52	−222 ± 8[c]

[a]Results in mg/day (mean ± SEM).
[b]Amorosa *et al.* (3).
[c]P<0.01 for appropriate comparison without POEC.

gesting a 4% fat diet. Uchida *et al.* (9) also reported the presence of an unidentified bile acid peak 10 which they considered to be ω-muricholic acid based on GLC/mass spectrometric analysis.

The increase in fecal weight associated with the 1.5% POEC and 2% cholesterol diets is, in part, due to the fecal content of POEC and increased steroids. POEC supplementation of the standard diet significantly increased chenodeoxycholic acid excretion. However, the excretion of lithocholic acid, the secondary bile acid derived by bacterial conversion of chenodeoxycholic acid, was so low in three of the six rats fed POEC that it was undetectable. This suggested that POEC might be impairing bacterial conversion or reducing the content of chenodeoxycholic acid in the intestine so that less is available for conversion to lithocholic acid. The latter is more likely because levels of deoxycholic acid, the secondary bile acid derived from cholic acid, were not depressed, indicating that POEC does not generally interfere with bacterial conversion of bile acids. Chenodeoxycholic acid, unlike cholic acid, affects the liver by decreasing synthesis and biliary excretion of cholesterol (10,11). Therefore, increased fecal loss of chenodeoxycholic acid may be partly responsible for the increased hepatic cholesterol synthesis and fecal losses of endogenous cholesterol previously observed (3).

There was a marked increase in excretion of all bile acids in response to a 2% cholesterol diet fed for three months consistent with the data of others (12,13). This can be related to the increase in hepatic cholesterol and its conversion to bile acids. The addition of POEC to the 2% cholesterol diet caused a further significant increase in total fecal bile acids by increasing each of the individual bile acids. The POEC effect on bile acid excretion in both the cholesterol free and enriched diets may be due to inhibition of bile acid reabsorption and/or increased bile acid synthesis. We speculate that the non-ionic surfactant and detergent properties of POEC may interfere with micellar function or reduce absorption of conjugated bile salts in the ileum. We do not know if POEC is competitively taken up by active bile acid transport mechanisms since we have been unable to measure POEC in tissues by high pressure liquid chromatography.

Fecal nitrogen was unaffected by POEC. This indicates that POEC does not cause gastrointestinal protein losses. Serum cholesterol was not altered by POEC and cholesterol feeding. This is due to the increase in hepatic cholesterol synthesis and excretion in response to POEC (3) and increased bile acid excretion in response to dietary cholesterol. This is confirmed by the cholesterol balance analysis of Table 3, which combines the current data with information previously published from the same experiments. Cholesterol balance can be obtained since serum cholesterol, rat and fecal weights within dietary groups were constant (13). The results show that POEC causes a negative cholesterol balance even with a 2% cholesterol intake. To maintain the serum cholesterol level in the presence of POEC requires net whole body cholesterol synthesis equal to the negative cholesterol balance.

The mechanisms by which POEC causes increased fecal losses of cholesterol and bile acids remain unclear. There is no evidence suggesting a non-specific toxic effect of POEC on intestinal mucosal cells since feeding behavior and nitrogen absorption are not significantly altered. Other compounds which inhibit cholesterol synthesis, such as simvastatin, ketoconazole, and 15-ketosterol derivatives have variable inhibitory effects on cholesterol absorption and intestinal ACAT activity, but are not known to significantly impair bile acid reabsorption (14-16). Thus, POEC combines the actions of two classes of drugs—those that interfere with cholesterol absorption and those that result in bile acid losses, such as cholestyramine. These actions in one compound may be therapeutically useful in the prevention and treatment of disorders of cholesterol metabolism.

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Species Variation in the Atherogenic Profile of Monkeys: Relationship Between Dietary Fats, Lipoproteins, and Platelet Aggregation

A. Pronczuk[a], G.M. Patton[b], Z.F. Stephan[a,1], and K.C. Hayes[a,*]
[a]Foster Biomedical Research Laboratory, Brandeis University, Waltham, Massachusetts 02254 and [b]Boston Veterans Administration Medical Center, Boston, Massachusetts 02130

Because lipoproteins and platelet aggregation have been implicated in atherogenesis, relative differences in the response of these variables to dietary fat saturation were compared in three species of monkeys differing in their susceptibility to atherosclerosis (cebus, rhesus, and squirrel monkeys). Both long-term (8–12 years) and short-term (8 weeks) responses to diets containing 31% fat calories were examined in the same monkeys. As expected, long-term feeding of coconut oil by comparison to corn oil produced significantly higher plasma concentrations of total cholesterol, LDL cholesterol, apoB, and triglycerides, as well as higher ratios of LDL/HDL cholesterol and apo B/apo A-I. These responses were characteristic of all species with cebus being most responsive and rhesus the least. The short-term plasma cholesterol response to animal fats (butter, lard, beef tallow) was significantly less than that to coconut oil. When fish oil was substituted for two-thirds of either corn oil or coconut oil, exceptional decreases occurred in plasma cholesterol and triglycerides, as well as in HDL cholesterol and apo A-I concentrations despite the fact that the fish oil diets contained more saturated fat and less polyenes than the corn oil diet. Platelet aggregation tended to increase with saturated fat consumption and greatly decreased with fish oil intake in all monkeys, although cebus monkeys were ten-fold more resistant to platelet aggregation than the other two species. The molecular species of platelet phosphatidylcholine (PC) varied with both the dietary fat fed and species of monkey. An inverse correlation ($r=-0.60$; $p<0.001$) was found between changes in one such PC molecular species (18:0-20:4) induced by diet and the platelet aggregation threshold. These results demonstrate that the lipemic and platelet responses to dietary saturated fat depend upon both the type of fat (*i.e.*, the specific combination of dietary fatty acids, including the chain length of saturated fatty acids and the degree of polyunsaturation) and the species of monkey (genetic component) in which the response is elicited.
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It is well documented that dietary saturated fat and cholesterol elevate plasma lipids and increase the risk of atherosclerosis, whereas polyunsaturated fat exerts the opposite effect (1–5). However, our evolving understanding of both lipoprotein metabolism and platelet aggregation in the process of atherogenesis (5,6), coupled with the questionable impact of monounsaturated fat intake on these processes (7), have raised questions concerning certain generalizations about dietary fat saturation and atherosclerosis. In humans not all "saturated fats" elevate plasma lipids to the same degree (1,8). On the other hand, highly polyunsaturated fats that lower serum cholesterol and low density lipoprotein (LDL) tend to lower high density lipoprotein (HDL) and apo A-I concentrations at high fat intakes (*i.e.*, 40% fat energy)(9,10). Recent findings suggest that although dietary monounsaturated fats may lower plasma cholesterol in humans under certain circumstances (7,11,12), this hypolipemic effect does not equal that of linoleic acid, under normal dietary exchanges (1,2,13), nor do monounsaturated fatty acids seem to reduce HDL (7,11,14).

In addition to the above caveats, it is well known that the plasma cholesterol response to dietary fat saturation among individuals and between animal species varies considerably (15–20). Furthermore, a variety of studies reported to date have addressed the issue under dissimilar experimental conditions, *e.g.*, normocholesterolemic or hypercholesterolemic subjects, and generally while evaluating one species of animal with individuals assigned to a single dietary treatment. These studies typically used either long-term (months) or short-term (weeks) feeding protocols, but seldom examined the relationship between both responses in the same individual.

For these reasons we designed a comprehensive study that considered the long-term and short-term effects of dietary fat saturation on plasma lipids, lipoproteins, and platelet aggregation in 24 monkeys representing three species known to differ in their susceptibility to atherosclerosis and in their cholesterolemic response to diet (21). We found significant differences between species of monkeys in their response to dietary saturated fat and in the *ex vivo* susceptibility of their platelets to aggregate under these dietary circumstances.

MATERIALS AND METHODS

The experimental plan and procedures involved in this study were approved by the Institutional Animal Use Committee. Twenty-four nonhuman primates (8 rhesus, 8 cebus and 8 squirrel monkeys; equal number of male and female of each species) had been raised (caged individually) from birth for 8–12 years with purified

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[1]Current address: Ciba-Geigy Corp., Summit, NJ.
*To whom correspondence should be addressed.

Abbreviations: ANOVA, analysis of variance; apo, apolipoprotein; COCO+, coconut oil and cholesterol; CORN+, corn oil and cholesterol; EDTA, ethylenediaminetetracetic acid; GLC, gas-liquid chromatography; HDL, high density lipoprotein; HDL-C, high density lipoprotein-cholesterol, HPLC, high performance liquid chromatography; LDL, low density lipoprotein; LDL-C, low density lipoprotein-cholesterol; LSD, least significant difference; M/S, monounsaturated/saturated fatty acid ratio; PC, phosphatidylcholine; PPP, platelet poor plasma; P/S, polyunsaturated/saturated fatty acid ratio; PRP, platelet-rich plasma; stdPRP, standardized platelet rich plasma; VLDL-C, very low density lipoprotein-cholesterol; THF, tetrahydrofuran.

diets containing 31% kcal as either corn oil or edible coconut oil without cholesterol (Table 1). The initial data obtained from these monkeys represent the long-term aspect of the dietary trial. Body weight averaged (± S.D.) 7.8 ± 0.7 kg, 2.2 ± 0.4 kg and 0.7 ± 0.1 kg for rhesus, cebus and squirrel monkeys, respectively. During subsequent short-term feeding trials, these monkeys received diets containing 31% calories as lard, followed by butter, fish oil (2/3 fish oil and 1/3 corn or coconut oil), beef tallow, or their original dietary fat (corn oil or coconut oil) plus 0.045% cholesterol (CORN+ and COCO+) for 8 week periods. The diets (Table 1) and their fatty acid composition (Table 2) are tabulated for comparison. The cholesterol added to the basal diets (0.11 mg/kcal) represented the amount contained in the diets containing 2/3 fish oil. Other diets contained slightly less cholesterol—butter (0.10 mg/kcal), tallow (0.06 mg/kcal) and lard (0.05 mg/kcal). Between each short-term diet, monkeys received their original corn oil or coconut oil (no added cholesterol) diet for 2-3 weeks in an attempt to partially "restore" the original reserves of fatty acids prior to each subsequent, new, short-term fat. From a pilot experiment we determined that 2-3 weeks allowed a 80-90% return to original plasma cholesterol values and that this was preferable to having a carry over effect from one diet to the next. Twice during the final 10 days of each 8 week trial, monkeys were fasted overnight, sedated with ketamine, and blood was collected by plastic syringe from femoral venipuncture into ethylenediamine tetraacetic acid (EDTA)-wetted glass tubes or into a plastic tube containing 3.8% sodium citrate solution (9 vol blood to 1 vol of citrate solution). EDTA plasma was separated by centrifuging the blood at 4°C for 30 min at 1000 × *g*. Samples were kept at −30°C until analyzed. Platelets isolated from citrated plasma were tested for aggregation immediately.

TABLE 1

Composition of Semipurified Diet[a]

Ingredients	Amounts
	g/100 g dry diet
Lactalbumin	20.0
Cornstarch	29.2
Sucrose	20.0
Fat[b]	15.0
Cholesterol	(0 or 0.045)[c]
Salt mix[d] (Ausman-Hayes)	5.0
Vitamin mix[e] (Newberne-Hayes)	0.5
Choline chloride	0.3
Cellulose	10.0

[a]Diets were prepared by combining 5 kg dry diet mix with 4 L of boiling water and 60 g agar.

[b]Either corn oil, coconut oil, butter, beef tallow, 2/3 fish oil + 1/3 corn oil or + 1/3 coconut oil. Fish oil was MAXEPA®, kindly provided by Scherer Corp. (Troy, MI).

[c]45 mg Cholesterol/100 g of diet (0.11/kcal) was added to corn oil and coconut oil diets to formulate basal diets for short-term study. Other diets contained the following amount of cholesterol (mg/kcal): 2/3 fish oil (+1/3 corn oil or coconut oil) 0.11; butter 0.10; tallow 0.06; lard 0.05.

[d]Salt mix contained (g/kg): magnesium oxide, 32.0; calcium carbonate, 290.5; potassium phosphate dibasic, 314.2; calcium phosphate dibasic, 72.6; magnesium sulfate, 98.7; sodium chloride, 162.4; ferric citrate, 27.0; potassium iodide, 0.077; manganese sulfate, 1.22; zinc chloride, 0.92; calcium phosphate tribasic, 0.50; cupric sulfate, 0.29; chromium acetate, 0.04; sodium fluoride, 0.02; sodium selenite, 0.0043.

[e]Vitamin mix contained (g/kg): thiamine HCl, 0.80; riboflavin, 1.60; pyridoxine HCl, 0.8; calcium pantothenate, 5.00; niacinamide, 8.00; folic acid, 0.80; biotin, 0.04; cyanocobalamin, 0.03; menadione, 0.1; d,l-α-tocopheryl acetate (500 IU/g), 20.00; vitamin A acetate (500,000 IU/g), 5.00; vitamin D_3 (400,000 IU/g), 0.62; ascorbic acid, 120; inositol, 10; taurine, 50; made to 1 kg with 776 g dextrin.

Total cholesterol, HDL-cholesterol (HDL-C) and total triglycerides were determined by enzymatic assays (Sigma kit procedures 351 and 336; Sigma Chemical Co., St. Louis, MO). HDL was obtained following sodium phosphotungstate-Mg^{++} precipitation of the apo B- and apo E-containing lipoproteins (22), a procedure standardized against separation of the lipoproteins by ultracentrifugation (data not included). Since preliminary testing did not show significant differences in HDL-cholesterol values for fresh and once frozen (kept 2 weeks at −30°C) monkey plasma, the frozen samples were used in all instances. Low density lipoprotein cholesterol (LDL-C) was estimated after adjusting for VLDL-C based on plasma triglycerides according to the procedure of Friedwald *et al.* (22).

The level of apo B and apo A-I was measured in whole plasma by an immunoturbidometric method (23) using polyclonal antibodies raised against each apoprotein in rabbits. Turbidity in plasma and in the appropriate apoprotein standard solutions was measured at 340 nm with a Perkin-Elmer spectrophotometer (Perkin-Elmer Co., Oak Brook Instrument Division, Oak Brook, IL).

Platelet aggregation was measured according to the method of Born as described previously (24). In brief, citrated blood was centrifuged at room temperature at 200 × *g* for 10 min. The top 1-2 mL of platelet-rich plasma (PRP) was collected and the remaining blood was recentrifuged at 2000 × *g* for 10 additional min to obtain platelet-poor plasma (PPP). The platelet concentration was determined with a hemocytometer and the PRP was diluted with PPP to obtain stdPRP (standardized platelet-rich plasma) containing 2.5 × 10^8 platelets/mL. Aggregation was performed with a Payton aggregometer Model 402 (Payton Scientific Inc., Buffalo, NY) using 0.4 mL of stdPRP. Following 5 min of incubation at 37°C in 1 mL siliconized cuvettes, a metal siliconized stir-bar was added, followed by a measured quantity of purified collagen (equine tendon, Hormon-Chemie GmbH, München, Germany). The aggregation induced was quantified turbidimetrically as the percent difference in light transmission between stdPRP and PPP. For each monkey sample an aggregation "threshold" was determined by stimulating platelets with different concentrations of collagen (4-5 levels) until 10% of the predetermined maximum aggregation was elicited. The "threshold" was expressed as the amount (μg) of collagen required for a 10% maximum platelet aggregation response per 1 mL stdPRP. Thus, a low threshold was characteristic of platelets that aggregated readily.

Molecular species of platelet phosphatidylcholine (PC) were determined by high performance liquid chro-

TABLE 2

Fatty Acid Composition of Dietary Fats[a]

Fatty acid	% Of total fatty acids						
	Corn oil	Coconut oil	Butter	Tallow	Lard	2/3 Fish oil 1/3 Corn oil	2/3 Fish oil 1/3 Coco oil
12:0	—	47.5	7.2	—	—	—	15.8
14:0	0.2	22.2	9.9	2.2	1.1	4.7	12.0
16:0	11.9	12.9	32.9	24.6	23.1	15.1	15.4
18:0	2.2	4.1	15.1	18.8	13.5	3.7	4.4
Total saturated	14.3	86.7	65.1	45.6	37.7	23.5	47.6
16:1	0.2	—	2.2	3.7	2.8	6.7	6.6
18:1	25.1	10.8	28.9	46.4	46.7	19.2	14.5
Total monounsaturated	25.3	10.8	31.1	50.1	49.5	25.9	21.1
18:2n-6	59.9	2.5	3.0	4.3	11.9	22.6	3.4
18:3n-3	0.6	—	0.8	—	0.9	0.3	0.2
20:4n-6	—	—	—	—	—	1.7	1.7
20:5n-3	—	—	—	—	—	13.7	13.7
22:5n-3	—	—	—	—	—	2.0	2.0
22:6n-3	—	—	—	—	—	10.3	10.3
Total polyunsaturated	60.4	2.5	3.8	4.3	12.8	50.6	31.3
P/S Ratio	4.22	0.03	0.06	0.09	0.34	2.15	0.66

[a]Fatty acids determined by GLC analysis of each fat as fed.

matography (HPLC) of the benzoyl esters as described previously (25). Platelets were separated from PRP and extracted with chloroform/methanol (2:1, v/v). Phosphatidylcholine was separated from the chloroform phase by HPLC on a silica column (Lichrosphere Si-100, 5μ). Next PC was cleaved to form diglycerides by phospholipase C hydrolysis and benzoyl esters were prepared. Finally, the benzoyl diglycerides were separated by HPLC on a silica column (Lichrosphere Si-100, 5μ) using 2.5% THF in hexane (1 mL/min) as solvent. Due to bleeding and analytical limitations, PC molecular species of platelets were analyzed for rhesus and cebus but not for squirrel monkeys, and only selected diets were included: basal diets (CORN+ and COCO+), fish oil (2/3 fish oil and 1/3 corn oil or coconut oil), and tallow.

Fatty acid analysis of formulated diets was accomplished by using the one-step transesterification method of Lapage and Roy (26). Gas-liquid chromatography (GLC) of fatty acid methyl esters was performed on a Hewlett-Packard 5790A GLC equipped with a flame ionization detector (Hewlett-Packard Co., Avondale, PA) and a 30-meter fused silica column (SP-2330, Supelco Inc., Bellefonte, PA).

Statistical analysis was performed on a Macintosh Plus® computer (Apple Computer Inc., Cupertino, CA) using appropriate statistical packages, Stat View™ 512+ and Cricket Graph 1.2™ (Cricket Software Inc., Philadelphia, PA). Statistical differences between the two original long-term dietary treatments and three species were assessed by two-factor analysis of variance. Since no fat times species interaction was noted, the data for all three species were collapsed and a direct dietary fat comparison was made using Student's *t*-test. Because all three species responded in the same fashion to the short-term dietary fat changes, data were combined and analyzed by repeated-measures analysis of variance (ANOVA) and Fisher's protected least significant difference (LSD) analysis (27).

RESULTS

Monkeys consumed their purified diet containing corn oil or coconut oil from birth without adverse effects in either group. They accepted the various fat substitutions without incident and without appreciable fluctuation in body weight over the 12 months required for these studies.

Fat saturation and plasma lipids. Several long-term effects of dietary fat saturation were apparent on plasma lipids and lipoproteins at the beginning of the study after long-term consumption (8–12 years) of either the corn oil or coconut oil diets. Although no sex or age effects were detected in the data by covariant analysis, analysis of variance did reveal that both fat and species affected the long-term responses (Table 3). Because no fat times species interactions were observed, within each dietary fat all three species were combined (n=12) for the comparison of dietary fat saturation (between corn oil and coconut oil). Within each species and for all monkeys, those raised on coconut oil tended to have higher plasma cholesterol and triglyceride concentrations than monkeys raised on corn oil. As for the species effect, the cebus plasma cholesterol and squirrel monkey triglyceride values were most severely elevated by coconut oil consumption. Other effects of long-term coconut oil intake included significantly higher LDL-cholesterol and apo B concentrations and higher LDL/HDL and apo B/apo A-I ratios. HDL-cholesterol values were not significantly affected by either oil (Table 3).

TABLE 3

Effect of Long-Term (8–12 years) Feeding of Dietary Corn Oil or Coconut Oil on Plasma Lipids and Apolipoproteins by Species[a]

	Long-term dietary treatment (8–12 years)							
	Corn oil				Coconut oil			
Criterion	Rhesus (4)[b]	Cebus (4)	Squirrel (4)	All (12)	Rhesus (4)	Cebus (4)	Squirrel (4)	All (12)
Plasma cholesterol								
Total (mg/dL)[c]	145 ± 20	152 ± 10	183 ± 7	160 ± 9[e]	200 ± 30	263 ± 25	244 ± 26	236 ± 16[e]
LDL-C (mg/dL)[c]	68 ± 14	63 ± 4	84 ± 4	72 ± 6[e]	125 ± 20	150 ± 18	143 ± 13	139 ± 11[e]
HDL-C (mg/dL)[d]	66 ± 7	82 ± 8	89 ± 8	79 ± 5	62 ± 8	100 ± 5	82 ± 11	81 ± 6
LDL-C/HDL-C[c]	1.07 ± .20	0.80 ± .10	0.98 ± .12	0.95 ± .10[e]	2.06 ± .33	1.46 ± .14	1.79 ± .09	1.78 ± .15[e]
Plasma triglycerides								
Total (mg/dL)	56 ± 10	52 ± 4	56 ± 6	55 ± 9[e]	64 ± 19	67 ± 4	99 ± 16	76 ± 8[e]
Apolipoproteins								
Apo B (mg/dL)[c,d]	87 ± 21	45 ± 3	56 ± 4	59 ± 7[e]	127 ± 23	84 ± 16	84 ± 6	98 ± 11[e]
Apo A-I (mg/dL)[d]	142 ± 18	208 ± 10	142 ± 12	160 ± 12	148 ± 20	231 ± 13	132 ± 4	170 ± 15
Apo B/apo A-I[c,d]	0.59 ± .13	0.22 ± .01	0.40 ± .04	0.39 ± .06[e]	0.87 ± .28	0.38 ± .10	0.63 ± .03	0.62 ± .08[e]

[a]Values are mean ± SEM; values for individual monkeys were derived from two plasma samples collected over the final 10 days.
[b]Indicates number of monkeys per group.
[c]Two-factor analysis of variance indicated significant effect of dietary fat.
[d]Two-factor analysis of variance indicated significant effect of species.
[e]Indicates significant differences between corn oil and coconut oil (all monkeys) by Student *t*-test ($p<0.05$).

To obtain a "basal" value for comparison with data from monkeys fed cholesterol-containing animal fats (lard, butter and beef tallow) and fish oil in the short-term studies, corn oil and coconut oil diets were supplemented with 0.11 mg/kcal of cholesterol (equivalent to that in diets with 2/3 fish oil). Adding cholesterol caused about a 10% rise in plasma total cholesterol, LDL cholesterol, and the LDL-C/HDL-C ratio for both diet groups. The LDL-C/HDL-C ratio was also significantly higher in COCO+ monkeys than in CORN+ monkeys. A small increase in plasma triglycerides was also noted in CORN+ monkeys, but a 20% average increase occurred in COCO+ monkeys. Minimal changes in plasma HDL-C and apolipoproteins were induced by cholesterol feeding (Tables 3 and 4).

In order to determine whether the diet of origin, *i.e.*, corn oil *vs.* coconut oil, exerted a residual impact on the short-term response (8 weeks) to other dietary fats or whether the species of monkey made a difference, covariant analysis was applied. Although corn oil-fed monkeys tended to have somewhat lower values than their coconut oil counterpart for each short-term diet (and for all species), neither the original fat nor monkey species revealed a statistically significant effect. In the final analysis, a one-factor ANOVA was used to examine the response to the four short-term fats (dietary fat effect) for each of the two original groups of monkeys (corn oil and coconut oil, n=12 per group) since they were so different in their long-term responses.

The short-term plasma lipid responses were most affected by consumption of butter fat and fish oil. Butter induced the greatest increases in monkeys raised on corn oil while fish oil caused the largest decline in monkeys raised on coconut oil (Table 4). In addition, the plasma cholesterol of monkeys fed COCO+ decreased significantly (20–23%) during the short-term response to animal fats (butter, lard, and tallow), whereas the plasma cholesterol of monkeys fed CORN+ collectively increased between 4–11% (n.s.) when fed these animal fats (Table 4). By contrast, the short-term consumption of fish oil decreased the plasma cholesterol 20% and 37% in corn oil-fed and coconut oil-fed monkeys, respectively.

The short-term triglyceride response was also linked to the previous long-term fat intake. Specifically, in CORN+ monkeys the short-term consumption of all animal fats substantially increased plasma triglyceride (23–40%), whereas essentially no change was observed in the plasma triglyceride response of the COCO+ monkeys during the short-term feeding of animal fats (Table 4). Fish oil supplementation caused modest (17% and 37%), but significant, decreases in plasma triglycerides for all three species independent of whether they previously received CORN+ or COCO+ long-term.

Another short-term effect of animal fat consumption was its impact on LDL and HDL cholesterol concentrations. The LDL-C pool was increased substantially by butter in those monkeys fed CORN+ (Table 4), whereas replacing two-thirds of corn oil or coconut oil with fish oil caused significant declines in LDL-C for all monkeys, independent of their original long-term dietary fat intake. Although the basal HDL-C value tended to be lower for CORN+ than COCO+ monkeys, the difference was not significant. HDL-C scarcely varied when the CORN+ monkeys consumed animal fats, but it dropped significantly (proportionally even more than LDL-C) when these monkeys received fish oil. In monkeys fed COCO+, the HDL-C decreased significantly when either butter fat or fish oil was fed, but not with lard or tallow, both of which contained appreciable 18:1. The decline in HDL-C associated with fish oil intake was almost twice as great as that attributed to butter fat.

Not surprisingly, these fluctuations in LDL and HDL cholesterol, induced by varying the short-term

TABLE 4

Effect of Short-Term (8 weeks) Feeding of Dietary Fats on Plasma Lipids and Apolipoproteins for All Monkeys Combined[a]

Criterion/original diet	Short-term dietary treatment (8 weeks)				
	Basal[b]	Lard	Butter	2/3 Fish oil	Tallow
Plasma cholesterol					
Total (mg/dL)[c]					
corn (12)[d]	174 ± 9[e,l]	181 ± 12[f]	193 ± 11[g]	138 ± 10[e,f,g,h]	189 ± 14[h]
coco (12)	256 ± 20[e,f,g,h]	199 ± 20[e,i]	199 ± 18[f,j]	157 ± 16[g,i,j,k]	204 ± 16[h,k]
LDL-C (mg/dL)[c]					
corn (12)	86 ± 7[e,l]	93 ± 11[f]	103 ± 7[e,g]	71 ± 9[f,g,h]	94 ± 10[h]
coco (12)	153 ± 17[e,f,g,h]	104 ± 15[e]	111 ± 13[f,i]	90 ± 12[g,i]	106 ± 11[e]
HDL-C (mg/dL)					
corn (12)	77 ± 5[e]	75 ± 5[f]	74 ± 7[g]	59 ± 4[e,f,g,h]	76 ± 6[h]
coco (12)	86 ± 7[e,f]	78 ± 8[g]	71 ± 6[e,h]	56 ± 3[f,g,h,i]	82 ± 7[i]
LDL-C/HDL-C[c]					
corn (12)	1.15 ± 0.11[e,l]	1.28 ± 0.15	1.50 ± 0.12[e]	1.27 ± 0.15	1.27 ± 0.11
coco (12)	1.88 ± 0.24[e,f]	1.33 ± 0.18[e]	1.59 ± 0.17	1.57 ± 0.16	1.33 ± 0.13[f]
Plasma triglycerides					
Total (mg/dL)					
corn (12)	59 ± 6[e,l]	72 ± 10[f]	76 ± 9[e,g]	48 ± 5[f,g,h]	78 ± 9[e,h]
coco (12)	91 ± 10[e]	93 ± 11[f]	91 ± 11[g]	56 ± 6[e,f,g,h]	83 ± 7[h]
Apolipoproteins					
Apo B (mg/dL)					
corn (12)	56 ± 8[e,l]	63 ± 9	70 ± 8[e]	65 ± 12	66 ± 9
coco (12)	96 ± 14	82 ± 8	78 ± 6	81 ± 10	89 ± 10
Apo A-I (mg/dL)					
corn (12)	149 ± 12[e]	146 ± 10[f]	150 ± 11[g]	112 ± 8[e,f,g,h]	157 ± 12[h]
coco (12)	180 ± 17[e,f,g,h]	153 ± 14[e,i]	156 ± 13[f,j]	120 ± 12[g,i,j,k]	159 ± 12[h,k]
Apo B/apo A-I					
corn (12)	0.41 ± 0.06[e]	0.46 ± 0.08[f]	0.49 ± 0.06	0.60 ± 0.13[e,f,g]	0.45 ± 0.06[g]
coco (12)	0.56 ± 0.10	0.60 ± 0.08	0.56 ± 0.08	0.73 ± 0.13	0.61 ± 0.08

[a]Values are mean ± SEM; values for individual monkeys were derived from two plasma samples collected over the final 10 days.
[b]Corn oil or coconut oil diet with 0.045% (0.11/kcal) cholesterol added (CORN+ and COCO+).
[c]Two-factor repeated-measures analysis of variance revealed significant interaction between original diet and short-term response to fats.
[d]Indicates number of monkeys per group.
[e,f,g,h,i,j,k]Means in rows sharing a common superscript significantly different by repeated-measured ANOVA and Fisher's protected LSD paired analysis ($p<0.05$).
[l]Indicates significant difference between basal corn oil (CORN+) and coconut oil (COCO+) by Student *t*-test ($t<0.05$).

fat intake, affected the LDL/HDL ratio. Animal fats tended to increase this ratio 20–35% in monkeys fed CORN+ and decreased it 10–25% in monkeys fed COCO+. Fish oil increased the LDL/HDL ratio about 25% in CORN+ monkeys but lowered the ratio 10% in COCO+ monkeys (Table 4).

The short-term responses in plasma apo B and apo A-I concentrations generally reflected those in LDL and HDL, respectively (Table 4). Accordingly, the low apo B in corn oil-fed monkeys became elevated when animal fats were fed to this group on a short-term basis. In contrast to LDL cholesterol, the apo B tended to rise with fish oil supplementation of corn oil-fed monkeys and decreased slightly during fish oil supplementation of monkeys raised on coconut oil, but neither shift was significant. Apo A-I tended to be higher in monkeys raised on coconut oil and did not change as much as apo B during the short-term feeding trials. Fish oil, however, caused a major drop in plasma apo A-I when it replaced a major portion of either corn oil or coconut oil. Plasma apo A-I also decreased when lard and butter were fed short-term by comparison to monkeys fed COCO+. These fluctuations in apolipoproteins caused the apo B/apo A-I ratio to increase significantly during fish oil consumption for all monkeys combined, while butter increased this ratio significantly only in the CORN+ monkeys.

Fat saturation and platelet aggregation. Monkeys fed coconut oil long-term (8–12 years) tended to be more sensitive to collagen-induced platelet aggregation than monkeys fed corn oil (Table 5). Rhesus and squirrel monkey platelets demonstrated similar aggregability whereas those from cebus monkeys were about ten-fold more resistant to aggregation. Adding cholesterol to these diets (*i.e.*, the basal diets) did not alter platelet aggregation in any of the three species.

As was the case for the long-term response, cebus platelets were also more resistant to aggregation than the other two species during the short-term trials (Table 5). Using the basal diet (original fat + cholesterol) as the reference threshold for aggregation, most short-term dietary fats except butter and tallow tended to increase the platelet aggregation threshold in all monkeys. In the CORN+ monkeys, butter caused a significant 42% decrease in platelet stability (*i.e.*, lowered the platelet aggregation threshold) while tallow caused an insignificant 13% decrease. Platelets from monkeys fed butter were also more sensitive to aggregation

TABLE 5

Effect of Long-Term (8–12 Years) and Short-Term (8 Weeks) Dietary Fat Treatment on Platelet Aggregation (Collagen Threshold) by Species[a]

Species	Diet	N	Long-term dietary treatment (8–12 years)	Short-term dietary treatment (8 weeks) Basal[b]	Lard	Butter	2/3 Fish oil	Tallow
				μg Collagen/mL PRP				
Rhesus	corn	4	1.72 ± 0.20	1.34 ± 0.11	1.41 ± 0.17[c]	0.80 ± 0.21[c,d]	1.74 ± 0.11[d,e]	1.14 ± 0.21[e]
	coco	4	1.13 ± 0.27	1.11 ± 0.19	1.13 ± 0.39	0.87 ± 0.16[c]	1.72 ± 0.67[c]	1.07 ± 0.20
Cebus[i]	corn	4	12.8 ± 2.4	11.8 ± 2.6	11.9 ± 1.4	8.4 ± 1.9[c]	15.5 ± 2.6[c]	12.7 ± 2.3
	coco	4	11.8 ± 3.6	11.1 ± 3.6[c]	13.3 ± 2.8	11.5 ± 3.5[d]	16.4 ± 2.5[c,d,e]	12.1 ± 3.6[e]
Squirrel	corn	4	1.44 ± 0.55	1.36 ± 0.44	1.02 ± 0.28[c]	0.48 ± 0.21[d]	2.50 ± 1.20[c,d,e]	0.75 ± 0.20[e]
	coco	4	0.80 ± 0.20	0.81 ± 0.43[c]	0.88 ± 0.22[d]	0.55 ± 0.17[e]	1.58 ± 0.64[c,d,e]	1.06 ± 0.44
					Percent change from basal value			
Combined Species	corn	12		basal value[c,d]	+8.2 ± 19.3[e,f]	−41.8 ± 9.2[c,e,g]	+42.7±11.8[d,f,g,h]	−13.4 ± 11.3[h]
	coco	12		basal value[c]	+26.8 ± 20.0[d]	−12.9 ± 9.6[d,e]	+73.2±25.4[c,e]	+13.2 ± 10.3[f]

[a]Values are mean ± SEM.
[b]Corn oil or coconut oil diet with 0.045% (0.11 mg/kcal) cholesterol added (CORN+ and COCO+).
[c,d,e,f,g,h]Means in rows showing a common superscript are significantly different by repeated-measures ANOVA and Fisher's protected LSD paired analysis ($p<0.05$).
[i]Cebus platelets significantly less sensitive than rhesus or squirrel monkeys.

than monkeys fed lard. In contrast to butter, fish oil exerted a significant, positive stabilizing effect on platelet aggregation, with the threshold for combined species increasing about 43% and 73% over basal values for CORN+ and COCO+, respectively.

Platelet PC molecular species. Cebus and rhesus monkeys, which were inherently different in their sensitivity to platelet aggregation, also displayed characteristic differences in platelet PC molecular species (Table 6). Cebus fed CORN+ maintained lower levels of the PC molecular species 18:1-18:2 and 18:0-18:2 and higher 18:0-20:4 than comparable rhesus platelets. Cebus monkeys fed COCO+ maintained higher 16:0-20:4 and 18:0-20:4 in their platelet PC while 16:0-18:1 was lower than corresponding values in rhesus platelets.

Dietary fat saturation had a significant impact on most of the platelet PC molecular species (Table 6). All monkeys fed the saturated, COCO+ basal diet revealed much lower 18:1-18:2 and 18:0-18:2 and higher 16:0-18:1 platelet PC molecular species than monkeys fed the basal CORN+. When compared to these basal diets, feeding tallow caused only small differences in the platelet PC molecular species. In both cebus and rhesus, 8-week supplementation with fish oil produced a most dramatic (average about 300%) increase in all 20:5 fatty acid-containing platelet PC molecular species (16:0-20:5, 18:1-20:5 and 18:0-20:5) and a concomitant decrease in the corresponding 20:4-containing and 18:2-containing PC molecular species.

When correlations between diet-induced changes in platelet PC molecular species and the platelet aggregation threshold were examind, the PC molecular species that best described the relationship was 18:0-20:4 ($r=-0.60$; $p=0.001$) (Fig. 1), *i.e.*, the greater the increase in 18:0-20:4, the greater the tendency to aggregate.

DISCUSSION

Species variation in lipemic response to dietary fat saturation. The present experiment was unique from two points of view. First, the repeated-measures design using the same monkey fed different fats in sequence is relatively unique among monkey studies. Thus, each animal served as its own control as the short-term dietary fats were varied, allowing us to control interindividual variation in the lipemic response within each species. Second, the design allowed us to assess interspecies variation between the three species of monkeys fed identical diets in sequence.

Examination of species differences indicates that the plasma lipids in all three species tended to respond in the same direction to any given fat, but that cebus were most sensitive to saturated fat consumption, while the rhesus were least affected. The variability between individuals within species and the major difference in the magnitude of the response between species emphasizes the significant, and to date uncategorized, genetic component associated with the metabolism of specific fatty acids. Variation between individual humans (15,18) and between other African and Asian monkey species (20) in their sensitivity to saturated fat can be as great as the cebus-rhesus diversity. Whether this variability reflects one or several related aspects of fatty acid metabolism involving phospholipids, triglycerides, or cholesteryl esters remains unresolved. This raises the question of the mechanism(s) controlling the response and whether sensitivity to dietary fat saturation may be a function of an individual and/or species-dependent "aberration or limitation" in lipid metabolism.

From these data the cebus would appear to be the more sensitive model for evaluating the mechanism of

TABLE 6

Platelet Phosphatidylcholine Molecular Species of Rhesus and Cebus Fed Corn Oil, Coconut Oil, Tallow and Fish Oil Diets[a]

	Short-term dietary treatment (8 weeks)					
	Basal[b]		Tallow		2/3 Fish oil	
Molecular species	Rhesus	Cebus	Rhesus	Cebus	Rhesus	Cebus
	% of Total					
16:0-20:5						
corn	0.9 ± 0.1	1.0 ± 0.1	1.7 ± 0.2	0.9 ± 0.1	9.6 ± 0.1[c]	7.6 ± 0.9[c]
coco	3.8 ± 1.1	1.2 ± 0.1	2.8 ± 0.6	1.2 ± 0.3	12.8 ± 1.0[c]	12.0 ± 2.0[c]
18:1-20:5						
corn	0.1 ± 0.1	1.3 ± 0.3	0.3 ± 0.1	0.3 ± 0.1	0.9 ± 0.4	1.2 ± 0.1
coco	0.3 ± 0.1	0.8 ± 0.1[d]	0.6 ± 0.2	0.4 ± 0.1	0.9 ± 0.1[c]	2.1 ± 0.2[c]
16:0-22:6						
corn	1.6 ± 0.2	0.9 ± 0.1	1.3 ± 0.4	2.4 ± 0.5[c]	3.1 ± 0.4[c]	2.4 ± 0.1[c]
coco	3.2 ± 0.5	2.9 ± 0.2	1.8 ± 0.4	2.9 ± 0.5	3.2 ± 0.4	3.7 ± 0.5
16:0-20:4						
corn	4.5 ± 0.2	7.7 ± 0.8	3.5 ± 0.1	6.7 ± 0.8	4.0 ± 0.1	4.5 ± 0.3[c]
coco	5.0 ± 0.3	12.7 ± 0.2[d]	3.0 ± 0.2	4.3 ± 0.5	3.9 ± 0.3	4.6 ± 0.7[c]
18:1-20:4						
corn	0.5 ± 0.1	2.0 ± 0.1[d]	2.9 ± 0.9	2.9 ± 0.5	0.6 ± 0.1	0.9 ± 0.1[c]
coco	0.7 ± 0.1	2.2 ± 0.2[d]	1.8 ± 0.4	2.2 ± 0.2	0.6 ± 0.1	0.8 ± 0.2[c]
18:1-18:2						
corn	25.5 ± 0.7	13.9 ± 0.8[d]	11.1 ± 0.6[c]	10.2 ± 1.0	13.4 ± 0.1[c]	10.7 ± 0.2
coco	12.2 ± 0.3	9.6 ± 1.2	6.8 ± 0.4[c]	9.1 ± 1.0	4.9 ± 0.1[c]	4.6 ± 0.2[c]
18:0-20:5						
corn	0.9 ± 0.1	1.3 ± 0.1	2.8 ± 0.3	1.9 ± 0.2	8.1 ± 0.6[c]	5.1 ± 1.2[c]
coco	2.9 ± 0.7	3.8 ± 0.2	3.4 ± 0.3	2.3 ± 0.1[c]	9.2 ± 1.2[c]	7.3 ± 0.7[c]
18:0-22:6 + 16:0-16:0						
corn	5.7 ± 0.6	5.9 ± 0.2	7.8 ± 1.6	7.0 ± 1.4	5.1 ± 0.3	6.9 ± 0.4
coco	4.9 ± 0.5	5.2 ± 0.3	8.7 ± 1.3	5.4 ± 0.8	6.9 ± 0.3	5.9 ± 0.5
16:0-18:1						
corn	17.6 ± 0.8	15.8 ± 0.5	24.8 ± 2.5	18.9 ± 1.2	24.9 ± 1.0[c]	26.4 ± 1.5[c]
coco	29.8 ± 0.9	20.0 ± 0.9[d]	27.0 ± 2.6	22.2 ± 0.9	31.7 ± 2.6	24.3 ± 2.2
18:0-20:4						
corn	4.8 ± 0.3	10.7 ± 0.6[d]	5.3 ± 0.6	10.7 ± 0.8[d]	3.8 ± 0.1	5.3 ± 0.2[c,d]
coco	4.4 ± 1.4	11.8 ± 0.5[d]	5.4 ± 0.6	10.0 ± 0.3[d]	3.4 ± 0.4	5.0 ± 0.4[c,d]
18:0-18:2						
corn	18.7 ± 0.7	13.0 ± 0.4[d]	9.3 ± 0.5	7.8 ± 0.9[c]	10.5 ± 0.4[c]	7.4 ± 0.1[c,d]
coco	7.9 ± 0.4	5.3 ± 0.2	5.9 ± 0.8	7.3 ± 0.6	3.1 ± 0.1[c]	3.1 ± 0.3[c]
22:5-20:3						
corn	0.3 ± 0.1	0.5 ± 0.1	1.1 ± 0.1[c]	0.9 ± 0.1	0.8 ± 0.2[c]	0.9 ± 0.1[c]
coco	0.4 ± 0.1	0.7 ± 0.1	1.5 ± 0.2	0.7 ± 0.1[d]	1.3 ± 0.2[c]	0.5 ± 0.2[d]
18:0-16:0						
corn	1.3 ± 0.1	2.9 ± 0.4[d]	2.5 ± 0.7	2.3 ± 0.6	0.5 ± 0.2[c]	1.3 ± 0.1[c,d]
coco	0.9 ± 0.1	1.4 ± 0.2	2.8 ± 0.6[†]	2.4 ± 0.2[c]	1.1 ± 0.1	1.7 ± 0.4
18:0-18:1						
corn	5.8 ± 0.4	8.6 ± 0.6	9.8 ± 1.3	8.2 ± 0.7	7.2 ± 1.3	8.6 ± 1.0
coco	8.9 ± 0.5	6.5 ± 0.4	10.2 ± 1.3	9.4 ± 0.2[c]	9.3 ± 0.2	9.2 ± 0.4[c]

[a]Values are mean ± SEM, N=4.
[b]Corn oil or coconut oil diet with 0.045% (0.11 mg/kcal) cholesterol added (CORN+ and COCO+).
[c,d]Significant effect of diet compared to basal ([c]) or between monkey species ([d]) by two-way ANOVA.

the cholesterol-elevating ability of specific dietary fats or fatty acids, even though cebus presented a relatively high HDL-C profile and the lowest LDL/HDL ratio. From these data and those reported by others in humans (4,15,28) and monkeys (17,20), the saturated fat-induced cholesterolemia typically reflects an expanded LDL pool, irrespective of whether the primate involved (man or monkey) maintains primarily an LDL or HDL transport system.

Although the qualitative response of cebus to saturated fat was similar to that in humans, the quantitative degree of the response was somewhat greater. For example, the typical differential in plasma cholesterol between coconut oil and safflower oil in humans was approximately 95 mg/dL (1), whereas the increment between CORN+ and COCO+ in cebus was on the order of 130 mg/dL. Cebus would also appear to have the greatest genetic propensity of the three species studied for production of HDL based on their plasma concentrations of apo A-I and HDL. A comparable contrast in the genetic influence over apo A-I and the plasma lipoprotein response elicited by saturated fat

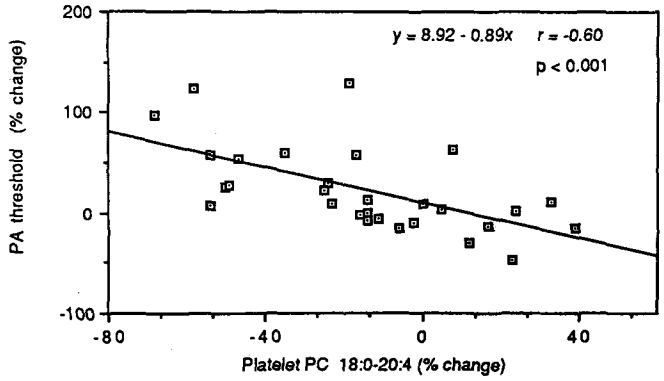


FIG. 1. Regression depicting the relationship between the observed changes in the platelet aggregation threshold and PC molecular species of 18:0-20:4 as affected by the various diet fat manipulations. The greater the diet-induced increase in 18:0-20:4, the lower the threshold.

and cholesterol feeding has been described between African green monkeys and cynomolgus macaques. The African green monkey, like the cebus, has a high HDL profile and would appear to be more resistant to atherosclerosis induction than the cynomolgus (20).

Specific dietary saturated fats and lipemia. From the data on total plasma cholesterol, triglycerides, and the LDL/HDL and apo B/apo A-I ratios, it was apparent that coconut oil (with 0.11 mg/kcal added cholesterol) elicited the greatest short-term lipemia. Not surprisingly, polyunsaturated corn oil produced much lower plasma lipid concentrations. Adding the human equivalent of 200–250 mg/day dietary cholesterol to either corn oil (CORN+) or coconut oil (COCO+) elicited a 10% increase in plasma cholesterol, which was reflected in the LDL cholesterol pool and a modest increase in the LDL/HDL ratio. We previously found this dietary cholesterol-induced expansion of plasma cholesterol to be associated with increased apolipoprotein E in VLDL and IDL of rhesus monkeys (29), in accord with the response in humans (30).

A second point emphasizes the notion that the term "saturated fat" must be qualified, at least when considering the lipemic response to a dietary fat that is accompanied by a low level of dietary cholesterol. This is supported by the fact that none of the so-called saturated animal fats were as hypercholesterolemic as coconut oil which contains large amounts of 12:0 and 14:0 fatty acids and minimal 18:2. Previous data in humans has indicated that myristic acid (14:0) may contribute most of the "saturated fat" effect (1). In our study neither lard nor tallow (rich in 16:0 and 18:0) differed appreciably from CORN+ (with 0.11/kcal cholesterol added) in terms of the total plasma cholesterol concentration produced when all three species were combined. Only the extremely sensitive cebus seemed to distinguish between these fats, and even these differences were not significant (data not shown). Part of this nonresponsiveness to lard and tallow may be attributed to the high 18:1 and 16:0 content of these two animal fats, a point that re-emphasizes the neutrality of 18:1 (1,2), or even its ability to lower cholesterol (under specific dietary and metabolic conditions) without depressing HDL-C (7,11,31), and the suggestion that 16:0 is not especially cholesterolemic (1,32). In this regard it is noteworthy that lard and tallow induced significant decreases in both total plasma cholesterol and LDL-C in monkeys raised on coconut oil without causing a significant depression in HDL-C. Accordingly, these were the only two fats to significantly improve the LDL/HDL ratio by comparison to COCO+, whereas butterfat (relatively rich in 14:0 and 12:0 and low in 18:2) was the only fat to raise this ratio in the CORN+ basal group.

The plasma triglyceride levels associated with lard, butter or tallow were similar to those for coconut oil, and all of these values were significantly greater than those observed when corn oil or fish oil were fed. Thus, an expanded triglyceride pool was consistently associated with consumption of all saturated fats, even when plasma cholesterol was not elevated. This interesting phenomenon may reflect an increased VLDL-TG secretion rate demonstrated *in vitro* by perfused livers from monkeys fed saturated fat compared to unsaturated fat (33) or saturated fat plus cholesterol as compared to polyunsaturated fat plus cholesterol (34). Another possibility includes decreased clearance of VLDL-TG by lipoprotein lipase, an observation that has been reported in humans consuming saturated fat (35).

A recurrent finding was the rise in plasma cholesterol concentration in the corn oil-raised monkeys during most short-term diets, whereas the high values attributed to the original coconut oil diet actually declined during the short-term dietary trials. Because of this convergence, the final concentrations between the two groups were similar for each short-term fat, although not completely equalized. One possible explanation for this failure to equilibrate may reflect adipose stores of fatty acids deposited during the long-term consumption of the original fat (*i.e.*, corn oil *vs* coconut oil) which exerted a residual effect on the plasma cholesterol level in the short-term. This same effect is apparent from the data from African green monkeys fed sequential diets, also for 8 week periods (36). Following a high-polyene intake (40% cal as fat), saturated fat consumption failed to raise the plasma cholesterol concentration to the original high level attained during a previous encounter with the same saturated fat. This putative difference in adipose fatty acids is documented for cebus monkeys fed corn oil or coconut oil. The combination of 12:0 and 14:0 represented more than 50% of the fatty acids accumulating in adipose tissue during clearance of chylomicrons absorbed during coconut oil consumption (37,38). We have subsequently found that the residual long-term fat effect on plasma cholesterol disappeared after 12 weeks of short-term fat feeding, as opposed to the 8 weeks used in this study (32), and that rhesus adipose maintains a much higher concentration of polyunsaturates than cebus when fed identical diets (unpublished data).

Response to fish oil. Fish oil was noteworthy because it caused the greatest decrease in total plasma cholesterol, an inconsistent response often observed in human and animal studies when a large amount of n-3 fatty acids are ingested (5,39–43). This decrease

was twice as great in the COCO+ monkeys as compared to those fed CORN+, more-or-less lowering plasma lipids in the two groups to equal concentrations. The decreases in plasma cholesterol attributable to fish oil were remarkable because the final concentrations for both the CORN-FISH and COCO-FISH periods were even lower than the CORN+ basal period. This depression in cholesterol occurred despite the fact that diets containing CORN-FISH, and especially COCO-FISH, had much higher total saturates and decidedly less total polyenes than the CORN+ basal diet. This confirms the exceptional ability of relatively large amounts of 20:5n-3 and 22:6n-3 to lower plasma cholesterol (44), even counteracting the sizable 12:0 and 14:0 contributed by the one-third portion of coconut oil. In essence, a diet with only 24% of its total fatty acids (about 6% total energy) as n-3 fatty acids (COCO-FISH) resulted in lower plasma cholesterol and triglycerides than the CORN+ basal diet contributing 60% of its fatty acids (about 18% total energy) as 18:2n-6 and containing only one-third the amount of saturates. This tendency for fish oil polyenes to substantially overshoot predictions generated by the Keys (2) or Hegsted (1) regression formulas (that treat all polyenes as equal) has been noted in the human response as well (45).

Also remarkable was the substantial drop in the plasma HDL-C and apo A-I concentrations when fish oil was fed. These decreases related to HDL were proportionally greater than those for LDL-C (in corn oil-raised cebus monkeys) or for apo B (in both corn oil- and coconut oil-raised monkeys), thereby increasing the LDL/HDL or apo B/apo A-I ratios during fish oil intake. Together these ratios would suggest an increase risk for atherogenesis. A similar adverse effect of fish oil on the distribution of lipoprotein cholesterol or apoproteins has been described by others for humans (42,43,46), monkeys (39), and pigs (47), and appears related to an increased rate of VLDL conversion to LDL (47) possibly coupled with reduced LDL clearance associated with decreased LDL receptor activity (48). The impact of these changes on atherogenesis has yet to be reconciled, but presumably the marked decrease in platelet aggregation in these monkeys and the lower transition temperature of their LDL cholesteryl esters (49) more than offsets the risk introduced by the adverse lipoprotein ratios.

Fish oil only modestly decreased the triglyceride level in these monkeys, unlike the rather striking decreases normally observed in humans (5,40,42), who typically have much higher initial plasma triglyceride concentrations. Presumably the lower plasma concentration reflects decreased triglyceride synthesis and secretion (43,50). Parks *et al.* (39) found slightly elevated triglycerides in African green monkeys fed fish oil and cholesterol and related this to a reduction of LDL particle size (49) which they previously found to be inversely correlated with the plasma triglyceride concentration. However, if that association were infallible, elevated triglyceride levels should have pertained as well in our monkeys fed fish oil, since their LDL size (based on apo B/LDL-C ratio) was also greatly reduced.

Platelet aggregation. The three species of monkeys differed inherently in their platelet sensitivity to aggregation as well as in their platelet response to changes in fat saturation. The species diversity in aggregation (cebus platelets were ten-fold more resistant to aggregation than rhesus or squirrel monkeys) was much more pronounced than the change in aggregation induced by dietary fat. On the other hand, differences in platelet PC molecular species induced by dietary fat were greater than the inherent differences in PC molecular species observed between cebus and rhesus monkeys, with the important exception of 18:0-20:4 which was consistently higher in cebus than rhesus. The extraordinary resistance of cebus (despite their inherently high 18:0-20:4) is in contrast to the enhanced platelet aggregation associated with increased 18:0-20:4 PC brought about by dietary fat in all monkeys. This implies that although the modest changes in aggregation attributed to dietary fat may have reflected the altered phospholipid fatty acid profiles, genetically modulated regulatory factors are much more important than dietary fatty acid-induced changes in platelet PC molecular species in terms of controlling the inherent aggregation difference between monkeys.

Consistent with data of others in humans and animals (40,51), fish oil (at the 20% energy level) significantly reduced platelet aggregability (increased threshold) in these monkeys. In fact, fish oil was the only fat to have a distinct effect on aggregation, although long-term coconut oil tended to enhance aggregation by comparison to corn oil, and butter tended to cause platelets to aggregate more quickly than most other fats. Reduced platelet aggregability associated with fish oil coincided with marked increase in all 20:5n-3 platelet PC molecular species and decrease in 20:4n-6 molecular species.

The exact mechanism responsible for the fish oil perturbation of platelet function is thought to relate to displacement of 20:4n-6 from platelet phospholipids by 20:5n-3 (51,52) which shifts the PGI_2/TXA_2 ratio to an antiaggregatory PGI_3/TXB_3 profile (53,54). The results of this study confirm this suggestion, *i.e.,* dietary fat-induced decreases in 18:0-20:4 PC molecular species reduced platelet sensitivity to aggregation.

In comparison to fish oil (rich in n-3 polyunsaturated fatty acids), the effect of other polyunsaturated fats (rich in n-6 fatty acids) on platelet aggregation is less well established (36,51,55-61). In this study corn oil seemed to exert a modest beneficial effect by comparison to coconut oil or butter fat. This unfavorable effect of saturated fats containing 14:0 on platelet aggregation is in agreement with Masi *et al.* (57), who reported that rabbit platelets were more sensitive to aggregation with collagen after a butter diet than corn oil. In addition, long-term diet modification of French farmers increased the dietary P/S ratio from 0.32 to 0.97 and produced a considerable decrease (81%) in platelet sensitivity to aggregation with thrombin (58). Lewis and co-workers (36,61), however, using ADP as agonist, failed to find a positive effect of polyunsaturated fat on platelet sensitivity in African green monkeys and, in some cases when the P/S ratio exceeded 1.50, noted an increase in platelet sensitivity which paralleled a significant drop in plasma HDL. In our experiment, a pilot study using collagen, thrombin, or ADP as agonist produced similar trends for all three

(data not included), with collagen being the most sensitive agonist and providing the most reproducible data.

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The Effect of Lysophosphatidylcholine on Coronary and Renal Circulation in the Rabbit[1]

A. Wolf, T. Saito, R. Dudek and R.J. Bing*
Huntington Medical Research Institutes, Department of Experimental Cardiology, Pasadena, California 91105

It has been shown that different lysophosphatidylcholines (LPC) possess relaxing properties in vascular strips. We report on the effect of intraatrial injection of LPC micelles into white New Zealand rabbits. Changes in renal and coronary flow were determined by radioactive microspheres. Coronary blood flow increased significantly following administration of LPC, while no significant changes were observed in renal blood flow, cardiac output and myocardial contractility. Coronary, renal and total vascular resistance, as well as mean arterial pressure, dropped significantly. Cardiac arrhythmia and hemolysis were not noted. The results demonstrate the vasorelaxant effect of LPC *in vivo*.
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The importance of endothelium dependent processes in the regulation of the caliber of arterioles and arteries has been well established. One of the compounds responsible for vasodilation is the endothelium-derived relaxing factor (EDRF) (1). Like nitrovasodilators such as nitroglycerin, EDRF acts through activation of guanylate cyclase and subsequent accumulation of cyclic-guanosine monophosphate (2). Lysophosphatidylcholine (LPC) is an endothelium dependent vascular smooth muscle relaxant. Relaxation of rabbit aortic strips induced by LPC is also accompanied by a rise of cyclic GMP content (3). LPC activates guanylate cyclase and is formed by phospholipase A_2 (PLA_2), which deacylates phosphatidylcholine (PC). LPC is a physiological constituent of cell membranes (4). During cardiac ischemia, LPC blood levels are diminished (5,6). Under certain conditions LPC is responsible for hemolysis and solubilization of membranes (7,8). It is known that LPC exerts certain physiological effects, such as inhibition of platelet aggregation (9), natriuresis and diuresis (10). The purpose of this study is to evaluate the *in situ* effects of LPC on coronary, renal and systemic hemodynamics and to investigate whether or not LPC causes hemolysis or myocardial damage under these experimental conditions.

MATERIALS AND METHODS

Eleven male white New Zealand rabbits weighing 2.0 to 2.8 kg were used for *in situ* experiments. After anesthesia with intravenous sodium pentobarbital (30 mg/kg body weight) and heparinization (600 IU/kg body weight) tracheotomy was performed. Ventilation was carried out by means of a Harvard Rodent ventilator (model 683, Harvard Apparatus, South Nattick, MA). The right femoral artery was then cannulated with an infant feeding tube (3.5 French, Mallinckrodt, Argyle, NY) which was connected to a pressure transducer (model P50, Gould Electronics Co., Cleveland, OH). After thoracotomy the heart was exposed, the pericardium incised and a catheter (Accumark, 5 French, Concord Laboratories Inc., Keene, NH) inserted into the left atrium and held in position by a tie. An angiocath (16 G, two inches, Deseret Medical Inc., Beckton Dickinson and Co., Sandy, UT) was inserted through the cardiac apex into the left ventricular cavity and connected to a pressure transducer (model P50). The signal of each transducer was processed by an amplifier (model 8188-3302-00, Gould Electronics). Systemic and left ventricular blood pressures were recorded and left ventricular dp/dt was determined using a differentiating circuit (model 8188-3302-00, Gould Electronics). To correct for changes in loading, contractility was expressed as dp/dt/IP, where IP equals the pressure at the end of the isovolumetric contraction (11). A blood sample was drawn from the right femoral artery for determination of blood gases, including pH, pO_2, pCO_2 and base excess using an acid base laboratory ABL 2a (Radiometer, Copenhagen NV, Denmark). Excessive variations in acid/base balance were corrected by adjustment of respiration or intravenous injection of sodium bicarbonate. Hemoglobin in heparinized plasma was determined with the method of Kohn and O'Kelly using tolidine reagent and peroxide (12). Coronary and renal blood flow, as well as cardiac output, were measured by means of radioactive microspheres using the method of Heymann *et al.* (13).

Two types of radioactive tracers (3M Company, Rochester, MN) 15 ± 3 micrometer in diameter were used — ^{141}Ce prior to and ^{51}Cr following injection of LPC. Microspheres were suspended in 0.05% Tween 80 in saline and vortexed for two minutes immediately prior to injection to prevent clumping. The reference sample was obtained from the femoral artery catheter by means of a Harvard Pump (model 901, Harvard Apparatus, Millis, MA), set at a speed of 1.25 mL per min. Fifteen seconds after commencing withdrawal of the reference sample, 0.3 mL of the ^{141}Ce microsphere suspension were injected through the atrial catheter over a period of 30 seconds and the catheter was flushed

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*To whom correspondence should be addressed at Huntington Memorial Hospital, 100 W. California Blvd., Pasadena, CA 91109.

Abbreviations: CBF, coronary blood flow; CO, cardiac output; CVR, coronary vascular resistance; cyclic GMP, cyclic guanosine monophosphate; EDRF, endothelium-derived relaxing factor; HE, hematoxilin-eosin; IP, pressure at end of isovolumic contraction; LPC, lysophosphatidylcholine; MAP, mean arterial pressure; OC, organ count; PC, phosphatidylcholine; pCO_2, partial pressure of carbon dioxide; PLA_2, phospholipase A_2; pO_2, partial pressure of oxygen; RBF, renal blood flowl; RC, counts/minute (reference sample); RVR, renal vascular resistance; TPR, total peripheral resistance; WR, reference blood flow withdrawal rate.

with 3 mL of saline. Withdrawal from the femoral artery (reference sample) continued for an additional 15 seconds (total withdrawal time 60 seconds). While both blood flows and cardiac output were determined only once prior to and again immediately following LPC injection, systemic and left ventricular blood pressures, as well as left ventricular dp/dt, were continuously recorded. Immediately after intraatrial injection of LPC micelles, ^{51}Cr microspheres were injected into the left atrium and a reference sample was again drawn as described above. The animals were then sacrificed by injection of potassium chloride, the heart and both kidneys were removed, weighed and divided into six specimens each. Samples were then counted in an automatic gamma counter (Gamma Trac 1191, Tracor Analytic, Elk Grove village, IL). Energy windows were set at 50-200 keV for ^{141}Ce and 280-360 keV for ^{51}Cr. Coronary or renal blood flows were calculated as follows:

$$\text{Coronary or renal blood flow (mL/min/g)} = \frac{\text{OC} \times \text{WR}}{\text{RC} \times \text{g}}$$

where OC (organ count) equals the total counts per min (CPM) of heart or kidney, WR is the reference blood flow withdrawal rate (1.25 mL/min) and RC is the CPM for the blood reference sample. Coronary and renal resistances (mm Hg/mL/min/g) were calculated as:

$$\text{Coronary (Renal) Resistance} = \frac{\text{Mean arterial pressure (mm Hg)}}{\text{Coronary (renal) blood flow (mL/min/g)}}$$

Cardiac output was obtained by a modified formula of Heymann *et al.* (13).

$$\text{Cardiac output (mL/min)} = \frac{\text{WR} \times \text{TIM}}{\text{RC}}$$

where WR is the reference sample withdrawal rate (mL/min), TIM is the CPM of the injectate and RC is the CPM of the reference sample. Total peripheral resistance (mm Hg/mL/min) was calculated as:

$$\text{Total Peripheral Resistance} = \frac{\text{Mean arterial pressure (mm Hg)}}{\text{Cardiac output (mL/min)}}$$

LPC micelles were prepared by suspending LPC (egg yolk) in 2 mL of saline, making a final dosage of 5 mg/kg. LPC derived from egg yolk was chosen because previous data demonstrated that it caused relaxation of arterial strips (14). Experiments with intravenous injection of LPC/cholesterol liposomes proved not to be successful, and were highly sensitive to small changes in the LPC/cholesterol ratio.

Specimens for histological examination were fixed in 10% formalin and stained with hematoxilin-eosin (HE). Statistical analyses were carried out according to Student's *t*-test for paired values. All chemical compounds were obtained from the Sigma Chemical Company (St. Louis, MO).

RESULTS

Intraatrial injection of 2 mL LPC micelles in saline caused a significant increase in coronary blood flow (CBF) (1.51 ± 12 to 1.93 ± 0.18 mL/min/g; Fig. 1) while coronary vascular resistance (CVR) decreased (58.4 ± 3.5 to 27.8 ± 6.3 mm Hg/mL/min/g; Fig. 1). As mean arterial pressure (MAP) declined (84.2 ± 3.0 to 69.9 ± 3.2 mm Hg; Fig. 2), the rise in coronary flow was mainly due to reduced coronary vascular resistance (correlation coefficient $r = 0.87$, $p<0.01$). Renal blood flow (RBF) showed no significant change (3.9 ± 0.4 to k3.8 ± 0.4 mL/min/g; Fig. 1), but renal vascular resistance (RVR) diminished (23.5 ± 2.3 to 20.6 ± 2.6 mm HKg/mL/min/g; Fig. 1). Systemic vascular resistance (SVR) also was significantly lower as compared to the control value (0.421 ± 0.039 to 0.323 ± 0.028 mm Hg/mL/min; Fig. 2.) No significant changes in cardiac output (CO), heart rate (HR) or contractility (dp/dt/IP) were recorded (Fig. 2). Intraatrial injection of LPC micelles did not result in hemolysis. Six samples drawn at one minute intervals prior to and following injection of LPC did not differ from the control samples ($n=4$; data not shown). Histological examination of HE-stained heart muscle in four animals showed no damage to the myocardium. Only minimal dilatation of epicardial veins and occasional extrusion of red cells into mycoardial parenchyma were noted. No cardiac arrhythmias were recorded after administration of LPC.

DISCUSSION

LPC is a physiological constituent of cell membranes (1.8% of total membrane lipid in rat liver plasma membranes) (4). It modifies the activity of membrane associated enzymes, such as guanylate cyclase, adenylate cyclase (15), sialytransferase (16) or galactosyltransferase (17). LPC may be involved in membrane transduction by diffusing through the lipid portion of membranes, thus activating enzymes at various locations in the cell (15). Furthermore, LPC changes general membrane properties such as fluidity and permeability (15). It is possible that the endothelium-dependent relaxation by LPC of isolated strips of rabbit aorta and bovine pulmonary arteries and veins is related to membrane alteration. LPC initiates the relaxation of endothelium-deprived strips of bovine pulmonary arteries upon superfusion from an endothelium intact bovine pulmonary artery (18).

Relaxation by LPC is inhibited by methylene blue and hemoglobin, indicating the involvement of cyclic GMP (14). Direct evidence of the activation of guanylate cyclase by LPC has recently been provided by Menon *et al.* (3). The decline in coronary and renal vascular resistance after injection of LPC is directly related to a smooth muscle relaxing effect on coronary and renal vessels.

LPC ($>10^{-6}$M) has also been shown to be cytolytic to red blood cells resulting in hemolysis (8) which appears to be the result of its detergent action (19). On the other hand, Menon *et al.* (3) have demonstrated that repeated administration of LPC (10^{-5}M) does not interfere with the functional integrity of the endothelium in rabbit aortic strips. In addition, the present study reveals that LPC (5 mg/k) does not cause *in vivo* hemolysis or marked histological damage of the myocardium.

Potential clinical effects of LPC have been investi-

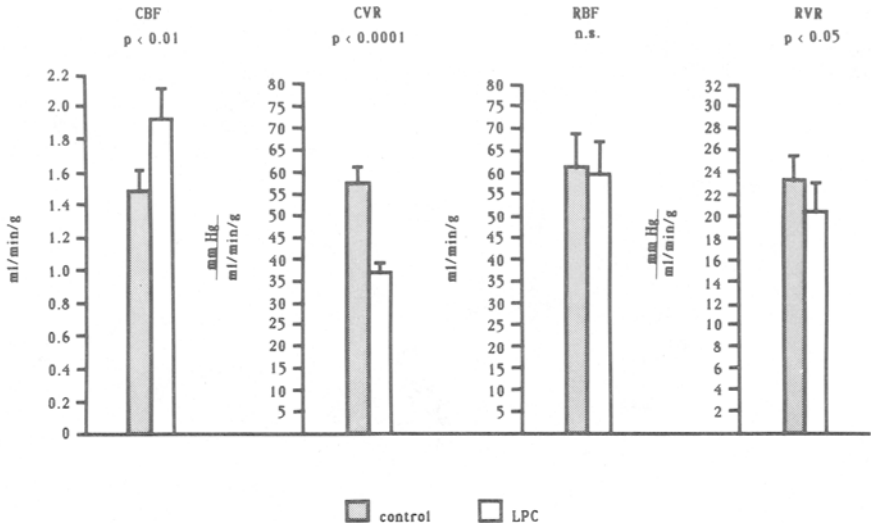


FIG. 1. Changes in coronary and renal blood flow and in coronary and renal vascular resistance following injection of LPC. The rise in coronary blood flow and the decline in coronary and renal vascular resistances are significant (n=11). Blood pressure was continuously recorded; determinations were performed simultaneously. Continuous blood pressure measurements allowed simultaneous determination of vascular resistances. CBF, coronary blood flow; CVR, coronary vascular resistance; RBF, renal blood flow; RVR, renal vascular resistance.

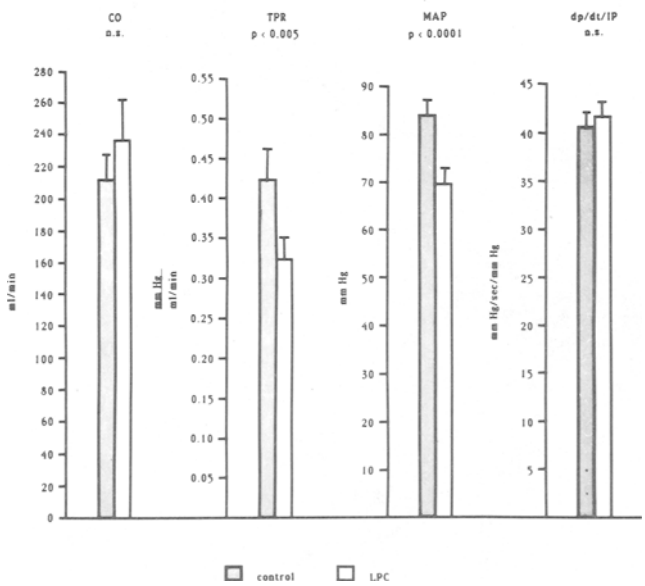


FIG. 2. Hemodynamic systemic changes following intraatrial injection of LPC micelles (5 mg/kg). The decline in total peripheral resistance (TPR) and mean arterial pressure (MAP) is significant (n=11). No significant changes in cardiac output (CO) and in cardiac contractility (dP/dt/IP) were noted.

gated by a number of workers; it has been established that LPC is diminished in plasma during myocardial ischemia (5,6). In our study no cardiac arrhythmias were recorded. The present study shows the vasodilating properties of exogenously administered LPC *in situ*. Administration results in a decline of coronary, renal and systemic vascular resistances. The question arises how LPC formed in vascular endothelium elicits vasodilation. Previous work indicates that the relaxation of arterial smooth muscle by LPC correlates with the deacylation of phosphatidylcholine (PC) to LPC (20). Deacylation of PC to LPC stimulated by mellitin, a potent activator of PLA_2, and the inhibition of the reacylation of LPC to PC by thimerosal, an inhibitor of lysolecithin acyltransferase, cause relaxation of arterial strips (20). Furthermore, Huang and Lee (21) and Bing and Saeed (22) demonstrated the relaxing effect of PLA_2 *in vitro*. Menon *et al.* (23) recently showed that activation of PLA_2 by thrombin results in production of LPC from PC in endothelium dependent relaxation.

The mechanisms of the action of LPC in the relaxation of blood vessels occurs *via* the activation of cyclic GMP. Phospholipase A_2 from various snake venoms, are activators of guanylate cyclase, especially the particulate form (24). This is probably the result of changes in membrane phospholipids and not of solubilization of the enzyme as was shown by Menon *et al.* (3) and by Zwiller *et al.* (24). Therefore, as previously proposed (3,14,24) conversion of PC to LPC by phospholipase A_2 may represent a mechanism in the regulation of vascular resistance. Ca^{2+} dependency of this mechanism is likely, since phospholipase A_2 requires Ca^{2+} for full activity. Although these results reveal that LPC infusion may cause coronary vasodilation, its therapeutic use is not suggested. In contrast to coronary vasodilators such as nitroglycerin and calcium blockers, the margin of safety of LPC appears limited.

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Analysis of Polymers from Autoxidized Marine Oils by Gel Permeation HPLC Using a Light-Scattering Detector

Ivan C. Burkow[a,*] and **R. James Henderson**[b]
[a]Institute of Fishery Technology Research, N-9001 Tromsø, Norway and [b]NERC Unit of Aquatic Biochemistry, University of Stirling, Stirling FK9 4LA, Scotland

A high-performance gel-permeation chromatographic method was developed for the isolation and quantification of polymers from autoxidized fish oils. The chromatographic system consists of a single pump, a 500 Å styrene/divinylbenzene column, and a light-scattering "mass" detector. Dichloromethane was used as the mobile phase, and quantifications were carried out using glycerol as internal standard. The polymer content of commercially available fish oils was found to be around 2%, but the value increased rapidly during oxidation at 35°C in air under light. The oxidized oils were also characterized by peroxide value, thiobarbituric acid value, ultraviolet (UV) measurements, thin-layer chromatography (TLC)/densitometry, and capillary gas chromatography. No correlation between polymer content and standard oxidation assessment parameters for the fish oils analyzed was found. However, a relationship between the polymer increase and the decrease of total fatty acids was observed, indicating that the polymers may be the main oxidation product in autoxidized marine oils.
Lipids 26, 227–231 (1991).

Due to their high degree of unsaturation, marine lipids are prone to autoxidation. If oxidation occurs in fish oil, loss of biologically important components, deterioration of product quality, and formation of potentially harmful substances may result (1–3). Possibly deleterious effects may be due to the formation of toxic compounds and their reactions, for example, with proteins, carbohydrates, nucleic acids, and vitamins. As a consequence, much work has been done to determine the chemical, nutritional, and metabolic implications of rancid fats and oils. However, the oxidation of polyunsaturated fatty acids of fish oils is still not well understood.

Most reports on the mechanisms and products of lipid autoxidation have concentrated on hydroperoxides, which are primarily formed (4), and on low molecular weight volatile secondary products (5,6). In addition, high molecular weight products are thought to be formed, but the existence of these compounds has only been documented for oligomeric and polymeric fatty acids (7–10) and for polymers from frying fats (11–14). Polymers from fish oils air-oxidized at ambient temperature have so far received little attention. The reason for this may partly be due to difficulties in the isolation and quantification of the complex structures present in marine oils. In addition, polymer formation until recently (15) has not been considered an extensive problem with unheated fish oils.

Among the various analytical methods used for determining oligomeric and polymeric materials, gel permeation chromatography (GPC) seems to be most promising. Several low-pressure systems have been described using gels such as cross-linked polystyrene and porous styrene-divinylbenzene (16,17), Sephadex LH-20 (18–21), and Bio-beads SX1, SX2, and SX3 (7,8,20–23). However, these methods are very time-consuming. In contrast, high-performance liquid chromatography (HPLC) on macroporous styrenedivinylbenzene copolymers is more convenient (8–10,12,24); it has previously been used for the separation of high molecular weight compounds from oxidized fatty acids and vegetable oils.

In the present study a simple and rapid method for the isolation and quantification of polymers by high-performance gel permeation chromatography is described. The polymer content of oxidized fish oils of different purities and degrees of unsaturation is also reported.

MATERIALS AND METHODS

Chemicals. Cod liver oil (CLO) and encapsulated fish oil concentrates (FOC) were purchased from a local pharmacy. Crude cod liver oil (CCLO) was an unrefined oil supplied by British Cod Liver Oils (Marfleet, Hull, U.K.). CPL Fish Oil 30 was obtained from Karlshamns AB (Karlshamn, Sweden). All chemicals used were of *p.a.* quality. Solvents were HPLC grade. Polystyrene standards (MW 2510, MW 4080 and MW 7030) were supplied by Millipore (Milford, MA).

General methods. Ultraviolet (UV) measurements were done on a Pye-Unicam SP 30 spectrophotometer using methanol or cyclohexane as solvent.

High-performance thin-layer chromatography was carried out using Si 60 pre-coated glass plates (Merck; Darmstadt, Germany) and chloroform/methanol/acetic acid/water (90:8:1:0.8, by vol) or hexane/diethyl ether/acetic acid (80:20:1, v/v/v) as developing solvent. After charring with cupric acetate (25), fractions were quantified with a Shimadzu dual-wavelength flying-spot scanner CS-9000 with control unit DR-13. The extent of oxidation was assessed by measuring the polar material of low R_f value (<0.35, one broad band).

*To whom correspondence should be addressed at the Institute of Fishery Technology Research, P.O. Box 677, N-9001 Tromsø, Norway.

Abbreviations: CCLO, crude cod liver oil; CLO, cod liver oil; CPL, chromatographically purified fish oil; FOC, fish oil concentrate; GPC, gel permeation chromatography; HPLC, high-performance liquid chromatography; PUFA, polyunsaturated fatty acids; PV, peroxide value; TBA, thiobarbituric acid; TLC, thin-layer chromatography; UV, ultraviolet.

Methyl esters of fatty acids were prepared by acid-catalyzed methylation (26) and subsequent purification using a Bond Elut NH_2 (Analytichem International; Harbor City, CA) with hexane as eluent. An additional thin-layer chromatography (TLC) purification was necessary with the methyl esters of highly oxidized samples using hexane/diethyl ether/acetic acid (90:10:1, v/v/v) as developing solvent. Fractions were made visible with iodine, the methyl ester band was scraped off, and the derivatized acids were extracted with chloroform/methanol (2:1, v/v). The samples were taken up in hexane, and fatty acid methyl esters were analyzed on a Packard 439 gas chromatograph equipped with flame ionization detector (FID) and a Chrompack CP Wax 51 fused silica capillary column (25 m, i.d. 0.25 mm). Hydrogen was used as carrier gas. The detector temperature was maintained at 250°C. Sample application was by on-column injection. The component peaks were identified by comparison with authentic standards and quantified relative to 23:0 methyl ester used as internal standard. Peak areas were measured with a Shimadzu C-R3A computing integrator.

Polymers were analyzed using a high-performance liquid chromatography system consisting of a Waters 501 pump with a U6K injector and an Applied Chromatography Systems 750/14 mass (light-scattering) detector. The mass detector was operated at an internal air pressure of 24 psi with the evaporator set at 20. Analyses were done on a Waters Ultrastyragel™ 500 Å (30 cm, i.d. 7.8 mm, particle size 10 μm) GPC column with dichloromethane (0.8 mL/min) as mobile phase. Sample concentration was 10–60 mg/mL in dichloromethane and injection volume was 5–50 μL. Glycerol was used as internal standard and peaks were integrated using a Hewlett-Packard 3390A integrator.

The oxidation experiments were carried out in an incubation cabinet kept at 35°C with irradiation from an artificial daylight fluorescent tube at 200 $\mu Em^{-2}s^{-1}$. The oils were oxidized either in open 2-mL vials (i.d. 0.8 mm, 20 μL sample) or in Petri-dishes (i.d. 95 mm, 2.5 g sample) without stirring.

Peroxide values (PV) were measured by iodometric titration (27). Thiobarbituric acid (TBA) values were determined as described by Ke and Woyewoda (28).

RESULTS AND DISCUSSION

Separation and quantification of polymers. To determine optimum chromatographic conditions for polymer separation and quantification, a number of exploratory experiments were carried out using cod liver oils (CLO) of different oxidation levels as test materials. Dichloromethane was used as mobile phase, mainly on the basis of its solvent properties, since the mobile phase affects separation by GPC only to a minor degree. Although the separation of the triglycerides from the oligomeric and polymeric material was best at a flow rate of 0.6 mL/min, a flow rate of 0.8 mL/min was considered optimal when analysis time was also taken into account. Using glycerol as internal standard (*vide infra*), total analysis time was less than 15 min (Fig. 1).

Optimization of the mass detector turned out to be very critical, requiring careful aerosol adjustment and accurate temperature control (29,30). The quality of the aerosol depends on the mobile phase composition and flow rate, as well as on the inlet air flow to the nebulizer (Table 1). In addition, the temperature setting turned out to be a most important operating parameter affecting the different compounds to different degrees (Table 2). For a sample containing approximately 2% polymers, 2% glycerol, and 96% triglycerides, an increase in the evaporator temperature was followed by an enhanced polymer response whereas the response of the internal standard decreased. As to the air flow optimalization, the triglyceride response was not influenced by the temperature setting in the normal working range.

The detector response was not linear, most likely due to the complex sample composition. Injection of 130 μg to 520 μg CLO containing 2% polymers, the increase of the polymer area and the triglyceride area was found to be 7.3- and 2.0-fold, respectively, instead of the expected four-fold increase. Although the amounts injected are expected to be in the linear range of the detector (31–33), no linearity was observed and quantification was therefore carried out using an internal standard. Among several standards tested, glycerol seemed to be the most suitable one. Due to its low

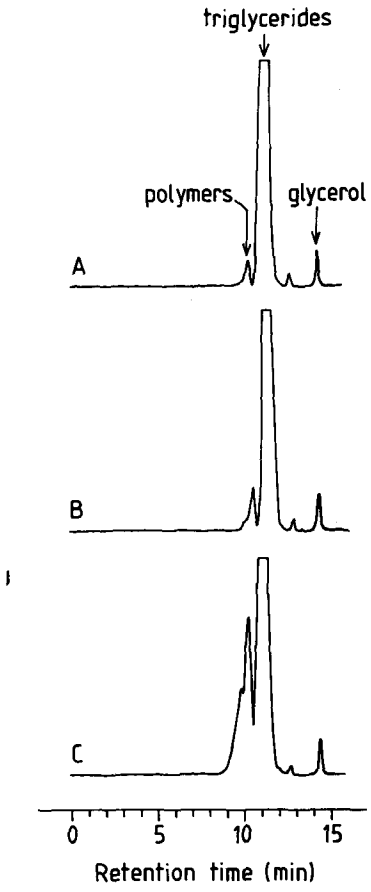


FIG. 1. GPC analyses of cod liver oil; A, starting sample; B, oxidized for two days; C, oxidized for four days. The oxidation was carried out at 35°C in light.

TABLE 1

Relative Response Factors as a Function of Nebulizer Air Pressure[a]

Pressure (psi)	Polymers	Triglycerides	Glycerol
20	1.03	0.98	1.04
24	1.00	1.00	1.00
27	0.81	0.99	0.78
30	0.74	1.01	0.74
35	0.70	1.01	0.62

[a]The response factors are measured with a cod liver oil sample containing approximately 2% polymers, 96% triglycerides, and 2% glycerol (analyzed by GPC). The other parameter settings of the light-scattering detector are as described in Materials and Methods.

TABLE 2

Relative Response Factors as a Function of Evaporation Tunnel Temperature[a]

Temperature[b]	Polymers	Triglycerides	Glycerol
15	0.73	1.00	1.00
20	1.00	1.00	1.00
25	1.14	1.00	0.73
30	1.38	1.00	0.53

[a]The response factors are measured with a cod liver oil sample containing approximately 2% polymers, 96% triglycerides, and 2% glycerol (analyzed by GPC). The other parameter settings of the light-scattering detector are as described in Materials and Methods.
[b]Arbitrary detector setting.

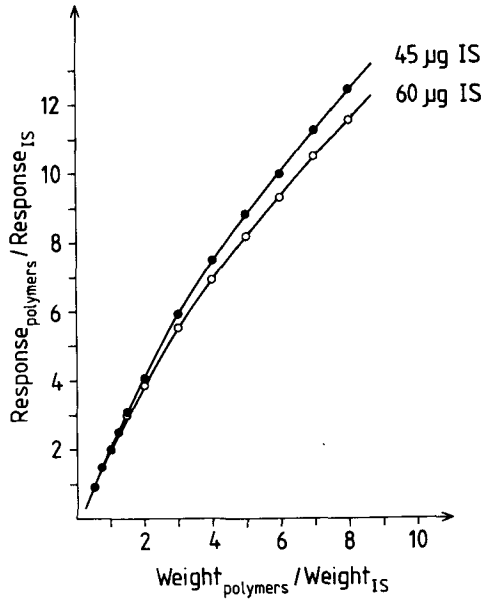


FIG. 2. Response ratio curve; ●, 45 µg internal standard; ○, 60 µg internal standard.

TABLE 3

Peroxide Value (PV), Thiobarbituric Acid (TBA) Value, Percent Oxidized Material, and Polymer Content of Starting Samples[a]

	PV (meq/kg)	TBA (µmol/g)	Oxidized[b] polymers[c] (%)	(%)
FOC	<1	0.5	2	2.1
CCLO	12.4	1.3	3	1.8
CLO	2.6	0.9	2	2.1
CPL	<1	0.5	ND[d]	0.4

[a]FOC, fish oil concentrate; CCLO, crude cod liver oil; CLO, cod liver oil; CPL, chromatographically purified fish oil.
[b]As measured by TLC.
[c]As measured by GPC.
[d]ND, not detected.

solubility in dichloromethane, methanol was used as cosolvent (2-4%). The response of glycerol was approximately linear in the range of 30-200 µg, with a detection limit of 4 µg. However, as a consequence of the non-linear response relationship between glycerol and polymer, quantifications were based on a constant amount of internal standard added to the samples. Then the ratio observed was corrected according to a calibration curve giving the response-weight relationship between the polymers and the internal standard (Fig. 2). The reasons for this non-linear behavior may be several, including aerosol particle size, refractive index, and opacity of the particles (34,35). However, other detection systems have been reported to be even more troublesome (36).

Polymers from oxidized fish oil. The amount of polymers in CLO, crude unrefined cod liver oil (CCLO), and encapsulated fish oil concentrate (FOC) was found to be around 2%, whereas the polymers in the chromatographically purified fish oil (CPL) amounted to 0.4% of the total weight (Table 3). Pure fish oils may not only include several percent of polymers, the results also show a lack of correlation between polymer content and standard methods for oxidative rancidity assessment. Even though an oil seems to be of high quality with low PV and TBA-values (37), substantial amounts of polymers may be present (see FOC, Table 3). This observation is important when quality evaluation of marine oils is carried out. In addition, oils with identical PV and TBA-values are not always identical with respect to oxidation, as is clearly demonstrated by comparing the polymer content in FOC and CPL (Table 3). The insufficiency of PV and TBA-values for quality assessment can also be concluded from TLC measurements, but separation and quantification of polymers is impossible with this technique.

The polymer content of all oils rapidly increased during oxidation in air with values reaching as high as 35% to 45% after four days (Fig. 3). Using polystyrene standards, the molecular weights of the polymers were measured to range from 2000 to 8000 depending on the level of oxidation. The compounds with molecular weights of about 2000 predominated when the total polymer content was less than 10%. Although the in-

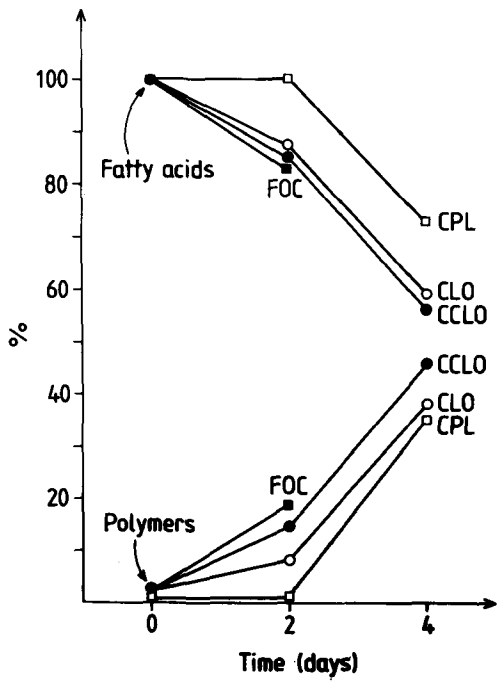


FIG. 3. Comparison of polymer amount and fatty acid content for autoxidized fish oil; ■, fish oil concentrate; ●, crude cod liver oil; °, cod liver oil; □, chromatographically purified fish oil.

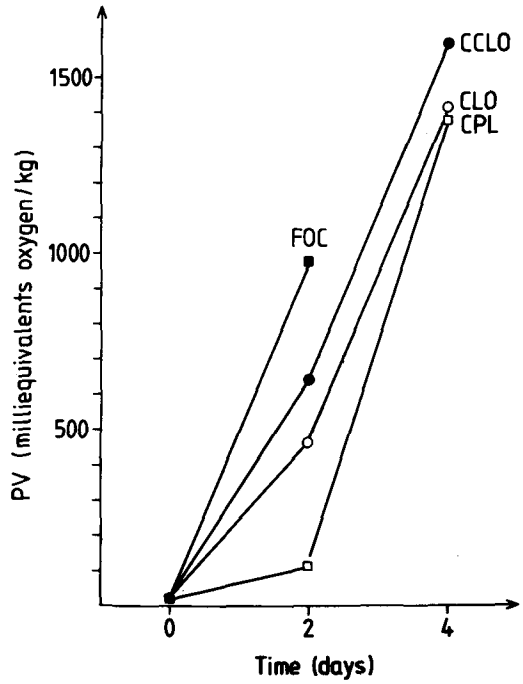


FIG. 4. Peroxide value of autoxidized fish oil; ■, fish oil concentrate; ●, crude cod liver oil; °, cod liver oil; □, chromatographically purified fish oil.

itial rate of polymer formation in CPL was lower than in the other oils, once initiated the rate was similar to that in the cod liver oils. For comparison, the standard quality assessment parameters, the PV values, TBA values, and the decrease of fatty acids are shown in Figures 3-5. The suceptibility towards oxidation was found to be in the order FOC>CCLO>CLO>CPL. FOC became so oxidized that the four-day sample was impossible to analyze. The differences observed can be explained on the basis of polyunsaturated fatty acid (PUFA) content and purity. The PUFA content was 29% of total fatty acids in the CCLO, 33% in the CLO, and 38% in the FOC and CPL. When the purity determined by TLC was taken into account, the oils could be classified as low PUFA oils with low and high purity, CCLO and CLO, respectively, and high PUFA oils with low and high purity, FOC and CPL, respectively.

The conditions employed in this study were deliberately chosen to induce autoxidation and are not representative of those employed normally for the storage of bulk oils. For this reason, and since the past history of the oils was not known, no conclusions can be drawn regarding the susceptibility of the different oils to autoxidation under commercial conditions.

An increase in the UV absorbance at 233 nm at the beginning of the reaction, due to formation of conjugated double bonds (37), was observed. Between the third and fourth day, a slight decrease in the absorbance was observed, a reduction which is probably caused by the formation of secondary oxidation products with a reduced number of double bonds. Whether

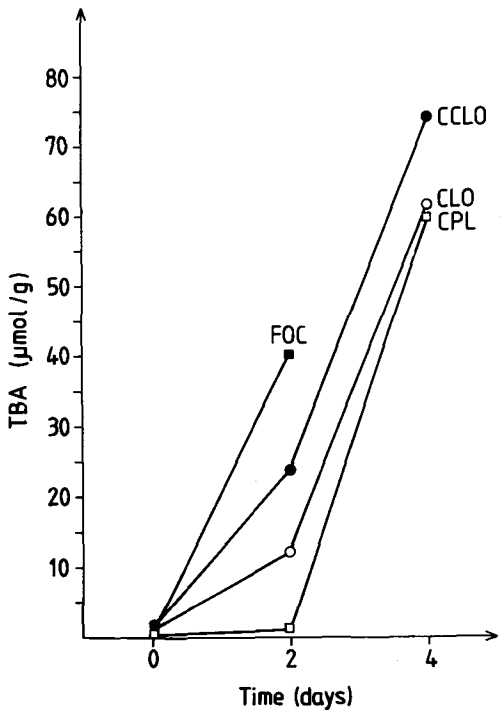


FIG. 5. Thiobarbituric acid value of autoxidized fish oils; ■, fish oil concentrate; ●, crude cod liver oil; °, cod liver oil; □, chromatographically purified fish oil.

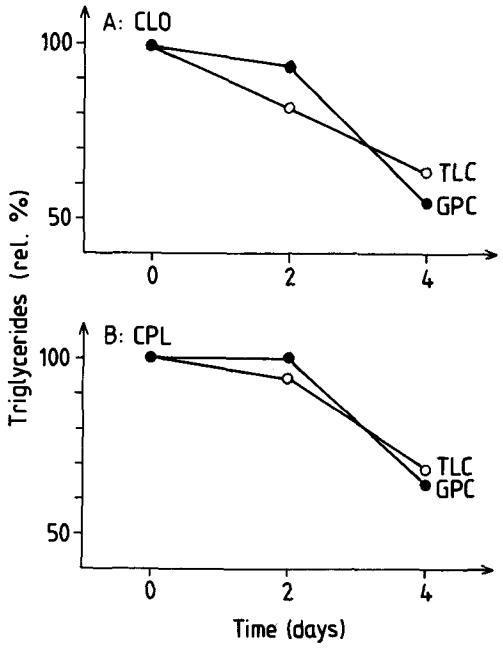


FIG. 6. Triglyceride content determined as sample weight minus polymer weight by GPC and nonpolar compounds minus polar compounds by TLC; A, cod liver oil; B, chromatographically purified fish oil.

these products also included polymers is difficult to determine from the UV measurements. However, a conspicuous relationship between the polymer increase and the decrease of the total amount of fatty acids was observed, indicating that the polymers may be the main oxidation product in autoxidized marine oils (Fig. 3).

TLC has been used for assessment of lipid oxidation measured as an increase in polar lipids (38). A comparison of triglyceride content determined as sample weight minus polymer weight (GPC) and nonpolar compounds minus polar compounds (TLC) are shown for CLO and CPL in Figure 6. As expected, in an early autoxidation state the triglyceride content is lower determined by the TLC-densitometry method, as peroxides would not be separated by the GPC method. However, after four days the TLC method gives a higher triglyceride value than the GPC method even though the autoxidized oil contains oxidation products other than polymers. The reason for this is most probably due to an inefficient staining of oxidation products caused by the lack of double bonds.

The method described here may find application in the routine assessment of fish oil quality. A more comprehensive study of polymer composition and structure is in progress.

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Malonaldehyde Determination in Tissues and Biological Fluids by Ion-Pairing High-Performance Liquid Chromatography[1]

Willy A. Behrens* and **René Madère**
Food Directorate, Bureau of Nutritional Sciences, Health Protection Branch, Health and Welfare Canada, Tunney's Pasture, Ottawa, Ontario, Canada K1A OL2

A method for the analysis of malonaldehyde by ion pairing high-performance liquid chromatography is described. The method is direct; no thiobarbiturate chromogen formation is required, and sample preparation is simple. After deproteinization with 50% ethanol and removal of particulate by centrifugation samples were passed through a small silica amino column to remove contaminants. Diluted samples (20 μL) were injected onto an octadecylsilane column (25 cm × 4.6 mm ID, 5 μm) which is eluted with 30 mM sodium phosphate buffer, pH 6.5 containing 30% ethanol and 1 mM tetradecyltrimethylammonium bromide. Detection was accomplished by monitoring absorbance at 267 nm. The lower limit for reliable quantification was 5 pmol per injection. The method has been successfully applied to the quantification of malonaldehyde present in plasma, urine and tissues of rats kept under different dietary conditions as well as after *in vivo* treatment with CCl_4 and iron-dextran. The method was also applied to the quantification of malonaldehyde during liver microsomal lipid peroxidation and was compared to the thiobarbituric acid test.
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Malonaldehyde (MA) is a volatile β-scission product formed from the peroxidation of certain polyunsaturated fatty acids and is considered an index of lipid peroxidation (1-3). MA has been implicated in aging (4), mutagenesis (5) and carcinogenesis (6). MA is a highly reactive compound and it is well documented that it undergoes reactions with amino acids (7,8) proteins (9-11) and nucleic acids (12,13).

Several methods have been developed to estimate the MA formed by lipid peroxidation either *in vivo* or *in vitro*. The most widely used method is the thiobarbituric acid (TBA) assay. In this procedure, heating of peroxidized lipids with TBA under acidic conditions produces a red pigment with an absorption maximum at 532 nm (14,15). The pigment has been considered to be the 1:2 adduct of malonaldehyde and TBA (16,17). However, the method is not specific for MA and often overestimates MA levels (18-22). More specific methods for MA determination have been developed using high-performance liquid chromatography (HPLC) (23-29) and gas chromatography (2,22,30). In spite of increased specificity and sensitivity, these methods are complex and time consuming; some attempted to separate the TBA-MA complex and others required the previous derivatization of MA. Others could not be reproduced in our laboratory nor were they able to measure MA in different biological fluids and tissues. For these reasons, a direct, simple, sensitive and specific HPLC method to measure MA in a variety of biological samples has been developed.

MATERIALS AND METHODS

Animals and diets. Male Wistar rats (Charles River Canada, St. Constant, Quebec, Canada) were used in the experiments and were fed a modified AIN (American Institute of Nutrition)-76 diet (31) for 2.5 months as follows: The diet for the basal group (group NE) contained casein, 20.0%; *dl*-methionine, 0.3%; corn starch, 15.0%; sucrose, 50.0%; cellulose, 5.0%; lard, 3.0%; corn oil-tocopherol stripped, 2.0%; AIN-76 mineral mixture, 3.5%; AIN-76A vitamin mixture, 1.0% (50 IU vitamin E/kg diet); and choline bitartrate, 0.2%. A second group of rats (group LE) was fed the same diet with vitamin E omitted from the vitamin mixture. In a third group (group HE), *d*-α-tocopherol acetate (ICN Nutritional Biochemicals, Cleveland, OH) was added to the vitamin mixture to give 250 IU vitamin E/kg diet.

Treatment of animals. CCl_4 (Sigma Chemical Co., St. Louis, MO) (2 mL/kg body weight) was administered by intraperitoneal injection in 50% tocopherol stripped corn oil. Control rats were given vehicle only. Animals were sacrificed after 2 hr. Iron dextran (Sigma Chemical Co.) (100 mg/kg body weight) was administered by gavage in 50% saline solution every second day. Animals were sacrificed after 7 days.

Blood and tissue collection. Blood was obtained by withdrawal from the abdominal aorta from animals anaesthetized with halothane (Fluothane, Ayerst Laboratory, Montreal, Canada) (2% in oxygen). The blood was transferred to a tube containing EDTA and centrifuged for 20 min at 2000 × *g*. Liver was perfused *in situ*, through the hepatic artery with 50-75 mL of cold saline. Spleen, white adipose tissue and muscle were also collected and kept at −75°C. Urine was obtained in metabolic cages during an overnight collection period.

Microsome preparation. Microsomes were prepared from a 25% liver homogenate (w/v) from rats fed the low vitamin diet (LE), according to Hogeboom (32). The final preparation was adjusted to 2 mg of protein/mL with 50 mM TRIS-HCl buffer, pH 7.5 containing 140 mM NaCl.

Microsomal lipid peroxidation system. The incuba-

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*To whom correspondence should be addressed.

Abbreviations: ADP, adenosine 5′-diphosphate; AIN-76 diet, American Institute of Nutrition-76 diet; HPLC, high-performance liquid chromatography; MA, malonaldehyde; TBA, 2-thiobarbituric acid; TBARS, TBA reactive substances.

tion mixture contained: 12 μM $FeCl_3$, 0.5 mM ascorbic acid, 0.2 mM ADP and 1 mg of microsomal protein. In some experiments ADP was omitted from the incubation mixture. Fifty mM TRIS-HCl, pH 7.5, containing 140 mM NaCl, was used to prepare all reagents and to bring the final volume of the incubation mixture to 2.0 mL. The reaction system was incubated in a shaking incubator for 45 min at 37°C.

Sample preparation for HPLC. Usually 1 mL of plasma was transferred to a 10-mL high-speed polycarbonate centrifuge tube containing 1 mL of absolute ethanol, mixed and kept cold for 1 hr, followed by centrifugation at 30,000 × *g* for 30 min in a refrigerated centrifuge. One mL of the clear deproteinized plasma was diluted with 3 mL of 30 mM sodium phosphate buffer, pH 5.5 containing 30% ethanol. One mL of this solution was cleaned by passing through a 1 mL silica-amino column (Supelclean LC-NH_2, Supelco Canada Ltd., Oakville, Ontario, Canada). This step was necessary to clean plasma of some interfering peaks, but mainly to eliminate some peaks that eluted very late in HPLC. Tissue homogenates and microsomes were treated in a similar manner. The urine extract was not cleaned. A 20-μL aliquot of deproteinized and cleaned sample was injected into the HPLC system. Recovery studies were performed by standard additions of MA to plasma, urine or tissue homogenates followed by 2 min of mixing. Between 15 to 20 min elapsed before the sample was deproteinized with 100% ethanol.

HPLC equipment and conditions. An HPLC system equipped with a Bas model PM-30A pump (Bioanalytical Systems Inc., W. Lafayette, IN), a model SP 8780 autosampler (Spectra-Physics, San Jose, CA), and a Linear model UVIS 204 variable-wavelength detector (Linear Instruments Corp., Reno, NV) was used for quantification of MA. The HPLC separations were performed on a reverse phase C18 column, 25 cm × 4.6 mm ID, 5 μm particle size (LC-18-DB Supelcosil, Supelco Canada Ltd.) with a mobile phase of 30 mM sodium phosphate buffer, pH 6.5 containing 30% ethanol and 1 mM tetradecyltrimethylammonium bromide (Sigma Chemical Co.) at a flow rate of 1.0 mL/min at ambient temperature. The absorbance was monitored at 267 nm. The detector was usually operated at a sensitivity of 0.006 AUFS. The data were recorded and electronically measured by a Spectra-Physics model SP4270 computing integrator.

Preparation of MA standard. A MA stock solution of approximately 10 mM was prepared by weighing 220 mg of malonaldehyde *bis*(dimethyl acetal) (Aldrich Chemical Co. Inc., Milwaukee, WI) into a 100-mL volumetric flask and diluting to the mark with 1% sulfuric acid. After standing overnight at 4–5°C, 1 mL of the solution was brought to a volume of 100 mL with 1% sulfuric acid and the MA concentration was determined by measuring the absorbance at 245 nm. A molar extinction coefficient of 13,700 was used (33). A solution of approximately 50 μM was prepared from the MA stock solution by dilution with phosphate buffer, pH 5.5 containing 30% ethanol. This solution was used to prepare a calibration curve in the range of 1 to 4 μM by dilution with the same buffer. Aliquots of 20 μL were injected into the HPLC system.

TBA test for rat liver microsomes. The TBA assay was performed according to Buege and Aust (14) with slight modifications. Standards diluted in 50 mM TRIS-HCl, pH 7.5, containing 140 mM NaCl and samples were heated at 80°C for 20 min. The mixture contained 0.1% butylated hydroxytoluene (Sigma Chemical Co.) in ethanol to prevent the formation of thiobarbituric reactive substances during color development (34). After cooling, the absorbance at 535 nm was measured for the quantification of MA.

Other analytical methods. Protein was estimated by the method of Bradford (35) using a protein assay reagent supplied by Bio-Rad Laboratories (Richmond, CA). Vitamin E (*d*-α-tocopherol) was estimated in plasma and tissues using the HPLC method of Thompson and Hatina (36), as previously described (37).

Statistical analysis. Student's *t*-test (38) was used for statistical evaluation of the results.

RESULTS AND DISCUSSION

A typical chromatogram of a pure MA standard is shown in Figure 1. The retention time remained constant for every batch of eluting solvent; variations from 8.0 to 8.5 min were observed and attributed to column aging. The lowest detection level of MA was set at 5.0 pmol, which is far greater than the generally accepted definition of chromatography detection limit, the amount of analyte which produces a peak height more than two times the noise level.

Figure 2 shows a typical MA calibration curve. The response was linear and detection, even at low levels, was accomplished with little variation. When two different standards were injected 15 times each to test reproducibility of the HPLC system, concentrations (pmol, mean ± SD) were 11.22 ± 0.26 and 22.5 ± 0.28.

MA was undetected or detected in trace amounts in plasma, urine and tissues from rats fed basal diet (AIN-76). Figure 3 shows representative chromatograms for plasma, liver and urine with and without standard added. Similar profiles were found for other tissues. Total time for each chromatogram was 10 min, with the exception of liver, which took 15 min due to a late

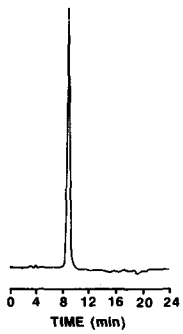


FIG. 1. Elution profile, at 267 nm absorbance, of a MA standard (51.6 pmol) on a C18 reverse phase column eluted with 30 mM sodium phosphate buffer, pH 6.5, containing 30% ethanol and 1 mM tetradecyltrimethylammonium bromide, at 1.0 mL/min.

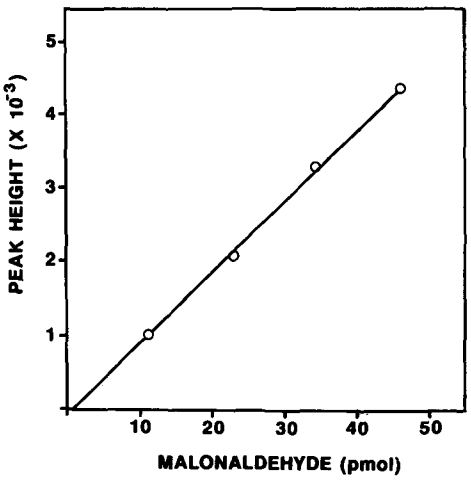


FIG. 2. Standard curve for HPLC of MA. The indicated amounts of MA standard were injected into the HPLC system (20 μL). Peak height *vs* injected amounts in pmol. Absorbance was measured at 267 nm.

non-MA peak. This short analysis time was achieved after cleaning the sample by passing it through a 1 mL silica-amino column to remove several contaminants that eluted very late (20–40 min), thus making the assay impractical in terms of timing. This clean-up procedure did not affect the recovery of MA. Table 1 shows the recoveries of various known concentrations of MA added directly to plasma, urine and tissue homogenates and carrying them through the entire procedure. It was found that extracted urine could be injected directly into the HPLC system without the cleaning step.

Of the tissue studied, muscle was the only one in which some difficulty was encountered due to the high content of ADP. This substance was not completely removed by the cleaning step, resulting in a relatively large peak which eluted immediately after MA. Nevertheless, preliminary experiments indicated that the interfering ADP peak could be successfully separated from the MA peak by decreasing the concentration of ion-pairing agent by 50%.

MA was found only in aged white adipose tissue homogenates kept under refrigeration for seven days. Extraction after that period showed a considerable amount of MA formed under these conditions (Fig. 4).

MA and vitamin E were measured in plasma and liver of rats fed low, normal and high vitamin E diets and after *in vivo* treatment with CCl_4 and iron-dextran. Different dietary levels of vitamin E produced the expected levels of vitamin in plasma and liver. These levels were not affected by treatment with CCl_4 and iron overload (Tables 2 and 3). Low levels of MA were detected in plasma of rats fed the low vitamin E diet, but could not be detected in rats fed the basal or high-vitamin E diet. Treatment with CCl_4 produced low levels of MA in plasma; however, no differences could be seen between rats fed low, normal or high vitamin E. No MA was found in liver under any circumstances despite the fact that obvious liver damage

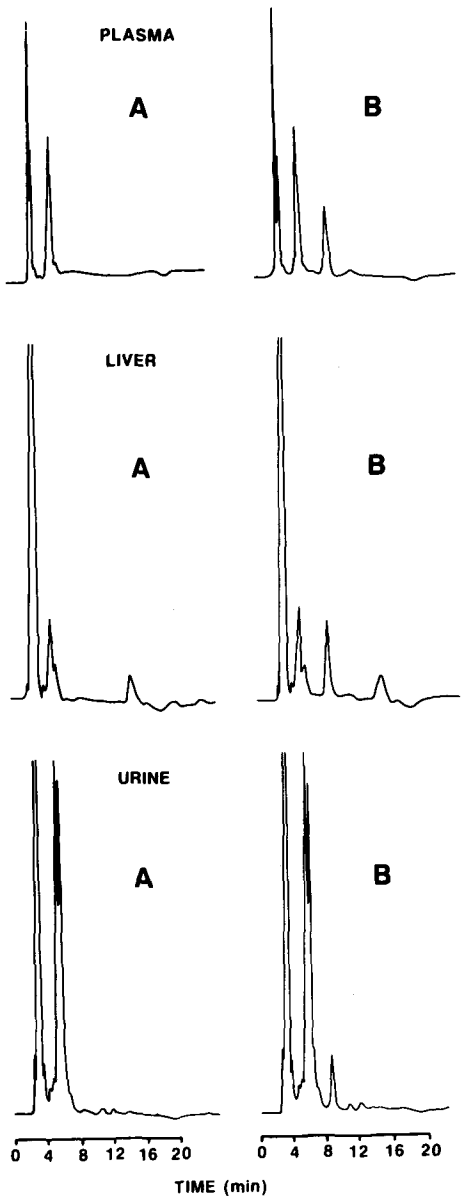


FIG. 3. Top panel. HPLC chromatogram for (A) plasma extract alone, and (B) with a 16.4 pmol MA standard added. Middle panel. HPLC chromratogram for (A) liver extract alone, and (B) with a 16.9 pmol MA standard added. Bottom panel. HPLC chromatogram for (A) urine extract alone, and (B) with a 10.5 pmol added.

TABLE 1

Recovery of Malonaldehyde Following Addition of Standards to Various Tissues

Tissue	(n)	Standard added pmol	% Recovery (range)
Plasma	6	19.3	97.7 (90–109)
Liver	6	17.5	94.3 (90–97)
Muscle	6	20.0	95.8 (93–104)
Spleen	6	17.7	100.7 (94–108)
White adipose	5	10.0	97.4 (92–101)

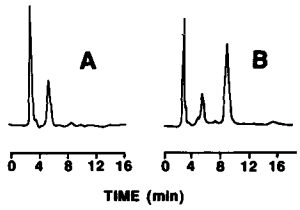


FIG. 4. HPLC chromatogram for (A) white adipose tissue extract and (B) white adipose tissue extract from a homogenate prepared seven days earlier. See text for details.

TABLE 2

Plasma Vitamin E and Malonaldehyde Levels of Rats Kept on Diets Varying in Vitamin E Content: Effect of CCl_4 and Iron-Overload

Treatment	Diet	LE[a]	NE[b]	HE[c]
Control	Vit. E.[d]	0.2 ± 0.0	19.0 ± 5.1	39.6 ± 4.3
	MA[e]	3.0 ± 0.6	ND[f]	ND
CCl_4	Vit. E	0.1 ± 0.0	19.0 ± 3.1	34.5 ± 5.5
	MA	2.5 ± 0.4	2.1 ± 0.4	2.0 ± 0.2
Iron	Vit. E.	—	19.0 ± 2.7	39.5 ± 4.5
	MA	—	ND	ND

[a]Low Vitamin E diet (LE): 0.0 *d*-α-tocopherol acetate/Kg diet.
[b]Normal Vitamin E diet (NE): 50 IU *d*-α-tocopherol acetate/kg diet.
[c]High Vitamin E diet (HE): 250 IU *d*-α-tocopherol acetate/kg diet.
[d]Vit. E. Vitamin E (μg/mL).
[e]MA. Malonaldehyde (nmol/ml). The values are expressed as mean ± SD. (n=4).
[f]ND, not detected.

TABLE 3

Liver Vitamin E and Malonaldehyde Levels of Rats Kept on Diets Varying in Vitamin E Content: Effect of CCl_4 and Iron-Overload

Treatment	Diet	LE[a]	NE[b]	HE[c]
Control	Vit. E.[d]	0.3 ± 0.2	12.6 ± 1.8	38.5 ± 9.1
	MA[e]	ND[f]	ND	ND
CCl_4	Vit. E	0.2 ± 0.1	12.5 ± 1.3	38.2 ± 6.5
	MA	ND	ND	ND
Iron	Vit. E.	—	11.3 ± 1.0	41.9 ± 13.0
	MA	—	ND	ND

[a]Low Vitamin E diet (LE): 0.0 *d*-α-tocopherol acetate/Kg diet.
[b]Normal Vitamin E diet (NE): 50 IU *d*-α-tocopherol acetate/kg diet.
[c]High Vitamin E diet (HE): 250 IU *d*-α-tocopherol acetate/kg diet.
[d]Vit. E. Vitamin E (μg/g).
[e]MA. Malonaldehyde (nmol/g). The values are expressed as mean ± SD. (n=4).
[f]ND, not detected.

was observed after CCl_4 treatment. The absence of MA, however, could be due to the short time (2 hr) that the chemical was allowed to act. Iron-overload resulted in death, within 48 hr, of all rats fed the low vitamin

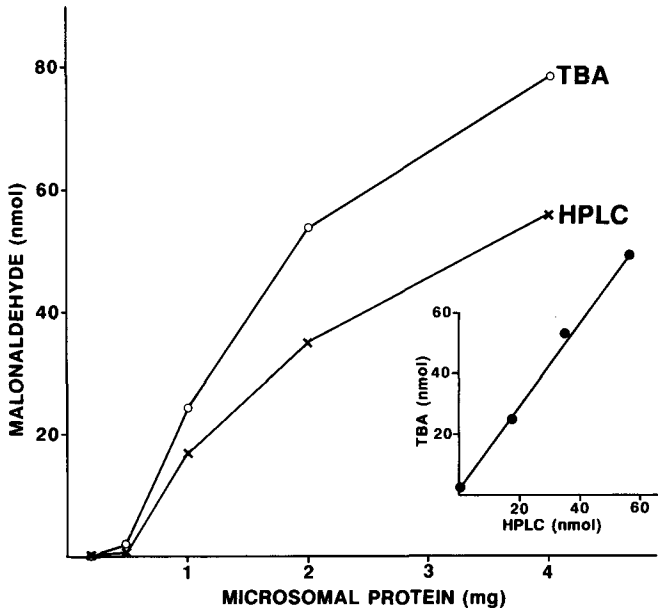


FIG. 5. MA produced during incubation of a liver microsomal preparation with ascorbate-Fe oxidative system. MA was estimated by the TBA assay and the HPLC method described herein.

E diet. However, no MA was detected in plasma or liver from rats fed normal and high vitamin E. The low levels or absence of MA in tissues of rats fed low vitamin E could reflect the fact that MA was not produced *in vivo* under these experimental conditions or the possibility that once produced, MA remained bound and could not be detected using the present method. To test this possibility, liver homogenates from rats fed the low vitamin E diet were submitted to acid hydrolysis according to Ichinose *et al.* (22) or to alkaline hydrolysis according to Lee and Csallany (39). These two procedures did not produce MA, indicating that it was not present at all, even in bound form. It appears from this that MA is not normally produced *in vivo* when vitamin E content is low. Only when an external chemical insult is added does production of MA take place. This is in apparent contradition to several reports that indicate that MA was produced in tissues (39) or excreted in urine (40) of rats fed diets depleted of vitamin E or after treatment with several chemicals (40,41). Moreover, Farris *et al.* (42) reported the absence of thiobarbituric acid-reactive substances in isolated rat hepatocytes during a toxic chemical insult. The reason for these contradictory results is not known, but probably could be found in the different methods used to estimate MA and in the diverse experimental conditions. The aim of the present work was to show the suitability of this HPLC method to measure MA in biological materials. The clarification of the contradictory results is beyond the scope of the objective of this study.

The HPLC method described herein measures MA without difficulty in a liver microsomal fraction obtained from rats fed the low-vitamin E diet and undergoing chemically induced lipid peroxidation *in vitro*

by an ascorbate-iron system. MA was also measured by the thiobarbituric acid assay in order to compare both methods (Fig. 5). The MA levels which were measured by the TBA method were 40-50% higher than those estimated by the HPLC method. The higher levels of TBA values may result from some substances other than MA, giving a positive TBA test. These results agree with the suggestion that results of the TBA assay should be referred to only as "TBA reactive substances" and should not be equated with MA levels (18-22). It should be indicated that microsomal chemical peroxidation was carried out in the absence of ADP, a common reactant in this type of experiment (34) because this compound interferes with the HPLC system as indicated above. As seen in Figure 5, the lack of ADP did not affect MA production. Previous experiments carried out in the presence of ADP gave higher values for MA with the HPLC method compared to the TBA assay.

In summary, a highly sensitive method for the analysis of MA by ion pairing HPLC is described. The method is direct without thiobarbiturate chromagen formation, sample preparation is simple and total time for each assay is short. The method is suitable for the quantification of MA in tissues, plasma and urine, as well as during chemically induced lipid peroxidation of microsomal fractions *in vitro*.

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Fish Oil Tetracosenoic Acid Isomers and GLC Analyses of Polyunsaturated Fatty Acids[1]

N.C. Shantha and **R.G. Ackman***
Department of Food Science and Technology, Technical University of Nova Scotia, Halifax, Nova Scotia, Canada B3J-2X4

The total tetracosenoic acid (24:1) levels in nine marine oils examined ranged from 0.4 to 1.1% of the total fatty acids. Gas-liquid chromatography (GLC) analysis of whole fish oil fatty acid methyl esters usually shows a single 24:1 peak, as the dominant (60–90%) isomer is 24:1ω9, and the minor peaks are not seen. Isolation and oxidative fission demonstrate that the lesser isomers present in these oils (in the first peak to elute) include 24:1ω15, 24:1ω13 and 24:1ω11, which are not resolvable from each other on open-tubular GLC; 24:1ω9 is followed by a peak for 24:1ω7. The complex first 24:1 isomer peak of fish oil fatty acids tends to coincide with or just follows the 22:6ω3 peak in GLC analyses carried out on Carbowax-20M type open-tubular (capillary) columns.
Lipids 26, 237–239 (1991).

A peak for tetracosenoic acid (24:1) is commonly observed in open-tubular gas-liquid chromatographic (GLC) analyses of fatty acids of marine oils. While the presence of isomers other than 24:1ω9 was reported in lipids of algae, fungi and higher animals (1), the only isomer reported in fish oils has been the ω9 isomer (2,3). An exception is the presence of trace quantities of ω11 and ω7 accompanying a similar level of the ω9 isomer in the 24:1 of salmon *(Salmo salar)* lipids; the structures of these acids have been confirmed by gas chromatography/mass spectrometry(GC/MS) and by hydrogenation of the main peak alone (1).

The proximity of the 24:1 components to the docosahexaenoic acid (22:6ω3) peak in GLC analyses on Carbowax-20M based capillary columns (4) required that we investigate the isomers in some detail since the only commonly available acid standard is nervonic acid (24:1ω9). This isomer can also be found in the fatty acid methyl esters derived from rapeseed oil, which is useful as a secondary GLC standard (4) since it also contains two eicosenoic acid isomers (20:1ω9 and ω7) and two docosenoic acid isomers (22:1ω9 and ω7) (5). The present investigation deals with the study of the 24:1 isomers in various marine oils. Some aspects of possible errors in calculating the different positional isomers based on the ozonolysis products and the biosynthetic pathway for the formation of these various isomers are also discussed.

MATERIALS AND METHODS

cis-15-Tetracosenoic acid was purchased from Serdary Research Laboratory Inc. (London, Ontario, Canada). The oil of jurel fish *(Trachurus murphyi)* was a gift from Professor L. Masson Salaue, Universidad de Chile, Santiago, Cod *(Gadus morhua)* (liver) and Atlantic herring *(Clupea harengus)* (body) were purchased from a local market and lipids were extracted by the method of Bligh and Dyer (6). The triacylglycerols were purified using conventional thin-layer chromatography. The esters were transesterified using BF_3/MeOH, and the methyl esters were recovered into hexane. The methyl esters were treated with urea in ethanol for adduct formation (7); the urea-complexing (UCF) and the non-urea-complexing fractions (NUCF) were separated and the methyl esters recovered (8). Both fractions were analyzed by gas-liquid chromatography for the presence of 24:1 methyl esters. No detectable amount of 24:1 ester was present in the NUCF. The 24:1 ester, found to be concentrated in the UCF, was purified by reversed-phase semi-preparative high-performance liquid chromatography (HPLC). The pure 24:1 esters thus isolated were further tested for purity and the presence of positional isomers by GLC. The esters were then subjected to oxidative ozonolysis for determination of double bond positions. Ozonolysis was performed as described elsewhere (9,10) and the ozonolysis products were analyzed by GLC and GC/MS. The proportions of different isomers were calculated from the weight percent of the dicarboxylic acid esters obtained upon ozonolysis (10). The areas of peaks for different chain lengths were corrected to weight percentages using the theoretical response factors calculated based on their ionizable carbon contents (11).

High-performance liquid chromatography. A Waters Associates (Milford, MA) HPLC system including 6000A pump, U6K injector, and R 401 refractive index detector was utilized. A C-18 μ Bondapak (8 mm = 10 cm) column was used with a RCM-100 radial compression module. Methanol/water (95:5, v/v) at a flow rate of 1.5 mL/min served as the mobile phase.

Gas-liquid chromatography. GLC analyses were performed on a Perkin-Elmer model 8420 gas chromatograph equipped with a digital integrator, using a SUPELCOWAX-10 (bonded Carbowax-20M) column (30 m × 0.32 mm i.d., phase thickness 0.25 μm; Supelco Inc., Bellefonte, PA). GLC analyses of fish oil fatty acid methyl esters were temperature-programmed from 195°C to 240°C at 3°C per min after an initial 8 min at 195°C and held at 240°C for 10 min. The split ratio was 1:32, helium flow rate was 1.2 mL/min and injection port temperature was 250°C. For the analysis of the monomethyl and the dimethyl fatty acid esters obtained from ozonolysis, the oven temperature was programmed from 120°C to 240°C at 3°C per min after an initial 8 min at 120°C and held at 240°C for 10 min. The other parameters were the same as above. The peak area output was recorded on a Perkin-Elmer GP-100 graphic printer.

Gas chromatography/mass spectrometry (GC/MS). Electron impact mass spectra were obtained on a model 700 Finnigan MAT Ion Trap Detector (ITD) system (Finnigan MAT, San Jose, CA) interfaced to a Perkin-Elmer

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*To whom correspondence should be addressed at the Department of Food Science and Technology, Technical University of Nova Scotia, P.O. Box 1000, Halifax, Nova Scotia, Canada B3J-2X4.

Abbreviations: GC/MS, gas chromatography/mass spectrometry; GLC, gas-liquid chromatography; HPLC, high-performance liquid chromatography; NUCF, non-urea complexing fraction; UCF, urea-complexing fraction.

METHODS

TABLE 1

Occurrence and Composition of 24:1 Isomers in Marine Oils

Marine oil	Weight % (% of total fatty acid)	Isomer distribution[a,b] (% of total 24:1)				
		ω15	ω13	ω11	ω9	ω7
Menhaden *(Brevoortia tyrannus)*	0.6	2.2	4.4	13.5	74.8	5.1
Jurel *(Trachurus murphyi)*	0.6	4.3	3.3	6.9	81.1	4.4
Tuna *(Thunnus alalunga)*	0.8	tr[c]	2.2	4.1	90.5	3.2
Herring *(Clupea harengus)*	0.9	3.0	6.5	17.7	71.1	1.7
Cod liver *(Gadus morhua)*	0.4	3.0	5.0	14.3	77.2	0.5
Dogfish liver *(Squalus acanthias)*	1.1	2.7	4.6	13.3	77.5	2.0
Redfish *(Sebastes marinus)*	0.8	5.5	11.2	15.6	66.3	1.3
Capelin *(Mallotus villosus)*	0.7	3.8	6.2	12.8	77.2	tr[c]
Whale *(Balaenoptera borealis)*	0.7	4.1	13.1	17.2	63.3	2.3

[a]Calculated on the basis of the wt% of dicarboxylic acid ester obtained by ozonolysis.

[b]Not corrected for secondary oxidation products (see text).

[c]Trace amounts (tr) are less than 0.1%.

model 990 gas chromatograph. The GLC fused silica capillary column was fed through a heated transfer line directly into the ITD gas inlet. For GC/MS, the separations were performed on a polyglycol DB-WAX column (30 m × 0.25 mm i.d., phase thickness 0.25 μm; J & W Scientific Inc., Folsom, CA). The oven temperature for analysis of 24:1 isomers was programmed from 180°C to 210°C at 3°C per min after an initial 8 min at 180°C, and held at 210°C for 48 min. For the analysis of ozonolysis products, the temperature was programmed from 110°C to 210°C at 3°C per min with an initial hold of 8 min at 110°C followed by a 48-min hold at 210°C. The helium pressure was 10 psi. The data system consisted of an IBM 286 compatible dtk TECH-1230 computer (Technida Ltd., Dartmouth, Canada). All studies were conducted with version 4.0 of the ITD software supplied by the Finnigan Corp. The ITD was tuned by using the procedure supplied by the manufacturer. The ITD was operated in the full scan mode and scanned with a 1-s cycle time.

RESULTS AND DISCUSSION

Table 1 gives the percentages of 24:1 fatty acids in the different fish oils and the percentages of different isomers present. In the fish oils examined, 24:1 was a minor component, ranging from about 0.4% of the total fatty acid methyl esters in cod liver oil to 1.1% in dogfish liver oil. In the ozonolysis products, the C_{15} diester and the C_9 monoester were always the major peaks. These fragments correspond to the 24:1ω9 isomer, calculated to be more than 60% of the total 24:1 in all the fish oils examined, and reaching 80–90% in the case of the jurel and tuna fish oils. The unresolved 24:1ω11 and ω13 were the next most prominent isomers, the ω11 exceeding the ω13 in all cases. The 24:1ω15 was also not resolved from the ω11 and ω13 isomers, but it and the ω7 isomer, identified as a separate GLC peak, were conspicuous in the ozonolysis products of all oils examined except for the capelin, where the 24:1ω7 was present in only trace amounts. The composition of different isomers in depot fat from sei whales, a mammal, did not differ much from that of the fish oils. This could well be related to the whales' marine fish diet (12) rather than being a species characteristic.

The various isomers were calculated from the weight percentage of the dicarboxylic acid diesters obtained upon ozonolysis. The corresponding monomethyl esters were used primarily as a qualitative match to verify that the complementary product was not an artifact since secondary oxidation products are known to be formed during oxidative ozonolysis (10). The 24:1ω9 isomer should give rise primarily to the C_9 monoester and the C_{15} diester upon ozonolysis. The C_{15} diester thus formed could undergo both α-oxidation to give rise to a C_{14} diester and β-oxidation to give rise to a C_{13} diester. α-Oxidation is known to usually amount to 2% (9). β-Oxidation is relatively minor, but the observed amount of C_{13} diester from 24:1ω11 could be slightly exaggerated, since the C_{15} diester present is the major product and will yield some C_{13} diester. A total correction of 2% must be added back to the C_{15} product. Similarly, some of the C_{11} and the C_9 diester could also be formed due to further secondary reactions. However, the values have not been corrected for added secondary oxidation products from longer chain lengths. The absence of a C_{10} monoester verifies that there is in fact no 24:1ω10 isomer such as might be deduced from the observed trace of C_{14} secondary diester product.

Under the conditions of the GLC analysis, the monomethyl and dimethyl esters are of similar equivalent chain lengths and tend to elute in close proximity. In order to avoid erroneous identifications, all ozonolysis products were subjected to GC/MS, and the peaks were identified based on their characteristic mass spectra. Ozonolysis with its various advantages is a well-accepted method for structure determination of unsaturated fatty acids (10). The formation of traces of the secondary products for most chain lengths complicates the chromatogram in one sense, but promotes identification in another. Usually the number of moles of the monomethyl and the corresponding dimethyl esters cannot be matched accurately due to the tendency of the shorter chain esters to be lost during extraction and/or concentration steps. Despite these mild disadvantages the formation of stable dicarboxylic and monocarboxylic acid products is advantageous and for quantitation of mixed isomer proportions is probably superior to sophisticated modern mass spectrometry (13,14).

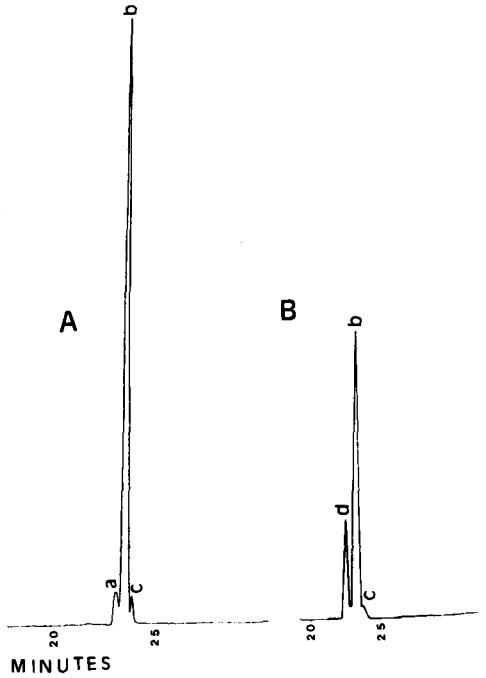


FIG. 1. (A) Gas-liquid chromatogram of methyl tetracosenoate (24:1) of the South Pacific fish jurel *(Trachurus murphyi)* showing resolution between its isomers. Peaks are: a, 24:1ω11+13+15; b, 24:1ω9; c, 24:1ω7. (B) Gas-liquid chromatogram of methyl tetracosenoate (24:1) of the South Pacific fish jurel (A) co-injected with methyl docosahexaenoate (22:6ω3) standard. Peaks are: d, 22:6ω3 + 24:1ω11+13+15; Other notations as in A.

Despite the presence of various isomers, only three peaks for 24:1 were observed on the SUPELCOWAX-10 column used (Fig. 1A). The composite peak for the 24:1ω11+13+15 isomers was possibly somewhat broader than one would expect for one component alone (Fig. 1A). It eluted before the 24:1ω9 isomer, and the 24:1ω7 isomer eluted after the 24:1ω9 isomer; however, there was no baseline separation between the three peaks which would correspond to the different isomers listed in Table 1. The lack of separation among the 24:1ω11, ω13 and the ω15 isomers would be expected since no separation is achieved between the ω11 and the ω13 isomers of 22:1 on polyester-based capillary columns of similar polarity (15). However, a 50-m methyl silicone column also separated 24:1ω11 and 24:1ω9 (14).

The 24:1 (ω11+13+15) peak coincided with the 22:6ω3 peak under our conditions of analysis on SUPELCOWAX-10, and on co-injection with 22:6ω3 the two emerged as a single peak (Fig. 1B). The overlap between 24:1ω11+13+15 isomers and 22:6ω3 on the SUPELCOWAX-10 column could potentially create a quantitation problem if high levels of the monoene isomers were present relative to 22:6ω3. Since 24:1 is a minor component in most fish oils, and ω11+13+15 isomers are much less conspicuous than ω9, this overlap could be overlooked. Equally, when working with mammalian brain lipid (16), which could have a considerable amount of 24:1 as well as 22:6ω3, care has to be taken in the analysis of the fatty acid methyl esters.

The structures of the monoethylenic fatty acids in fish oil were determined and inter-related over twenty years ago for herring oil, a common fish depot fat, in light of the information then available (17). The original discovery (18,19) that the important 22:1ω11 isomer originated in copepod fatty alcohols, oxidized to the corresponding fatty acids when ingested by fish, was later extended by detailed investigation of several marine fish oils (20,21). From Table 1 it is clear that 24:1ω9 is the dominant isomer and presumably accumulates for reasons not associated with chain extension from the marine 22:1 fatty acids which are known to be dominated by 22:1ω11. The menhaden is a short-lived planktonivorous fish and yet has a high proportion of 24:1ω9, similar to that of the longer-lived carnivorous Atlantic cod. Despite one report of algal 24:1ω9 from Antarctic waters (14), there is no obvious geographical factor for distribution of 24:1 isomers, as shown by the North Atlantic and South Pacific species analyses, and our comparisons thus suggest that the principal isomer, 24:1ω9, is biosynthesized specifically in fish, all other 24:1 isomers being inadvertent additions.

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Plasma Lipid Profiling by Liquid Chromatography with Chloride-Attachment Mass Spectrometry

A. Kuksis*, L. Marai and J.J. Myher
Banting and Best Department of Medical Research, University of Toronto, Toronto, Canada M5G 1L6

A sensitive high-performance liquid chromatographic assay was developed using chloride attachment negative chemical ionization mass spectrometry for detection of glyceryl esters and ceramides, and positive chemical ionization mass spectrometry for detection of free cholesterol and cholesteryl esters in minimal quantities of plasma. The novel technique was validated by high temperature gas-liquid chromatography with flame ionization detection. Sample preparation was achieved by phospholipase C digestion of whole plasma, total lipid extraction and derivatization of any free carboxyl and hydroxyl groups by trimethyl- or *tert*-butyldimethylchlorosilane. The lipids were separated by reverse phase HPLC with 20–90% propionitrile in acetonitrile containing 1% dichloromethane, which served as the reagent and the source of chloride. Negative chemical ionization with chloride attachment is estimated to provide about 100 times higher response for the triacylglycerols and the trimethylsilyl or *tert*-butyldimethylsilyl ethers of diacylglycerols, and about 500 times higher response for the trimethylsilyl or *tert*-butyldimethylsilyl ethers of ceramides than positive chemical ionization mass spectrometry. Determination of the full negative chemical ionization mass spectra showed that each glycerolipid and ceramide species yielded a single ionic species corresponding to the chloride-attachment product of the parent ion. The cholesteryl esters and ethers failed to attach chloride and remained undetected by negative chemical ionization. However, the cholesteryl esters and ethers gave a high response for the steroid nucleus in positive chemical ionization mass spectrometry. Chloride attachment negative chemical ionization mass spectrometry is suitable for the unequivocal identification of plasma glycerolipids and ceramides in high-performance liquid chromatography and for the quantitation of molecular species in any unresolved peaks following appropriate calibration of the instrument response.
***Lipids 26,* 240–246 (1991).**

The application of reverse phase high-performance liquid chromatography (HPLC) for plasma lipid profiling has been explored only to a limited extent (1–8) because lipids lack chromophores which can be detected spectrophotometrically. Ultraviolet (UV) detection at 200–215 nm has been used most extensively (1,2,6) followed by differential refractometry (3,4), although very few solvents are entirely satisfactory for these methods. Free cholesterol and cholesteryl esters have been monitored by optical activity (5). Mass spectrometry coupled with reverse phase HPLC would seem to provide an ideal method for the detection and quantitation of molecular species in HPLC effluents, but difficulties have arisen as a result of a highly variable positive chemical ionization of different plasma lipid classes in the propionitrile/acetonitrile solvent system (7,8) when used with direct liquid inlet interface (9). The present study shows that under the same HPLC conditions chloride-attachment negative chemical ionization mass spectrometry (NCIMS) results in much more uniform response and greatly increased sensitivity of detection of ceramides as well as of glycerolipids, which yield $[M + 35]^-$ and $[M + 37]^-$ as the only readily detectable negative ions. However, the trimethylsilyl (TMS) and *tert*-butyldimethylsilyl (TBDMS) ethers and fatty acid esters of cholesterol do not yield negative ions with chloride attachment and must be determined in the positive chemical ionization mode.

MATERIALS AND METHODS

Various synthetic neutral lipid mixtures were available in the laboratory from previous studies (10). Normal human plasma (0.5 mL) was digested with phospholipase C *(Clostridium welchii)* and the lipids recovered by extracting the reaction mixture with chloroform/methanol as previously described (10). The extracts were dried and silylated with trimethylchlorosilane (TMSCl) or *tert*-butyldimethylchlorosilane (11) as previously described in detail. The overall workup of the sample including trimethylsilylation is summarized in Figure 1. For a rapid and complete digestion of the minor acidic phospholipids also present in plasma, a total lipid extract is first prepared and phospholipase C from *Bacillus cereus* is used (11).

The HPLC analyses were performed with a Hewlett-Packard Model 1084B liquid chromatograph (Hewlett-Packard Co., Palo Alto, CA) equipped with a 25 cm ×

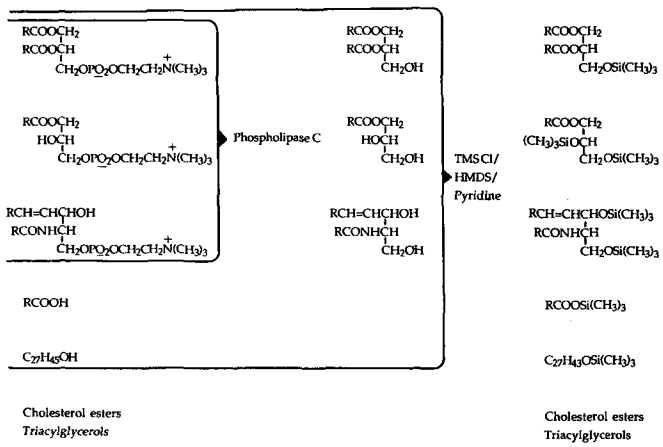


FIG. 1. Summary of sample preparation. TMSCl, trimethylchlorosilane; HMDS, hexamethyldisilazane.

*To whom correspondence should be addressed.

Abbreviations: CI, chemical ionization; ECN, equivalent carbon number; FID, flame ionization detector; GLC, gas-liquid chromatography; HMDS, hexamethyldisilazane; HPLC, high-performance liquid chromatography; LC/MS, liquid chromatography with mass spectrometry; NCI, negative chemical ionization; PCI, positive chemical ionization; TBDMS, tertiary butyldimethylsilyl; TMS, trimethylsilyl; TMSCl, trimethylchlorosilane; UV, ultraviolet.

0.46 cm OD Supelcosil LC-18 column (Supelco, Bellefonte, PA), using a gradient of 20–90% propionitrile in acetonitrile (8,9). The solvent mixture contained a constant concentration of 1% dichloromethane (12). The columns were operated at a flow rate of 1.5 mL/min and 30°C oven temperature. The mass spectrometry was done on a Hewlett-Packard Model 5985B quadrupole mass spectrometer equipped with a Hewlett-Packard direct liquid inlet interface based on the Baldwin-McLafferty split chemical ionization (CI) approach (8,9). The mass spectrometer scans were restricted to masses above 200 and were taken with a scan rate of 4.7 sec (*m/z* 200–950) over the entire elution profile. In some instances background subtractions were performed by the computer to compensate for impurities in the solvents. The positive and negative chemical ionization spectra were recorded at 210 eV from separate liquid chromatography/mass spectrometry (LC/MS) analyses. The ion source temperature was 200°C and 150°C for positive chemical ionization mass spectrometry (PCIMS) and negative chemical ionization mass spectrometry (NCIMS), respectively.

Control gas-liquid chromatography (GLC) analyses of TMS ethers of diacylglycerols were performed on polar (SP-2330, Supelco) capillary columns (10,11) while those of ceramides, cholesteryl esters and triacylglycerols were performed on polarizable (OV-22, Quadrex, New Haven, CT) capillary columns (13), as previously described.

RESULTS

Comparison of LC/PCIMS and LC/NCIMS profiles of plasma lipids. Figure 2 shows the total ion current profiles obtained by LC/MS for the TMS ethers of plasma lipids in the negative (A) and positive (B) chemical ionization. In the positive mode, the major peaks correspond to the TMS ether of free cholesterol (13.5 min) and the 20:4 (19.5 min), 18:2 (22 min), 18:1 (25 min) and 16:0 (25.5 min) esters of cholesterol. The TMS and TBDMS ethers of diacylglycerols as well as the triacylglycerols can be monitored by LC/MS in the positive chemical ionization mode, but the signal intensity is about 100 times lower than that recorded for the fatty acid esters of cholesterol (7,8). The diacylglycerols and triacylglycerols yield the $[MH - RCOOH]^+$ ions from which the structure of the molecular species can be deduced. In the chloride-attachment negative ionization mode, the triacylglycerols and the TMS ethers of both diacylglycerols and ceramides yield greatly increased response. The early peaks in Figure 2A correspond to the TMS ethers of the diacylglycerols, while the later peaks correspond to various common triacylglycerols, with the ceramides appearing in both diacylglycerol and triacylglycerol elution regions (see below).

A comparable LC/MS profile was obtained for the TBDMS ethers of plasma lipids in the negative and positive chemical ionization mode (chromatograms not shown). In the positive mode, only the TBDMS ether of cholesterol (19 min), which was eluted just before cholesteryl arachidonate, and the fatty acid esters of cholesterol are detected. In the chloride attachment negative chemical ionization, the triacylglycerols and TBDMS ethers of diacylglycerols and ceramides were detected with increased sensitivity. The early peaks corresponded to the TBDMS ethers of diacylglycerols, while the later peaks were due to the TBDMS ethers of ceramides, with the triacylglycerols appearing in both diacylglycerol and ceramide elution regions (see below). This behavior of the silyl ethers of glycerolipids and ceramides on reverse phase HPLC is similar to that observed previously with the propionitrile/acetonitrile system in the absence of dichloromethane (7–9). The LC/MS spectrum of cholesteryl linoleate as obtained in the positive CI mode for the TMS- and TBDMS-treated plasma lipids gave only the ion for the steroid nucleus (*m/z* 369) in the 300 to 900 mass range. The free cholesterol and the molecular species of the cholesteryl esters were identified from their relative retention times.

Nature of chloride-attachment NCI spectra. Figure 3A depicts the full mass spectrum of a diacylglycerol TMS peak taken at 13 min in Figure 2A. The diacylglycerols are identified by the number of their acyl carbons:number of double bonds. In addition to the major components

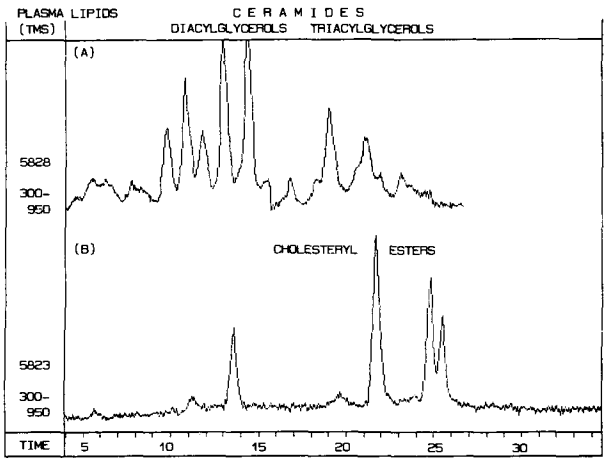


FIG. 2. Total ion current profiles obtained by LC/MS for the TMS ethers of plasma lipids with NCI (A) and PCI (B). Peak identification, as given in figure and explained in text. LC/MS conditions as given in text.

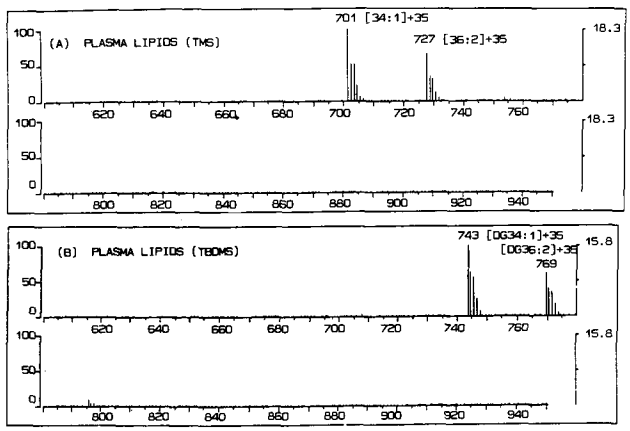


FIG. 3. Full mass spectra of corresponding diacylglycerol peaks as obtained by LC/MS of the TMS (13 min) (A) and TBDMS (16 min) (B) ethers in chloride attachment NCI. LC/MS conditions as given in Figure 2.

[DG34:1] + 35 (*m/z* 701) and [DG36:2] + 35 (*m/z* 727), minor components are seen for the chloride 37 attachment products [DG34:1] + 37 (*m/z* 703) and [DG36:2] + 37 (*m/z* 729) within the ion clusters accompanying the parent ions. No other negative ions are seen for these diacylglycerols or any other components in the 600–950 mass range. Figure 3B shows the full mass spectrum for the corresponding TBDMS diacylglycerol peak. Both DG34:1 and DG36:2 are seen as the $[M + 35]^-$ and the $[M + 37]^-$ ions at the corresponding *m/z* values. The chlorine isotopes 35 and 37 occur naturally in the ratio 3 to 1 (14).

The ceramide peaks are identified by the total number of carbons:number of double bonds. The full mass spectra of the ceramide TMS peaks also showed only one molecular species in most instances. Thus, the peak emerging at 13.5 min in Figure 2A showed the masses at *m/z* 716 [CER34:1] + 35 and *m/z* 718 [CER34:1] + 37. Likewise, the TBDMS ether of the same ceramide peak also yielded a single negative ion for each isotope species corresponding to $[M + 35]^-$ and $[M + 37]^-$ of CER34:1.

Figure 4 shows that each species of triacylglycerols yields only the chloride attachment ions $[M + 35]^-$ and $[M + 37]^-$ from both TMS- (Fig. 4A) and TBDMS- (Fig. 4B) treated plasma lipids. However, the triacylglycerol peaks in both TMS- and TBDMS-treated plasma lipids overlap with the ceramide peaks, as already noted. Thus, the triacylglycerol peak [TG52:2] + 35 (*m/z* 893) in the TMS plasma (23.5 min) overlaps with the ceramide peak [CER41:1] + 35 (*m/z* 814), while the same triacylglycerol peak [TG52:2] + 35 (*m/z* 893) in the TBDMS plasma (23.5 min) overlaps with the ceramides [CER36:1] + 35 (*m/z* 828) and [CER38:2] + 35 (*m/z* 854). Of course, each triacylglycerol and ceramide peak also is accompanied by the chlorine-37 peak, as explained above.

Identification of molecular species. Figure 5 shows the total ion current profile of the TMS ethers of plasma lipids along with the single ion plots for the major diacylglycerol species as obtained by LC/MS with chloride-attachment negative chemical ionization. Most *m/z* values represent two species of diacylglycerols differing by two mass units, one representing $[M + 35]^-$, the other $[M + 37]^-$ of a corresponding species having a molecular weight lower by two mass units. Thus, the [DG32:0] + 35 ion has the same mass as the [DG32:1] + 37 ion. The [DG32:1] + 37 (*m/z* 675) corresponds in retention time to [DG32:1] + 35 (*m/z* 673). The diacylglycerol species are eluted in order of increasing equivalent carbon number (ECN). All peaks of sufficient intensity represent Gaussian curves with minimum distortion. A similar single ion plot was obtained for the major diacylglycerol species when analyzed as the TBDMS ethers (figure not shown), which also yielded only one negative ion for each isotope species corresponding to the $[M + 35]^-$ and $[M + 37]^-$ ions of the TBDMS ethers of diacylglycerols, as shown in Figure 3.

Figure 6 shows the total ion current profile of the TBDMS ethers of plasma lipids along with the single ion plots for the major ceramide species as obtained by LC/MS with chloride attachment negative chemical ionization. Each species yields only one negative ion corresponding to $[M + 35]^-$ and one corresponding to the satellite isotope $[M + 37]^-$. All peaks, regardless of the molecular weight of the component species, are seen to tail, especially the less abundant ones. This is not due to overlap among species, which are eluted in order of increasing ECN values. A similar single ion plot was obtained for the major ceramide species when analyzed as the TMS ethers, which also yielded only one negative ion for each isotope (figure not shown).

Figure 7 shows the total ion current profile of the TBDMS plasma lipids along with the single ion plots for the major triacylglycerol species as obtained in negative chemical ionization with chloride attachment. The triacylglycerol species, which are identified by the number of acyl carbons: number of double bonds, range from 48:1 to 54:4. They emerge in order of increasing ECN values. Due to the rather low concentration of the solutes, the

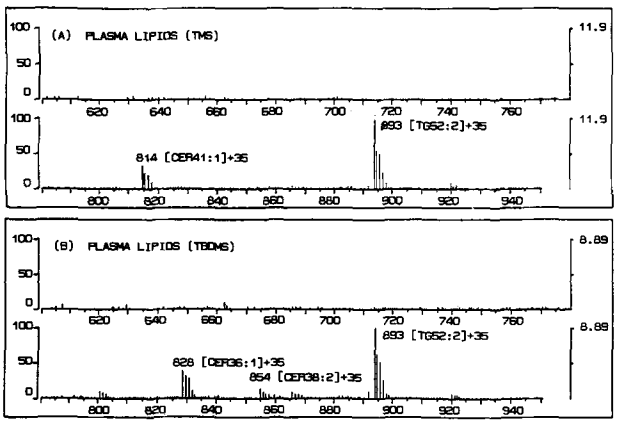


FIG. 4. Full mass spectra of the corresponding triacylglycerol species as obtained by LC/MS with chloride attachment NCI for the TMS- (A) and TBDMS- (B) treated plasma lipids. LC/MS conditions, as in Figure 2.

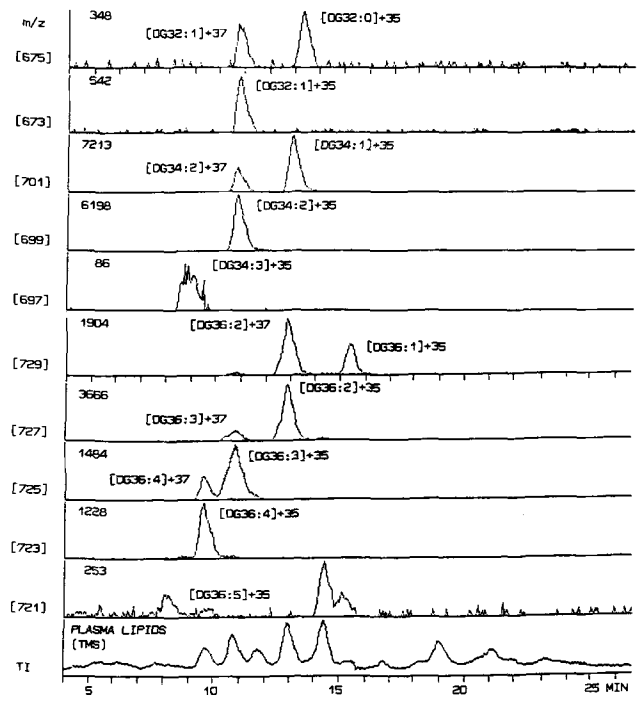


FIG. 5. Total ion current profile (TI) of the TMS-treated plasma lipids with single ion plots (*m/z*) for the major diacylglycerol species as obtained by LC/MS with chloride attachment NCI. Peak identification, as given in figure. LC/MS conditions as in Figure 2.

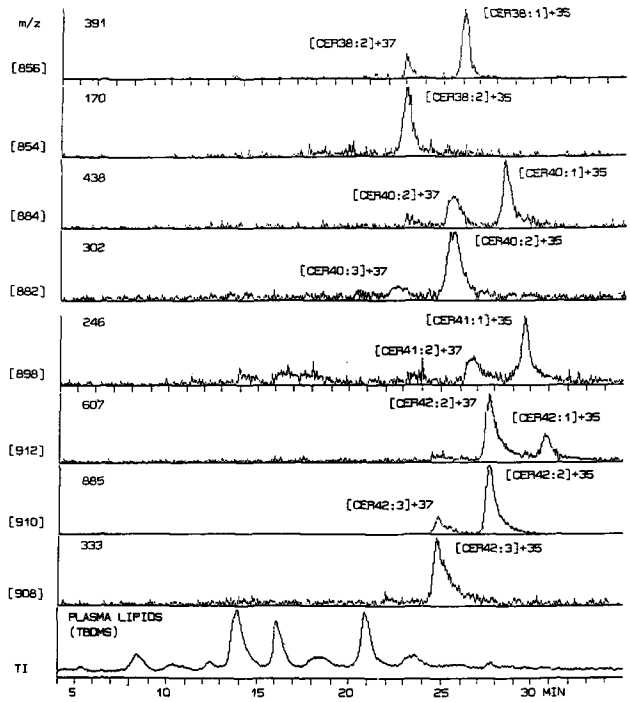


FIG. 6. Total ion current profile (TI) of the TBDMS-treated plasma lipids with single ion plots *(m/z)* for the major ceramide species as obtained by LC/MS with chloride attachment NCI. Peak identification, as given in figure. LC/MS conditions, as given in Figure 2.

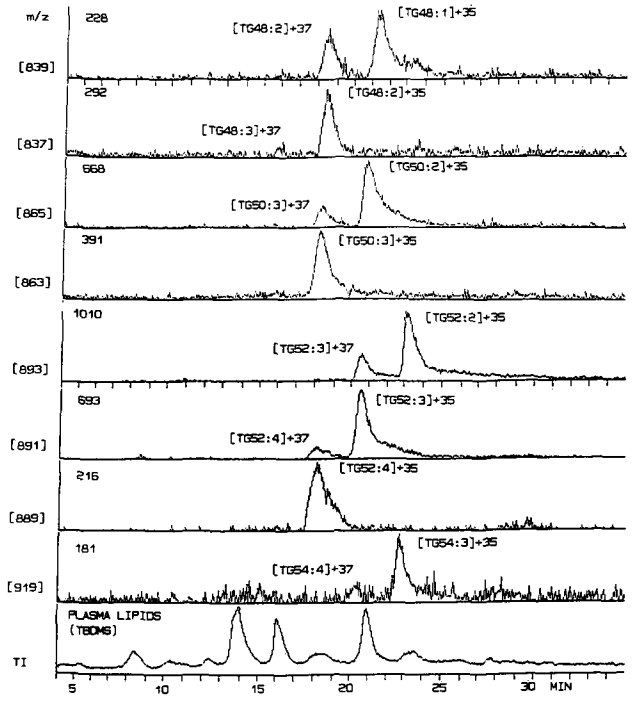


FIG. 7. Total ion current profile (TI) of the TBDMS-treated plasma lipids along with the single ion plots *(m/z)* for the major triacylglycerol species as obtained by LC/MS with chloride attachment NCI. Peak identification, as given in figure. LC/MS conditions, as given in Figure 2.

peaks show somewhat more tailing than the more abundant diacylglycerols and ceramides examined above. An identical single ion plot was obtained for the TMS plasma triacylglycerols (figure not shown).

Quantitation of molecular species. The areas under the various single ion peaks can be calculated and expressed as a percentage of the uncorrected total peak area representing the sum of the peak areas of all the species belonging to each lipid class. Table 1 demonstrates that the quantitative estimates for molecular species of the TMS and TBDMS ethers of diacylglycerols agree closely with each other and deviate little from the control estimates derived by polar capillary GLC of the TMS ethers. For a more accurate comparison to the results of the GLC analysis with flame ionization detection, the negative ion current response would need to be calibrated using appropriate reference standards.

Table 2 shows an equally close agreement for the quantitative estimates of the molecular species of triacylglycerols between the TMS- and TBDMS-treated plasma lipids and those obtained by control GLC analyses on polarizable capillary column with flame ionization detection. These results indicate that chloride-attachment negative chemical ionization yields nearly correct quantitative response for the common glycerolipid species under the present working conditions and instrumentation.

Table 3 compares the recoveries of the ceramides as the TMS and TBDMS ethers, as obtained by LC/MS with chloride-attachment negative CI. The longer chain ceramides appear to have been recovered to a somewhat lower extent when analyzed as the TBDMS rather than the TMS ethers. However, the recoveries of the long chain ceramides as the TMS ethers are also significantly lower, when compared to those obtained by polarizable (13) or non-polar capillary GLC (15). It is unlikely, that the longer chain homologues of the ceramides would form the chloride adducts less readily than the shorter chain homologues, and other possibilities, such as "cold trapping" of the species of higher molecular weight and greater degree of saturation must be considered. Previously, "cold trapping" has been observed (16) in GC/CIMS analysis of cholesteryl esters. It was manifested in peak tailing and by loss of steryl esters of higher molecular weight than cholesteryl palmitate.

As pointed out above, the silyl ethers and fatty acid esters of cholesterol do not attach chloride and are not detected in the negative CI mode. Therefore, they were quantitated in the positive CI mode. Table 4 gives the relative response for the various cholesteryl esters in the TMS- and TBDMS-treated plasma lipid samples. It is seen that the LC/MS estimates for the individual molecular species agree closely with each other and with the control GLC analyses, except for cholesteryl arachidonate, which is not recovered from the polarizable capillary column (13). The silyl ethers of free cholesterol, however, give a lower response in LC/MS than in GLC/flame ionization detector (FID). Thus, the LC/MS estimates for the TMS (17%) and TBDMS (8.5%) ethers are significantly lower than the GLC/FID determinations (22%) for the free cholesterol in the sample. Nevertheless, these ion yields are reproducible and can be utilized in quantitative measurements following appropriate calibration of the instrument.

TABLE 1

Relative Recoveries of Plasma Phosphatidylcholines (as Diacylglycerols) by LC/MS with Chloride-Attachment NCI and by Polar Capillary GLC

Molecular species	Uncorrected area%[a]				
	TMS ethers		TBDMS ethers		TMS ethers
	Mass[b]	LC/MS	Mass[b]	LC/MS	GLC
32:0	675	1.3	717	1.5	0.8
32:1	673	2.0	715	2.2	0.7
34:1	701	28.3	743	26.9	29.4
34:2	699	24.5	741	23.1	19.4
34:3	697	0.4	739	0.5	0.3
36:1	729	3.8	771	4.5	2.7
36:2	727	16.4	769	15.3	16.7
36:3	725	8.1	767	8.2	7.5
36:4	723	4.9	765	5.3	7.7
36:5	721	0.5	763	0.6	1.9
38:2	755	0.3	797	0.1	
38:3	753	2.5	795	2.5	6.7
38:4	751	3.4	793	3.7	
38:5	749	1.2	791	1.5	1.3
38:6	747	1.6	789	2.6	3.5
40:6	775	0.8	817	1.4	1.5

[a]Average of two injections.
[b]*m/z*, M + 35.

TABLE 2

Relative Recoveries of Plasma Triacylglycerols by LC/MS with Chloride-Attachment NCI and by Polarizable Capillary GLC

Molecular species	Mass[b]	Uncorrected area%[a]		
		LC/MS (TMS)	LC/MS (TBDMS)	GLC (TMS)
48:0	841	1.0	1.0	
48:1	839	5.8	4.1	3.0
48:2	837	1.7	3.5	2.4
50:1	867	10.6	8.6	9.1
50:2	865	18.7	13.9	14.2
50:3	863	6.5	6.5	3.5
52:1	895		2.2	1.8
52:2[c]	893	25.5	18.5	25.5
52:2				7.0
52:3	891	16.7	13.7	18.6
52:4	889	4.7	4.2	
54:2	921	2.0	0.5	1.3
54:3	919	2.9	2.2	3.7
54:3				2.2
54:4	917	1.5	1.3	1.5
Other		2.4	3.8	6.2

[a]Average of two injections.
[b]*m/z*, M + 35.
[c]Braces indicate only one estimate was obtained for the two types of 52:2.

TABLE 3

Relative Recoveries of Plasma Sphingomyelins (as Ceramides) by LC/MS with Chloride-Attachment NCI and by Polarizable Capillary GLC

Molecular species	Uncorrected area%[a]				
	TMS ethers		TBDMS ethers		TMS ethers
	Mass[b]	LC/MS	Mass[b]	LC/MS	GLC
CER32:1	688	3.7	772	7.3	1.9
CER33:1					1.2
CER34:1	716	44.8	800	49.7	25.4
CER34:2	714	5.9	798	5.2	2.7
CER35:1					0.6
CER36:1	744	5.8	828	7.6	6.4
CER36:2	742	2.9	826	3.4	2.1
CER36:3	740	0.3	824	0.3	0.0
CER37					0.5
CER38:1	772	2.6	856	2.3	5.1
CER38:2	770	1.4	854	1.4	0.1
CER39:1					1.0
CER40:1	800	5.1	884	3.4	8.4
CER40:2	798	3.5	882	3.5	5.3
CER40:3	796	0.4	880	0.5	0.0
CER41:1	814	2.9	898	2.0	3.8
CER41:2					2.3
CER42:1	828	2.4	912	2.0	6.0
CER42:2	826	13.6	910	7.9	18.7
CER42:3	824	4.5	908	3.6	4.8
CER42:4					1.1

[a]Average of two injections. [b]*m/z*, M + 35.

TABLE 4

Relative Recoveries of Plasma Free Cholesterol and Cholesteryl Esters by LC/MS with PCI and by Polarizable Capillary GLC

Molecular species	Uncorrected area%[a]				
	LC/MS		LC/MS		
	Mass	(TMS plasma)	Mass	(TBDMS plasma)	GLC
CHOL14:0	369	0.6	369	0.7	1.9
CHOL16:0	369	20.3	369	22.1	20.0
CHOL16:1	369	1.1	369	1.0	4.4
CHOL18:0	369	1.1	369	1.0	1.1
CHOL18:1	369	28.5	369	25.7	25.0
CHOL18:2	369	43.1	369	44.8	45.9
CHOL18:3	369	0.6	369	0.4	n.d.[b]
CHOL20:4	369	3.5	369	3.9	n.d.
Free cholesterol (%) of total)		17		8.5	22.0

[a]Average of two injections. [b]n.d., not determined.

DISCUSSION

Parker *et al.* (14) have shown that successful chloride-attachment NCIMS, developed by Dougherty (17) can be adopted to the analysis of various biologically important substances by LC/MS with direct liquid introduction through the addition of a chlorinated solvent modifier. The present study shows that glyceryl esters and ceramide ethers can be readily detected by this method during

LC/MS, although other neutral lipids, such as cholesteryl esters and ethers, do not yield negative ions. Previously paraffinic hydrocarbons and methyl stearate had failed to give chloride attachment spectra when examined by direct probe mass spectrometry (17). The chloride-attachment NCIMS provides a significant improvement in the sensitivity of detection, identification and quantitation of glycerolipids and ceramides in the effluents of HPLC columns when compared to PCIMS. The increase in sensitivity stems from the high yield of the negative ions following chloride attachment and the apparent absence of their fragmentation under the direct liquid inlet chemical ionization conditions. It was estimated that the overall increase in sensitivity of detection over positive chemical ionization for the glycerolipids was about 100-fold, and for the ceramides about 500-fold.

The formation of a single chloride-attachment ion provided a ready indication of the molecular weight of each solute, which, together with the knowledge of the retention time, was usually sufficient for peak identification. In the absence of significant further fragmentation, however, it was not possible to confirm the exact combinations of the fatty acids in each diacylglycerol and triacylglycerol molecule. The formation of a readily detectable single chloride-attachment product in the NCIMS also simplified the quantitation of the molecular species, which appeared to be recovered in nearly correct mass proportions. The only exceptions were provided by the more saturated short chain ceramides which were overestimated and the long chain ceramides, which were underestimated. This effect, however, did not appear to be due to any difficulty in chloride attachment or in NCIMS, but to "cold trapping" of the less soluble higher molecular weight components in the 150°C ion source or to tailing in the direct liquid inlet probe. Increasing the temperature of the ion source resulted in increased relative recoveries of these components. However, the overall sample recovery (ionization) was reduced (Marai, L., Kuksis, A., and Myher, J.J., 1990, unpublished results).

The major shortcoming of the LC/MS with chloride-attachment NCI is the absence of response to the TMS and TBDMS ethers as well as the fatty acid esters of cholesterol (12,18). These cholesterol derivatives require PCI for detection, which is most effective due to high yield of the ion at *m/z* 369, corresponding to the steroid nucleus of the cholesterol molecule (7,8). The glycerolipids give much lower response than the cholesteryl esters in the PCI, while the TMS and TBDMS ethers of ceramides remain largely undetectable under normal conditions of plasma lipid profiling (7,8). The formation of the $[MH - RCOOH]^+$ ions in the positive CI spectra, however, is important for the exact determination of the pairing of the fatty acids in the diacylglycerol and triacylglycerol molecules, and we have employed this method for the LC/MS identification and quantitation of molecular species of natural glycerolipids in the past (19,20).

All our LC/MS studies have been performed with the direct liquid inlet probe, which allows admission of only 1% of the column effluent to the mass spectrometer. This decreases the overall sensitivity of detection of any component about 100-fold. The use of narrower bore HPLC columns would allow the admission of a higher proportion of the total solvent flow to the mass spectrometer and could increase the sensitivity of detection. Likewise, the use of other LC/MS interfaces (21), including thermospray (22), that permit the transfer of the entire HPLC column effluent to the mass spectrometer should increase the sensitivity of detection of all solutes. It is not known, however, whether or not these interfaces also yield negative ions in the chloride-attachment mode. It must be recognized that simultaneous determination of all the lipid classes present in plasma does not allow optimization of the analytical parameters for any one of the lipid classes or molecular species. A generally acceptable working routine must be found if the method is to be practical. With such limited objectives, chloride attachment negative CIMS provides a much improved method for the identification and quantitation of molecular species of the neutral glycerolipids, and the neutral lipid moieties of glycerophospholipids and sphingomyelins present in plasma. A parallel LC/PCIMS run would allow the determination of all the major lipid components of plasma. In view of the limited resolution and extensive peak overlap, current methods of reverse phase HPLC require interfacing with mass spectrometry for reliable peak identification and quantitation. It cannot be substituted by such universal detectors as flame ionization (23) and light scattering (24).

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COMMUNICATIONS

Identification of Ergosta-6(7),8(14),25(27)-trien-3β-ol and Ergosta-5(6),7(8),25(27)-trien-3β-ol, Two New Steroidal Trienes Synthesized by *Prototheca wickerhamii*

Robert A. Norton and **W. David Nes***
Plant and Fungal Lipid Research, Microbial Products Research Unit, Richard B. Russell Research Center, Athens, Georgia 30613

Ergosta-6(7),8(14),25(27)-trien-3β-ol, ergosta-5(6),7(8),25(27)-trien-3β-ol, ergosterol, 25(27)-dehydroergosterol (prototheca-sterol), ergosta-7,25(27)-dien-3β-ol and ergost-7-en-3β-ol have been isolated from cells of *Prototheca wickerhamii*. The structures of the compounds have been determined in stereochemical detail by a series of chromatographic and spectral techniques. The results are interpreted to imply that whereas *P. wickerhamii* is non-photosynthetic and yeast-like in appearance and synthesizes ergosterol, the organism can be biochemically distinguished from yeast and considered an alga in part through the detection of other sterols described herein, notably those with $\Delta^{25(27)}$ side chains.
Lipids 26, 247-249 (1991).

Recent studies show the kinds and amounts of 24-alkylated sterols which plants produce may vary during phylogeny and development (1-3). In those plants where ontogenetic regulation of sterol biosynthesis occurs, specific 24-alkyl sterols may play multiple stage-specific roles *e.g.,* hormonal and membranous (4-6). We considered the possibility that some of the novel minor sterols recently identified in fungi (7-9) and vascular plants (1,5,10) may be acting hormonally rather than acting as intermediates in the pathway and may have phylogenetic significance. For this reason we now have extended our chemotaxonomic survey of phytosterols to a unicellular alga, *Prototheca wickerhamii.* The major sterols of actively proliferating cells of *P. wickerhamii* were previously identified as ergosterol and 25(27)-dehydroergosterol (11). In this study we characterized four additional *P. wickerhamii* sterols, one of which (ergosta-6(7),8(14),25(27)-trienol) occurred at hormonal levels in the cells and for biosynthetic reasons seemingly is not a precursor of ergosterol (12).

MATERIALS AND METHODS

Prototheca wickerhamii, strain NRPL Y-6870, was obtained from Dr. C. P. Kurtzman, USDA Northern Regional Research Center, Peoria, IL. *P. wickerhamii* is a non-photosynthetic alga which is yeast-like in culture (ref. 11, and refs. cited therein). The cultures were grown on a medium of 1% Bacto-yeast extract, 2% Bacto-peptone and 2% glucose. Cultures (300 mL medium in 1-L flasks) were inoculated with *ca.* 0.5×10^6 cells/mL and shaken continuously for 48 hr (growth arrest) to 100 hr at 180 rpm on a G10 gyratory shaker. At stationary phase, *ca.* 6.5×10^7 cells/mL, the cell yield per flask was *ca.* 3.9 g fresh weight of pelleted cells. Cells at different stages of stationary phase were pooled and the combined pellets saponified by refluxing in a solution of 10% KOH, 10% H_2O, 80% MeOH (w/v/v) for 2 hr. A non-saponifiable lipid fraction (NLF) was obtained by partitioning the cooled solution between diethyl ether and water. NLF was chromatographed on Silica gel G plates using benzene/diethyl ether (85:15, v/v) to separate sterols into classes based on the degree of C-4 substitution (13,14). Final purification of the minor compounds from bulk sterols and from one another was accomplished by high-performance liquid chromatography (HPLC) column chromatography. An initial separation was achieved by reversed-phase HPLC using an Alltech Econosil C_{18} 25-cm semi-preparative (10 μm) column (Alltech Associates, Deerfield, IL), which was eluted with 4% aq. MeOH at ambient temperature, followed by injection of select HPLC fractions into a 25-cm Whatman partisphere reversed phase C_{18} analytical (5 μm) column (Whatman, Ltd., Maidstone, England), which were eluted with 4% aq. MeOH at 40°C (cf., Results). The relative retention time (RRT_c) and α_c values are given relative to cholesterol. Gas-liquid chromatography (GLC) (packed 3% SE-30 column operated at 245°), gas chromatography (GC) (30-m capillary SE-30 like column, DB-1701-mass spectrometry (MS) and ^1H nuclear magnetic resonance (NMR) were performed as described (12-14).

RESULTS AND DISCUSSION

Cells from a series of growth experiments, where the cells were harvested at various times from the logarithmic and resting phases of growth, were combined and saponified, and the resulting NLF chromatographed by thin-layer chromatography (TLC). From the GLC profile of the 4,4-desmethyl sterol TLC band, we observed 3 peaks, none of which was fully separated from the others, at RRT 1.20, 1.35, 1.42 in a ratio of 63:27:10, respectively. When the sterol mixture was examined by GC-MS, the first peak was shown to possess two components corresponding in retention time and fragmentation pattern to ergosterol and prototheca-sterol (25(27)-dehydroergosterol) (11). The second peak also contained two components corresponding to an ergostadienol and an ergostatrienol isomer of ergosterol. The third peak contained one component and it was tentatively identified

*To whom correspondence should be addressed at Plant and Fungal Lipid Research, Microbial Products Research Unit, Richard B. Russell Research Center, P.O. Box 5677, 950 College Station Road, Athens, GA 30613.

Abbreviations: DAD, diode-array detector; GC, gas chromatography; GLC, gas-liquid chromatography; ^1H NMR, proton nuclear magnetic resonance spectroscopy; MS, mass spectroscopy; NLF, nonsaponifiable lipid fraction; RPHPLC, reversed-phase high pressure liquid chromatography; RRT, relative retention time; TLC, thin-layer chromatography; UV, ultraviolet.

as the monoene, ergost-7-enol. When the sterol mixture was examined by reversed-phase high pressure liquid chromatography (RPHPLC) using 4% aq. MeOH as the eluant and the column oven operated at 40°C, a series of peaks was evident at α_c 0.66, 0.74, 0.84 and 1.10. However, using the multiple wavelength diode-array detector (DAD) and the peak purity method (14) to monitor each of the four peaks, we determined that six rather than four sterols were present in the mixture. The early eluting peak was due to a single component with a chromophore for the $\Delta^{5,7}$-system. The second peak represented two compounds with the ultraviolet (UV) absorption characteristic of a $\Delta^{5,7}$-system and a $\Delta^{6,8(14)}$ system (12). The third peak also represented a mixture of two compounds, one of which possessed UV absorption for a $\Delta^{5,7}$-system, one possessed end absorption. The late (fourth) eluting peak at α_c 1.10 possessed an end absorption spectrum similar in shape to the end absorption spectrum observed for peak 3. The end absorption spectra for monoenic sterols are not sufficiently descriptive to differentiate between Δ^8- or Δ^7-systems, but the shape of the curves are different when compared to an end absorption spectrum for a Δ^5-sterol (8,14).

The sterols purified by TLC were further separated by preparative HPLC into four fractions. Each one of these fractions, referred to as fractions 1 to 4 in order of polarity beginning with the least polar, was examined by GC-MS. Consistent with our previous determination using the DAD, fractions 1 and 4 appeared to be uniform while fractions 2 and 3 consisted of mixtures of sterols. In order to resolve the mixtures, fractions 2 and 3 were injected into an analytical HPLC C_{18}-column where several parameters were changed from those used to assess α_c values. In the new separation system, the column was operated at 25°C and the eluant was changed to a higher percentage (6%) of water. These small changes have a profound influence on the resolving power of the column. As shown in Figure 1, the steroidal trienes which cochromatographed in the preliminary analysis of the sterol mixture at α_c 0.74 (fraction 2) were easily separated with base line resolution. In order to reduce the level of ergosta-5,7,25(27)-trienol, the front portion of peak 2 was shaved during the preparative scale-up of the fractionation and the early eluting fraction was then reinjected into the HPLC operating under the new conditions. The same protocol was used to separate the sterols in fraction 3. At that point, we had obtained six pure sterols which could be further examined by ^{1}H NMR (Fig. 2). The total amount of sterol in the cells was determined to be *ca.* 73 fg/cell, whereas the level of ergosta-6(7),8(14),25(27)-trienol was two orders of magnitude lower at 0.52 fg/cell.

Comparison of the chromatographic behavior, and of mass, UV and ^{1}H NMR spectra (Table 1) of the six sterols with literature data (12,15,16) indicated that compounds 1, 2 and 5 (cf. Fig. 1) were new sterols. The configuration at C-24 was established by comparing the spectra of the Δ^{22}- and $\Delta^{25(27)}$-sterols isolated from *P. wickerhamii* with those previously reported for ergosterol and 24-epiergosterol (17) and of codisterol and 24-epicodisterol (18,19). The chemical shift for the olefinic methyl signal at δ 1.64 in 5 (Fig. 1) and the C-21 doublet at δ 1.048 in 3 supports the 24β-epimer orientation. The isolation of a $\Delta^{6,8(14)}$-sterol is rather unusual. To our knowledge this is the first time a $\Delta^{6,8(14)}$-sterol has been

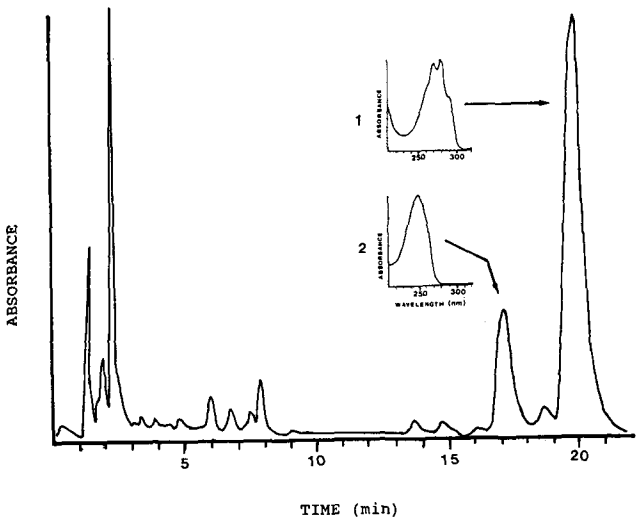


FIG. 1. HPLC chromatogram of a subfraction (peak 2 from preparative scale-up of sterol mixture, see text for details) containing two ergostatrienes. UV spectra were obtained on each compound by monitoring the eluant with the multiple wavelength diode array detector. HPLC conditions: solvent, 6% aq. methanol; pump speed, 1.5 mL/min; ambient temperature; Whatman C_{18}-column (5 μ); detector set at 205 nm.

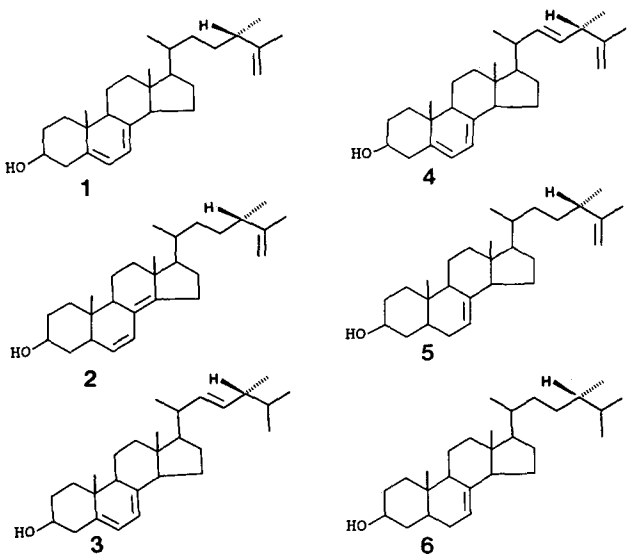


FIG. 2. Structures of sterols isolated from *Prototheca wickerhamii*.

isolated from algae and the second time it has been observed in nature, the other instance being the isolation from the fungus *Gibberella fujikuroi* which synthesizes ergosta-6,8(14),22-trienol (12). For mechanistic reasons, $\Delta^{6,8(14)}$-sterols are not assumed to be intermediates to $\Delta^{5,7}$-sterols (12). Alternatively, biosynthetic interrelationships of the other five sterols seem possible based on their structural similarities and on sterol transformation studies performed on related algae using sterols with similar functional groups as those now found to be synthesized by *P. wickerhamii* (20). Compound 2 (Fig. 2) was not detected in actively growing cultures of *Prototheca* (11, Norton, R.A., and Nes, W.D., unpublished data).

TABLE 1

Chromatographic, Spectral, and Quantitative Data for Sterols of *Prototheca wickerhamii*

Analysis[a]		Values for sterols					
		(1)	(2)	(3)	(4)	(5)	(6)
^{1}H NMR	H-18	0.615(*s*)	0.641(*s*)	0.632(*s*)	0.629(s)	0.527(*s*)	0.531(*s*)
	H-19	0.948(*s*)	0.885(*s*)	0.948(*s*)	0.945(*s*)	0.793(*s*)	0.794(*s*)
	H-21	0.938(*d*)	0.996(*d*)	1.038(*d*)	1.086(*d*)	0.916(*d*)	0.947(*d*)
	H-26	1.640(*s*)	1.650(*s*)	0.821(*d*)	1.680(*s*)	1.637(*s*)	0.643(*d*)
	H-27	4.665(*s*)	4.680(*s*)	0.844(*d*)	4.700(*s*)	4.660(*s*)	0.643(*d*)
	H-28	0.998(*d*)	0.946(*d*)	0.919(*d*)	1.039(*d*)	0.993(*d*)	0.855(*d*)
	H-22/23	—	—	5.235(*dd*)/ 5.167(*dd*)	5.235(*dd*)/ 5.167(*dd*)	—	—
EIMS	M^+	396, 86%	396, 68%	396, 88%	394, 82%	398, 23%	400, 100%
	M^+-Me	381, 8%	381, 34%	381, 0%	379, 0%	383, 27%	385, 31%
	M^+-H_2O	378, 16%	378, 0%	378, 7%	376, 0%	380, 0%	387, 6%
	M^+-Me-H_2O	363, 100%	363, 12%	363, 100%	361, 100%	365, 6%	367, 7%
	M^+-SC	271, 9%	271, 100%	271, 14%	269, 12%	271, 100%	273, 25%
	M^+-SC-H_2O	255, 3%	255, 8%	255, 0%	253, 34%	255, 27%	255, 71%
	M^+-SC-H_2O-2H	253, 30%	253, 48%	253, 24%	251, 0%	253, 7%	253, 2%
	GLC RRT	1.33	1.31	1.22	1.20	1.37	1.43
	TLC R_f	0.31	0.31	0.31	0.31	0.31	0.31
	HPLC α_c	0.74	0.72	0.82	0.66	0.84	1.10
	UV λ_{max}	282 nm	250 nm	282 nm	282 nm	E.A.	E.A.
As % total sterol		16	0.8	12	50	10	12

[a] ^{1}H NMR: Nicolet 200 MHz spectrometer operating at ambient temperature in the Fourier transform mode; solvent, $CDCL_3$; δ values are relative to Me_4Si. EIMS was obtained on an HP model 5970 table-top mass detector coupled to a GLC 30-m capillary column DB-1701 operated from 130°C to 260°C (10°C/min), then held at 260°C for another 10 min. SC = side chain and a dash indicates not present. GLC was obtained on an HP 5890 equipped for flame ionization detection, 3% SE-30 packed columns on gas chrom Q 100/120 mesh, 0.25-in. spiral glass columns; ionization detector temperature, 300°C; oven temperature, 245°C; flow rate of He (carrier) 30 mL min^{-1}. RRT = relative retention time. TLC was performed on Silica Gel G plates (250 μ, Analtech) with benzene/diethyl ether (85:15, v/v) as the developing solvent. HPLC, Whatman C_{18}-column (5 μ), 4.0 mm i.d. by 250 mm. Solvent, 4% aq MeOH; flow rate, 1.5 mL min^{-1}; Detector, 205 nm; temperature of oven, 40°C. UV, the samples were dissolved in 4% aq MeOH and the spectra obtained from the diode-array detector (UV/vis diode-array detector 1040A Hewlett-Packard) after passage thru the C_{18}-column.

Therefore, it is unlikely to be an artifact of the isolation methods.

The cyclopropyl steroid precursor which gave rise to the $\Delta^{25(27)}$-double bond in *P. wickerhamii* was earlier shown by us to be cyclolaudenol (11). *Chlorella*, its photosynthetic alga progenitor (11), also synthesized cyclolaudenol and ergosterol (20). *Chlorella*, however, reduces the side chain at an early step in the conversion of cyclolaudenol to ergosterol (20), whereas in *Prototheca* reduction of the $\Delta^{25(27)}$-double bond occurs at a late step, or not at all, *e.g.*, as in 2. Presumably, that is why prototheca-sterol and related 4,4-desmethyl $\Delta^{25(27)}$-sterols were not detected in *Chlorella*. Studies in progress with transition state sterol biosynthesis inhibitors incubated with *P. wickerhamii* at several stages of the algal life history should resolve whether 2 has functional importance.

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The Solubilization of Calcium Soaps by Fatty Acids

R.J. Jandacek
The Procter & Gamble Company, Miami Valley Laboratories, Cincinnati, Ohio 45239-8707

The solubilization of the calcium soaps of long chain fatty acids by liquid fatty acids was observed. The solubilities of calcium palmitate, calcium laurate, and calcium oleate were 15.6, 22.8, and 53.3 wt%, respectively, in oleic acid at 40°C. The formation of an acid-calcium soap complex was demonstrated by x-ray diffraction studies of calcium laurate, lauric acid, and a mixture of these compounds that had been heated. Similar evidence was obtained for a calcium oleate-oleic acid complex. The solubility of calcium oleate in a bile salt micellar system was enhanced by oleic acid. The solubilization of calcium soaps by liquid fatty acids may explain the unexpectedly high bioavailability of some calcium soaps.
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Dietary calcium can increase the excretion of ingested fat, presumably by the formation of insoluble calcium soaps that are unavailable for absorption from the intestine (1). Calcium primarily affects the absorption of long chain, saturated fatty acids, and has little effect on medium chain or unsaturated fatty acids (1).

It is generally assumed that the insolubility of calcium soaps in aqueous systems is the cause of their minimal absorption. This assumption is consistent with the low solubilities of calcium palmitate and calcium stearate in water (3-4 mg/100 mL) (2). We cannot, however, ignore the low solubility of the calcium soaps of lauric acid (4 mg/100 mL) and oleic acid (40 mg/100 mL) (2). In spite of the low solubility of the shorter chain and unsaturated fatty acid calcium soaps, dietary calcium has virtually no effect on the absorption of these fatty acids (1).

In addition to the observed effect of dietary calcium on fatty acid absorption, there is the possibility that dietary fatty acids interfere with dietary calcium absorption. A typical adult human ingests more than 100 g of triglyceride per day, and approximately two-thirds of that mass is converted to ionized fatty acids in the small intestine. This amount is in excess of that required to precipitate the 600-800 mg of ingested calcium as insoluble, nonabsorbable calcium soaps. Although most dietary calcium is typically not absorbed (3), one might predict that the formation of calcium soaps would prevent the absorption of calcium.

Two questions then arise from consideration of the physical properties and the biological behavior of calcium, fatty acids, and calcium soaps: (i) why are the unsaturated fatty acids not precipitated by calcium in the intestine? and (ii) why don't fatty acids precipitate all dietary calcium and prevent its absorption?

The milieu of the intestinal lumen contains many substances that might alter the chemical activity of calcium and fatty acid ions (*e.g.*, bile acids, chelators, sugars, bicarbonate) and thus hinder the formation of calcium soap precipitates. We found one class of compounds that does indeed markedly alter the solubility of calcium soaps—the fatty acids themselves. We report here our observations on the solubilization of calcium soaps by fatty acids and discuss possible implications in intestinal events related to calcium and fat absorption.

Abbreviations: AA, atomic absorption; GC, gas chromatography.

MATERIALS AND METHODS

Materials. Oleic acid (Sigma Chemical Co., St. Louis, MO) was shown by gas chromatographic (GC) analysis of its methyl ester to have a purity of >99%. Calcium oleate was prepared by dissolution of 0.02 moles oleic acid in 50 mL absolute ethanol, followed by the addition of 20 mL of 0.1N aqueous NaOH. This solution was added to a solution of 0.01 moles $CaCl_2.2H_2O$ in 50 mL absolute ethanol. The resulting milky suspension was centrifuged and the pellet was washed by repeated suspension in distilled water, centrifugation, and discarding of the supernatant. The sample was then frozen and lyophilized. Calcium palmitate and calcium laurate were obtained from Pfaltz and Bauer (Waterbury, CT). Triolein, lauric acid, and octanoic acid (all 99%) and sodium glycodeoxycholate were purchased from Sigma. A sample of a mixture of calcium stearate and calcium palmitate was purchased from Baxter Scientific Products (McGraw, IL).

The compositional analysis of the soaps was determined after dissociating the soap into aqueous and organic phases. Approximately 100 mg of the soap was dispersed into 80 mL of 1:1 (v/v) ethanol and water that was adjusted to pH of 2.0 with HCl. This suspension was extracted in a separatory funnel 3 times with 100 mL of hexane. The calcium in the aqueous phase was assayed by atomic absorption spectroscopy (AA). The results were: calcium laurate, 9.1% wt found (9.1% theory); calcium palmitate, 6.7% wt found (7.3% theory); calcium oleate, 6.7% wt (6.7% theory); and calcium stearate-palmitate mixture, 7.2% wt (7.1% theory). The fatty acids in the hexane phase were measured by GC analyses of the methyl esters, and the following fatty acid compositions resulted: Calcium oleate, 99.8% oleic acid; calcium laurate, 95% lauric, 4% decanoic acid; calcium palmitate, 92% palmitic, 6% stearic, 2% myristic acid; calcium stearate mixture, 67% stearic, 26% palmitic, 2% myristic, 2% heptadecanoic, 1% oleic, 1% arachidic acid.

Solubility measurements. The solubility of calcium soaps was determined by suspending an excess of the soap in the organic liquid in a capped vial and mixing on a rotary mixer for 48 hr in a room held at 39-40°C. After mixing, the samples were centrifuged at this temperature to precipitate the soap crystals. Examination of the supernatant by polarized light microscopy confirmed the absence of soap crystals in the liquid. The supernatant was then partitioned between the acidic aqueous ethanol and the hexane phases described above. The aqueous phase was analyzed for calcium by AA, and that value was converted to mass of calcium soap in the organic phase in question. The fatty acid composition of the hexane phase was also measured in the laurate, palmitate, and stearate-palmitate soaps. This method was validated by the measurement of calcium in systems in which known amounts of calcium soap were dissolved. Duplicate or triplicate determinations were used except for the

TABLE 1

The Solubility of Calcium Soaps in Organic Phases at 39–40°C Based on Recovery of Calcium

	Solubility (wt %; mean ± SD)			
	Ca laurate	Ca palmitate	Ca oleate	Ca stearate-palmitate mixture
Oleic acid	22.8 ± 1.6 (23.5 ± 0.6)[a]	15.6 ± 0.9 (14.7 ± 0.3	53.3 ± 1.6	18.3 ± 0.1 (16.6 ± 0.2)
Oleic acid/triolein (75:25)	18.1 ± 0.3 (17.1 ± 0.8)	10.4 ± 0.6 (10.1 ± 0.2)	49.5 ± 0.7	14.3 ± 1.0 (12.4 ± 0.2)
Oleic acid/triolein (50:50)	11.3 ± 1.0 (10.3 ± 0.4)	5.8 ± 0.4 (5.9 ± 0.1)	30.6 ± 0.5	8.9 ± 0.1 (7.4 ± 0.1)
Oleic acid/triolein	5.0 ± 0.0 (4.2 ± 0.2)	2.3 ± 1.4 (2.6 ± 0.1)	17.0 ± 0.1	4.1 ± 0.2 (2.8 ± 0.6)
Triolein	0.6 ± 0.1 (0.4 ± 0.2)	0.2 ± 0.1 (0.4 ± 0.3)	0.3 ± 0.1	0.1 ± 0.2 (0.8 ± 0.3)
Heptane	0.5			
Heptane/octanoic acid (50:50)	13.0			

[a]Solubility based on fatty acid recovery in parentheses.

heptane-octanoic acid system on which a single measurement was made.

Calcium oleate solubility in an aqueous bile salt solution was measured by mixing an excess of calcium oleate with water or solutions of 0.01 M sodium glycodeoxycholate with or without oleic acid (3.6 mM). Mixing conditions and centrifugation were the same as described above except that the supernatant was filtered with a 0.45 micron filter and then analyzed by AA for calcium.

X-ray diffraction studies. The complexes of fatty acids and calcium soaps were prepared by raising the temperature of the mixtures to 150°C to form a translucent phase. The samples were then cooled and powder diffraction patterns obtained with a diffractometer using CuKα radiation at 25°C. This procedure has been previously shown to form fatty acid-potassium soap complexes (4). Resolution of the peaks allowed differences of 0.3 Å to be determined when second and third orders were used. The first order peaks allowed differences of 1.5 Å to be resolved.

RESULTS

The solubilities of calcium soaps in triolein, heptane, oleic acid, and octanoic acid with heptane (50/50 wt %) are given in Table 1. Measurements based on calcium and on the oil's fatty acid composition are presented. The solubility of all soaps in triolein was less than 1%. There was, however, a high level of miscibility of the soaps in oleic acid, with a maximum of 53% for calcium oleate in oleic acid. Both oleic and octanoic acids, liquids that are miscible with nonpolar phases such as heptane and triolein, solubilized calcium soaps in the nonpolar phase. The determination of solubility based on calcium was in agreement with that found by measurement of the fatty acid composition of the oil phase.

All calcium soaps were solubilized by oleic acid, and the solubility followed a trend related to the melting points of the fatty acids. Calcium oleate and laurate were thus more soluble than the soaps of the higher melting palmitic and stearic acids.

The fatty acid compositions of the oil phase showed that there was no preferential dissolution of the impurities of the calcium laurate or calcium palmitate in the oil. Lauric acid accounted for 95.0% of the fatty acids (other than oleic acid) in the oil phase of the calcium laurate soap solutions, unchanged from the added calcium laurate solute. Similarly palmitic acid was 90.3–91.4% of the fatty acids (other than oleic acid), compared with a value of 92% for the calcium palmitate solute.

The relatively high solubility of the mixed calcium stearate-palmitate soaps is consistent with the reduced melting point of the mixture. In addition the fatty acid composition of the oil phase from the soap mixture showed preferential solubility of the shorter chain, lower melting acids. The ratio of stearic/palmitic acids in the undissolved soap was 2.6. In the soap dissolved in the oil phase, however, this ratio decreased by 30% to 1.8, demonstrating a higher solubility of the palmitate compared with the stearate. Similarly the ratio of stearic/myristic acids decreased by 50% from 33.5 in the undissolved soap to 16.2 in the dissolved soap.

To determine whether or not a fatty acid would enhance the solubilization of a calcium soap by a micellar bile salt solution, we measured the solubility of calcium oleate in a solution of 0.1 M sodium glycodeoxycholate with or without 3.6 mM oleic acid present. The presence of oleic acid increased the calcium oleate concentration from 138 ± 5 to 309 ± 33 μg/mL (n=3, mean ± SEM).

We investigated the possibility of acid soap formation in the calcium laurate-lauric acid and the calcium oleate-oleic acid systems. The powder x-ray diffraction patterns showed evidence of the formation of an acid soap, and these are displayed in Figure 1. As shown in Table 2, the long spacing from lauric acid (27.6 Å) is distinct from that of calcium laurate (34.0 Å). Both values are consistent with those previously reported (27.6 Å, 34.8 Å,

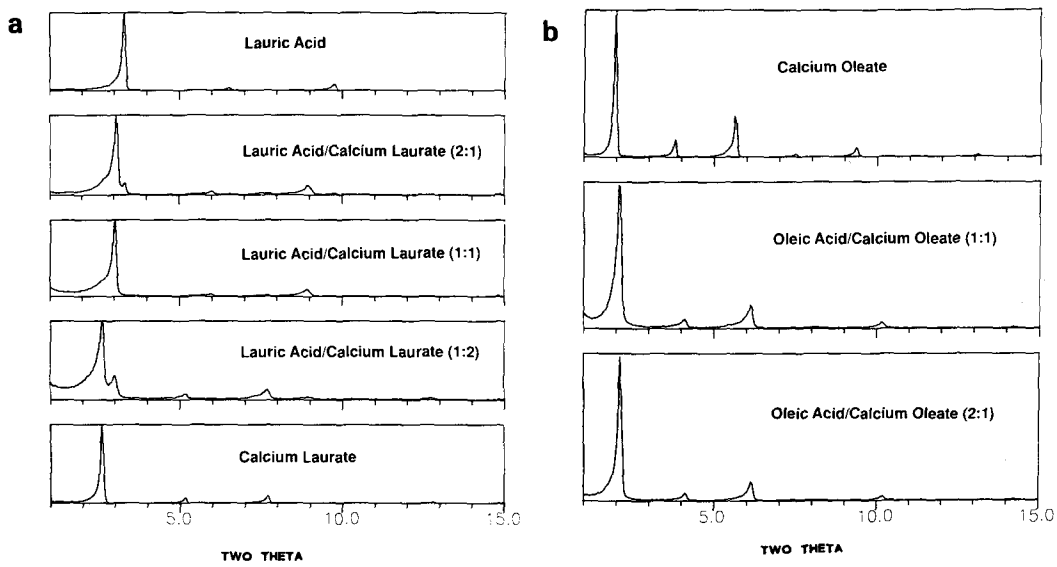


FIG. 1. (a) The x-ray diffraction pattern from lauric acid, calcium laurate, and varying molar ratios of the acid and soap that had been melted and cooled to 25°C. The 1:1 mixture displays a long spacing of 29.4 Å, distinct from the long spacing of 34.0 Å for calcium laurate and 27.6 Å for lauric acid, indicating a complex of the acid and soap. The pattern of the 1:2 mixture includes the spacings of the soap and the complex, while that of the 1:2 mixture includes those of the acid and the complex. (b) The x-ray diffraction pattern of calcium oleate and mixtures of 2:1 and 1:1 molar ratios of oleic acid and calcium oleate that had been melted and cooled to 25°C. The long spacing of both mixtures (42.1 Å) is distinct from that of calcium oleate (44.2 Å), suggesting the formation of a complex of the acid and soap of indeterminate stoichiometry.

TABLE 2

Diffraction Spacings (Å) from Calcium Soaps, Fatty Acids, and Mixtures After Melting and Cooling to Room Temperature

Calcium laurate	Lauric acid	Calcium laurate/lauric acid molar ratio			Calcium oleate	Calcium oleate/oleic acid molar ratio	
		2:1	1:1	1:2		2:1	1:2
34.0				34.0	44.2		
		29.4	29.4	29.4		42.1	42.1
	27.6	27.6			23.3		
17.3				17.3		21.6	21.6
		14.7	14.7		15.5		
	13.6					14.4	14.4
11.5		11.8		11.5		8.71	8.71
		9.9	9.9	9.9			6.22
	9.1						
8.7							

respectively) (5). When mixtures of lauric acid and calcium laurate were heated to 150°C and then cooled, a long spacing appeared at 29.4 A, and those at 27.6 A and 34.0 A disappeared. Similarly the long spacing of calcium oleate shifted from 44.2 Å to 42.1 Å when it was heated with oleic acid.

DISCUSSION

The data presented are evidence for the solubilization of calcium soaps by low-melting fatty acids. This solubilization has not been reported previously and was observed both in an oil phase and in a bile salt micellar phase. The solubility of the soaps in oleic acid decreased with increasing melting point. In addition, the solubilities of oleate and laurate soaps in oleic acid suggest that this solubilization might prevent the precipitation of calcium oleate and calcium laurate in the intestine.

The solubilization effect was observed both for oleic acid and for octanoic acid and occurred in the presence of a liquid triglyceride or an organic solvent, heptane. Presumably, calcium soaps can dissolve in many liquid fatty acids or their solutions. Dietary linoleic acid, oleic acid, α-linolenic acid, octanoic acid, decanoic acid, and lauric acid would be expected to solubilize calcium soaps in the intestine.

The normal digestion process for dietary fat takes place at the surface of emulsified triglyceride droplets with lipase splitting interfacial triglycerides into

monoglyceride and free fatty acid. This interfacial region includes a high concentration of free fatty acids that may hinder the precipitation of calcium soaps. In addition, bile salt mixed micelles that include free fatty acids can partially solubilize calcium soaps. The inhibition of calcium soap precipitation and the solubilization of the soaps by low melting fatty acids may therefore enhance the pool of calcium and fat in the intestinal lumen that is available for absorption.

The x-ray data provide evidence for the previously unreported fatty acid-calcium soap complex formation. The melting method used to form the complex has been shown to form fatty acid-potassium soap complexes (4). The distinct diffraction lines for the acid-calcium soap mixture are evidence of a crystalline array that is distinct from either the calcium soap or the fatty acid. The multiple orders of diffraction d-spacing ratios of 1:1/2:1/3 correspond to a fatty acid bilayer structure. The dimension of the bilayer of the complex is distinctly different from those of the calcium soap and the fatty acid. The data suggest a complex of 1:1 molar ratio of lauric acid to calcium laurate since peaks corresponding to uncomplexed lauric acid appeared in the 2:1 mixture and peaks corresponding to uncomplexed calcium laurate, in the 1:2 mixture. The stoichiometry of the oleic acid calcium oleate complex is not indicated by the data given here since the 1:1 and 2:1 mixtures gave similar diffraction patterns. The pattern from the 2:1 mixture may, however, be explained by a 1:1 complex and an excess of amorphous liquid oleic acid.

The demonstration of the existence of fatty acid complexes with calcium soaps is consistent with the insertion of the acid into the soap lattice with the carboxyl group attracted to the calcium region in the crystal. Presumably the carboxyl groups of liquid fatty acids similarly approach the calcium atoms in the dissolution of calcium soaps. Disruption of the crystalline array of the fatty acid chains and formation of a nonpolar environment of fatty acids surrounding the calcium facilitate the dissolution of calcium soaps by fatty acids.

The solubility and x-ray diffraction data presented here describe two previously unreported interactions between fatty acids and calcium soaps—a high level of solubility of these soaps in fatty acids, and the formation of an acid soap complex. This evidence for calcium soap compatibility with fatty acids can explain, in part, the efficient absorption of fatty acids from a milieu that would apparently favor the formation of insoluble, nonabsorbable calcium soaps.

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Identification of Diacylglycero-4′-*O*-(*N,N,N*-trimethyl)homoserine in Mushrooms

Victor E. Vaskovsky[a], Svetlana V. Khotimchenko[a] and Andrew A. Benson[b,*]

[a]Institute of Marine Biology, Far East Branch, Academy of Sciences of the USSR, Vladivostok 690032 USSR and [b]Scripps Institution of Oceanography, La Jolla, California 92093

An unusual polar lipid component was found in some mushrooms and was isolated from *Boletus edulis*. It has been identified as diacylglycero-4′-*O*-(*N,N,N*-trimethyl)-homoserine (DGTS) by its thin-layer chromatography (TLC) mobilities and staining behavior as well as by means of infrared (IR) and nuclear magnetic resonance (^{1}H NMR) spectroscopy and mass spectrometry. Identification of this lipid in mushrooms indicated that DGTS is widely distributed among a diverse group of lower plants and may have chemotaxonomic significance for fungi. It is interesting that DGTS is a component of some edible mushrooms commonly consumed in food.
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During investigation of the lipid composition of lower plants, an unusual polar lipid in mushrooms attracted our attention. It was not any of the well-known plant polar lipids, yet had the same chromatographic characteristics as diacylglycero-4′-*O*-(*N,N,N*-trimethyl)homoserine (DGTS), which we had identified in the green alga *Ulva fenestrata* (1). DGTS, Figure 1, was first discovered in the golden brown alga, *Ochromonas danica* (2), and subsequently in micro- and macroalgae (1,3-9), lower plants (10-13) and soil amoebae (14). Nothing was known of the presence of this lipid in mushrooms (15-19). We report here identification of this "homoserine" lipid in the mushroom *Boletus edulis*, for example, by means of thin-layer chromatography (TLC) characteristics, infrared (IR) nuclear magnetic resonance (^{1}H NMR) and mass spectrometry.

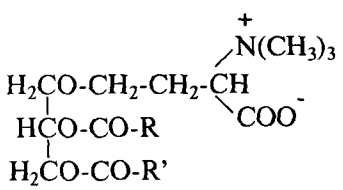


FIG. 1. Structure of DGTS (diacylglyceryltrimethylhomoserine ether).

MATERIALS AND METHODS

Materials. Mushrooms were harvested in summer and autumn in the maritime region near Vladivostok and Nakhodka. They were cleaned, cut into small pieces and heated in boiling water 2-3 min for inactivation of phospholipase D. Lipids were extracted using a modified Bligh and Dyer method (20) as described earlier (21).

Separation and detection. Lipid extracts were analyzed by two-dimensional TLC on silica gel plates (22) using solvent systems for separation of plant polar lipids (21) and phospholipids (23). Lipids were detected using the following spray reagents: 10% H_2SO_4 in methanol followed by charring for detection of all lipids, Dragendorff reagent for choline lipids (24), 0.25% ninhydrin in acetone for amino-containing lipids, anthrone reagent for glycolipids (25), molybdate reagent for phospholipids (26) and malachite-green reagent for phosphorus-containing lipids (27).

Identification. Total lipids (1.1 g) were extracted by the above procedure from 130 g *B. edulis*. The lipid X was isolated from the total lipids by column chromatography on silica gel. The column was washed with chloroform and lipid X was eluted with chloroform/methanol (4:1, v/v). It was purified by preparative TLC in a solvent system of chloroform/acetone/methanol/formic acid/water (100:40:20:20:8, by vol). Yield of the purified lipid was 90 mg.

Fatty acid methyl esters were obtained (28) and subjected to gas-liquid chromatography on a Shimadzu GC-9A instrument (Shimadzu Corp., Kyoto, Japan) equipped with flame ionization detector. A glass column (25 m × 0.25 mm) with Silar-5CP (SERVA, Heidelberg, Germany) was used. The column temperature was 190°; the flow rate of the carrier gas (helium) was 40 mL/min. Fatty acids were identified by comparison of the retention times with those of authentic standards and by equivalent chain length (29).

The infrared spectrum was recorded using a Specord 75 IR spectrometer (Jenoptik, Jena, Germany) with a sample prepared in chloroform. The NMR spectrum was obtained on a Bruker WM-250 (Bruker Instruments, Karlsruhe, Germany) spectrometer ($CDCl_3$ solution). Proton chemical shifts are given in ppm downfield from internal tetramethylsilane. Mass spectra of the lipid were obtained by the direct inlet mode using an LKB 9000 (LKB Produkter, Bromma, Sweden) instrument. The source temperature was 150° and the ionization voltage was 7 eV.

RESULTS

TLC demonstrated that all mushrooms contained well-known polar lipids (15-19). Phosphatidylcholine, phosphatidylethanolamine and phosphatidylserine were present as main components and phosphatidylinositol, phosphatidic acid and glycolipids as minor components (Fig. 2). Sugai *et al.* (19) reported pyrophosphatidic acid in some mushrooms, but we did not detect this lipid in any mushroom species investigated, even though we used high-performance thin-layer chromatography (HPTLC) with several solvent systems and a sensitive reagent for detection of phosphorus-containing substances (27). Besides the usual lipids, several unidentified mushrooms contained the lipid X as one of their major lipid components (see Fig. 2).

*To whom correspondence should be addressed.

Abbreviations: DGTS, diacylglycero-4′-*O*-(*N,N,N*-trimethyl)homoserine; TLC, thin-layer chromatography; HPTLC, high-performance thin-layer chromatography; IR, infrared; NMR, nuclear magnetic resonance.

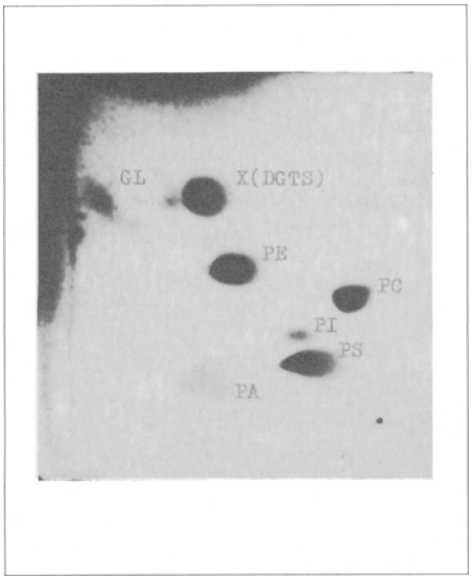


FIG. 2. Two-dimensional thin-layer chromatogram of polar lipids from a mushroom, *Boletus edulis*, containing DGTS. Solvent systems: first direction (vertical), chloroform/methanol/benzene/28% NH_4OH (65:30:10:6, by vol), second direction, chloroform/methanol/benzene/acetone/acetic acid/water (70:30:10:5:4:1, by vol). Visualization was by charring after spraying with 10% H_2SO_4 in methanol. Abbreviations: DGTS, diacylglycerotrimethylhomoserine; GL, glycolipids; PA, phosphatidic acid; PC, phosphatidylcholine; PE, phosphatidylethanolamine; PI, phosphatidylinositol; PS, phosphatidylserine; O, origin.

The lipid X had a higher R_f than phosphatidylethanolamine. It was found that the lipid gave a positive reaction with Dragendorff reagent and was not stained with the anthrone reagent (25). With the molybdate reagent (26) it was stained as phospholipid, yet it did not give a positive reaction with the more specific malachite-green reagent for phosphorus-containing substances (27). Thus, the lipid was neither a phospholipid, a glycolipid nor an amino-lipid, but contained a quaternary ammonium group. Mobility on two-dimensional TLC and the color reactions with specific spray reagents pointed to the similarity of the lipid X to the DGTS in other organisms (1,3,5,7).

To identify the lipid X it was isolated from *Boletus edulis* by column and thin-layer chromatography and subjected to spectral analysis. The infrared spectrum of lipid X showed absorption bands at 3384cm^{-1} (OH, bonded), 3000cm^{-1} (HC = CH), 2940, 2870, 1370cm^{-1} (CH_2, CH_3), 1736cm^{-1} (C = O, ester), 1632cm^{-1} (COO-), 1470cm^{-1} (CH_2), 1174cm^{-1} (C-O-C, ester), and 970cm^{-1} $N(CH_3)_3$. This spectrum was identical to that reported for the DGTS in *Ulva fenestrata* (1) and in other organisms (3,5,7).

The proton NMR spectrum of lipid X showed signals at δ 0.88 ppm ($C\underline{H}_3$), 0.97 ppm (*t*, terminal $C\underline{H}_3$ of fatty acids), 1.30 ppm (*m*, $C\underline{H}_2$), 1.62 ppm (*m*, -$C\underline{H}_2$-CH_2-COO), 2.05 ppm (*m*, allylic $C\underline{H}_2$), 2.30 ppm (*m*, -$\underline{H}_2$C-C=O), 2.78 ppm (*m*, $C\underline{H}_2$ between two double bonds), 3.67 ppm (*m*, -$\underline{H}_2$C-O-$C\underline{H}_2$ and -O-CH_2-CH_2-$C\underline{H}$(N-)-COO^-), 4.16 ppm and 4.32 ppm (*m*, $C\underline{H}_2$, glycerol), 5.20 ppm (*m*, $C\underline{H}$, glycerol) and 5.35 ppm (*t*, $\underline{H}C = C\underline{H}$). A prominent singlet signal at δ 3.30 ppm indicated the presence of a $N^+(CH_3)_3$ group in the lipid. The NMR spectrum of the lipid X was virtually identical to that of DGTS from *Ulva fenestrata* (1). The observed chemical shifts for lipid X were compatible with those of DGTS from other organisms (5,10,30).

TABLE 1

Fatty Acid Composition of Total Lipids from *Boletus edulis* and DGTS (% of total fatty acids)

	16:0	16:1	18:0	18:1	18:2n-6	Other
Total lipids	9.2	0.5	0.3	41.9	46.3	1.8
DGTS	34.2	0.9	1.5	10.6	47.9	4.9

The mass spectrum of the lipid X showed ions corresponding to the polar group *N,N,N*-trimethylhomoserine (*m/z* 162), its dehydrated product (*m/z* 144), its dehydrated and demethylated product (*m/z* 130), its decarboxylated product (*m/z* 118), its decarboxylated, dehydrated and demethylated product (*m/z* 86) and finally to $(CH_3)_2$-N-CH_2^+ (*m/z* 58). The mass spectrum of lipid X also showed peaks corresponding to molecular ions at *m/z* 736 (a major peak), 759, 738, 734 and peaks at *m/z* 677, 700, 678, 675 $[M-59]^+$. It suggested that the lipid X was a mixture of several molecular species with fatty acids differing in their chain length and their degree of unsaturation. Thus, our results demonstrated that the lipid X is diacylglycero-4′-*O*-(*N,N,N*-trimethyl)homoserine.

Table 1 shows the composition of fatty acids of total lipids and DGTS from *B. edulis*. Major fatty acids in both the lipid extract and in DGTS were palmitic, oleic and linoleic acids. But in DGTS the 16:0 acid was present in much higher and the 18:1n-9 acid in much lower proportions than in the total lipids. On the basis of mass-spectral data revealing the molecular ion $[M+1]^+$ *m/z* 736 indicative for the 16:0-18:2 species and fatty acid analysis we propose that the major molecular species is palmitoyl,linoleoyl-glycero-4′-*O*-(*N,N,N*-trimethyl)-homoserine.

DISCUSSION

DGTS may play an important role in membrane biogenesis in plants and could be a substrate for desaturation of 18:1 and 18:2 fatty acids in the green alga *Chlamydomonas reinhardtii* (31). It was suggested that DGTS could be involved in biosynthesis of γ-18:3 acid (3) or in formation of the 18:4n-3 acid (1). The role of DGTS in mushroom membrane development remains to be clarified.

Although a large group of plant species was examined for DGTS (11-13), the mushrooms were not investigated. When Sugai *et al.* (19) described the occurrence of pyrophosphatidic acid in mushrooms, it is quite possible that they also observed DGTS in their lipid extracts as the TLC shown in Figure 1 of their paper revealed one major unidentified polar lipid. Previously, DGTS was found in a human pathogenic fungus, *Epidermophyton floccosum* (class Ascomycetes) (30). *Boletus edulis* and the majority of other edible mushrooms belong to Basidiomycetes. The results obtained in this paper are of chemotoxonomic and practical interest. Published data

(11-13) and our results (32) on DGTS distribution among plants showed that DGTS is absent in all investigated species of one plant taxon (Rhodophyta, Phaeophyta, Angiosperms, Gymnosperms) and present in all species of another (Pteridophyta, Bryophyta, Chlorophyta). Mushrooms were found to be an exception: all analyzed species of Boletales contained DGTS and the lipid was not found in representatives of the Russulales.

With the exception of several green algae used as food in Asia, DGTS had been found only in inedible organisms. Mushrooms from the family Boletaceae, however, are widely used as foodstuffs. In preliminary experiments we have found DGTS in fried and salted mushrooms. Thus, man may consume with a mushroom dish tens of milligrams of DGTS. Therefore, it is of interest to study the taste, metabolism, and biological activities of DGTS and its deacylated derivative.

ACKNOWLEDGMENTS

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Platelet-Activating Factor Stimulates Expression of IL-1 beta mRNA in THP-1 Cells

Roger A. Barthelson* and **Frank H. Valone**
Department of Medicine, University of California San Francisco, and the Veterans Administration Medical Center, San Francisco, California 94121-1598

A human monocytic cell line (THP-1) was used to study the effects of PAF (platelet-activating factor) on the expression of IL-1 beta mRNA. THP-1 cells were incubated with 10 pM PAF in the presence or absence of 0.1 μg/mL endotoxin for 4 hr, after which cytoplasmic RNA was extracted and subjected to Northern hybridizations. PAF, alone and in combination with endotoxin, caused an increase in mRNA levels for IL-1 beta. The magnitude of the effects of PAF on IL-1 beta mRNA levels matched closely the effects seen at the level of protein synthesis, suggesting that the effects of PAF on IL-1 beta release may result largely from its effects on IL-1 beta mRNA levels.
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Platelet-activating factor (PAF) is a potent lipid mediator of allergic reactions and other inflammatory conditions (1–3). PAF administered *in vivo* causes both short and long-term effects that may resemble asthma, anaphylaxis, acute serum sickness, or endotoxin shock (4–7). The long-term effects probably result from secondary mediators produced by immune cells in response to PAF, because PAF itself has a half-life of less than five min *in vivo* (2). These secondary mediators may include TNF alpha and IL-1 beta (8–13).

PAF stimulates the release of IL-1 beta and TNF alpha by human monocytes and macrophages, which are a major source of these cytokines (8–11). PAF alone has a relatively small effect on the release of IL-1 beta by monocytes, but in the presence of a co-stimulus, such as gamma-interferon, PAF has a substanital effect (8). Similarly, endotoxin is more potent than PAF for stimulating IL-1 beta release by THP-1 cells, and when PAF and endotoxin are combined, the two stimuli interact synergistically to yield even higher levels of IL-1 beta release (14). Studies with THP-1 cells demonstrate that PAF increases IL-1 beta release by increasing IL-1 beta protein synthesis and that the combination of endotoxin and PAF increases IL-1 synthesis synergistically (14). Increased IL-1 beta protein synthesis can result from increased levels of IL-1 beta mRNA or increased translation of pre-existing mRNA. This communication demonstrates that PAF increases levels of IL-1 beta mRNA.

*To whom correspondence should be addressed at the VA Medical Center, 111H, 4150 Clement Street, San Francisco, CA 94121.

Abbreviations: GAPDH, glyceraldehyde-3-phosphate dehydrogenase; LPS, lipopolysaccharide from E. coli, endotoxin; PAF, platelet-activating factor, 1-0-hexadecyl-2-acetyl-*sn*-glycero-3-phosphocholine; SSC, 0.15 M sodium chloride, 0.015 M sodium citrate buffer, pH 7.0.

MATERIALS AND METHODS

PAF (1-*O*-hexadecyl-2-acetyl-*sn*-glycero-3-phosphocholine) was obtained from Bachem (Torrance, CA). cDNA to IL-1 beta inserted in a pSP64 plasmid at the PST I site was a gift from the Genetics Institute (Cambridge, MA). The cDNA to the glyceraldehyde-3-phosphate dehydrogenase gene was a gift from Dr. Alfredo Lopez (VA Medical Center, San Francisco, CA). The Multiprime random primer extension kit and ^{125}I-streptavidin were purchased from Amersham (Arlington Heights, IL). [^{32}P]dCTP (3000 Ci/mmole) was obtained from NEN/DuPont (Wilmington, DE). A Transvac TE80 transfer apparatus, Nylon 66 Plus blotting membrane, and Seakem GTG agarose were from Hoeffer Scientific (San Francisco, CA). Nitrocellulose membrane was purchased from Schleicher and Schuell (Keene, NH). Restriction endonucleases and ribonuclease-free deoxyribonuclease I were purchased from Promega (Madison, WI). Vanadyl ribonucleosides and proteinase K were from Bethesda Research Laboratories (Bethesda, MD). Fetal bovine serum was from Hyclone (Logan, UT). Bovine serum albumin (BSA) was certified low in endotoxin (<0.5 ng/mg) and came from Boehringer-Mannheim (Indianapolis, IN). LPS (endotoxin from Escherichia coli, serotype 0127:B8) was obtained from Sigma Chemical Co. (St. Louis, MO). Anti-IL-1 beta antibodies were from Endogen (Boston, MA). Biotinylated goat anti-rabbit antibodies were from Zymed (South San Francisco, CA). All other reagents were of the highest purity available and certified for use for molecular biology when possible.

Incubation of THP-1 cells. THP-1 cells were cultured and incubated as described (14). The cells were washed, suspended at 1 × 10^6/mL in RPMI 1640 containing 50 μM 2-mercaptoethanol, and 0.15% BSA, and then were incubated with control buffer, 10 pM PAF, 0.1 μg/mL LPS, and LPS + PAF. These concentrations of stimuli stimulate IL-1 beta synthesis optimally (14). After 30 min, fetal bovine serum was added to achieve a final concentration of 10% and the incubation was continued for 4 hr.

Western dot blots. THP-1 cells were centrifuged, resuspended at 2 × 10^6/mL in PBS, submitted to three cycles of freezing and thawing, and centrifuged again at 9000 × *g*. The resulting supernatants were applied directly to a nitrocellulose membrane (100 μL or approximately 2 × 10^5 cell equivalents) with a BioRad dot blot vacuum apparatus. The resulting blot was incubated with 5% non-fat milk as a blocking agent, rabbit anti-IL-1 beta antibodies, biotinylated goat anti-rabbit antibodies, and finally [^{125}I]streptavidin (20–40 μCi/μg) (14). All incubations contained PBS with 5% non-fat milk. The blot was rinsed in PBS, covered in plastic wrap, and exposed to x-ray film overnight with intensifying screens (14).

Preparation of RNA. Cytoplasmic RNA was pre-

pared by the NP-40 lysis method (15). Briefly, a cytoplasmic extract from 3×10^6 cells was digested with proteinase K, extracted with phenol/chloroform, and then incubated with deoxyribonuclease I. Vanadyl ribonucleoside complexes (10 mM) were present throughout the preparation to prevent degradation from ribonucleases. The final RNA samples were extracted twice with phenol/chloroform (1:1), and precipitated with ethanol to remove traces of the vanadyl ribonucleoside complexes. The RNA was quantitated by measuring the absorbance at 260 nM.

Preparation of cDNA. E. coli bearing plasmids were grown in LB media with the appropriate antibiotic (ampicillin and tetracycline for IL-1 beta and GAPDH, respectively). Chloramphenicol (34 μg/mL) was used for amplification of the plasmid DNA. The plasmid DNA was purified by alkaline lysis (15). The IL-1 beta cDNA insert was removed from the plasmid with PST I restriction endonuclease, and purified by electrophoresis on a 1% agarose gel made with TRIS/acetate/EDTA buffer (15). The appropriate band was located by ethidium bromide staining and was excised from the gel. The cDNA was eluted from the gel fragment and purified by using powdered glass (Geneclean kit, Bio 101, La Jolla, CA). The cDNA for GAPDH was isolated in the same manner except that two restriction enzymes, PST I and Bgl I, were used to excise a 949 base-pair fragment of the cDNA (16). The use of Bgl I was necessitated by a small mutation in the plasmid that had removed one PST I site.

Labelling of cDNA. cDNA was radiolabelled to be used as a hybridization probe for Northern blots. Labelling was accomplished by using the random primer extension method (Amersham Multiprime kit) and alpha-[^{32}P]dCTP. Labelled cDNA was separated from smaller contaminants by G-50 Sephadex chromatography.

Northern blots. RNA samples (20 μg) were heated to 65°C for 5 min and cooled rapidly on ice. The samples were applied to a 0.9% agarose gel for electrophoresis in formaldehyde-containing buffer (15). The resulting gel was stained for 10 min with 30 μg/mL acridine orange in 10 mM phosphate buffer, pH 7.0. The gel was rinsed 4–5 times in phosphate buffer over a period of 1 hr to destain. The separated RNA was seen by UV transillumination. The RNA was transferred to a nylon membrane with a Transvac vacuum blotting apparatus using 20X SSC (1X SSC = 0.15M NaCl, 0.015M Na citrate, pH 7.0) as the buffer and a vacuum of 4 mm Hg for 45–60 min. The blot membrane was rinsed in 5X SSC for 5 min and then dried at room temperature. The RNA was crosslinked to the membrane by exposure to a UV transilluminator for 5 min, and the membrane was incubated overnight with shaking at room temperature with approximately 1 mL/10 cm^2 prehybridization solution in a sealed plastic bag. The prehybridization solution consisted of 50% deionized formamide, 5X SSC, 1X Denhardt's, 0.2% SDS, 0.2 mg/mL heat-denatured salmon-sperm DNA and 5% dextran sulfate. Approximately 1×10^6 cpm/mL of ^{32}P-labelled, heat-denatured cDNA was added to the prehybridization mixture and the plastic bag was resealed and incubated at 42°C overnight with shaking. Then the hybridization solution was removed, and the membrane was washed three times with 2X SSC at room temperature for 10 min per wash. The final wash consisted of a 40 min incubation in 0.5X SSC plus 0.1% SDS at 55°C and a 20 min wash in 0.15X SSC plus 0.1% SDS at 55°C. The membrane was blotted with filter paper to remove excess moisture, wrapped in plastic wrap and exposed to x-ray film overnight.

RNA dot blots were performed in the same manner except that the RNA was not heated or submitted to electrophoresis. Instead, it was diluted into 150 μL final volume of 10 mM NaOH and 1 mM EDTA. The RNA was applied directly to the nylon membrane with a 96-well, vacuum blotting apparatus with a vacuum of 4 mm Hg. Autoradiographs of blots were quantitated by scanning with a BioRad densitometer.

Stripping blots. Northern blots were treated with a 1:1 solution of deionized formamide and 5X SSC at 65°C for 45 min to remove the ^{32}P-labelled IL-1 beta cDNA. The stripped blot was exposed to film overnight to confirm that all of the label had been removed before incubating it with a new cDNA probe.

RESULTS AND DISCUSSION

The human monocytic cell line THP-1 responds to stimulation by LPS by synthesizing IL-1 beta and releasing it (17). When 10 pM PAF and 0.1 μg/mL LPS are incubated together with THP-1 cells, the two stimuli interact synergistically to elevate IL-1 beta synthesis, as seen in Figure 1. PAF alone also causes a small increase in IL-1 beta synthesis over the buffer control. These data confirm previous studies that showed that human monocytes (8,10) and THP-1 cells (14) synthesize and release IL-1 beta in response to PAF, and that PAF and LPS interact synergistically to give a greater response. The increased synthesis of IL-1 beta in response to LPS results, in part, from increased expression of IL-1 beta mRNA by THP-1 cells, which peaks at about 3 hr (17). Similarly, at 3 hr (data not shown) or 4 hr (Fig. 2), 10 pM PAF increases the levels of IL-1 beta mRNA in THP-1 cells that are already stimulated with LPS. In the same experiment, PAF had no apparent effect on the levels of the mRNA of the normally unregulated gene, glyceraldehyde-3-phosphate dehydrogenase. This suggests that the differences in IL-1 beta mRNA levels were not due to variations in the amount of RNA loaded on the gel, but that PAF is specifically regulating the expression of IL-1 beta. Apparently the synergistic interaction between PAF and LPS in stimulation of IL-1 beta synthesis and release is due at least partly to synergy at the level of mRNA.

Earlier studies show that PAF, without any added co-stimulus, can stimulate IL-1 beta synthesis in THP-1 cells and in human monocytes (8,14). It is not clear if the relatively small effects of PAF alone are dependent on minute amounts of endotoxin that may be found in the delipidated BSA used as a carrier for PAF in the media, or in the fetal bovine serum. In any case, PAF alone under the conditions of these experiments does cause the synthesis (Fig. 1) and release (14) of IL-1 beta from THP-1 cells. The data in Table 1 suggest that 10 pM PAF may cause an increase in IL-1 beta synthesis in THP-1 cells by increasing IL-1 beta mRNA levels. For these same experiments, PAF had no effect

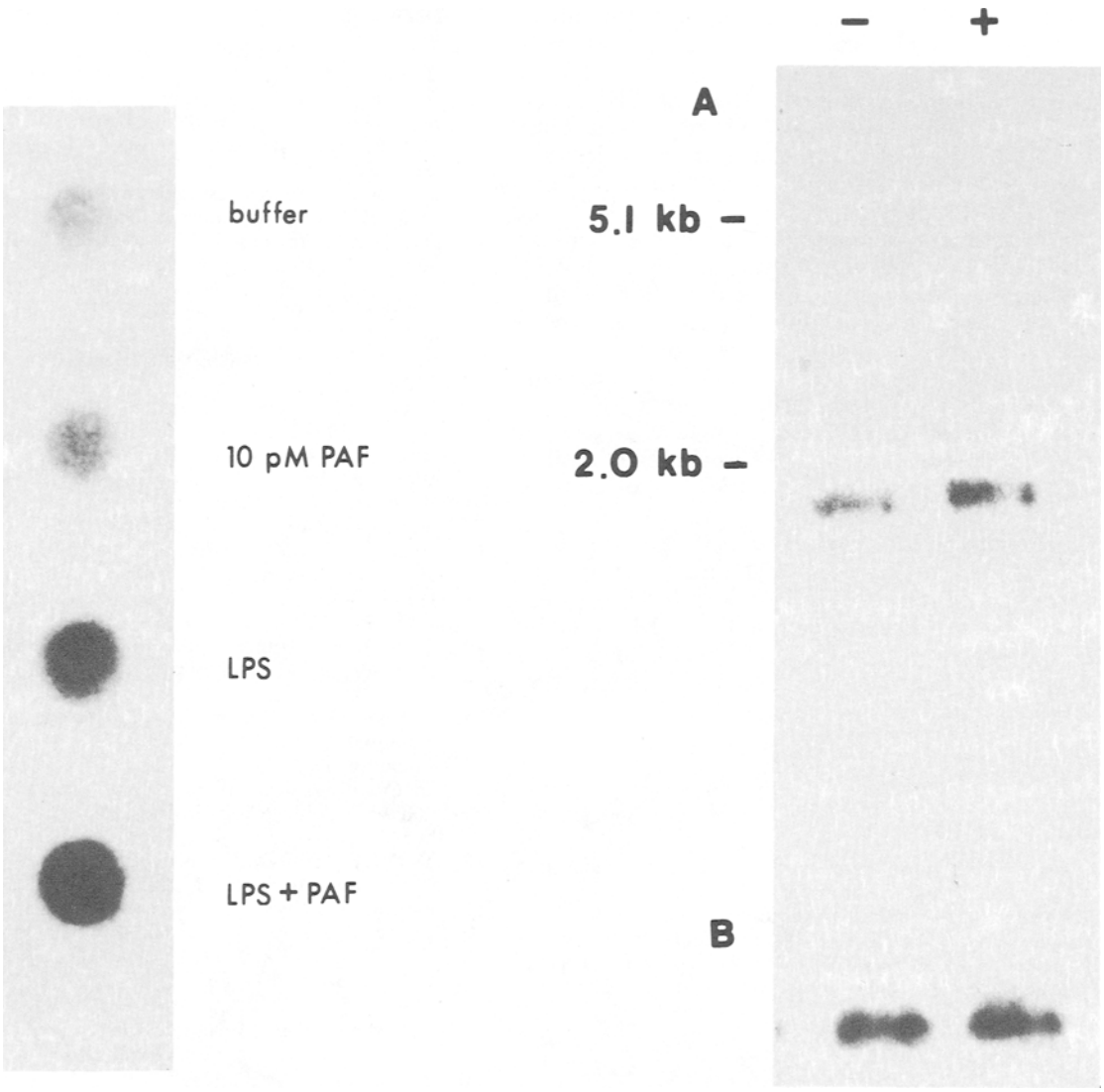


FIG. 1. Autoradiograph from a Western dot blot of THP-1 cell extracts. The THP-1 cells were incubated for 4 hr with buffer, 10 pm PAF, 0.1 μg/mL LPS or LPS + PAF, as indicated. Cell protein extracts were spotted directly on a nitrocellulose membrane for immunoassay using anti-IL-1 beta antibodies, a biotinylated secondary antibody, and [^{125}I]streptavidin.

FIG. 2. Autoradiographs of Northern blots of RNA from THP-1 cells. THP-1 cells were incubated in the presence of LPS (0.1 μg/mL), and in the absence (−) or presence (+) of PAF (10 pM) for 4 hr before the cells were harvested for extraction of RNA. The RNA (20 μg) was separated by electrophoresis on an agarose gel, transferred to a nylon membrane and hybridized with ^{32}P-labelled cDNA for (A) IL-1 beta and (B) GAPDH.

TABLE 1

mRNA on Northern Blot Autoradiographs (% of Control)[a]

Condition	IL-1 beta	GAPDH	IL-1 beta/GAPDH
Control	100	100	100[b]
+ 10 pM PAF	138[c] ± 25	95 ± 11	143[b] ± 19

[a]THP-1 cells were incubated for 4 hr before harvesting for preparation of RNA. The RNA was spotted on a nylon membrane, and probed with ^{32}P-labelled cDNA to IL-1 beta. After stripping, the blots were reprobed with labelled cDNA to glyceraldehyde-3-phosphate dehydrogenase as a control for total RNA.
[b]$P<0.05$, paired Student's t-test, n = 10.
[c]Values are means ± SEM.

on the mRNA levels for glyceraldehyde-3-phosphate dehydrogenase (GAPDH), which was used as an unregulated control. When the relative amount of GAPDH in each sample is used to normalize the mRNA levels for IL-1 beta, the resulting values should reflect the effects of PAF without the effects of variations in the amounts of RNA on the blot membrane. These values, given in the third column on Table 1, indicate again that PAF causes a significant increase in IL-1 beta mRNA levels in THP-1 cells.

PAF causes only a 40% increase in IL-1 beta mRNA levels, but this increase matches the 40% increase in IL-1 beta protein synthesis described previously for

THP-1 cells (14). This small percentage increase nonetheless can yield a biologically significant net increase in IL-1 beta protein when a co-stimulus such as LPS is present. It also should be noted that the THP-1 cell line is not as responsive to PAF as are human monocytes. PAF causes a 100–200% increase in IL-1 beta release in monocytes as compared to a 50–70% increase in THP-1 cells (8,14).

These experiments suggest that PAF may increase IL-1 beta release largely by increasing mRNA levels, and that synergy between PAF and LPS may also occur at this level. It is not clear from these experiments whether PAF increases transcription of IL-1 beta mRNA or if it decreases the rate of turnover of the IL-1 beta mRNA. For the studies with PAF alone, the extent of the effect at the mRNA level is enough to account for the increase at the protein level, so it is not likely that PAF alone affects the rate of translation.

PAF's ability to induce the production of IL-1 beta may be important to the persistent inflammation that results from administration of PAF *in vivo*. IL-1 beta can induce the synthesis of more PAF (18), thereby completing a cycle of a positive feedback loop. This positive feedback may provide sustained inflammation in the absence of any new stimulus.

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The Effect of Dietary n-3 Polyunsaturated Fatty Acids on HDL Cholesterol in Chukot Residents *vs* Muscovites

E. Gerasimova*[a], N. Perova[a], I. Ozerova[a], V. Polessky[a], V. Metelskaya[a], I. Sherbakova[a], M. Levachev[b], S. Kulakova[b], Yu. Nikitin[c] and T. Astakhova[c]

[a]Department of Biochemistry, Institute of Noncommunicable Disease Prevention of the USSR Research Centre for Preventive Medicine, Moscow 101953, the [b]Institute of Nutrition, Moscow 109240 and the [c]Institute of Therapy, Novosibirsk 630003, USSR

Native Chukot Peninsula residents, in contrast to Muscovites, consume a diet rich in n-3 polyunsaturated fatty acids. This dietary peculiarity is reflected in differences in plasma lipid and apolipoprotein contents. The Chukot residents have lower contents of total cholesterol, triglyceride, LDL (low density lipoprotein) cholesterol and apolipoprotein B, but higher HDL (high density lipoprotein) cholesterol levels than do Muscovites. The apolipoprotein A-I levels were identical in both groups. A higher HDL cholesterol to apolipoprotein A-I ratio was determined in the coastline Chukot residents (0.52 ± 0.01) than in Muscovites (0.43 ± 0.01; $p < 0.01$). In contrast to Muscovites, the coastline Chukot residents also had higher n-3 and lower n-6 polyunsaturated fatty acid percentages in plasma and erythrocyte lipids, and lower phosphatidylcholine and higher sphingomyelin or phosphatidylethanolamine levels in HDL_{2b} and HDL_3. The higher HDL cholesterol levels in the plasma of the coastline Chukot residents appears to reflect the higher cholesterol-scavenging capacity of their HDL.

We conclude from this study that the regular consumption of dietary n-3 polyunsaturated fatty acids by the coastline Chukot residents decreased LDL cholesterol transfer from plasma to peripheral cells, and enhanced cholesterol efflux from cellular membranes toward HDL. *Lipids 26,* **261–265 (1991).**

Since the study of the effects of dietary n-3 polyunsaturated fatty acids (PUFA) on the plasma lipid content of Greenland and Danish Eskimos (1), the hypolipidemic effects of n-3 PUFA, which occur abundantly in marine organisms, have become a focus of considerable attention. Laboratory experiments and human trials have shown that seafood dietary supplements and "max-Epa" type drugs, that are rich in n-3 PUFA, decrease total cholesterol (Chol), triglyceride (TG), LDL cholesterol, and apoprotein B (apoB) contents of plasma (2–4). However, the data reported on the effects of dietary n-3 PUFA on HDL Chol levels are not consistent (3,5–7), and whether and how various n-3 PUFA supplements can be used to correct atherogenic dyslipoproteinemias remains an open question (5).

The present study was undertaken to determine the effect of dietary n-3 PUFA on lipoprotein profiles and fatty acid compositions of plasma and erythrocyte lipids of native male residents of the coastline Chucot Peninsula and of Muscovites. Both groups vary greatly in dietary n-3 PUFA consumption (8–10). Plasma Chol, TG, LDL Chol, HDL Chol, apo B and apo A-I levels were determined in both groups, and HDL phospholipid composition and plasma and erythrocyte fatty acid profiles were monitored. Erythrocytes were chosen as cellular model because their phospholipids and PUFA are known to be modulated by lipoproteins (11–13), and because the efflux of Chol from the erythrocyte membrane is known to depend on HDL lipid composition and unsaturation (14–16).

MATERIALS AND METHODS

Blood samples were obtained from 30- to 59-year-old males randomly chosen from Moscow residents (n = 650) and from coastline area residents of the Chukot Peninsula (n = 261). Plasma apolipoprotein B (apo B) and apo A-I levels were determined on 110 Muscovites and 157 Chukot residents. Fatty acid composition of plasma and erythrocytes were measured on 41 Muscovites and 61 Chukot residents. The composition of high density lipoprotein (HDL) phospholipids was analyzed in samples from 10 Muscovites and 58 Chukot residents.

Fasting blood was drawn from vein using Vacutainer tubes containing solid disodium EDTA (Sigma, St. Louis, MO; 1 mg/1 mL blood). Chol and TG levels were measured on a Technicon AutoAnalyzer-II (Technicon Ltd., Dublin, Ireland). HDL Chol was determined after heparin-manganese precipitation of low density lipoprotein (LDL) and very low density lipoprotein (VLDL) (17). LDL Chol levels were calculated as the difference between total plasma Chol content and the sum of HDL Chol and one fifth of total plasma TG (18). Lipid measurements standardizations were performed using a program available from the Center for Disease Control, Atlanta, GA (19). An electroimmunoassay was used to determine the concentrations of the major apolipoproteins, namely, apo B and apo A-I (20,21). HDL_{2b} and HDL_3 were separated by preparative ultracentrifugation at d 1.063–1.100 g/mL and d 1.125–1.210 g/mL, respectively (22). Lipids were extracted from HDL according to established procedure (23), and phospholipids were fractioned by two-dimensional thin-layer chromatography (TLC) using Solvent Systems A, chloroform/methanol/water (65:25:4, v/v/v) and B, chloroform/methanol/aqueous ammonia (14:6:1, v/v/v) (24). Lipid phosphorus was measured in each fraction (25), and the relative content of each phospholipid class was expressed as percentage of the total. Erythrocytes were treated as previously described (26). Plasma lipids (PL, Chol esters, TG) were separated by TLC on Silica Gel plates (24) using hexane/diethyl ether/acetic acid (85:15:3, v/v/v) as developing solvent; lipid fractions were scraped into tubes and methyl esters were prepared by transesterification (24).

*To whom correspondence should be addressed at the Department of Biochemistry, Institute of NCD Prevention, 10, Petroverigsky Str., Moscow 101953, USSR.

Abbreviations: Apo A-I, apolipoprotein A-I; apo B, apolipoprotein B; Chol, cholesterol; HDL, high density lipoproteins; LDL, low density lipoproteins; MUFA, monounsaturated fatty acids; PC, phosphatidylcholine; PE, phosphatidylethanolamine; PL, phospholipids; P/S, polyunsaturated fatty acid to saturated fatty acid ratio; PUFA, polyunsaturated fatty acids; SEM, standard error of the mean; SPH, sphingomyelin; TLC, thin-layer chromatography; TG, triglycerides; VLDL, very low density lipoproteins.

Gas-liquid chromatography of methyl esters was performed on an "Intersmat" gas chromatograph (JLD-Instruments, France) equipped with flame ionization detector and a 0.4 × 240 cm glass column with 10% Silar-10C on Chromosorb W/AW, 100–200 mesh (Serva, Heidelberg, Germany). Nitrogen was used as carrier gas at a flow rate of 40 mL/min. The temperatures of the column, the injector and the detector were held at 175°C, 230°C and 240°C, respectively. Fatty acids were identified by comparing the retention times with those of standards. Individual fatty acid levels are given as a percentage of the total.

Student's *t*-test was used for statistical data treatment. The tabulated data are given as mean ± SEM.

RESULTS

Table 1 shows data on the lipid composition of plasma of representative Muscovites (Group 1) and Chukot residents (Group 2). It is evident that the levels of plasma Chol, TG, and LDL Chol were lower in Chukot residents as compared to Muscovites, while the HDL Chol levels in Chukot residents were higher. Male subgroups which had representative plasma lipid levels (Chol, TG, HDL Chol) revealed lower apo B levels in the Chukot residents as compared to Muscovites. No differences in apo A-I levels were apparent between the two groups (Table 2). Moreover, the Chukot residents had a higher HDL Chol to apo A-I ratio (Table 2).

Dietary differences between Chukot residents and Muscovites were analyzed using a 24-hr recall method of food patterns (8). Total caloric intake was 3157 ± 82.9 kcal for the coastline Chukot residents and 2568 ± 32.6 kcal for Muscovites with respective intakes of protein of 32 ± 1.0% and 15 ± 0.1%; carbohydrates of 26 ± 1.0% and 43 ± 3.7%; and fats of 36 ± 0.9% and 38 ± 0.4%. In spite of the rather similar total fat intake by the two groups, considerable differences in the fat composition were detected. Thus, the dietary polyunsaturated fatty acid to saturated fatty acid (P/S) ratios were 0.52 and 0.31 for Chukot residents and Muscovites, respectively. PUFA sources also differed—Chukot residents consumed n-3 PUFA rich lipids from sea animals (whale, walrus, seal) and from ocean fish (salmon), while Muscovites used plant oils as the major n-6 PUFA source. Therefore, it seemed of interest to determine whether the observed differences in plasma lipid and apoprotein contents were related to the changes in fatty acid composition of plasma and erythrocyte lipids. Table 3 and Figure 1 show the composition of the major fatty acids from plasma and erythrocyte lipids. It is evident that Chukot residents, when compared to Muscovites, had lower levels of major n-6 PUFA (18:2 and 20:4) in plasma phospholipids and increased n-3 PUFA levels due to 20:5. In addition, in Chukot residents a lower content of 18:0 and higher levels of MUFA (monounsaturated fatty acids) (16:1 and 18:1) were observed in plasma phospholipids. Similar changes in PUFA composition, namely, lower n-6 PUFA levels

TABLE 1

Composition of Plasma Lipids in Muscovites and Coastline Chukot Residents[a]

Group	Chol	TG	HDL Chol	LDL Chol
	mg/dL			
Muscovites	203 ± 1.4	102 ± 2.4	53 ± 0.6	133 ± 4.1
Chukot residents	190 ± 1.5	79 ± 2.8	63 ± 1.1	115 ± 2.0
P[b]	<0.001	<0.001	<0.001	<0.001

[a] Blood samples were collected from 30- to 59-year-old men chosen randomly from the Moscow district (n = 650) and from the Chukot Peninsula (n = 261). Total plasma cholesterol (Chol), triglycerides (TG), HDL cholesterol (HDL Chol) and LDL cholesterol (LDL Chol) were determined as described in the Methods Section. Data are presented as mean ± SEM.

[b] P denotes the significance of the difference between groups according to Student's *t*-test. NS, not statistically significant (p > 0.1).

TABLE 2

Content of HDL Cholesterol and Apolipoproteins in Plasma of Muscovites and Coastline Chukot Residents[a]

Group	HDL Chol	apo B	apo A-I	HDL Chol / apo A-I
	mg/dL			
Muscovites	53 ± 1.2	119 ± 1.9	135 ± 2.2	0.43 ± 0.01
Chukot residents	65 ± 1.3	99 ± 2.3	133 ± 1.1	0.52 ± 0.01
P[b]	<0.001	<0.001	NS	<0.001

[a] Blood was collected from 30- to 59-year-old Muscovites (n = 110) and coastline Chukot residents (n = 157). The contents of HDL cholesterol (HDL Chol), apolipoprotein B (apo B) and apolipoprotein A-I (apo A-I) were determined as described in the Methods Section. Data are presented as mean ± SEM.

[b] P denotes significance of the difference between groups according to Student's *t*-test. NS, not statistically significant (p > 0.1).

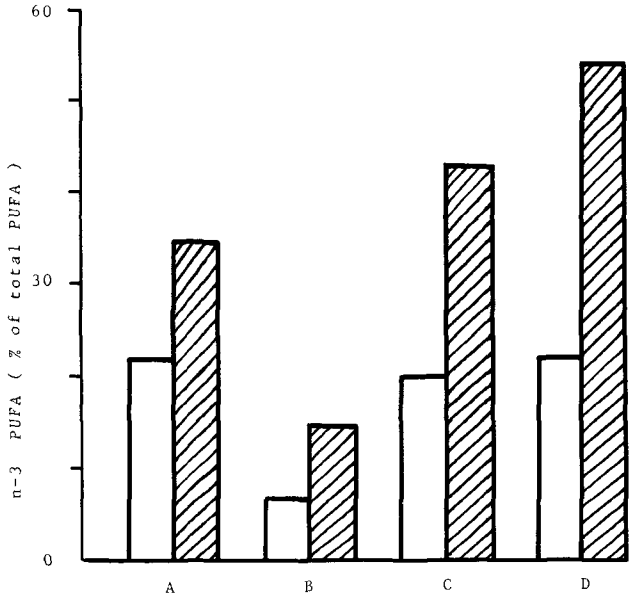


FIG. 1. n-3 PUFA contribution, expressed as percentage of total PUFA, in plasma phospholipids (A), plasma cholesterol esters (B), plasma triglycerides (C) and erythrocyte lipids (D) of Muscovites (open bars) and Chukot residents (hatched bars). Experimental details are given in the footnote to Table 3.

TABLE 3

Fatty Acid Composition of Plasma and Erythrocyte Lipids in Muscovites and Coastline Chukot Residents[a]

Fatty acid %	Plasma lipids						Erythrocyte lipids	
	PL		Chol esters		TG			
	Musc. (11)	Chuc. (34)	Musc. (6)	Chuc. (39)	Musc. (6)	Chuc. (10)	Musc. (41)	Chuc. (61)
16:0	26.7 ± 0.72	28.6 ± 1.10	11.2 ± 0.37	17.6 ± 0.89*	26.2 ± 1.69	24.3 ± 0.99	21.7 ± 0.16	25.9 ± 0.49*
16:1	1.1 ± 0.13	4.4 ± 0.47*	4.1 ± 0.59	7.4 ± 0.64*	6.2 ± 0.45	5.2 ± 0.40	3.7 ± 0.35	1.9 ± 0.45*
18:0	13.5 ± 0.29	11.2 ± 0.48*	1.3 ± 0.09	2.5 ± 0.38*	4.0 ± 0.53	6.8 ± 0.89*	11.2 ± 0.64	13.7 ± 0.17*
18:1	12.4 ± 0.65	16.8 ± 0.99*	19.0 ± 1.05	20.9 ± 0.93	35.7 ± 1.61	36.6 ± 1.25	13.2 ± 0.89	15.6 ± 0.28*
18:2	21.7 ± 1.10	12.3 ± 1.80*	50.4 ± 1.96	29.5 ± 2.51*	16.0 ± 1.64	13.6 ± 2.39	11.2 ± 1.20	6.7 ± 0.53*
20:4	10.9 ± 0.74	4.6 ± 0.53*	7.1 ± 0.42	3.2 ± 0.33*	1.5 ± 0.19	1.2 ± 0.15	13.3 ± 0.88	7.2 ± 0.28*
20:5	1.9 ± 0.44	5.4 ± 0.45*	1.9 ± 0.16	3.2 ± 0.51*	0.9 ± 0.36	2.9 ± 0.82*	1.2 ± 0.09	6.1 ± 0.37*
22:5	1.4 ± 0.19	1.5 ± 0.22	traces	1.0 ± 0.18	1.0 ± 0.17	2.4 ± 0.40*	2.9 ± 0.23	3.2 ± 0.15
22:6	5.2 ± 0.67	5.5 ± 0.52	0.8 ± 0.05	1.1 ± 0.17	2.1 ± 0.70	2.4 ± 0.37	4.4 ± 0.26	5.3 ± 0.25

[a]Blood was collected from 30- to 59-year-old Muscovites and coastline Chukot residents. Plasma lipids: phospholipids (PL), Cholesterol esters (Chol esters), triglycerides (TG) were isolated by TLC, and fatty acid composition was determined as described in the Methods Section. Data are presented as mean ± SEM.

*$P < 0.01$ in Muscovites *vs* coastline Chukot residents. Number of cases is given in parentheses.

TABLE 4

Relationship Between HDL Cholesterol and Apolipoprotein A-I Contents and 20:5 and n-3 PUFA Levels in Erythrocyte Lipids of Coastline Chukot Residents[a]

Group	Erythrocyte		Plasma		HDL Chol
	20:5	n-3 PUFA	HDL Chol	Apo A-I	
	%		mg/dL		apo A-I
1 n=11	2.8 ± 0.39	11.1 ± 0.70	49.0 ± 4.5	129 ± 6.9	0.38 ± 0.020
2 n=19	6.2 ± 0.26	14.2 ± 0.61	72.0 ± 4.9	132 ± 5.7	0.54 ± 0.037
3 n=11	8.9 ± 0.21	18.6 ± 0.79	77.0 ± 5.8	135 ± 7.8	0.56 ± 0.043
P 1-2		<0.001	<0.01	NS	<0.001
P 1-3		<0.001	<0.01	NS	<0.001
P 2-3		<0.001	NS	NS	NS

[a]Blood samples were collected from 30- to 59-year-old men chosen randomly from coastline Chukot residents (n = 41). HDL cholesterol (HDL Chol), apolipoprotein A-I (apo A-I) in plasma and also level 20:5 and n-3 PUFA in erythrocytes were determined as described in the Methods Section. Chukot residents were split into three groups according to the 20:5 contents in erythrocytes. Group 1 consisted of 25% of the samples with the lowest levels of erythrocyte 20:5; Group 2 consisted of 50% of the samples with medium values of 20:5. Group 3 consisted of 25% of the samples with the highest proportion of erythrocytes 20:5. Data are presented as mean ± SEM. P denotes significance of the difference between groups according to Student's *t*-test. NS, not statistically significant ($P > 0.1$).

(18:2 and 20:4) and a higher n-3 PUFA levels, mainly due to 20:5, were found in serum Chol esters and TG as well as in erythrocyte lipids (Table 3).

It should also be mentioned that the relative amounts of eicosapentaenoic acid (20:5) in erythrocytes and the HDL Chol levels considerably varied among the Chukot residents, namely from 1.3% to 10.4% and from 36 mg/dL to 126 mg/dL, respectively. To determine whether the differences in HDL Chol levels were related to the 20:5 changes in erythrocyte lipids, 41 samples obtained from Chukot residents were split into three groups according to their eicosapentaenoic acid contents. Group 1 consisted of 25% of the samples with the lowest levels of erythrocyte 20:5; Group 2 consisted of 50% of the samples with medium values for 20:5; Group 3 consisted of 25% of the samples with the highest proportion of erythrocyte 20:5. Table 4 shows that Group 3, with the highest 20:5 levels in erythrocyte lipids also had the highest HDL Chol levels. Since the apo A-I concentrations were similar in all three groups, the HDL Chol to apo A-I ratios were the highest for the subjects with the highest 20:5 contents in their erythrocyte lipids (Table 4).

We also studied the composition of HDL phospholipids, because in model systems the interaction between apo A-I and Chol was shown to depend upon HDL phospholipids (16,27). Table 5 shows that the Chukot residents, as compared to Muscovites, had lower percentage of phosphatidylcholine (PC) and higher percentage of sphingomyelin (SPH) and phosphatidylethanolamine (PE) in HDL_{2b} and HDL_3.

TABLE 5

Phospholipid Composition of HDL Subfractions in Muscovites and Coastline Chukot Residents[a]

Group	HDL Chol (mg/dL)	HDL_{2b} PC	HDL_{2b} SPH	HDL_{2b} PE	HDL_3 PC	HDL_3 SPH	HDL_3 PE
		%					
Muscovites	53 ± 2.1	75.0 ± 1.5	10.0 ± 0.5	6.6 ± 0.9	79.2 ± 2.3	9.8 ± 1.1	5.4 ± 0.3
Chukot residents	62 ± 1.9	67.8 ± 0.8	14.0 ± 0.5	9.0 ± 0.4	68.8 ± 0.8	13.1 ± 0.3	8.9 ± 0.3
P	<0.01	<0.01	<0.01	<0.02	<0.001	<0.01	<0.001

[a]Blood was collected from 30- to 59-year-old Muscovites (n=10) and Chukot residents (n=58). HDL_{2b} and HDL_3 were isolated by preparative ultracentrifugation at d 1.063-1.100 g/mL and d 1.125-1.210 g/mL, respectively. Lipids were extracted from HDL_{2b} and HDL_3 according to the Folch's procedure (23), and phospholipids were separated by TLC as described in the Methods Section. Data are presented as mean ± SEM. P denotes significance of the difference between groups according to Student's *t*-test.

DISCUSSION

The present comparative study of plasma lipids of randomly chosen native coastline Chukot male residents *vs* Muscovites has demonstrated that the lower plasma total Chol content in Chukot residents is due to both lower LDL and VLDL levels, *i.e.*, the lipoprotein classes that are responsible for Chol transport into peripheral cells. This is supported by our observation that the Chukot residents had both lower lipoprotein Chol and apo B levels (Tables 1 and 2). This is consistent with the results of other studies which similarly revealed that apo B-containing lipoproteins decrease in population groups whose dietary intake includes considerable amount of seafood rich in n-3 PUFA (3,4). Considering the evidence that n-3 PUFA lowers the synthesis of apo B and VLDL in liver (2,4), one could suggest that this mechanism is essential in lowering plasma levels of apo B and TG, the main components of VLDL, and of LDL Chol with apo B as its main apoprotein component. However, a contribution from a more active Chol transport from apo B-containing to apo A-I-containing lipoproteins presently cannot be excluded as the Chukot residents as compared to Muscovites had higher HDL Chol levels without having elevated apo A-I concentrations (Table 2). This suggests that the higher HDL Chol levels in the Chukot residents are predominantly due to an increased portion of HDL particles rich in Chol.

The lipid and protein profiles observed for Chukot coastline residents and Muscovites cannot be explained merely by the differences in total daily caloric intake or in protein and carbohydrate consumption. According to Eisenberg (28), carbohydrate enrichment of the daily dietary intake resulted in a 20% increase of apo A-I and 32% increase of HDL Chol levels. The Chukot residents, consuming less carbohydrates, had the same apo A-I levels, but higher HDL Chol levels as compared to Muscovites.

The data suggest that the high intake of dietary n-3 PUFA by coastline Chukot residents could contribute to the increased Chol transport and, probably, to the increased Chol scavenging capacity of their HDL particles. Table 3 shows that the n-3 PUFA levels in PL, Chol esters, and TG of plasma and erythrocytes were higher in the Chukot residents as compared to Muscovites. It was found that plasma phospholipids from Chukot residents were enriched with MUFA and n-3 PUFA (Table 3). It had previously been demonstrated that the Chukot residents had higher 20:5 contents in HDL phospholipids as compared to Muscovites. It appears likely that accumulation of phospholipids containing MUFA and n-3 PUFA in the surface monolayer of HDL particles would increase fluidity. This, in turn, could result in a higher capacity of the particles to accept Chol.

Studies performed on model systems have demonstrated that Chol interaction with apo A-I in HDL is dependent upon the composition and the degree of unsaturation of the phospholipids involved (15,16,27). We had earlier shown (29,30) that Muscovites with higher HDL Chol levels also had higher PC levels with higher 18:2 and 20:4 contents and lower levels of SPH and PE. The Chukot residents with higher HDL Chol levels as compared to subjects with lower HDL Chol levels showed similar tendencies in changes in phospholipid composition not only in regard to n-6 PUFA, but also with respect to n-3 PUFA (20:5) (29,30). The Chukot residents had higher HDL Chol levels (Table 1) than the Muscovites that had lower PC levels and larger amounts of SPH and PE in the HDL subfractions (Table 4). The HDL phospholipids in the Chukot residents were enriched with n-3 PUFA and MUFA (Table 3), even when they had lower PC and higher PE contents as compared to Muscovites. Elevated levels of n-3 PUFA and MUFA would probably increase the fluidity of the HDL surface monolayer and thus enhance its ability to accept Chol, as is manifested in the higher HDL Chol level in the Chukot residents as compared to the Muscovites (Table 5).

We propose that the lower PC levels in HDL of Chukot residents *vs* Muscovites is due to decreased PC synthesis in the liver due to low dietary n-6 PUFA. In fact, it had been shown that the presence of n-6 PUFA and, in particular, of 18:2 in phosphatidic acid makes it a better substrate for PC synthesis (31,32).

We previously demonstrated that increased HDL Chol levels are associated with increased fluidity of the HDL surface monolayer (33), as we determined with spinlabeled probes (34) and by following changes in HDL particle molecular patterns (using fluorescent derivatives of phospholipids) (35). In addition, it has been shown that SPH and PE have a high capacity for complex formation with Chol when compared to PC (16). Therefore, we believe that the enrichment of HDL phospholipids with

n-3 PUFA ensures greater fluidity and, hence, capacity to accept Chol, and thus can explain the higher HDL Chol levels in the coastline Chukot residents as compared to Muscovites.

Moreover, n-3 PUFA enrichment of lipoprotein core lipids, namely Chol esters and TG, makes the lipoprotein a better substrate for lipoprotein lipase (36), which may explain the low plasma TG levels in Chukot residents.

The results of our study permit us to conclude that regular consumption of n-3 PUFA contributes to a decline in Chol transport to cells *via* LDL and to an increase in Chol efflux from the cells *via* HDL particles.

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Rat Liver Outer Mitochondrial Carnitine Palmitoyltransferase Activity Towards Long-Chain Polyunsaturated Fatty Acids and Their CoA Esters

Grace R. Gavino and **Victor C. Gavino***
Department of Nutrition, Université de Montreal, Montreal, Quebec, Canada H3C 3J7

The activity of the overt form of rat liver mitochondrial carnitine palmitoyltransferase or CPT_0 (EC 2.3.1.21) towards different fatty acid substrates was studied. The following non-esterified fatty acids (NEFA) and their CoA esters in the presence of 1% bovine serum albumin (BSA) were tested: 16:0, 18:0, 18:1, 18:2, 18:3ω3, 20:4, 20:5ω3 and 22:6ω3. The data fit a square hyperbolic model for enzyme catalysis ($p<0.001$, non-linear regression). Asymptotic V_{max} and $K_{0.5}$, substrate concentration at one-half V_{max}, were calculated using total concentrations of acyl-CoA, or unbound concentrations of NEFA. BSA was found to act as a true substrate reservoir for NEFA in that the dissociation of the NEFA-BSA complex was 10–330 times faster than the CPT_0 reaction. Regardless of form (NEFA or CoA ester), 18:3ω3 gave the highest, while 22:6ω3 and 18:0 gave the lowest rates of acylcarnitine synthesis. Except for 18:3ω3 and 18:2, V_{max} for NEFA was generally lower than for acyl-CoA, with the greatest differences observed for 20:4, 20:5ω3 and 22:6ω3, suggesting that acyl-CoA synthesis may also be important in the control of the entry of these fatty acids into the mitochondria. The data provide an enzymatic rationale for the relatively low content of 18:3ω3 in esterified lipid.
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The overt form of carnitine palmitoyltransferase (EC 2.3.1.21), or CPT_0, regulates the entry of long-chain fatty acyl-CoA into the mitochondria for β-oxidation (1–4). In a review on the partition of fatty acyl equivalents between oxidation and esterification, Zammit (5) noted that triglyceride synthesis increases upon attainment of the maximum capacity for the β-oxidation of fatty acids, indicating that CPT_0 plays a role in the control of this branchpoint (Fig. 1). Physiologically, the flux through acylcarnitine synthesis (Fig. 1) involves a mixture of different fatty acid substrates, each with potentially different kinetic properties towards CPT_0. These differences may affect the availability of the various types of fatty acids for glyceride synthesis. We therefore tested the rates of mitochondrial CPT_0-catalyzed acylcarnitine synthesis for a series of 16-, 18-, 20- and 22-carbon fatty acids both as nonesterified fatty acids (NEFA) and as acyl-CoA. Our results show that CPT_0 activity is highest towards 18:3ω3, and is relatively lower for 18:0 and the 20- and 22-carbon polyunsaturated fatty acids. By measuring the rate of dissociation of NEFA from bovine serum albumin (BSA), we further show that BSA, commonly used in assays involving lipophilic substrates, is not likely to affect NEFA-CPT_0 kinetics. Finally, the data suggest that mitochondrial fatty acyl-CoA synthetase (EC 6.2.1.3) may limit acylcarnitine synthesis from 20- and 22-carbon polyunsaturated fatty acids in NEFA form.

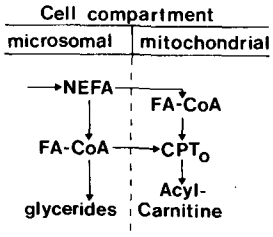


FIG. 1. Partitioning of fatty acids between acylcarnitine synthesis and glyceride formation. Acyl-CoA synthesis can occur in both microsomal and mitochondrial compartments.

MATERIALS AND METHODS

Chemicals. L-[^{3}H-*methyl*]Carnitine was from Amersham (Oakville, Ontario, Canada) and [1-^{14}C]fatty acids were from Dupont (Mississauga, Ontario, Canada). All other NEFA were from NuChek Prep (Elysian, MN). BSA (fatty acid-free), CoA esters of 16:0, 18:0 and 18:1, and other biochemicals were from Sigma (St. Louis, MO). The CoA esters of 18:2, 18:3ω3, 20:4, 20:5ω3 and 22:6ω3 were synthesized according to Singh *et al.* (6). The CoA ester of 18:1 was also synthesized for comparison with commercially obtained 18:1-CoA. The purity of the commercial and synthesized fatty acyl-CoA were assayed by thin-layer chromatography (TLC) and by gas-liquid chromatography (GLC) after transesterification to methyl esters with $NaOCH_3$. Transesterification was essentially complete as verified with synthesized [1-^{14}C]18:1-CoA. All acyl-CoA preparations used in the experiments were better than 98% pure. Affi-Gel was from BioRad (Richmond, CA). All other chemicals were reagent grade or better.

Preparation of mitochondria and CPT_0 assay. Liver mitochondria from 200–250 g male Sprague Dawley rats (Charles River, Montreal, Quebec Canada) were prepared according to McGarry *et al.* (7) using "Method C", which was reported to yield minimal contamination from other subcellular fractions. This method for obtaining a mitochondrial preparation was used without modification. The rats were fed *ad libitum* and sacrificed at 8:00 a.m. The livers were immediately perfused *in situ* with 150 mL ice-cold 0.25 M (pH 7.4) sucrose through the portal vein before homogenization. Using catalase (8) and glutamate dehydrogenase (9) as marker enzymes for peroxisomes and mitochondria, respectively, peroxisomal contamination of the mitochondrial preparation was determined to have been reduced 17–18 times relative to the crude homogenate. The conditions for the CPT_0 assay was as previously described (7), and the substrates used were either fatty acyl-CoA or NEFA. The overt CPT activity in these preparations was 93%, inhibited by 20 μM

*To whom correspondence should be addressed at Department of Nutrition, University of Montreal, P.O. Box 6128, Station A, Montreal, Quebec, Canada H3C 3J7.

Abbreviations: Acyl-CoA, fatty acid esters of coenzyme A; BSA, bovine serum albumin; CPT_0, overt form of mitochondrial carnitine palmitoyltransferase; GLC, gas-liquid chromatography; $K_{0.5}$, kinetic parameter designating the substrate concentration at half maximal velocity V_{max}; NEFA, non-esterified fatty acids; TLC, thin-layer chromatography.

malonyl-CoA, indicating intactness of the isolated mitochondria. When the substrate was NEFA, 12.5 μM CoA was included in the assay mixture. This concentration was found to be optimal for CPT_0 activity with NEFA. Carnitine concentration in the assay mixture was set at 0.2 mM for the following reasons: i) the K_m values for carnitine reported for the type of assay used (7) fall below 100 μM (7,10–12); and ii) Miyazawa *et al.* (12) reported that when fatty acid chain length increases from 2 to 18 carbons, the K_m for carnitine decreases from 100 μM to 40 μM. All lipid substrates were prepared from freshly opened bottles of neat fatty acid and used within 24 hr. The neat fatty acids were consistently more than 99% pure as determined by GLC. Fatty acyl-CoA or NEFA were initially dissolved at the appropriate concentrations in 105 mM Tris buffer (pH 7.4) containing 10% BSA. The exact substrate concentrations were determined by GLC after transmethylation of acyl-CoA with $NaOCH_3$ or methylation of NEFA with diazomethane. Mitochondria (0.5 mg protein) were pre-incubated for 2 min in 0.925 mL of assay mixture. The reaction was started by the addition of 75 μL substrate solution. The final concentration of the assay components (7) were: 4 mM $MgCl_2$, 15 mM KCl, 2 mM KCN, 0.25 mM glutathione, 4 mM ATP, 40 μg rotenone, 1% BSA, 105 mM Tris·HCl, 0.2 mM L-carnitine and 0.25 μCi of ^{3}H-labelled carnitine. Reactions were terminated by the addition of 1 mL of 1.2 N HCl. The radioactive acylcarnitine product was extracted with n-butanol and counted as described by McGarry *et al.* (13).

Determination of unbound NEFA in the presence of BSA. Spector *et al.* (14) used the general stepwise equilibrium model to describe the binding of the fatty acids 16:0, 18:0, 18:1 and 18:2 to BSA. We repeated and confirmed his data under conditions identical to the CPT_0 assays for selected concentrations of 18:1, using CPT_0 assay buffers at pH 7.4, 30°C, and with 1% (w/v) BSA. We therefore used the reported binding coefficients directly. We experimentally determined the BSA-binding isotherms under CPT_0 assay conditions for 18:3ω3, 20:4, 20:5ω3 and 22:6ω3, for which data are not yet available.

BSA-crosslinking to Affi-Gel 10. Two grams of BSA were incubated with 50 mL of Affi-Gel 10 in 80 mL of 0.1 N HEPES and 80 mM $CaCl_2$ at pH 7.5 for 4 hr at 4°C with gentle shaking. The reaction was stopped by an additional 1 hr incubation with 5 mL 1 N ethanolamine-HCl at pH 8.0. The Affi-Gel was washed free of excess BSA by filtration. The amount of crosslinked BSA was estimated to be 20 mg/mL Affi-Gel.

Rate of NEFA dissociation from BSA. The initial rate of dissociation of NEFA from the fatty acid-BSA complex was estimated using a method similar to that reported previously (15) which is based on the measurement of the exchange rate of NEFA between soluble and Affi-Gel-linked BSA, extrapolated to zero time. Briefly, [1-^{14}C]NEFA complexed to 150 μM BSA was added to an equal volume of crosslinked NEFA-free BSA under conditions identical to the CPT_0 assay. At short time intervals, crosslinked BSA was separated from soluble BSA by rapid filtration. The rate of disappearance of radioactivity from the filtrates extrapolated to zero time, was taken as the rate of dissociation of NEFA from the soluble NEFA-BSA complex.

Calculations and miscellaneous analytical procedures. GLC was performed on a Hewlett-Packard (Mississauga, Ontario, Canada) Model 5890 gas chromatograph equipped with a 30-m bonded phase Carbowax capillary column (Supelco, Oakville, Ontario, Canada) with H_2 as the carrier gas. Samples were injected at a split ratio of 1:50, and the column temperature was programmed from 80°C to 210°C. Detection was by flame ionization and peak areas were measured by electronic integration using a Hewlett-Packard Integrator Model 3392A. The system was calibrated using gas chromatograph standards obtained from NuChek Prep, and the internal standard technique was used for sample quantitation.

Kinetic constants were derived by non-linear regression of initial velocity data directly to a square hyperbola as described by Sagnella (16). Protein was measured according to Lowry *et al.* (17).

RESULTS AND DISCUSSION

Acylcarnitine production was linear during the incubation period (up to 6 min) at all concentrations for all substrates tested. Table 1 shows the results of the regression of data pairs of initial velocity and acyl-CoA concentration to a square hyperbola. The Michaelis-Menten model for enzyme catalysis is mathematically also a square hyperbola. The use of this function for the non-linear regression to obtain V_{max} and apparent $K_{0.5}$ is empirical and is not intended to ascribe Michaelis-Menten behavior to CPT_0. Figure 2 shows actual data points superimposed on substrate velocity plots generated from the calculated regression coefficients. Correlation coefficients ranged from 0.91 to 0.99, except for 20:4 and 22:6ω3. Although all regressions were statistically significant ($p<0.001$), we have arbitrarily chosen $r=0.80$ as the lower limit for an acceptable data fit. The low r values for 20:4 and 22:6ω3 were due in part to the high degree of data scatter for these substrates. Any sigmoidicity in the kinetic data (18) would also result in a poor fit to the hyperbolic model but we could not ascertain this from the data. Table 1 shows that the CPT_0-V_{max}, the asymptote of the square hyperbola, as well as the $K_{0.5}$, vary according to the fatty acid substrate. For example, at 10 μM total acyl-CoA, the reactivities in decreasing order are: 18:3ω3 > 18:2 > 20:5ω3 ≈ 16:0 > 20:4 > 18:1 > 22:6ω3 > 18:0. The concentration 10 μM is included within the experimental range of all the acyl-CoA substrates tested

TABLE 1

CPT_0 Apparent $K_{0.5}$ and V_{max} for Fatty Acyl-CoA Substrates[a]

Fatty acyl-CoA	$K_{0.5}$ M × 10^6	V_{max} nmol/mg × min	r	n
16:0-CoA	25 ± 8	4.6 ± 0.6	0.91	30
18:0-CoA	51 ± 12	1.9 ± 0.2	0.96	23
18:1-CoA	40 ± 7	4.4 ± 0.4	0.92	56
18:2-CoA	16 ± 3	5.0 ± 0.3	0.99	23
18:3-CoA	15 ± 3	7.1 ± 0.8	0.98	23
20:4-CoA	4 ± 2	1.5 ± 0.1	0.71	15
20:5-CoA	20 ± 4	4.1 ± 0.3	0.95	15
22:6-CoA	8 ± 3	1.2 ± 0.1	0.71	15

[a]Values were derived from non-linear regression and represent mean ± S.E. for n data points. The correlation coefficient r was significant at $p<0.001$.

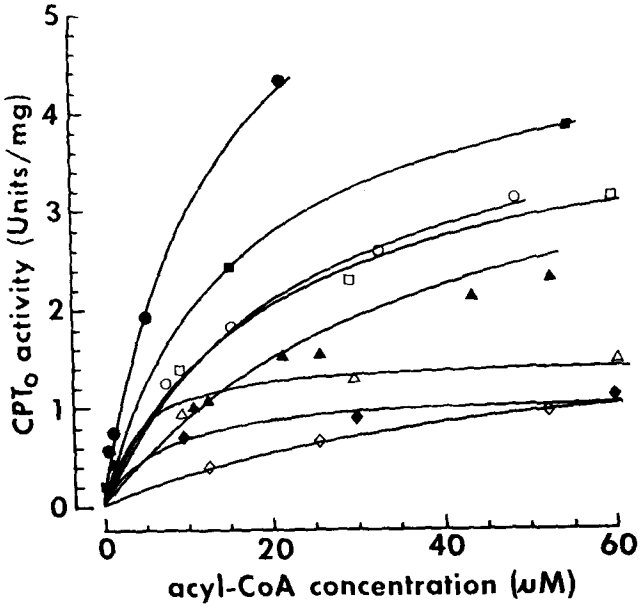


FIG. 2. CPT$_0$ initial velocity as a function of acyl-CoA substrate concentrations. Curves were generated from V_{max} and apparent $K_{0.5}$ values calculated by non-linear regression. Each point represents the average of 5 experiments. The fatty acyl-CoA substrates are: (○) 16:0; (◆) 18:0; (▲) 18:1; (■) 18:2; (●) 18:3ω3; (△) 20:4; (□) 20:5ω3; (◆) 22:6ω3.

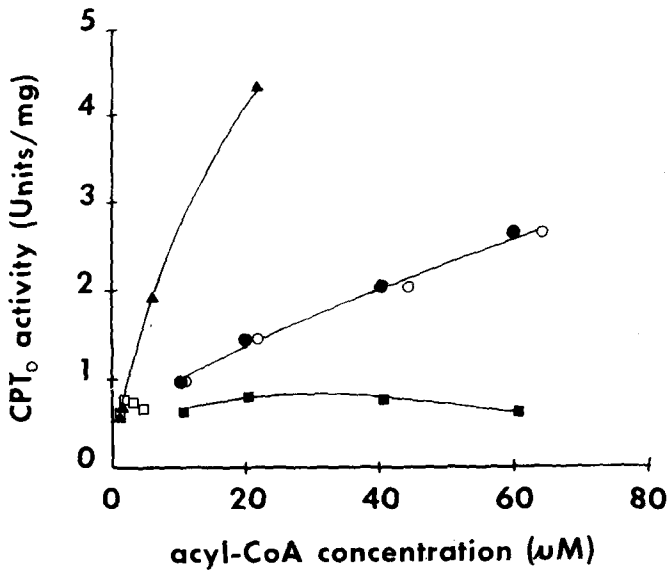


FIG. 3. Apparent differences in the CPT$_0$ substrate-velocity plots due to use of nominal concentrations of impure 18:3ω3-CoA preparations. The curves are: i) nominal (●) and analyzed (○) concentrations of 18:1-CoA; ii) commercial nominal (■) and analyzed (□) concentrations of 18:3ω3-CoA; and iii) (▲) analyzed concentration of synthesized 18:3-CoA.

and is therefore an interpolation rather than an extrapolation. The data agree with those of Borgeson *et al.* (19) for 18-carbon acyl-CoA up to 18:3ω3-CoA and provide an enzymatic rationale for the observation by Björntorp (20) and by Reid and Husbands (21) that the ketogenic potential of fatty acids increases with increasing unsaturation for the 18-carbon fatty acids. In contrast to our data, Clouet *et al.* (22) reported that CPT$_0$ activity for 18:3ω3-CoA was consistently lower than for either 18:1- or 18:2-CoA. We do not know the reason for the discrepancy. We have found, however, that all commercial preparations of 18:2-CoA and 18:3ω3-CoA we received were not more than 20% pure, analyzed immediately upon receipt (data not shown). Figure 3, for example, shows that the use of the commercial preparation of 18:3ω3-CoA at nominal concentrations yields data similar to those of Clouet *et al.* (22). Analysis of the true concentrations of the commercial 18:3ω3-CoA in the assay mixture and correction of the data for actual purity shows a different trend. In contrast, 18:3ω3-CoA that we synthesized and that we determined to be over 98% pure yields activities that are consistently higher than any of the various acyl-CoA tested. Finally, commercial 18:1-CoA that was more than 98% pure when received yielded identical results to laboratory-synthesized 18:1-CoA.

We tested the corresponding NEFA forms of the fatty acid substrates to determine whether mitochondrial fatty acyl-CoA synthetase, an obligatory enzyme when the substrate is NEFA, affects the order of reactivities determined for the analogous acyl-CoA substrates. As with fatty acyl-CoA, reaction progress curves were linear within the 6-min measurement period. The results of the regression analysis on the initial velocity-NEFA concentration data are shown in Table 2. As indicated by the correlation coefficients, the kinetic data for NEFA, similarly to those for acyl-CoA (Table 1), fit a hyperbolic

TABLE 2

CPT$_0$ Apparent $K_{0.5}$ and V_{max} for Non-Esterified Fatty Acid Substrates[a]

Fatty acid	$K_{0.5}$ M × 10^8	V_{max} nmol/mg × min	r	n
16:0	4.9 ± 0.8	3.4 ± 0.3	0.93	36
18:0	19 ± 9	1.2 ± 0.4	0.84	31
18:1	11 ± 3	3.2 ± 0.6	0.92	35
18:2	10 ± 2	5.1 ± 0.7	0.95	33
18:3ω3	12 ± 5	9.0 ± 2.5	0.91	32
20:4	4 ± 2	0.8 ± 0.2	0.57	31
20:5ω3	7 ± 2	1.7 ± 0.1	0.92	16
22:6ω3	1.3 ± 0.4	0.28 ± 0.02	0.80	15

[a]Values were derived from non-linear regression and represent mean ± S.E. for n data points. The correlation coefficient r was significant at $p<0.001$.

model except for 20:4. At 0.10 μM concentrations of unbound NEFA, the reactivities in decreasing order are: 18:3ω3 > 18:2 > 16:0 > 18:1 > 20:5ω3 > 20:4 > 18:0 > 22:6ω3. The comparison was made at 0.10 μM substrate, a concentration included within the range of all the NEFA tested. The results for the 18-carbon NEFA are consistent with data reported by Clouet *et al.* (22). We did not test for possible sigmoidicity; however, important aspects of this experiment are that: i) BSA-binding of NEFA was taken into account in the calculations; and ii) the kinetics of dissociation of NEFA from BSA was ruled out as a factor in overall rates of acylcarnitine synthesis. First, we verified and confirmed the data of Spector *et al.* (14) on BSA-binding of NEFA under CPT$_0$ assay conditions. We then evaluated BSA-binding for 18:3ω3, 20:4, 20:5ω3 and 22:6ω3, for which data are not available, using the

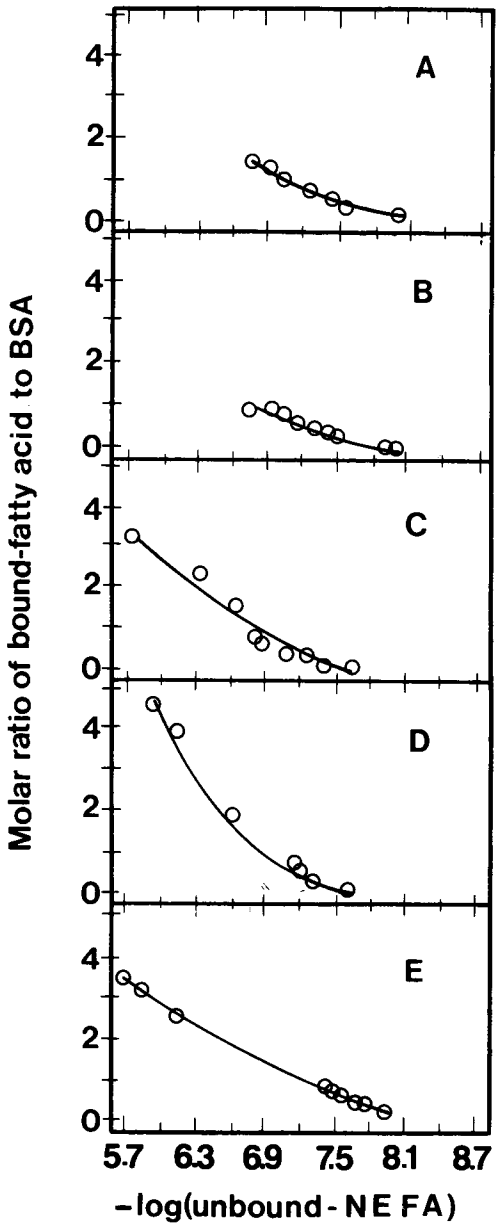


FIG. 4. Fatty acid binding to BSA. The heptane partition method was used to determine unbound-NEFA molar concentration as a function of the bound-NEFA to BSA ratio (14) for the following NEFA: A, 18:1; B, 18:3ω3; C, 20:4; D, 20:5ω3; E, 22:6ω3. Each point represents a duplicate determination.

heptane equilibrium partition method (14) under our CPT_0 assay conditions (Fig. 4). These data allowed us to use unbound NEFA instead of total NEFA concentration for the evaluation of apparent $K_{0.5}$ (Table 2). We did not take the further step of evaluating NEFA binding to mitochondrial proteins or membranes because we considered this phenomenon to be integral to acylcarnitine synthesis by intact mitochondria and, hence, physiologically relevant. Second, we verified that the dissociation rate of NEFA from the BSA complex was not limiting acylcarnitine synthesis. Table 3 shows that the rate of dissociation of NEFA from the BSA complex ranged from 10 to 330 times the rate of acylcarnitine synthesis.

In comparing kinetic data from acyl-CoA to those from NEFA experiments, it is understood that the apparent $K_{0.5}$ values for various acyl-CoA are overestimates. Therefore, in reality, the V_{max} for each of these acyl-CoA substrates is approached more rapidly than would be predicted from $K_{0.5}$ listed in Table 1. In contrast, the estimates for apparent $K_{0.5}$ for NEFA are devoid of BSA effects and is therefore more indicative of the physiological situation. The NEFA and corresponding acyl-CoA forms of either 18:3ω3 or 18:2 have similar V_{max}, suggesting that mitochondria can utilize both forms of these substrates with equal facility. We observed larger discrepancies between the acyl-CoA and NEFA forms of the 20- and 22-carbon polyunsaturated fatty acids. The ratio of V_{max} between the acyl-CoA and NEFA forms are 4.3 for 22:6ω3; 2.5 for 20:5ω3; and 1.8 for 20:4. This suggests that mitochondria use the cellular pools of the acyl-CoA and NEFA forms of these fatty acids differentially, and that mitochondrial fatty acyl-CoA synthetase, an additional obligatory enzyme in acylcarnitine synthesis from NEFA, may be responsible for this effect.

Derrick and Ramsay (23) reported that peroxisomal CPT accounts for 20% of the total activity in liver from adult fed rats. Peroxisomal contamination of our mitochondrial preparations was reduced to 6% relative to the homogenate, confirming the report of McGarry *et al.* (7). Therefore, peroxisomal contribution to the CPT activity in our final mitochondrial preparations should be no more than 1.2%. Furthermore, Clouet *et al.* (22), in comparing classical mitochondrial preparations to purified preparations devoid of peroxisomes reported that purification did not change the order of reactivities for the lipid substrates tested. Thus, it is unlikely that peroxisomal contamination was a problem in our experiments.

Figure 2 suggests that the absolute rates of acylcarnitine synthesis from the various fatty acid substrates will depend not only on substrate availability but also on the proportion of each fatty acid relative to the total substrate pool. Thus, the absolute rate of acylcarnitine synthesis from 18:3ω3 would be lower than for 16:0 if the proportion of 16:0 far exceeded that of 18:3ω3. However, when individual fatty acid pool sizes are taken into account, the turnover rate for 18:3ω3 is actually always faster than that for 16:0 within the concentration range used in our experiments. The reactivity of 18:3ω3 to CPT_0 is consistent with the well-known observation that 18:3ω3 is generally found only in minor quantities in esterified cellular lipid even when the diet contains significant quantities of this fatty acid (24).

Acylcarnitine synthesis is lowest for the 20- and 22-carbon polyunsaturated fatty acids. This is to be expected from a teleological point of view—the cell expends significant energy in chain elongation and desaturation of the shorter chain precursors of these 20- and 22-carbon polyunsaturated fatty acids and, therefore, they are to be protected from oxidation. It is understood that acylcarnitine synthesis is only one factor in the control of the hepatic flux of NEFA (Fig. 1). Other factors, such as desaturation of 18:0 to 18:1 and phospholipid synthesis from 18:1, 18:2 and the 20- and 22-carbon polyunsaturated fatty acids, may pre-empt oxidation.

The combined kinetic behavior of systems of enzymes acting on a common substrate, referred to as the branch-

TABLE 3

Comparison of CPT_0 Activity with the Initial Rates of Dissociation of NEFA from BSA

Fatty acid	FFA/BSA molar ratio	Dissociation rate (μmol/min)[b]	CPT_0[a] (μmol/min)	Ratio of rates dissociation/CPT_0
16:0	1.49	59.9 ± 15.1	1.16	51.6
16:0	0.27	19.7 ± 7.8	0.28	68.9
18:1	0.88	77.4 ± 15.4	0.77	100
18:3ω3	0.73	31.7 ± 5.4	1.81	17.5
18:3ω3	0.076	3.7 ± 0.9	0.37	9.9
20:4	0.93	39.3 ± 11.7	0.32	123
20:5ω3	1.49	74.2 ± 5.4	0.51	146
22:6ω3	0.68	29.7 ± 5.6	0.09	331
22:6ω3	0.062	1.60 ± 0.04	0.03	53.3

[a]Corresponding CPT_0 activity for each unbound NEFA concentration was interpolated from the data in Table 2 using the derived V_{max} and apparent $K_{0.5}$.

[b]Mean ± S.E.M. (n=6).

point effect, is beginning to be appreciated (25). An appreciation of the significance of the branchpoint effect to systems of lipid enzymes confronted with multiple analogous but kinetically different substrates requires data such as is reported in this paper. An understanding of the kinetics of such integrated lipid metabolism will provide an insight to the control of the partitioning and channelling of fatty acyl equivalents by the liver. This aspect will be pursued in future studies.

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Eicosapentaenoic Acid Inhibits Tube Formation of Vascular Endothelial Cells *in Vitro*

Toshie Kanayasu[a,*], Ikuo Morita[a], Junko Nakao-Hayashi[b], Noriko Asuwa[c], Chiho Fujisawa[a], Toshiharu Ishii[c], Hideki Ito[b] and Sei-itsu Murota[a]

[a]Department of Physiological Chemistry, Faculty of Dentistry, Tokyo Medical and Dental University, Yushima, Bunkyo-ku, Tokyo 113, [b]Department of Endocrinology and Metabolism, Tokyo Metropolitan Geriatric Hospital, Tokyo, and [c]Department of Pathology, Tokyo Medical College, Tokyo, Japan

We previously have described a quantitative angiogenesis *in vitro* model, in which endothelial cells are cultured between two layers of type I collagen gel and become organized into tube-like structures. Using this model, the effect of eicosapentaenoic acid (20:5n-3) on tube formation was investigated. When the endothelial cells isolated from bovine carotid artery were treated for 2 days with 5 μg/mL of arachidonic acid (20:4n-6), eicosapentaenoic acid or docosahexaenoic acid (22:6n-3), these polyunsaturated fatty acids were extensively incorporated into cellular phospholipids. The content of arachidonic, eicosapentaenoic and docosahexaenoic acid increased from 9.58% to 23.29%, from 0.98% to 11.76% and from 6.88% to 18.40%, respectively. When the eicosapentaenoic acid-treated cells were cultured between collagen gels, the tube-forming ability of the cells was markedly inhibited. The inhibition was dose-dependent between 1.0 and 5.0 μg/mL of eicosapentaenoic acid. At 5.0 μg/mL of eicosapentaenoic acid the inhibition reached 76%. By contrast, arachidonic acid increased tube formation, and docosahexaenoic acid had no effect. To elucidate the mechanism of eicosapentaenoic acid induced inhibition of *in vitro* tube formation, we examined the effect of the acid on the proliferation of endothelial cells. Eicosapentaenoic acid at any dose (<5.0 μg/mL) had no effect on the proliferation of endothelial cells cultured on plastic plates without collagen gel. However, when the cells were cultured between collagen gels, eicosapentaenoic acid inhibited cell growth in a dose-dependent manner with maximum inhibition being observed at 2.5 μg/mL. These data suggest that eicosapentaenoic acid suppresses tube formation of endothelial cells, at least in part, *via* its inhibitory effect on cellular proliferation. Thus eicosapentaenoic acid may act as an endogenous inhibitor of angiogenesis under various pathological conditions, including tumor growth and chronic inflammation.
***Lipids* 26, 271–276 (1991).**

Angiogenesis, the generation of new blood vessels, is required not only in normal phenomena such as embryonic development, but also in pathological conditions such as tumor growth, proliferation of connective tissue, chronic inflammation, and in certain immune reactions (1). Therefore, it is important to find reliable and effective inhibitors to control pathological angiogenesis. A number of potential inhibitors were previously extracted from vascular tissues (2). Also protamine (3), angiostatic steroids (4,5), and a cancer-suppressor gene product (6) were reported to suppress neovascularization. However, there are few cases in which therapeutic or beneficial uses of these substances were demonstrated.

There are a number of papers, however, showing that eicosapentaenoic acid (EPA) affects various biological functions of endothelial cells. For example, EPA increased the generation of endothelium-derived relaxing factor (7) and decreased platelet-derived growth factor-like protein (8). It is commonly accepted that angiogenesis depends on endothelial cell functions, such as migration and proliferation. In the present paper, we demonstrate that EPA suppresses the tube-forming activity of vascular endothelial cells *in vitro* which may be beneficial for treatment of some angiogenic diseases.

MATERIALS AND METHODS

EPA and docosahexaenoic acid (DHA) were kindly supplied by Nippon Oil & Fat Co. (Tokyo, Japan). The purity of EPA and DHA was determined by capillary gas chromatography. EPA was 95% pure with contaminations consisting of 1.4% arachidonic acid (AA), 1.2% DHA, 1.3% 18:4, 0.7% 18:3 and 0.4% 18:2. DHA was 94.6% pure with contaminations consisting of 1.0% AA, 0.5% EPA and 3.9% 22:5. The stock solutions of EPA and DHA were kept under nitrogen gas to minimize oxidation. Vitrogen-100 was obtained from Collagen Co. (Palo Alto, CA). Eagle's minimum essential medium (MEM) was purchased from Gibco (Grand Island, NY). Fetal bovine serum (FBS) was obtained from Irvine Scientific (Santa Ana, CA).

Culture of endothelial cells and tube formation analysis. Endothelial cells were isolated from bovine carotid arteries and maintained as described previously (9). At confluence, the aortic endothelial cells showed the typical cobblestone patterned monolayer and stained positively with 1,1′-dioctadecyl-[3,3,3′,3′-tetramethylindo]carbocyanine perchlorate labeled acetylated low-density lipoprotein. Endothelial cells with passage numbers 7–14 were used for the present study.

The method used for examining tube formation of endothelial cells was previously reported (10). Briefly, the experiments on tube formation were conducted in triplicate in 12-multiwell dishes (Terumo Corp., Tokyo, Japan). Onto 0.75 mL of collagen gel, 1×10^5 endothelial cells in 2 mL of culture medium supplemented with 10% FBS were inoculated and incubated for 24 hr. Then the medium was aspirated, 0.75 mL of collagen gel was overlaid, and 2.0 mL of culture medium supplemented with 10% FBS was added. On the second day, the morphological changes were observed under a phase contrast microscope. Each well was photographed in 4 different fields at random, and tube length was measured quantitatively with an image analyzer (revealed as mm/mm^2).

*To whom correspondence should be addressed.

Abbreviations: AA, arachidonic acid; DHA, docosahexaenoic acid; EDTA, ethylenediaminetetraacetic acid; EPA, eicosapentaenoic acid; FBS, fetal bovine serum; L, lumen; MEM, Eagle's minimum essential medium; PG, prostaglandin; PGE_2, prostaglandin E_2; RIA, radioimmunoassay; TGF-β, transforming growth factor-β.

The culture medium was collected and used to determine prostaglandin E_2 (PGE_2) and 6-keto $PGF_{1\alpha}$ production. The quantitative determination of PGE_2 and 6-keto $PGF_{1\alpha}$ was carried out by the radioimmunoassay as previously reported (9,11).

Treatment of the cells with polyunsaturated fatty acids and lipid analysis. Endothelial cells at confluence in 75 cm^2 plastic dishes (Falcon, Oxnard, CA) were treated with various concentrations of polyunsaturated fatty acid in 10% FBS-MEM. The medium was changed the next day. After 48-hr treatment with polyunsaturated fatty acid, the incubation was terminated by removing the medium and by washing the monolayer with 3 mL of ice-cold phosphate-buffered saline solution. Polyunsaturated fatty acid-treated endothelial cells were harvested with 0.05% trypsin and 0.05% ethylenediaminetetraacetic acid (EDTA) solution and resuspended in 10% FBS-MEM for tube formation assay. For fatty acid analysis of cellular phospholipids, cells were harvested by scraping, and lipids were extracted by the methods of Bligh and Dyer (12). Separation of phospholipids was by thin-layer chromatography on silica gel (E. Merck, Darmstadt, Germany) using n-hexane/diethyl ether/acetic acid (90:10:1, v/v/v) as solvent. The phospholipids were transesterified in a mixture containing 1 mL of 5% HCl-methanol. After heating at 80°C for 2 hr, the fatty acid methyl esters were extracted with hexane and separated by gas-liquid chromatography using a 0.25 mm × 30 m column with Supelcowax 10 (Hewlett-Packard 5890A, Supelco, Inc., Bellefonte, PA). The temperatures of the injector, detector and column were 250°C, 270°C and 185°C, respectively.

Electron microscopy. For electron microscopic analysis, the cells in collagen culture were fixed on the second day *in situ* with 2% paraformaldehyde and 2.5% glutaraldehyde in 0.1 M cacodylate buffer (pH 7.2) at 4°C. The collagen culture was cut into small fragments perpendicular to the surface of the gel sheet, washed with 3 changes of the same buffer at 4°C, and postfixed in 1% osmium tetroxide in the same buffer at 4°C. Samples were dehydrated by serial passage through ethanol, then were transferred to n-butyl glycidylether, and embedded in Poly/Bed 812 (Polyscience Inc., Warrington, PA). Ultrathin sections were stained with uranyl acetate and lead citrate and observed under a JEM-100B electron microscope.

Effects of EPA on cell proliferation. Effects of EPA on cell proliferation were examined under two experimental conditions. Cells were grown in a monolayer culture on a plastic plate or in three-dimensional culture between collagen gels. Cells were treated with EPA as described in the tube formation analysis. In the monolayer culture, 1×10^5 cells were inoculated and incubated in the medium supplemented with 10% FBS in 12-multiwell plastic plates. On the second day after seeding, cell numbers were determined. The cell layer was washed with 1 mL of phosphate buffered saline; then a mixture of 0.4 mL of 0.05% trypsin and 0.05% EDTA was used to remove the endothelial cells. The reactions were terminated by the addition of 0.6 mL of 10% FBS. The cells were counted with a Coulter Counter ZM (Coulter Electronics LTD., Luton Beds, England). In three-dimensional culture, 1×10^5 cells were grown between collagen gels according to the methods described in the tube formation analysis. On the second day, the medium was aspirated, the gel was transferred to a test tube, and 0.5 mL of 1% collagenase (Wako Pure Chemical Industries, Osaka, Japan) in MEM was added to the gel. The mixture was then incubated at 37°C for 15 min. After vigorous pipetting, the cells were sedimented at 1000 rpm for 5 min at 4°C. Then 0.5 mL of 0.05% trypsin and 0.05% EDTA in phosphate buffered saline was added to the cell pellet, and the mixture was incubated at 37°C for 5 min. The incubation was terminated by the addition of 0.5 mL of 10% FBS. Single-cell suspensions were obtained after vigorous pipetting. The cells were sedimented at 1000 rpm for 5 min at 4°C, resuspended in 0.3 mL of 10% FBS, and counted using a hemocytometer.

Statistical analysis. The data were analyzed by Student's t-test.

RESULTS

Effects of polyunsaturated fatty acids on tube formation. Figure 1a shows a photomicrograph of bovine carotid artery endothelial cells cultured between collagen gels. Typical tubular network formation was observed on the second day. Figure 2a shows an electron photomicrograph of part of a longitudinally sectioned tube and Figure 2b shows a cross-sectioned tube composed of three cells. As shown in Figure 2a, the tubular structure had a lumen (L) composed of several endothelial cells linked to each other by some junctional complex (arrowheads) containing pinocytotic vesicles. The effects of various polyunsaturated fatty acids on tube formation were tested in this system. When the cells were treated with AA (5 μg/mL) and applied to this system, the total tube lengths increased (Fig. 1b). In contrast, as shown in Figure 1c, EPA treatment (5 μg/mL) resulted in a decrease while DHA failed to show any effect (Fig. 1d). When tube lengths were measured with an image analyzer, AA treatment was shown to increase tube formation up to 2.5 fold ($p<0.01$). EPA treatment decreased total tube lengths by 24% relative to controls. The data are summarized in Figure 3.

EPA dose response of the inhibition of tube formation. As EPA was found to have an inhibitory effect on tube formation, we examined the dose dependency of the effect. As shown in Figure 4, EPA inhibited tube formation in a dose-dependent fashion with significant inhibition occurring at concentrations over 1.0 μg/mL. The maximum inhibition of 75% occurred at 5.0 μg/mL.

The fatty acid compositions of the phospholipids from endothelial cells, which had been treated for 48 hr with 5.0 μg/mL of each of the polyunsaturated fatty acids, are summarized in Table 1. After EPA treatment, the content of EPA in cell phospholipids increased from 0.98% to 11.76%. The content of docosapentaenoic acid (22:5n-3), a product of EPA elongation, increased from 4.21% to 16.22%, while the content of AA decreased from 9.58% to 6.57%. After AA treatment of the cells, AA content increased from 9.58% to 23.29%. After DHA treatment, DHA content increased from 6.88% to 18.4%, while EPA content increased from 0.98% to 4.33%, and AA content decreased from 9.58% to 7.59%.

Effects of EPA on endothelial cell proliferation and prostaglandin (PG) production. To shed light on the mechanism of inhibition of tube formation by EPA, we tested whether EPA does affect endothelial cell proliferation and

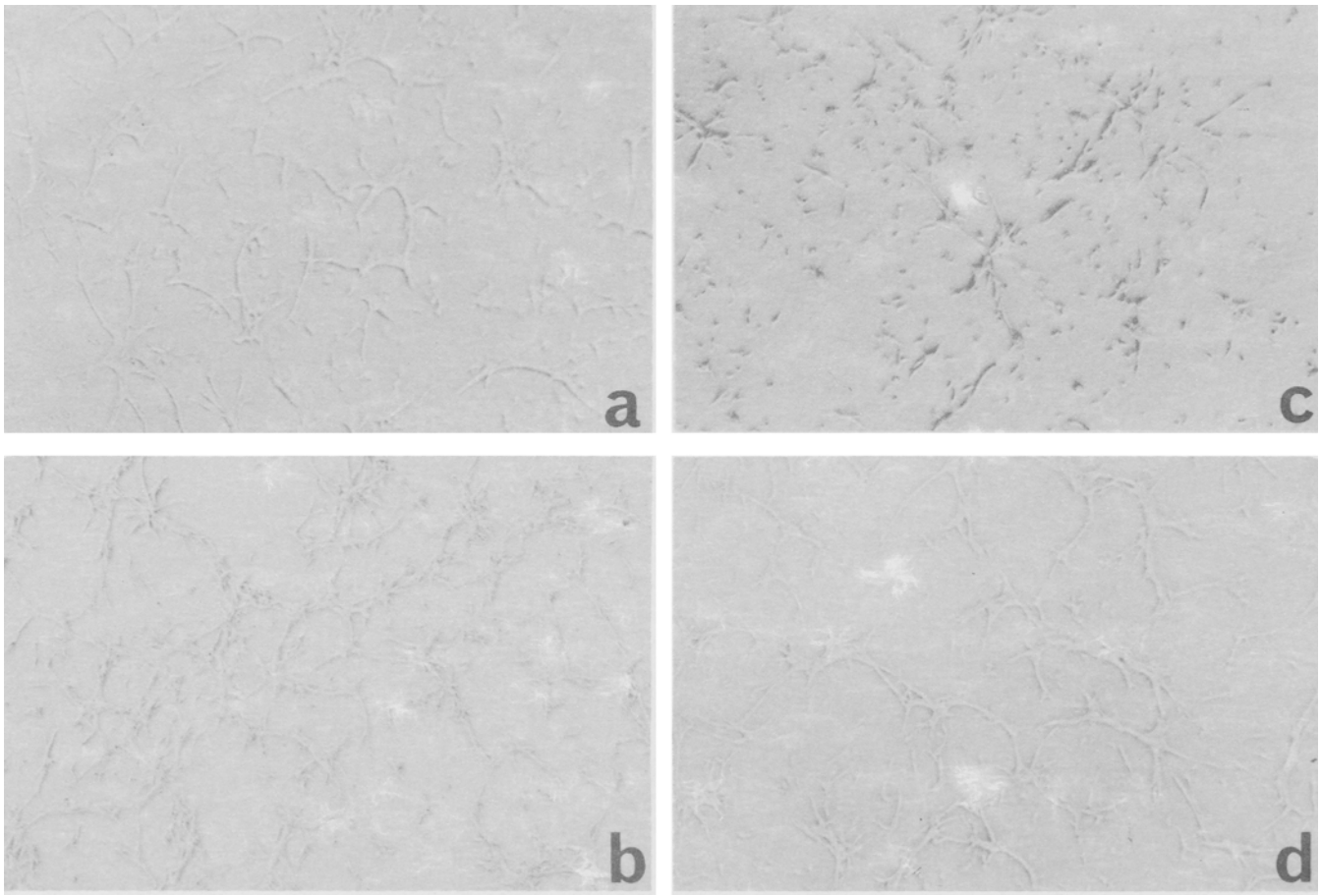


FIG. 1. ***In vitro*** **tube formation of bovine arterial endothelial cells. Endothelial cells were inoculated at the density of 1×10^5 cells/well and incubated in MEM containing 10% FBS as described in Materials and Methods. Microphotographs were taken on the 2nd day of incubation (phase contrast). a) Control, b) AA, c) EPA, d) DHA. Cells were treated with a fatty acid concentration of 5μg/mL.**

TABLE 1

Fatty Acid Composition of Endothelial Cells After Treatment with Polyunsaturated Fatty Acids[a]

	Fatty acid composition (%)							
	16:0	18:0	18:1	18:2n-6	20:4n-6	20:5n-3	22:5n-3	22:6n-3
Control	22.87	19.10	27.83	3.32	9.58	0.98	4.21	6.88
AA	29.13	20.40	16.88	2.97	23.29	n.d.	3.28	4.05
EPA	26.86	20.14	12.38	2.68	6.57	11.76	16.22	3.39
DHA	25.94	20.39	15.99	2.67	7.59	4.33	4.69	18.40

[a]Confluent endothelial cells were treated each with polyunsaturated fatty acid (5 μg/mL) for 48 hr, and fatty acid compositions in phospholipids were analyzed by capillary gas chromatography. The data represent the mean of duplicate assays of two (control) or three (polyunsaturated fatty acids) dishes. Values are the molar percentages of total fatty acids in the phospholipid fraction.

PG production. For this purpose, we first examined the effects of EPA on the proliferation of endothelial cells in two different systems, namely in monolayer culture on plastic plates and in three-dimensional culture between collagen gels. In the monolayer culture, EPA had no effect on cellular proliferation at any dose examined (Table 2). In the three-dimensional culture, cell proliferation was reduced in a dose-dependent manner by EPA treatment. Significant inhibition of cell growth was observed upon EPA treatment at doses over 2.5 μg/mL ($P<0.05$). Cell viability was checked by a dye exclusion method using 0.02% erythrosine-B; only a few of the cells were stained. By contrast, AA increased proliferation of endothelial cells in the three-dimensional culture. AA at 5 μg/mL stimulated cell growth 1.7-fold over controls (from $1.50 \pm 0.15 \times 10^5$ to $2.37 \pm 0.03 \times 10^5$, $P<0.01$).

Next, we examined the effects of pretreatment with EPA on PG production in three-dimensional culture,

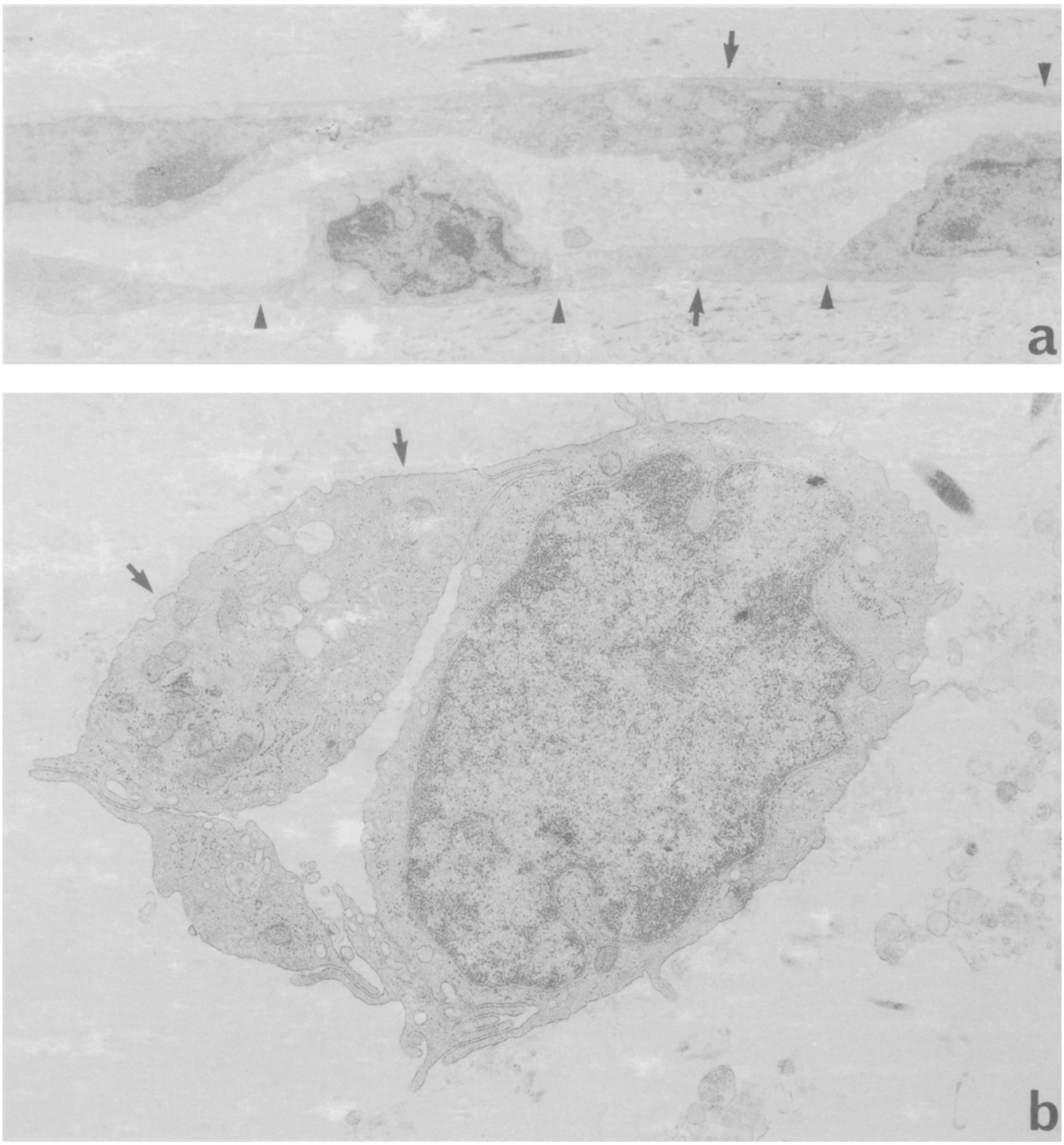


FIG. 2. Panel (a) : Part of a longitudinally sectioned tube on the 2nd day of culture between collagen gels. Endothelial cells are arranged around the narrow lumen (L) by overlapping cells (arrowheads). A number of pinocytotic vesicles are seen at the luminal side of the cells. The arrow indicates basal lamina-like material (× 12600). Panel (b) : A cross-sectioned endothelial cell tube composed of three cells. Pinocytotic vesicles are present in the peripheral part of the cells. Thin basal lamina-like material (arrows) is seen between the abluminal side of the endothelial cells and the collagen gel(× 20000).

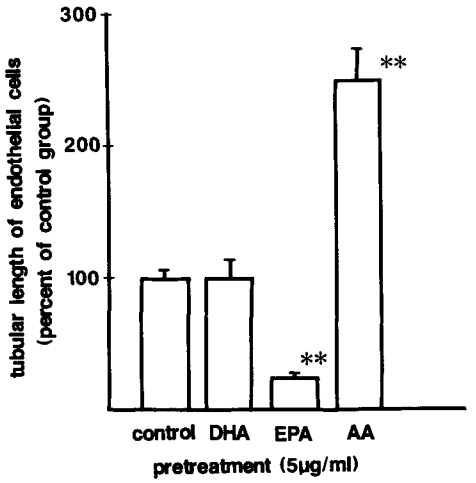


FIG. 3. Effects of EPA, DHA and AA on tube formation (image analyzer). Culture conditions were identical to those given in the legend of Figure 1. Each bar represents the mean value and SE (** P<0.01) of 4 analyses performed on 3 individual wells (n=12).

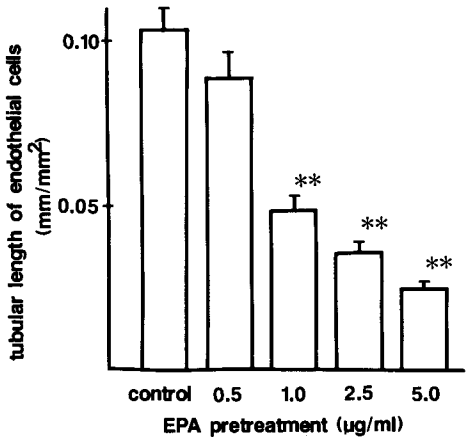


FIG. 4. Dose-dependency of the inhibitory effect of EPA on tube formation (image analyzer). Culture conditions were identical to those given in the legend of Figure 1. Each bar represents the mean value and SE (** P<0.01) of 4 analyses performed on 3 individual wells (n=12).

TABLE 2

Effects of EPA on the Proliferation of Endothelial Cells Grown in Monolayer or Three-Dimensional Cultures[a]

EPA concentration μg/mL	Cell number (× 10^5/well)	
	Monolayer culture[b]	Collagen gel culture[c]
0	6.94 ± 0.27	1.90 ± 0.15
0.5	6.54 ± 0.32	2.37 ± 0.17
1.0	6.01 ± 0.33	1.73 ± 0.18
2.5	6.72 ± 0.27	1.10 ± 0.17[d]
5.0	6.69 ± 0.46	1.13 ± 0.22[d]

[a]Data represent mean ± SE for three individual values.

[b]Cells treated with EPA were inoculated at the density of 1 × 10^5 cells/well in a plastic dish and incubated for 2 days.

[c]Cells treated with EPA were inoculated at the density of 1 × 10^5 cells/well onto collagen gel. After 1 day of incubation the cells were overlaid with collagen gel and incubated for another 2 days.

[d]Significantly different from the control, P<0.05.

TABLE 3

Effects of AA and EPA Treatment on the Release of PG from Endothelial Cells in Three-Dimensional Culture[a]

	6-keto $PGF_{1\alpha}$ (ng/mL)	PGE_2 (pg/mL)
Control	0.63 ± 0.16	13.26 ± 0.98
AA (5 μg/mL)	0.94 ± 0.17	14.06 ± 0.36
EPA (5 μg/mL)	0.72 ± 0.24	12.68 ± 0.27

[a]Cells treated with AA or EPA were inoculated at the density of 1 × 10^5 cells/well onto collagen gel. After 1 day of incubation, the cells were overlaid with collagen gel and incubated for another 2 days. On the second day, the medium was collected and 6-keto $PGF_{1\alpha}$ and PGE_2 were determined by radioimmunoassay (RIA). The data represent mean ± SE of ten (control) or five (polyunsaturated fatty acids) wells.

because some prostaglandins are known to stimulate angiogenesis (13,14). On the second day in three-dimensional culture, medium was collected and used to measure PGE_2 and 6-keto $PGF_{1\alpha}$ levels. As shown in Table 3, EPA-treated cells failed to decrease the release of both PG. AA treatment also failed to affect the level of both PG in the three-dimensional culture, but stimulated the release of 6-keto $PGF_{1\alpha}$ in the monolayer culture on plastic plates (data not shown).

DISCUSSION

It is of pathological importance that neovascularization occurs in processes such as solid tumor growth, microangiopathy, inflammation, and others. Therefore, many investigators have been searching for effective inhibitors to control these pathological phenomena. For example, D-penicillamine and gold compounds, therapeutic drugs for rheumatoid arthritis, were reported to inhibit angiogenesis (15,16). It has also been reported that fish oil ingestion resulted in the alleviation of subjective symptoms of active rheumatoid arthritis (17), and that the growth of some transplanted tumors was inhibited in rats given fish oil (18). Both diseases are accompanied by angiogenesis, and the improvement of symptoms may be due to the inhibitory effect of fish oil on neovascularization. The present data, which show that EPA inhibits *in vitro* angiogenesis, support this possibility. The mechanism of the inhibitory effect of EPA on tube formation *in vitro* still remains unclear. It is commonly accepted that several processes are essential for neovascularization, namely destruction of basement membrane by proteases, and migration and proliferation of endothelial cells and rearrangement of the tube structure. We therefore examined the effects of EPA on endothelial cell proliferation and migration. It is of interest that EPA treatment did not inhibit cell proliferation in the monolayer culture, but inhibited it in the three-dimensional collagen culture. This phenomenon was also observed with the transforming growth factor (TGF)-β, one of the well known angiogenic factors *in vivo*. Madri *et al.* (19) reported that TGF-β inhibited endothelial cell proliferation in monolayer culture; however, in three-dimensional culture TGF-β did not inhibit cell proliferation, but elicited rapid and extensive tube formation. Therefore, EPA inhibition on tube formation may be due to its effect on endothelial cell

proliferation. However, the minimum effective dose of EPA on cell proliferation was higher than that on tube formation, *i.e.*, 2.5 μg/mL and 1.0 μg/mL, respectively. It is therefore suggested that mechanisms other than the inhibition of cell proliferation may be involved in the inhibition on tube formation by EPA. However, we found no inhibitory effect of EPA on migration of endothelial cells (data not shown). Reich *et al.* (20) reported that EPA reduced invasive and metastatic activities of malignant tumor cells by a decrease in collagenase production. Therefore, specific changes in protein synthesis by EPA treatment cannot be ruled out. In contrast to EPA, AA stimulated tube formation (Fig. 1b and 3). In our previous paper (10), we reported that PGE_2 as well as leukotriene C_4 had stimulatory effects on tube formation of endothelial cells. Ohtsu *et al.* (13) reported that PGI_2 also stimulated angiogenesis in chicken chorioallantoic membranes (13). Therefore, we examined whether the inhibitory effect of EPA on tube formation was due to the inhibition of PG production. As shown in Table 3, however, the pretreatment of EPA as well as AA had no effect on PGE_2 and PGI_2 production in the three-dimensional culture. The data indicate that the inhibitory effect of EPA on tube formation might not be connected with PG production. Further experiments to elucidate the mechanism of *in vitro* inhibition of tube formation by EPA are required.

Recent studies have shown that the growth inhibitor of vascular endothelial cells *in vitro* also suppresses angiogenesis *in vivo*. For example, Maione *et al.* (21) reported that platelet factor-4 inhibited cell proliferation *in vitro* and also suppressed angiogenesis *in vivo*. We plan to examine the effects of EPA on angiogenesis *in vivo*.

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Thermal Adaptation in Biological Membranes: Functional Significance of Changes in Phospholipid Molecular Species Composition

Jeffrey R. Hazel*, E. Eugene Williams, Rochelle Livermore and **Nancy Mozingo**
Department of Zoology, Arizona State University, Tempe, Arizona 85287

Quantities of 1-palmitoyl 2-docosahexaenoyl phosphatidylcholine (16:0/22:6-PC or PDPC) increase from 24 to 40 weight percent as a consequence of cold acclimation in mitochondrial membranes of rainbow trout liver (*J. Comp. Physiol. 156,* 665–674, 1986). The present study was undertaken to assess the impact of such a large change in the proportions of a single molecular species on the fluidity, lateral packing (as sensed by phospholipase A_2), and permeability of biological membranes. These properties were examined in multilamellar liposomes prepared from binary mixtures of dipalmitoyl phosphatidylcholine (DPPC) and PDPC in proportions increasing from 10 to 40 mole% PDPC. Glucose permeability was positively correlated with both assay temperature and PDPC content. The temperature dependence of Na^+ permeability declined steadily as the mole fraction of PDPC increased; consequently, sodium permeability was positively correlated with PDPC content at 5°C, but inversely correlated at 20°C. Phospholipase A_2 activity was independent of both assay temperature and vesicle composition. Vesicles of all compositions displayed a single transition in the temperature dependence of 1,6 diphenyl-1,3,5-hexatriene (DPH) fluorescence polarization, which shifted to lower temperature and broadened as proportions of PDPC increased. At temperatures below the transition, fluidity was positively correlated with the mole fraction of PDPC, but interfacial and deeper regions of the bilayer were affected differently by variations in PDPC content. Nonelectrolyte permeability was the only index of membrane structure or function to be significantly correlated with the fluidity of the bilayer interior. The tendencies of PDPC to both fluidize the membrane and to reduce the temperature sensitivity of electrolyte permeation may promote the adaptation of membrane function to low temperature.
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Temperature acclimation in biological membranes of poikilotherms is mediated, in part, by changes in the molecular species composition of phospholipids. Growth at cold temperature generally promotes the replacement of phospholipid species possessing short-chain mono- and dienoic fatty acids with species bearing long-chain polyunsaturated fatty acids (PUFA) (1). The retailoring of membrane lipid composition permits the physical properties of structural lipids to be matched to the prevailing ambient temperature and provides a mechanism by which thermal compensation (a degree of temperature independence in fully-acclimated animals) of membrane fluidity (*i.e.,* homeoviscous adaptation) and function are attained in a fluctuating thermal environment (2).

We have shown previously that the complex patterns of acclimational change in fatty acid composition of trout hepatocyte membranes can be accounted for by adjustments in the abundance of relatively few molecular species (1). Most strikingly, the proportions of 1-palmitoyl-2-docosahexaenoyl phosphatidylcholine (16:0/22:6-PC or PDPC) increased dramatically following cold acclimation in both mitochondrial (from 24 ± 1.9 to 38 ± 1.3%) and plasma membranes (from 28 ± 1.9 to 38 ± 2.2%). In addition, didocosahexaenoyl species (DDPC) account for from 15% to 20% of the molecular species of phosphatidylethanol (PE) and phosphatidylserine (PS), respectively, in brain phospholipids of trout acclimated to 4–6°C (3). These data suggest that modulation of the content of molecular species of phospholipid enriched in 22:6 (such as PDPC) may be an important mechanism of thermal adaptation and provide the basis for thermal compensation of membrane function (1).

Although PDPC is highly unsaturated, its impact on membrane organization and function is difficult to predict *a priori* because significant changes in the level of fatty acid unsaturation are not necessarily reflected in altered physical properties (4). For example, the chain melting point of PDPC (−12°C) is almost identical to that of 16:0/16:1-PC (−10°C) (5), perhaps due to the tendency of 22:6 to adopt a helical or 'angle-iron' conformation, with a consequent reduction in cross-sectional area and effective chain length (6). Furthermore, since all PUFA are fluid at temperatures well below those normally encountered by biological systems (7), the particular advantages imparted by selective enrichment with PDPC are not evident.

Consequently, the present study was undertaken to explore the physical and functional consequences of changes in the proportions of PDPC in liposomes of defined chemical composition. Physical properties were assessed by measurements of i) membrane fluidity [fluorescence polarization of diphenylhexatriene (DPH)] fluorescence polarization; and ii) the activity of phospholipase A_2 from *Crotalus atrox.* Passive permeability to both electrolytes (Na^+) and nonelectrolytes (glucose) was employed to assess membrane function. These properties were studied in two-component liposomes of dipalmitoyl phosphatidylcholine (PC) (DPPC; 16:0/16:0-PC) and PDPC mixed in proportions that match and extend the range of PDPC content encountered in hepatocyte membranes of thermally-acclimated trout (1). The results identify several features of membranes containing elevated proportions of PDPC that we consider to be cold adaptive.

MATERIALS AND METHODS

Materials. DPPC was obtained from Sigma Chemical Co. (St. Louis, MO) and used without further purification; thin-layer chromatography (see below) revealed no

*To whom correspondence should be addressed.

Abbreviations: DDPC, didocosahexaenoyl phosphatidylcholine; DPH, 1,6-diphenyl-1,3,5-hexatriene; DPPC, dipalmitoyl phosphatidylcholine; HEPES *N*-[2-hydroxyethyl]piperazine-*N'*-[2-ethansulfonic acid]; LPC, lysophosphatidylcholine; PDPC, 1-palmitoyl 2-docosahexaenoyl phosphatidylcholine; PE, phosphatidylethanolamine; PS, phosphatidylserine; PUFA, polyunsaturated fatty acid; SPLV, stable, plurilamellar vesicles.

detectable levels of lysophosphatidylcholine (LPC) and fatty acid analysis confirmed the acyl chain composition. [^{3}H-*methyl*]DPPC (60 Ci/mmole, NET-654) and $^{22}Na^+$ (946 mCi/mmole, NEZ-081) were products of New England Nuclear (Boston, MA); [U-^{14}C]glucose (270 mCi/mmole) was from Amersham (Arlington Heights, IL). PDPC was from Avanti Polar Lipids (Pelham, AL) (results of phospholipase A_2 digestion indicated that >97% of the 22:6*n*-3 was located at the *sn*-2 position). Phospholipase A_2 was purified from the venom of *Crotalus atrox* (Sigma) as described by Wells and Hanahan (8). Diphenylhexatriene (DPH) was from Molecular Probes (Eugene, OR). Spectrapore dialysis tubing (12–14,000 MW cutoff) was obtained from American Scientific Products (McGaw Park, IL). Organic solvents were freshly redistilled prior to use (9). All other chemicals were of analytical grade and aqueous solutions were prepared in glass-distilled, deionized water. Silica gel G and Ready-Safe™ liquid scintillation cocktail were from Sigma and Beckman (Fullerton, CA), respectively.

Liposome preparation. Stable, plurilamellar vesicles (SPLV) were selected for studies of solute permeation because of their stability, uniform solute distribution, and large entrapped solute volumes, and were prepared as described by Gruner *et al.* (10). SPLV containing 10, 20, 30 and 40 mole% PDPC in DPPC were prepared by drying a total of 25 mg of lipid in $CHCl_3$ solution under a stream of nitrogen and redissolving the residue in 5 mL of diethyl ether. After adding 0.3 mL of aqueous buffer (10 mM HEPES, pH 7.4, containing 72.5 mM of both NaCl and KCl—buffer A), the ether was removed under a stream of nitrogen as the mixture was gently agitated in a bath-type sonicator. Temperature was maintained at 45°C throughout the liposome preparation to facilitate vesicle formation by DPPC. The resulting paste was resuspended in 2.0 mL of buffer A and allowed to equilibrate at room temperature for 2 hr while gently shaken. All preparative steps were performed under a nitrogen atmosphere. Finally, the SPLV preparation was diluted with an additional 3 mL of buffer A and collected by centrifugation at 10,000 × *g* for 10 min. The resulting SPLV pellet was then washed an additional two times before being resuspended in 16 mL of buffer A. Vesicles of all composition behaved as simple osmometers in concentrations of KCl ranging from 50–400 mOsm and displayed similar optical properties at 520 nm, suggesting that they were of similar size, a conclusion consistent with the small effect of variations in acyl chain length (>C_{15}) on the size of multilamellar vesicles (11).

Fluorescence polarization. The fluorescent probe 1,6-diphenyl-1,3,5-hexatriene (DPH; 2 mM in tetrahydrofuran solution) was added to SPLV at a final probe-to-phospholipid molar ratio of 0.001. Following incubation at room temperature for 1 hr (under nitrogen), steady state depolarization of fluorescence was monitored employing a Perkin-Elmer (Norwalk, CT) LS-50 luminescence spectrophotometer. Excitation and emission monochromators were set at 356 and 430 nm, respectively. The temperature of the cuvette contents was varied by means of a water-jacketed cell holder and monitored to within ± 0.1°C. Fluidity as assessed by the fluorescence polarization of DPH reflects "the rate and extent of phospholipid acyl chain excursion away from some initial chain orientation during the lifetime of the fluorescence excited state" and does not imply isotropic probe motion in an isotropic liquid hydroarbon bilayer (12).

For establishing correlations between the fluidity of vesicle lipids and various indices of membrane function, polarization data were expressed in terms of an anisotropy parameter (Eq. [1]). This parameter has been shown to be a useful indicator of relative changes in fluidity (13).

$$\text{Anisotropy Parameter} = [(r_0/r)-1]^{-1} \qquad [1]$$

In equation [1], r is the fluorescence anisotropy and r_0 is the limiting value of r at infinite viscosity. A value of 0.395 was employed for r_0 of DPH (12).

Assay of phospholipase A_2 activity. For assays of phospholipase activity, 10 μCi of [^{3}H]DPPC in chloroform was added to 25 mg of nonradioactive phospholipid prior to removal of the chloroform. A sufficient volume of 100 mM $CaCl_2$ was then added to the final resuspension of SPLV to bring the concentration in the reaction mixture to 1.6 mM. The reaction was initiated by the addition of 7 μg of purified phospholipase to an SPLV suspension containing 5 mg of phospholipid in a total volume of 3.0 mL. The reaction was stopped after 15 min by transferring triplicate (0.5 mL) aliquots of the reaction mixture into 2 mL of methanol/chloroform/water (2:1:0.8, v/v/v). Lipids were extracted by the procedure of Bligh and Dyer (14) and total lipid extracts spotted onto 500-μm plates of silica gel G (prerun in hexane/acetone, 75:25, v/v, and preactivated at 110°C for 1 hr) and developed in $CHCl_3$/MeOH/H_2O/NH_4OH (120:75:6:2, by vol). Spots were visualized with iodine vapor and regions corresponding to authentic standards of PC and LPC were scraped into liquid scintillation vials. Radioactivity was determined by liquid scintillation counting (Beckman LS-7000) employing Ready-Safe™ scintillation cocktail. CPM were converted to DPM by quench correction.

Determination of vesicle permeability (15). For determination of vesicle permeabilities, the aqueous buffer added to the ether solution of phospholipid contained either 8 μCi of ^{22}NaCl or 2 μCi of [^{14}C]glucose (at a total glucose concentration of 5 mM). After washing (in nonradiolabeled medium), the SPLV suspension (0.60 mL) was pipetted into dialysis bags which were then placed in medium (1.3 mL) of a composition identical to the vesicle contents except for the absence of isotope. The appearance of radioactivity in the medium outside the bag was determined in 100 μL aliquots by liquid scintillation counting. The initial rate of isotope diffusion (v_0) from the interior of the vesicles to the medium outside the dialysis bag was computed using the KINFIT computer program of Knack and Rohm (16). No hydrolysis of PC to LPC was detected during the first 6 hr of these experiments; later sampling times were not assayed for LPC content.

Statistics. The statistical significance of experimental treatments (assay temperature and vesicle composition) was established by one-way analysis of variance. Differences between means (in vesicles of differing composition at a single assay temperature or of single composition at variable assay temperature) were tested employing an independent *t*-test. All statistics were computed using the ABSTAT™ (Anderson-Bell, Parker, CO) statistical package.

RESULTS

Vesicle permeability. As illustrated by representative time course data obtained at 25°C (Fig. 1), solute efflux for both Na^+ and glucose increased steadily for nearly 9 hr, before declining significantly at 20 hr. Similar kinetics were observed at lower assay temperatures (data not shown). Solute effluxes (used as an index of bilayer permeability) reported in subsequent figures are expressed as the initial rate of efflux computed as a percentage of the initially trapped radioisotope.

Vesicle permeability to glucose (Fig. 2, top) increased both as a function of rising temperature and increasing content of PDPC. The latter trend was significant at all assay temperatures above 10°C. The temperature dependence of glucose permeability (*i.e.*, the slope of Arrhenius plots of v_0 *vs* 1/°K, data not shown), however, was not significantly influenced by PDPC content and activation energies ranged between 9–13 kcal/mole.

Patterns of sodium ion permeability were complex (Fig. 2, bottom) and varied with the temperature range. At 5°C, Na^+ permeability was positively correlated with the content of PDPC. However, at higher assay temperatures (20° and 25°C), Na^+ efflux was inversely correlated with PDPC content. Consistent with this pattern, the temperature dependence of Na^+ permeation was monotonically reduced as a function of PDPC content (Fig. 3). Activation energies for Na^+ permeation varied from 16 kcal/mole at 10% PDPC to 3.6 kcal/mole at 40% PDPC (intermediate values were 9.6 and 7.0 kcal/mole for 20 and 30% PDPC, respectively).

Phospholipase A_2 activity. Phospholipase A_2 from *Crotalus atrox* was employed as a probe of membrane organization since the activity of this enzyme has been shown to depend on the lateral packing of bilayer

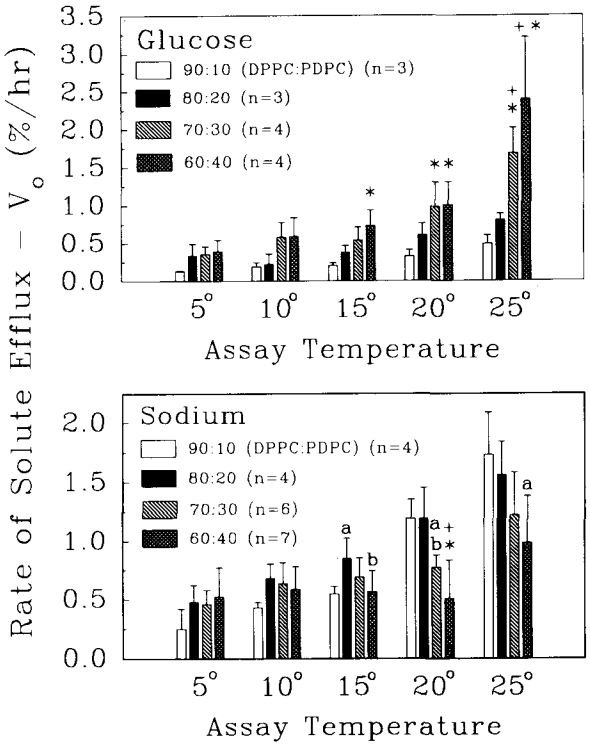


FIG. 2. Dependence of solute efflux (top, glucose; bottom, Na^+) from stable plurilamellar vesicles on assay temperature and vesicle composition. Results are presented as mean ± SD. An asterisk indicates a significant difference ($p < 0.05$) between the designated mean and vesicles comprised of 90:10 DPPC/PDPC; a plus sign indicates a significant difference in comparison to vesicles comprised of 80:20 DPPC/PDPC. Means indicated by an a or b are marginally ($0.10 > p > 0.05$) different from vesicles of 90:10 or 80:20, respectively.

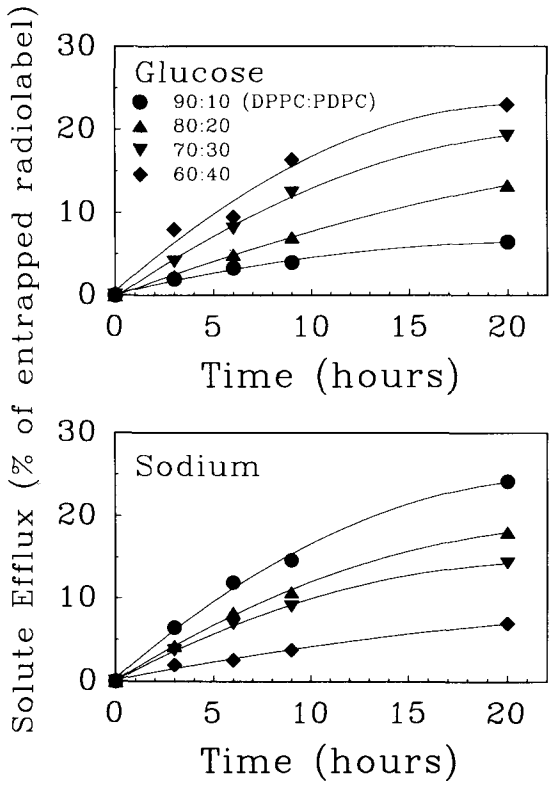


FIG. 1. Time course of solute efflux (top, glucose; bottom, Na^+) from stable plurilamellar vesicles comprised of binary mixtures of DPPC/PDPC. Results of a single typical experiment; assay temperature, 25°C.

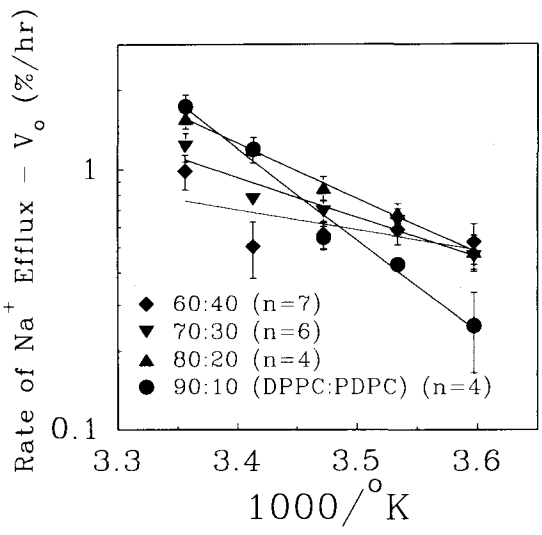


FIG. 3. Arrhenius plots for rates of Na^+ efflux from plurilamellar vesicles of DPPC/PDPC. Data are presented as mean ± SEM (n = 5). Least-square linear regression lines were calculated by SigmaPlot™ (Jandel Scientific, Corte Madera, CA).

phospholipids (17). Under the assay conditions adopted, the amount of LPC formed from vesicle preparations increased with both time and enzyme concentration (Fig. 4, top). Based on these observations, an enzyme concentration of 7 μg/assay and a time of 15 min were employed for the routine assay of phospholipase A_2 (PLA_2). PLA_2 activity was not significantly influenced by temperature in the range from 5–25°C (Fig. 4, bottom). Neither did activity of PLA_2 vary consistently with vesicle composition. Although there was a tendency for activity to decline with increasing contents of PDPC between 10 and 30%, at 40%, activity did not differ significantly from the 10% value. Consequently, PLA_2 displayed minimal activity in vesicles comprised of 30% PDPC at all assay temperatures (Fig. 4, bottom).

Fluorescence polarization measurements. The steady-state fluorescence polarization of DPH was strongly influenced by assay temperature in vesicles of all compositions (Fig. 5, top). A single transition was observed in all cases; however, the midpoint of the transition decreased (being 41.5°, 39°, 37°, 33°, and 32°C for vesicles containing 0, 10, 20, 30 and 40% PDPC, respectively) and the range of the transition broadened (from 2–3°C to 20°C) with increasing amounts of PDPC. In addition, the steady state polarization of DPH fluorescence declined as a linear function of PDPC content (up to 40%) at temperatures below 25°C (Fig. 5, bottom) and was negatively correlated with PDPC levels in all temperature ranges.

DISCUSSION

Correlations between acyl chain unsaturation and various indices of membrane function, including nonelectrolyte permeability (18), rates of cell growth (19), and rates of membrane-associated enzyme activities (20,21), have been reported. Furthermore, changes in acyl chain unsaturation generally alter membrane fluidity (22), and thus may contribute to the regulation/modulation of those membrane activities that are fluidity-dependent, including lipid (23) and water (24) permeabilities, and rates of both Na^+/H^+ exchange (25) and Ca^{++} transport in the sarcoplasmic reticulum (26). However, the relationship between the degree of acyl chain unsaturation and the physical properties of membrane lipids is not a simple function of double bond content (4). For example, whereas the melting point of dieicosanoyl PC (20:0/20:0-PC) is lowered 50°C by the introduction of a single double bond (20:0/20:1-PC) and reduced an additional 22°C by a second double bond (20:0/20:2-PC), further increases in unsaturation (in the form of 20:0/20:3-PC or 20:0/20:4-PC) have no significant additional effect on melting behavior (27). Furthermore, anomalous physical properties frequently emerge at extreme levels of acyl chain unsaturation. For example, 16:0/18:3-PC occupies a greater molecular area

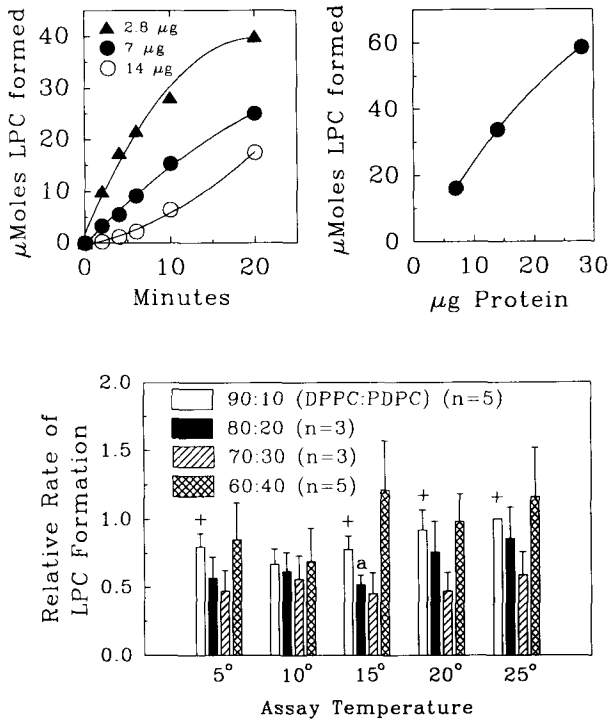


FIG. 4. Top: Lysophosphatidylcholine formation due to the action of phospholipase A_2 from *Crotalus atrox* acting on vesicles of 90:10 DPPC/PDPC at 20°C as a function of assay time (left) and enzyme concentration (right—at a fixed time of 15 min). Bottom: The influence of assay temperature and vesicle composition on rates of phospholipase A_2 activity expressed relative to the activity measured in vesicles of 90:10 DPPC/PDPC assayed at 25°C. Data are presented as mean ± SD. A plus sign indicates a marginal difference ($0.10 > p > 0.05$) between the mean designated and the mean for vesicles comprised of 70:30 DPPC/PDPC; similarly, the letter a indicates a difference ($0.10 > p > 0.05$) between the designated mean and the mean for vesicles of 90:10 composition.

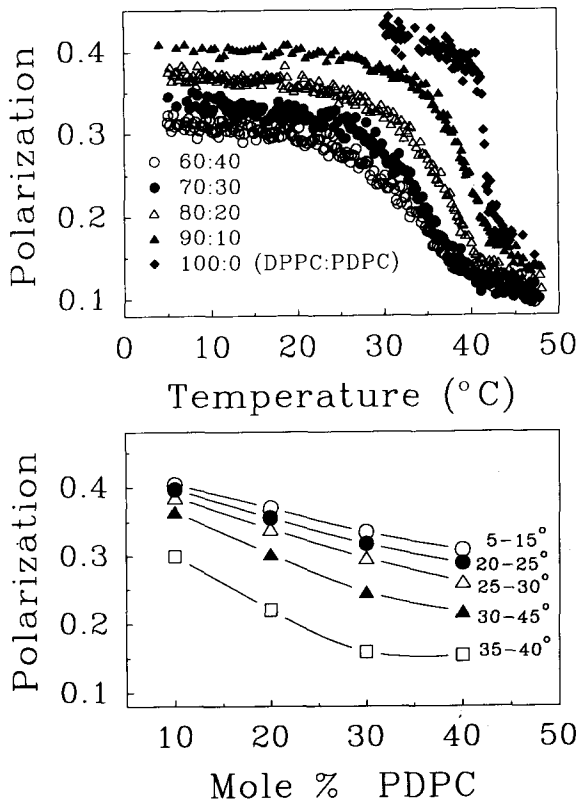


FIG. 5. Top: Effect of temperature on the fluorescence polarization of DPH in plurilamellar vesicles of DPPC and PDPC. Bottom: Effect of the mole percentage of PDPC on the fluorescence polarization of DPH at various assay temperatures.

than 16:0/20:4-PC (4) and 16:0/22:6-PC occupies less area than 18:3/18:3-PC (28), suggesting that some effective ordering is induced in unsaturated acyl chains by the presence of multiple double bonds (29). Docosahexaenoic acid (22:6*n*-3), in particular, appears to assume a compact conformation in phospholipids (28), perhaps due to a helical arrangement of the acyl chain (6). Therefore, it is not surprising that in *Dictostylium*, enrichment with fatty acids possessing three or four, rather than two double bonds did not markedly increase membrane fluidity (30). Nevertheless, the presence of highly unsaturated fatty acids in high concentration in some tissues (*e.g.*, muscle, brain, testes and retina) and their tenacious retention during dietary deprivation, indicate that long-chain PUFA play an essential role in membrane function. Temperature-induced adjustments in phospholipid molecular species composition (1) also suggest a unique role for PUFA-containing molecular species in the thermal adaptation of membrane function. The present studies identify several possible ways in which elevated proportions of PDPC may contribute to maintained membrane function at low temperature.

Fluorescence polarization data (Fig. 5, top) reveal a single, well-defined transition for all binary mixtures of DPPC/PDPC, the temperature of which declines significantly as a function of PDPC content. Polarization values at temperatures below the transition also decline steadily as a function of PDPC content, indicating that PDPC exerts a fluidizing effect. Furthermore, the fluidity of the membrane interior is a nearly linear function of PDPC content (Fig. 5, bottom) in the range of temperatures normally encountered by rainbow trout. It is not clear from the data collected whether DPPC and PDPC mix ideally, or whether the single transition reflects the melting of a DPPC-enriched phase, with the transition of a PDPC-enriched phase occurring only at subzero temperatures (31). Regardless, it is clear that PDPC has a significant effect on membrane properties, especially at temperatures below the transition. In fact, within the physiological temperature range of trout (0–25°C), PDPC content has a much more significant impact on membrane fluidity than temperature, suggesting that changes observed in the PDPC content of trout hepatocyte membranes (1) are more than adequate to counteract the direct effects of temperature on membrane fluidity. In addition, mitochondrial membranes, for which the efficacy of homeoviscous adaptation is greatest (31), also experience the largest temperature-induced changes in PDPC content (1). Elevated proportions of PDPC may also contribute to the maintenance of fluidity in biological membranes at low temperature due to a reduced tendency for PDPC (compared to more saturated species) to interact with cholesterol (28). It is worth noting that the native membranes of trout hepatocytes contain a mixture of phospholipid species much more complex than those present in the SPLV preparations. While this may account for the greater temperature dependence of DPH fluorescence polarization in native membranes (32), it should not substantially alter qualitative tendencies in membrane properties attributed to differences in PDPC content.

In order to distinguish between the effects of temperature and the fluidity of the bilayer on membrane function, indices of function are related to the anisotropy parameter in Figure 6. Of the membrane functions assayed, only

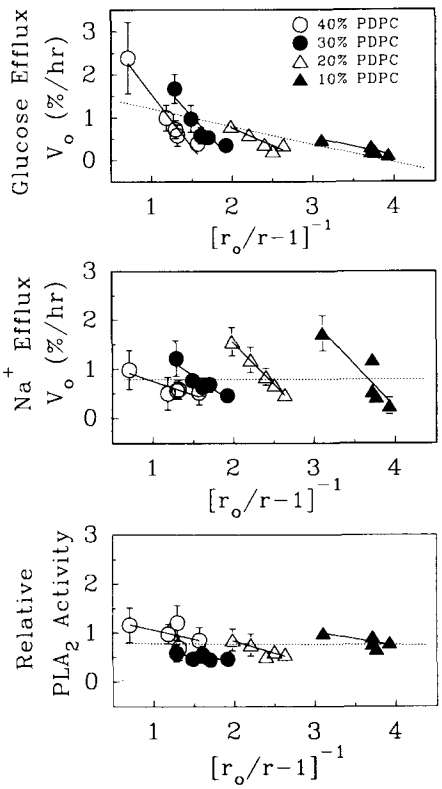


FIG. 6. Dependence of the rates of glucose efflux (top), Na^+ efflux (middle), and phospholipase A_2 activity (bottom) on the physical properties of the bilayer interior (expressed as an anisotropy parameter). Each data point within the data set for vesicles of similar composition was obtained at a different assay temperature. Data are presented as mean ± SD. The dashed line corresponds to the regression for all the data in the graph, regardless of assay temperature or vesicle compostion.

nonelectrolyte (glucose) permeation was significantly correlated ($r^2 = 0.6924$) with the physical properties of the acyl domain. For vesicles containing 10% and 20% PDPC, rates of glucose permeation closely tracked temperature-induced changes in membrane fluidity. However, in vesicles containing 30% and 40% PDPC, glucose permeation was more temperature-dependent than fluidity. Regardless, glucose permeation is negatively correlated with fluidity, suggesting that modulation of PDPC content may play a significant role in the thermal compensation of nonelectrolyte permeability. Similar correlations between the physical state of membrane lipids and bilayer permeability have been reported for lipid (23) and osmotic water fluxes (24,25).

In contrast, electrolyte (Na^+) permeation, although temperature dependent (Fig. 2, bottom) did not vary with membrane fluidity. Rates of sodium ion efflux varied in excess of three-fold at comparable anisotropy values, while similar rates of Na^+ permeation were observed at widely different membrane fluidities. Most remarkably, Na^+ permeation became significantly less temperature dependent as the concentration of PDPC increased, whereas membranes comprised of 40% PDPC were most permeable to Na^+ at 5°C, the opposite was true at 25°C. Although not causally linked to fluidity, elevated rates of Na^+ permeation, characteristic of membranes enriched in PDPC, may be a significant feature of membrane

adaptation to low temperature. Given the role of Na^+ permeation in determining the membrane potentials of excitable tissues (33), modulation of PDPC content may be a primary means of regulating electrical excitability in poikilotherms as body temperature changes.

In contrast to passive permeability, the activity of PLA_2 was not consistently influenced by either assay temperature or membrane lipid composition, which is surprising in light of reports that activity of this enzyme is sensitive to alterations in phospholipid packing (17). Although lipase activity declined with increasing PDPC content between 10 and 30%, activity rose abruptly (but not significantly) between 30 and 40% PDPC. We conclude either that: i) the phospholipase is not sensitive to changes in the fluidity of its environment; or ii) it does not experience the fluidity of the bulk phase phospholipid as detected by DPH fluorescence polarization. Since the former conclusion is not consistent with other data, it seems more likely that characteristics of the bilayer/water interface (to which the enzyme binds) are less (or differentially) affected by variations in PDPC content than those of the bilayer interior, a suggestion consistent with the dependence of nonelectrolyte permeability on both the content of PDPC and membrane fluidity.

In summary, we conclude that increased proportions of PDPC may contribute to the low-temperature acclimation of membrane function by: i) increasing the fluidity of the bilayer interior and thereby elevating nonelectrolyte (*i.e.,* glucose) permeation; and ii) decreasing the thermal sensitivity of transmembrane ion flux so that ion (*i.e.,* Na^+) permeability is higher in the cold than would be predicted from the temperature dependence of Na^+ permeation in membranes possessing only small quantitites (*i.e.,* 10%) of PDPC. Furthermore, variations in PDPC content influence interfacial phenomena (*i.e.,* PLA_2 activity) and those processes (*i.e.,* nonelectrolyte permeation) mediated by the bilayer interior in different ways.

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Hydrolysis of Neutral Lipid Substrates by Rat Hepatic Lipase

Rebecca W. Wilcox*, Tom Thuren, Patricia Sisson, Gregory L. Kucera and Moseley Waite
Department of Biochemistry, Bowman Gray School of Medicine, Wake Forest University, Winston-Salem, North Carolina 27103

Rat hepatic lipase, an enzyme whose involvement in the catabolism of lipoproteins remains poorly defined, has both neutral lipid and phospholipid hydrolyzing activity. We determined the substrate specificity of hepatic lipase for 1-oleoyl-*sn*-glycerol, 1,2-dioleoyl-*sn*-glycerol, and 1,3-dioleoyl-*sn*-glycerol in the Triton X-100 mixed micellar state, and compared these results to those obtained previously in our laboratory for the phospholipid substrates phosphatidic acid (PA), phosphatidylethanolamine (PE), and phosphatidylcholine (PC). V_{max} values were determined by diluting the substrate concentration in the surface of the micelle by Triton X-100. The V_{max} values obtained were 144 μmol/min/mg for 1-oleoyl-*sn*-glycerol, 163 μmol/min/mg for 1,2-dioleoyl-*sn*-glycerol, and 145 μmol/min/mg for 1,3-dioleoyl-*sn*-glycerol. These values were higher than those obtained earlier for phospholipids which were 67 μmol/min/mg for PA, 50 μmol/min/mg for PE and 4 μmol/min/mg for PC. In addition, the mole fraction of lipid substrate at half maximal velocity (K) in the surface dilution plot was lower for the neutral lipid substrates as compared to those obtained for the phospholipid substrates. When the hydrolysis of 1,3-dioleoyl-*sn*-glycerol mixed micelles was studied as a function of time, cleavage at the *sn*-1 and *sn*-3 positions occurred at the same rate, suggesting that hepatic lipase is not stereoselective with respect to 1,3-diacyl-*sn*-glycerol substrates. To determine if the presence of one lipid could affect the hydrolysis of the other, all possible dual combinations of 1-oleoyl-*sn*-glycerol, 1,2-dioleoyl-*sn*-glycerol, and 1,3-dioleoyl-*sn*-glycerol in the same micelle were made and the hydrolysis rate of each substrate was determined. Interaction occurred only for the 1,2-dioleoyl-*sn*-glycerol/1,3-dioleoyl-*sn*-glycerol mixture where the hydrolysis of 1,2-dioleoyl-*sn*-glycerol was slightly inhibited and that of 1,3-dioleoyl-*sn*-glycerol slightly activated compared to the predicted theoretical rate. These findings demonstrate that when presented in similar physical states, the neutral lipid substrates tested were hydrolyzed at a higher rate by hepatic lipase than the phospholipid substrates.
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Rat hepatic lipase (E.C. 3.1.1.32; HL) is located on the surface of endothelial cells lining the vascular lumen of the liver (1). The enzyme is heparin releasable and has a broad substrate specificity, hydrolyzing monoacylglycerols, diacylglycerols, and triacylglycerols as well as phospholipids *in vitro*. In some studies, *in vitro* activity was reported to be highest for trioleoylglycerol while in others phosphatidylethanolamine was found to be the preferred substrate (2–5). In addition to the hydrolysis of neutral lipid and phospholipid substrates, rat HL also has transacylase activity (6). The best acyl donors for transacylation were reported to be 1(3)-monoacylglycerol and phosphatidylethanolamine. The preferred acyl acceptor was 1(3)-monoacylglycerol although a number of primary and secondary alcohols were found to be acyl acceptors. When monoacylglycerol served as both the acyl donor and acceptor molecule, the products formed by transacylation were diacylglycerol and glycerol (7). The transacylation reaction occurred readily when pure lipids were used as substrates. In contrast, lipids in the presence of Triton X-100 (6) and in lipoproteins exhibited insignificant rates of transacylation *in vitro* (8).

The *in vivo* substrates for rat HL are plasma lipoproteins, although the exact role of HL in lipoprotein metabolism remains poorly defined. HL has been shown to hydrolyze phospholipids on the surface of HDL_2 particles *in vitro* (9), and a role for the enzyme in the conversion of HDL_2 to HDL_3 has been suggested (10,11). Involvement of the enzyme in the conversion of IDL to LDL in the VLDL-IDL-LDL cascade (12) and in the metabolism of chylomicron remnants (13–15) has also been proposed.

Here we report a study of the substrate specificity of rat HL employing 1-oleoyl-*sn*-glycerol, 1,2-dioleoyl-*sn*-glycerol, and 1,3-dioleoyl-*sn*-glycerol in mixed micelles with Triton X-100 as models for the hydrolysis of neutral lipids. Our laboratory has previously studied the substrate specificity of this enzyme for phospholipid mixed micelles (16). In mixed micelles the substrates are in relatively uniform physical states and are easily comparable. It is our goal to compare the current results to those obtained earlier with phospholipids in the same type of substrate preparation. The fact that both neutral lipid and phospholipid substrates are hydrolyzed by HL has been well established. However, to our knowledge this is the first report in which the hydrolysis of both lipid classes has been studied in a substrate system where each lipid tested has a similar physical state.

MATERIALS AND METHODS

1-Oleoyl-*sn*-glycerol, 1,2-dioleoyl-*sn*-glycerol, and 1,3-dioleoyl-*sn*-glycerol were obtained from Serdary Research Laboratories Inc. (Port Huron, MI). Triton X-100 and sodium azide were obtained from Fisher Scientific (Raleigh, NC). Tris-HCl, ethylenediaminetetraacetic acid (EDTA), and phospholipase A_2 from *Crotalus adamanteus* were obtained from Sigma Chemical Company (St. Louis, MO). Phospholipase C from *Bacillus cereus* was obtained from Boehringer Mannheim (Indianapolis, IN). Sephacryl S-200 was obtained from Pharmacia (Piscataway, NJ). Silica gel H (Merck, Darmstadt, Germany) was used for thin-layer chromatography. Silicic acid column chromatography was performed with Kieselgel 60 from EM Science (Cherry Hill, NJ). All neutral lipids were quantitated by the method of Sardesai and Manning (17). Briefly, samples were saponified and the glycerol moiety oxidized to formaldehyde. The formaldehyde was further converted to 3,5-diacetyl-1,4-dihydrolutidine, a yellow-

*To whom correspondence should be addressed at the Department of Biochemistry, Bowman Gray School of Medicine, Wake Forest University, 300 South Hawthorne Rd., Winston-Salem, North Carolina 27103.

Abbreviations: DG, diacylglycerol; HDL, high density lipoprotein; HL, hepatic lipase; IDL, intermediate density lipoprotein; LDL, low density lipoprotein; PA, phosphatidic acid; PC, phosphatidylcholine; PE, phosphatidylethanolamine; TG, triacylglycerol; VLDL, very low density lipoprotein.

colored compound which was quantitated spectrophotometrically.

Enzyme purification. Rat HL was purified using a modification (18) of the method of Jensen and Bensadoun (19). The hepatic heparin perfusate was collected in a beaker containing heparin-Sepharose in equilibration buffer. The heparin-Sepharose/perfusate mixture was then transferred to a sintered glass funnel and washed using a gentle vacuum. After the washes, the gel was packed into a column, and the enzyme was eluted with a linear gradient between 0.5 and 1.8 M NaCl. The conductivity of the fractions was determined and those fractions containing between 0.65 and 1.0 M NaCl were pooled and dialyzed. After dialysis, the enzyme was further purified by ion-exchange chromatography on DEAE-Sephacel. The specific activity of the purified enzyme preparation determined using 1-oleoyl-*sn*-[2-^{3}H]glycerol (1.5×10^3 dpm/nmol) as substrate averaged 190 μmol/min/mg.

Preparation of radiolabeled substrates. Phosphatidylcholine was purified from rabbit liver (16) and hydrolyzed by phospholipase A_2 from *Crotalus adamanteus.* The lysophosphatidylcholine was acylated with either [^{14}C]-oleic acid or [^{3}H]oleic acid using the acyl-CoA acyltransferase of rat liver microsomes to form phosphatidylcholine radiolabeled in the acyl chain primarily at the *sn*-2 position (20). After acylation, greater than 99% of the ^{14}C-labeled fatty acid and greater than 95% of the ^{3}H-labeled fatty acid was located at the *sn*-2 position as determined by snake venom phospholipase A_2 cleavage (20). 1,2-Diacyl-*sn*-glycerol labeled at the *sn*-2 position was then prepared from radiolabeled phosphatidylcholine by phospholipase C cleavage (16). To prepare 1,3-diacyl-*sn*-glycerol radiolabeled at the *sn*-3 position, purified 1,2-diacyl-*sn*-glycerol with radiolabeled fatty acid at the *sn*-2 position was applied to a silicic acid column equilibrated with $CHCl_3$. The sample was left overnight to promote migration of the acyl group from the *sn*-2 to the *sn*-3 position. Diacylglycerols were then eluted with $CHCl_3$ and purified by thin-layer chromatography in a hexane/diethyl ether/formic acid system (90:60:4, v/v/v) that separated 1,2- and 1,3-diacyl-*sn*-glycerols. Radioactivity was localized with a Bioscan radiochromatogram imaging system. The migration of authentic standards was detected by iodine staining. Regions that migrated with 1,2- and 1,3-diacyl-*sn*-glycerol standards and contained radiolabel were scraped off and extracted by the method of Bligh and Dyer (21). The radiospecific activity of 1-acyl-2-[^{14}C]-oleoyl-*sn*-glycerol was 2.4×10^4 dpm/nmol.

1-Oleoyl-*sn*-[2-^{3}H]glycerol (1.5×10^3 dpm/nmol) used in the specific activity determination for purified HL preparations and for monoacylglycerol kinetic studies was synthesized by Dr. Craig Miller (Salem College, Winston-Salem, NC).

Characterization of mixed micellar substrates. In order to determine the size characteristics of the micelles, gel filtration chromatography of mixed micellar substrates was performed on Sephacryl S-200 (2.5 × 44 cm) at a flow rate of 42 mL/hr at room temperature (V_0 = 66 mL). The column was equilibrated and run in 100 mM Tris, pH 9.0, 2 mM EDTA, 0.12% sodium azide, and 0.3 mM Triton X-100. Triton X-100 was added to the buffer at its critical micellar concentration of 0.3 mM (22) to prevent a decrease in the lipid to Triton X-100 ratio that would result from the dissociation of Triton X-100 from the mixed micelles. Each substrate was prepared at two different mole fractions of lipid, 0.1 and 0.01, and the total lipid concentration in each sample was 2 mM. Samples were applied to the column in a total volume of 0.5 mL, and 3.6-mL fractions were collected. The elution of Triton X-100 was monitored by the absorbance at 280 nm. Radiolabeled lipid (2×10^4 dpm) was added to each sample and the elution of lipid was followed by liquid scintillation counting of a 0.5-mL aliquot from each fraction.

Light-scattering analyses of micelles prepared at lipid mole fractions of 0.1 and 0.01 were carried out by Dr. Roy Hantgan (Bowman Gray School of Medicine, Winston-Salem, NC).

Enzyme assay. The hydrolysis experiments were performed as described by Kucera *et al.* (16) for phospholipid/Triton X-100 mixed micelles. Each radiolabeled substrate (5×10^4 dpm/incubation) was dried under N_2, resuspended in an aqueous solution of Triton X-100 as indicated for each experiment, vortexed, and sonicated until a homogeneous dispersion was obtained. Additional Triton X-100 was added to each substrate preparation at its critical micellar concentration of 0.3 mM (22) to maintain the appropriate molar ratio of lipid to Triton X-100 in the micelles. When mixed micelles containing two different lipid substrates were hydrolyzed, one substrate was labeled with ^{3}H (5×10^4 dpm/incubation) and the other with ^{14}C (5×10^4 dpm/incubation). Assays were run in a total volume of 0.5 mL with 100 mM Tris-HCl, pH 9.0 at 37°C for 30 min. The purpose of the hydrolysis experiments was to obtain a V_{max} value for each substrate assayed under the same conditions. An incubation time of 30 min was necessary to obtain sufficient hydrolysis over the range of substrate concentrations tested. Therefore the V_{max} values obtained are relative values for the conditions employed since the reaction was not strictly linear with time during the incubation, as will be illustrated later (Fig. 2). However, hydrolysis was directly proportional to protein concentration over the 30-min incubation period. Hydrolysis was started by the addition of enzyme and stopped by the addition of 1.5 mL $CHCl_3$/MeOH (1:2, v/v). For the diacylglycerol studies, reaction mixtures were then extracted by the method of Bligh and Dyer (21) and the radiolabeled products separated by thin-layer chromatography in a hexane/diethyl ether/formic acid system (90:60:4, v/v/v). Monoacylglycerol, diacylglycerol, and fatty acid standards were visualized by iodine staining. The corresponding regions in each sample were scraped off and radioactivity was measured by liquid scintillation counting. Hydrolysis of 1-oleoyl-*sn*-[2-^{3}H]glycerol resulted in the release of [2-^{3}H]glycerol which partitioned into the methanol/water layer of the Bligh and Dyer (21) extract while 1-oleoyl-*sn*-[2-^{3}H]glycerol partitioned into the $CHCl_3$ layer. Glycerol liberation was measured by liquid scintillation counting of an aliquot from both phases of the Bligh and Dyer (21) extraction. The $CHCl_3$ aliquot was dried under N_2 before counting.

Kinetic model. Triton X-100 mixed micelles were used as substrates to determine the specificity of HL for neutral lipids in order to compare these substrates with the phospholipids studied previously in our laboratory (16). First a saturating bulk substrate concentration was determined with mixed micelles at a constant lipid mole fraction of 0.09. For these experiments the ratio of lipid

to Triton X-100 remained constant. The total amount of each was increased proportionally resulting in an increase in the total number of micelles while the surface concentration of lipid remained the same. Then experiments were conducted at a constant saturating bulk substrate concentration in which the surface concentration of lipid was varied by changing the concentration of Triton X-100. V_{max} values were determined from the plot in which the surface concentration of lipid was diluted with Triton X-100 by nonlinear regression analysis using the program EZ-FIT (23). The substrate specificity of HL for the mixed micellar substrates was expressed in terms of V_{max} values. In addition to comparing V_{max} values, a relative affinity value (K) was determined as the mole fraction of lipid at half maximal velocity in which the surface concentration of lipid was diluted with Triton X-100. This experimental approach is similar to that originally described by Hendrickson and Dennis (24) except that the assumptions and calculations concerning the binding to and hydrolysis of substrates made earlier were not applied here.

RESULTS

Characterization of Triton X-100 mixed micellar substrates. Gel filtration chromatography and light-scattering analysis were used to determine the size characteristics of the 1-oleoyl-*sn*-glycerol, 1,2-dioleoyl-*sn*-glycerol, and 1,3-dioleoyl-*sn*-glycerol mixed micellar substrates. It was observed that micelles with higher lipid content were larger in size as determined by gel filtration chromatography. The average relative molecular mass of the neutral lipid micelles prepared at a lipid mole fraction of 0.01 was 70,000. The average relative molecular mass of the neutral lipid micelles prepared at a lipid mole fraction of 0.1 was 170,000. As an example, the elution of lipid and Triton X-100 for 1,2-dioleoyl-*sn*-glycerol micelles prepared at a lipid mole fraction of 0.01 is shown in Figure 1. A slight enrichment of lipid at the peak front was observed. This did not appear to affect results because hydrolysis of mixed micellar preparations was linear with time (Fig. 2) and individual lipid-containing fractions were hydrolyzed by HL at approximately the same rate (data not shown).

Light-scattering analysis of the three substrates showed that mixed micelles of 0.01 and 0.1 lipid mole

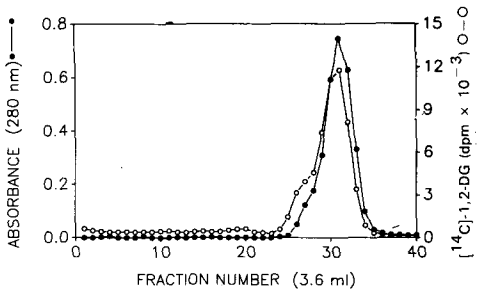


FIG. 1. Gel filtration chromatography of 1,2-dioleoyl-*sn*-glycerol mixed micelles. 1-Acyl-2-[^{14}C]oleoyl-*sn*-glycerol (2×10^4 dpm, 0.8 nmol) was added to unlabeled 1,2-dioleoyl-*sn*-glycerol (1 μmol) during mixed micelle preparation to follow lipid elution from the column. 1-Acyl-2-[^{14}C]oleoyl-*sn*-glycerol (open circles) and Triton X-100 (closed circles) were measured as described in Materials and Methods. [^{14}C]-1,2-DG, 1-acyl-2-[^{14}C]oleoyl-*sn*-glycerol.

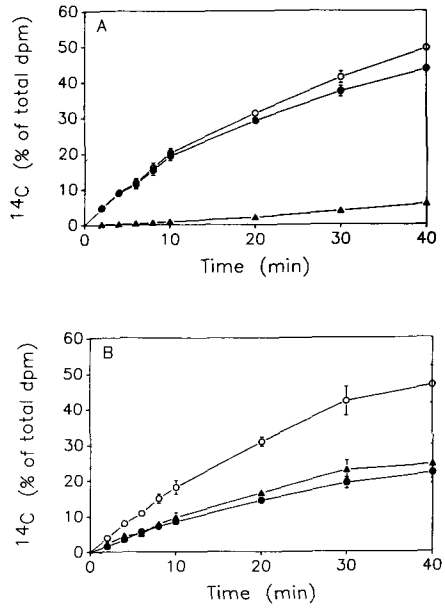


FIG. 2. Time-dependent hydrolysis by rat HL of A) 1,2-dioleoyl-*sn*-glycerol/Triton X-100 mixed micelles, and B) 1,3-dioleoyl-*sn*-glycerol/Triton X-100 mixed micelles. At each point, the initial bulk dioleoylglycerol concentration was 2 mM and the molar ratio of lipid to Triton X-100 was 0.1. Assay conditions were as described in Materials and Methods; the enzyme concentration used was 185 ng/mL. The radiolabeled substrates 1-acyl-2-[^{14}C]oleoyl-*sn*-glycerol (5×10^4 dpm, 2 nmol) and 1-acyl-3-[^{14}C]oleoyl-*sn*-glycerol (5×10^4 dpm, 2 nmol) were used to follow the hydrolysis of 1,2-dioleoyl-*sn*-glycerol and 1,3-dioleoyl-*sn*-glycerol, respectively. Enzyme activity is expressed as dpm of total diacylglycerol added to each assay recovered as monoacylglycerol (closed circles) or as free fatty acid (closed triangles); open circles show the recovery of both products in dpm. Each symbol represents the mean ± SD of two experiments done in duplicate.

fraction were homogeneous in size at each mole fraction although the 0.1 mole fraction preparations (10.4 ± 0.5 nm) were larger on average than the 0.01 preparations (7.9 ± 0.1 nm).

*Time-dependent hydrolysis of 1,2-dioleoyl-*sn*-glycerol and 1,3-dioleoyl-*sn*-glycerol mixed micelles.* Time course studies of the hydrolysis of 1,2-dioleoyl-*sn*-glycerol and 1,3-dioleoyl-*sn*-glycerol mixed micelles were conducted to determine the identity and temporal relationship of the radiolabeled products released. The hydrolysis of 1,2-dioleoyl-*sn*-glycerol was linear for the first 10 min after which the rate decreased somewhat (Fig. 2A). At 40 min, 44% of the radiolabeled 1,2-dioleoyl-*sn*-glycerol substrate was converted to radiolabeled monoacylglycerol, and 6% appeared as radiolabeled fatty acid. The release of radiolabeled fatty acid was apparently due to acyl migration from the *sn*-2 position to a primary position of the glycerol backbone of monoacylglycerol since greater than 99% of the labeled fatty acid had been determined to be at the *sn*-2 position of the original 1,2-diacyl-*sn*-glycerol substrate. We could not study the hydrolysis of triacylglycerol (TG) in this system because this substrate does not form micelles under the conditions used. However, diacylglycerol (DG) is a natural product of TG hydrolysis and is readily hydrolyzed by HL releasing 2-monoacylglycerol.

The hydrolysis of 1,3-dioleoyl-*sn*-glycerol had kinetics similar to that of 1,2-dioleoyl-*sn*-glycerol as shown by the total percentage of radiolabeled product released

(Fig. 2B). In this case [^{14}C]oleate was in position 3 since 1,3-dioleoyl-*sn*-glycerol was produced from 1-acyl-2-[^{14}C]oleoyl-*sn*-glycerol by acyl migration. Nearly equal amounts of [^{14}C]oleate and mono-[^{14}C]oleoylglycerol were formed, which suggests that the enzyme attacked positions 1 and 3 of 1,3-diacyl-*sn*-glycerol equally.

Kinetic analysis. Kinetic analysis of rat HL with 1-oleoyl-*sn*-glycerol, 1,2-dioleoyl-*sn*-glycerol and 1,3-dioleoyl-*sn*-glycerol mixed micellar substrates was performed using the surface dilution kinetic model described in Materials and Methods. When the bulk concentration of 1,2-dioleoyl-*sn*-glycerol mixed micelles with a constant lipid mole fraction of 0.09 was increased, a hyperbolic curve was obtained which approached saturation at 2 mM substrate (Fig. 3). Therefore, 2 mM lipid was chosen as a saturating concentration and used in obtaining the plot shown in Figure 4. In this plot the surface concentration of lipid was varied by maintaining the concentration of substrate at 2 mM and varying the Triton X-100 concentration (0.008 to 0.12 lipid mole fraction). The double reciprocal plots for each set of conditions were linear (mean correlation coefficient, 0.98) and are shown as insets in Figures 3 and 4. Plots resembling those in Figures 3 and 4 were also obtained for 1-oleoyl-*sn*-glycerol and 1,3-dioleoyl-*sn*-glycerol mixed micelles (data not shown); Table 1 shows the V_{max} values obtained for these three substrates. Those values for 1-oleoyl-*sn*-glycerol, 1,2-dioleoyl-*sn*-glycerol, and 1,3-dioleoyl-*sn*-glycerol were 144, 163, and 145 μmol/min/mg, respectively. We recalculated the data obtained earlier for phosphatidic acid (PA), phosphatidylethanolamine (PE), and phosphatidylcholine (PC) using the program EZ-FIT so that direct comparisons of neutral lipids and phospholipids could be made (Table 1). The V_{max} values obtained for the neutral lipid substrates were higher than those obtained for the phospholipid substrates which were 67 μmol/min/mg for PA, 50 μmol/min/mg for PE, and 4 μmol/min/mg for PC. Since we have analyzed each substrate under the same set of conditions, these V_{max} values should be a meaningful way to compare the hydrolysis of neutral lipid and phospholipid substrates realizing that these are relative values and apply only under the conditions employed. Relative affinity values were obtained for each substrate as described in Materials and Methods and are shown in Table 1. A K value of 0.02 mole fraction was obtained for both 1,2-dioleoyl-*sn*-glycerol and 1,3-dioleoyl-*sn*-glycerol. The K determined for 1-oleoyl-*sn*-glycerol was 0.05 mole fraction, somewhat higher than the value obtained for the diacylglycerol substrates. The relative affinity values for the phospholipid substrates were higher than those obtained for the neutral lipid substrates, 0.1, 0.14, and 0.11 mole fraction for PA, PE, and PC, respectively. The K values, which are the mole fractions of substrate at half maximal velocity in the surface dilution plot, suggest that the enzyme has a greater binding affinity for neutral lipid substrates reflecting the higher V_{max} values obtained.

Hydrolysis of two different lipids present in the same mixed micelle. The hydrolysis of two different lipids present in the same mixed micelle was examined to determine whether the presence of one lipid could affect the hydrolysis of the other since we had observed this earlier for phospholipid mixtures (16,25). All possible dual combinations of 1-oleoyl-*sn*-glycerol, 1,2-dioleoyl-*sn*-glycerol, and 1,3-dioleoyl-*sn*-glycerol were made. Enzyme activity plotted against the mole percent of 1,2-dioleoyl-*sn*-glycerol and 1,3-dioleoyl-*sn*-glycerol in the mixed micelles is shown in Figure 5. The total concentration of diacylglycerol was constant at each point (2 mM). Dashed lines were drawn

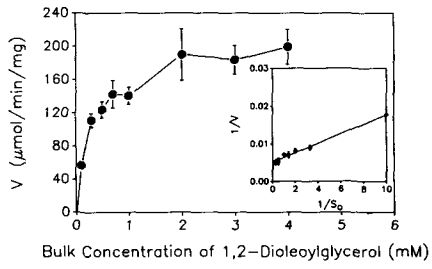


FIG. 3. Hydrolysis of 1,2-dioleoyl-*sn*-glycerol mixed micelles by rat HL as a function of the bulk 1,2-dioleoyl-*sn*-glycerol concentration. The mole fraction of dioleoylglycerol was held constant at 0.09. Assay conditions were as described in Materials and Methods; the enzyme concentration used was 18.5 ng/mL. The radiolabeled substrate 1-acyl-2-[^{14}C]oleoyl-*sn*-glycerol (5 × 10^4 dpm, 2 nmol) was added to follow the hydrolysis of 1,2-dioleoyl-*sn*-glycerol. Each symbol represents the mean ± SD of two experiments done in duplicate. The inset is the double reciprocal plot of the velocity *vs* bulk 1,2-dioleoyl-*sn*-glycerol concentration curve.

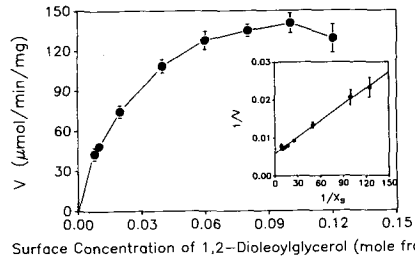


FIG. 4. Hydrolysis of 1,2-dioleoyl-*sn*-glycerol mixed micelles by rat HL as a function of the surface concentration of 1,2-dioleoyl-*sn*-glycerol. The bulk 1,2-dioleoyl-*sn*-glycerol concentration was held constant at 2 mM while the substrate concentration on the surface of the micelle was diluted with Triton X-100. Assay conditions were as described in Materials and Methods; the enzyme concentration used was 185 ng/mL. The radiolabeled substrate 1-acyl-2-[^{14}C]oleoyl-*sn*-glycerol (5 × 10^4 dpm, 2 nmol) was added to follow the hydrolysis of 1,2-dioleoyl-*sn*-glycerol. Each symbol represents the mean ± SD of three experiments done in duplicate. The inset is the double reciprocal plot of the velocity *vs* mole fraction of 1,2-dioleoyl-*sn*-glycerol curve.

TABLE 1

Summary of Rat HL Kinetic Values

Substrate	V_{max} (μmol/min/mg)	K (mole fraction)
MO[a]	144	0.05
1,2-DO	163	0.02
1,3-DO	145	0.02
PA	67	0.10
PE	50	0.14
PC	4	0.11

[a]Abbreviations: MO, 1-monooleoyl-*sn*-glycerol; 1,2-DO, 1,2-dioleoyl-*sn*-glycerol; 1,3-DO, 1,3-dioleoyl-*sn*-glycerol; PA, phosphatidic acid; PE, phosphatidylethanolamine; and PC, phosphatidylcholine.

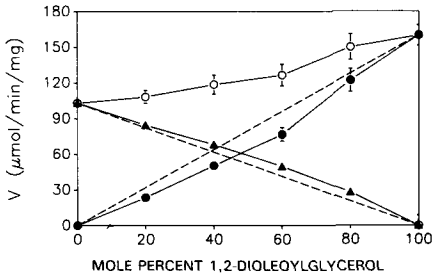


FIG. 5. Hydrolysis by rat HL of 1,2-dioleoyl-*sn*-glycerol and 1,3-dioleoyl-*sn*-glycerol present in the same mixed micelle. Mixed micelles were prepared with a constant total lipid concentration of 2 mM and diacylglycerols were mixed at different mole percents while maintaining the mole fraction of total lipid constant at 0.09. The enzyme concentration used was 100 ng/mL. The radiolabeled substrates 1-acyl-2-[^{14}C]oleoyl-*sn*-glycerol (5×10^4 dpm, 2 nmol) and 1-acyl-3-[^{3}H]oleoyl-*sn*-glycerol (5×10^4 dpm, 2 nmol) were used to follow the hydrolysis of 1,2-dioleoyl-*sn*-glycerol and 1,3-dioleoyl-*sn*-glycerol, respectively. The hydrolysis of 1,2-dioleoyl-*sn*-glycerol (closed circles), 1,3-dioleoyl-*sn*-glycerol (closed triangles), and total hydrolysis (open circles) calculated as the sum of the two is shown. Each symbol represents the mean ± SD of two experiments done in duplicate. Dashed lines are drawn from activity at 0 mole percent of each substrate to 100 mole percent.

to represent the predicted rate of hydrolysis of each substrate due to dilution by the addition of the alternate substrate. The hydrolysis of 1,2-dioleoyl-*sn*-glycerol increased with increasing mole percent to a final value of 150 μmol/min/mg at 100 mole percent. This hydrolysis appeared to be inhibited slightly by the presence of 1,3-dioleoyl-*sn*-glycerol since it fell below the predicted rate. The hydrolysis rate of 100 mole percent 1,3-dioleoyl-*sn*-glycerol was slightly lower than that of 100 mole percent 1,2-dioleoyl-*sn*-glycerol similar to the results reported in Table 1. 1,3-Dioleoyl-*sn*-glycerol hydrolysis decreased with increasing mole percent 1,2-dioleoyl-*sn*-glycerol at a slightly activated rate. In the other substrate combinations, 1-oleoyl-*sn*-glycerol plus 1,2-dioleoyl-*sn*-glycerol and 1-oleoyl-*sn*-glycerol plus 1,3-oleoyl-*sn*-glycerol, hydrolysis rates fell along the theoretical lines representing the predicted rate of hydrolysis due to substrate dilution by the addition of the alternate substrate (data not shown).

DISCUSSION

Here we report the substrate specificity of HL for the neutral lipid substrates 1-oleoyl-*sn*-glycerol, 1,2-dioleoyl-*sn*-glycerol, and 1,3-dioleoyl-*sn*-glycerol in Triton X-100 mixed micelles and compare the results with our previous findings on phospholipid substrates (16). The physical properties of neutral lipid mixed micelles have not been studied extensively in the past although phospholipid mixed micelles have been characterized rigorously (26–28). Using gel filtration chromatography and light-scattering analysis, we found that monooleoylglycerol and dioleoylglycerol mixed micelles prepared with the same lipid mole fraction were relatively homogeneous in size although micelles which had a higher lipid mole fraction were larger than those containing less lipid. Dennis (27) reported that phospholipid micelles also changed in apparent size when the ratio of lipid to Triton X-100 was varied, and this might influence the total surface area available for hydrolysis. However, based on the phospholipid substrates tested previously (16), this does not appear to affect our results noticeably. The emphasis here is on the comparison of lipids present in a relatively uniform physical state, and since the neutral lipid mixed micelles were found to be comparable in size and homogeneity to the phospholipid mixed micelles, we reasoned that these substrates were suitable for comparison of HL action on a range of substrates.

The V_{max} values obtained for the neutral lipids were higher than those found with phospholipids although both lipid classes were hydrolyzed readily by the enzyme. The bulk lipid concentration dependent affinity of HL did not differ significantly between the two lipid classes tested (data not shown). However, the affinity of HL for substrate diluted in the surface of the micelle was greater for the neutral lipid substrates, an additional reflection of the substrate specificity of HL. In preliminary monolayer studies conducted in our laboratory, HL demonstrated the same specificity based on V_{max} values which were ranked in the following order: 1,2-diacyl-*sn*-glycerol (DG) > 1,3-DG > PE > PA > PC.

Conflicting reports have been published concerning the stereoselectivity of rat HL in rat liver post-heparin perfusate preparations with respect to the primary ester bonds. Assmann and co-workers (29) compared the hydrolysis of 1-acyl-2,3-di-*O*-alkyl-*sn*-glycerol and 3-acyl-1,2-di-*O*-alkyl-*sn*-glycerol by rat liver post-heparin perfusate and found no difference in the hydrolysis of the 1- and 3-acyl residues of these triradylglycerols. Contrary to this finding, Åkesson and co-workers (30) compared the hydrolysis of 1-*O*-alkyl-2,3-diacyl-*sn*-glycerol and 3-*O*-alkyl-1,2-diacyl-*sn*-glycerol by rat liver post-heparin perfusate and found that the ester bond at the *sn*-1 position of triradylglycerol was preferentially hydrolyzed. However, when 1-*O*-alkyl-3-acyl-*sn*-glycerol and 1-acyl-3-*O*-alkyl-*sn*-glycerol were compared (30), hydrolysis rates were the same. This suggests that the hydrolysis of diradylglycerol substrates by the post-heparin perfusate was not stereoselective with respect to the primary ester bonds. Kucera *et al.* (31) observed a stereoselectivity for the ester bond at the *sn*-1 position of phosphatidic acid since a racemic mixture of monothioester phosphatidic acid was hydrolyzed at a 10-fold higher rate than the corresponding unnatural isomer. Those results suggested that the enzyme is not stereospecific with respect to the primary ester bonds of phospholipid substrates but stereoselective, hydrolyzing the ester bond at the *sn*-1 position preferentially. Our time course study of 1,3-dioleoyl-*sn*-glycerol mixed micelles using a purified rat HL preparation suggests that the hydrolysis of diacylglycerol substrates is nonselective between primary ester bonds. Therefore it appears from the various studies described above that the enzyme is stereoselective if all three glycerol hydroxyls are esterified, but if one glycerol hydroxyl is free the enzyme does not exhibit stereoselectivity. Porcine pancreatic lipase, an enzyme related to HL, was shown to be stereoselective for the *sn*-3 ester bond during the hydrolysis of monomolecular films of 1(3)-acyl-2-amino-2-deoxy-*sn*-glycerol (diradylglycerol). This stereoselectivity increased with decreasing surface pressure. However, when triradylglycerol analogs were studied, the enzyme no longer showed a preference for the *sn*-3 position (32). Thus, it appears that the stereoselectivity of many lipases may be regulated by the number of esterified hydroxyls on the glycerol backbone.

This is the first study to compare the hydrolysis by HL of neutral lipid and phospholipid substrates present in relatively uniform physical states and demonstrates that in a model system the neutral lipid hydrolase and phospholipase A_1 activities of HL are both active. These data suggest that physiologically both phospholipid and neutral lipid in lipoproteins could be hydrolyzed by HL, and support the postulate that HL could participate in the catabolism of triacylglycerol-rich lipoproteins in the VLDL-IDL-LDL cascade as well as in the catabolism of phospholipid in the conversion of HDL_2 to HDL_3. Further, the data suggest that factors other than lipid structure itself determine which substrate molecules are hydrolyzed most efficiently by HL. Possible factors participating in the regulation of HL are lipid composition, lipid packing, and the presence of apolipoproteins.

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Lipoprotein Lipase in Rats Fed Fish Oil: Apparent Relationship to Plasma Insulin Levels

Janet K. Baltzell*, Joseph T. Wooten and **David A. Otto**
Department of Research, Baptist Medical Centers, Birmingham, Alabama 35211

To investigate the role of lipoprotein lipase (LPL) and hepatic lipase in the triacylglycerol lowering effects of fish oil, rats were fed lard (L), corn oil (CO) or menhaden oil (MO) as the primary fat source in otherwise identical diets. After 2 weeks, soleus muscle LPL differed between groups (MO>CO>L). Hepatic lipase did not differ between CO- and MO-fed rats but was elevated in L-fed rats. Adipose LPL did not differ between diet groups. Total epididymal fat weight was reduced in MO-fed rats. There was a significant positive correlation between adipose tissue weight and plasma free fatty acids. MO-fed rats had lower plasma insulin levels. Insulin was directly correlated with plasma triacylglycerol and glucose, consistent with a hyperinsulinemic, insulin-resistant state in CO- and L-fed rats, and a protective effect with MO feeding. In addition, insulin was directly correlated with adipose LPL. A negative relationship between soleus muscle LPL and insulin approached significance. Soleus muscle LPL was significantly inversely correlated with triacylglycerol. The data indicate that increased skeletal muscle LPL, in response to MO or a MO-induced decrease in insulin, may contribute to the triacylglycerol-lowering effects of fish oil. Decreased fat weight and adipose LPL and increased soleus muscle LPL and decreased plasma triacylglycerol suggest a shift from fat deposition to oxidation with MO feeding. The lack of response of hepatic lipase to MO feeding suggests that this enzyme does not contribute to the fish oil-stimulated lowering of plasma triacylglycerol *via* hepatic reuptake of very low density lipoproteins or other triacylglycerol-rich lipoproteins.
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The documented beneficial effects of fish oil consumption by both human and the rat model include a lowering of plasma triacylglycerol (TAG) and often a lowering of plasma cholesterol (CH) (1). Thus the rat is a useful model for investigating potential mechanisms underlying the observed effects of fish oil feeding. Both skeletal muscle and adipose lipoprotein lipase (LPL) and hepatic lipase (HL) have a role in regulating the level of circulating plasma lipids (2). Fish oil-induced alterations in the activities of HL or LPL could contribute significantly to the observed effects of these oils. This would be especially true regarding any changes in LPL of skeletal muscle, a tissue which represents a major proportion of total body weight. Previous reports have shown no alterations in adipose tissue and post-heparin lipoprotein lipase in response to fish oil feeding in rats (3) and humans (4,5), respectively. However, Herzberg and Rogerson (6) have recently reported increased hind leg skeletal muscle LPL in rats fed fish oil.

Current evidence suggests that fish oil intake may in some way alter insulin metabolism (7–9). We have previously observed decreased plasma insulin levels in rats fed fish oil (9) and have suggested that this may have a role in the TAG-lowering effects of fish oil. Because insulin is of importance in regulating the activities of LPL (10), it is possible that ingestion of fish oil could alter the enzyme activities. For these reasons, the present study investigating the effects of fish oil consumption on the activities of HL and LPL was undertaken.

METHODS

Feeding study. Male Sprague-Dawley rats (225–250 g; Charles River Laboratories, Inc., Wilmington, MA) were housed in suspended transparent polycarbonate cages with stainless steel wire bottoms in an animal facility maintained at 22 ± 1°C, 70% humidity, with a 12 hr light/dark cycle. Animals were acclimated to surroundings and fed a standard purified diet (AIN-76A) (11) (Research Diets, Inc., New Brunswick, NJ) for one week. Animals had free access to deionized water at all times. Following the acclimation period, rats were matched for weight and assigned to one of three diet groups (six rats/group). The fish oil diet was prepared in the laboratory by addition of menhaden oil to the basal diet (Research Diets) as previously described (12). All other diets were custom-prepared by the manufacturer. The purified diets were based on the AIN-76A diet (11) (Table 1) and provided 30% of calories (14.4% by weight) as fat. The diets contained identical ingredients, except for the source of fat. Fat calories were balanced by reduction in carbohydrate (sucrose/corn starch, 2:1, w/w). The fat sources (Table 2) were menhaden oil (MO) (vacuum stripped menhaden oil provided by the Fish Oil Test Materials Program of the National Institutes of Health and the National Marine Fisheries Service), corn oil (CO), and lard (L). Corn oil was added to the MO and L diets (corn oil providing 4% of the 30% fat calories) to prevent essential fatty acid deficiency. As previously described (9,12) the diets were balanced for cholesterol (4.10 mg/g oil), β-sitosterol (9.68 mg/g oil) and antioxidant content (α- and γ-tocopherols, and *tert*-butylhydroquinone, 1.40, 1.20, and 0.2 mg/g, respectively). Rats consumed the diets *ad libitum* for two weeks, and were fasted overnight prior to sacrifice. We have shown two weeks to be sufficient time to observe MO-induced changes (9).

The rats were killed by exsanguination from the abdominal aorta following nembutal anesthesia (5 mg/100 g body weight, IP). Plasma was collected as described previously (9) from whole blood following centrifugation at 500 x *g* for 20 min at 4°C. Livers were removed, weighed, freeze-clamped, and ground into a powder under liquid nitrogen. Soleus muscles and epididymal fat pads were collected and immediately frozen in liquid nitrogen. All samples were stored at −80°C prior to assay.

*To whom correspondence should be addressed at Baptist Medical Centers, Department of Research, 701 Princeton Avenue, Birmingham, AL 35211.

Abbreviations: CH, cholesterol; CO, corn oil; FFA, free fatty acids; G6PDH, glucose-6-phosphate dehydrogenase; HL, hepatic lipase; L, lard; LPL, lipoprotein lipase; MO, menhaden oil; TAG, triacylglycerol; VLDL, very low density lipoproteins.

TABLE 1

Composition of Purified Diets[a]

Ingredients	Menhaden oil		Corn oil		Lard	
	% by wt	% of calories	% by wt	% of calories	% by wt	% of calories
Casein (alcohol extracted)	22.2	20.5	22.5	20.5	22.2	20.5
DL-Methionine	0.3	0.0	0.3	0.0	0.3	0.0
Salt mix[b]	3.9	0.4[c]	3.9	0.4[c]	3.9	0.4[c]
Vitamin mix[d]	1.1	1.0[c]	1.1	1.0[c]	1.1	1.0[c]
Choline bitartrate	0.2	0.0	0.2	0.0	0.2	0.0
Cellulose	5.6	0.0	5.6	0.0	5.6	0.0
Corn starch	17.4	16.0	17.4	16.0	17.4	16.0
Sucrose	34.5	32.1	34.5	32.1	34.5	32.1
Fish oil	12.5	26.0	0.0	0.0	0.0	0.0
Corn oil	1.9	4.0	14.4	30.0	1.9	4.0
Lard	0.0	0.0	0.0	0.0	12.5	26.0

[a]Diets are based on the AIN-76A diet with substitution of fat for carbohydrate calories, thus the nutrient (protein, vitamin and minerals)/calorie ratios were unchanged from the basal diet.

[b]Salt mix (in g/kg of mixture) calcium phosphate, dibasic, 500; magnesium oxide, 24; potassium citrate, monohydrate, 220; potassium sulfate, 52; sodium chloride, 74; chromium potassium sulfate, 0.55; cupric carbonate, 0.3; potassium iodate, 0.01; ferric citrate, 6.0; manganous carbonate, 3.5; sodium selenite, 0.01; zinc carbonate, 1.6; sucrose, 118.03.

[c]As sucrose.

[d]Vitamin mix (in g/kg of mixture) Vitamin A palmitate (500,000 IU/g), 0.8; Vitamin D_3 (400,000 IU/g), 0.25; Vitamin E acetate (500 IU/g), 10.0; menadione sodium bisulfate, 0.08; biotin (1%), 2.0; cyanocobalamin (0.1%), 1.0; folic acid, 0.2; niacin, 3.0; calcium pantothenate, 1.6; pyridoxine HCl, 0.7; riboflavin, 0.6; thiamin HCl, 0.6; sucrose, 979.17.

TABLE 2

Fatty Acid Composition of Menhaden Oil, Corn Oil, and Lard

Fatty acid	Menhaden oil	Corn oil	Lard
	% of total fatty acids		
14:0	7.6	Trace	1.4
14:1	0.1	—	0.1
16:0	16.6	10.7	24.5
16:1n-9	0.2	0.2	3.3
16:1n-7	10.3	—	—
18:0	3.1	2.1	15.0
18:1n-9	8.1	24.9	42.8
18:2n-6	1.2	59.9	10.5
18:3n-3	1.2	0.4	1.0
20:1	2.3	1.6	0.6
20:5n-3	14.8	—	—
22:1n-9 + n-11	0.5	—	—
22:5n-3	2.5	—	—
22:6n-3	8.7	—	—
Saturated	27.3	12.8	40.9
Monounsaturated	21.5	26.7	46.8
Polyunsaturated			
n-6	1.2	59.9	10.5
n-3	27.2	0.4	1.0
P/S ratio	1.04	4.71	0.28

Enzyme assays. LPL activities were determined using a modification of the method of Nilsson-Ehle and Schotz (13). HL was determined by the method of Nilsson-Ehle and Ekman (14). For each tissue assay system, separate trials were run to determine the ideal buffer, tissue concentration, and incubation time to achieve linearity (15). In the LPL assay, appropriate blanks, adjusted to 1 M NaCl, were run with each sample (15). All samples were run in duplicate. Partitioning coefficients of oleic acid into the fatty acid separation system were determined for each sample by including a small amount (500 dpm) of [^{14}C]-oleic acid in each sample system. Soleus muscle (approximately 50 mg) was homogenized on ice (Tissumizer, Tekmar Company, Cincinnati, OH) for four seconds (setting #65) in 80 volumes of 0.2 M Tris-HCl buffer, pH 8.3, containing 15 U heparin/mL. The homogenates were kept on ice for 30 min and then centrifuged for 15 min (300 × *g*, 4°C). Aliquots of this supernatant (100 μL) were then used for LPL determination. Epididymal fat (150–200 mg) was homogenized on ice in the same manner as the muscle tissue in 10 volumes of Krebs-Ringer-HEPES buffer (16), pH 7.4, with 15 U heparin/mL. Following chilling on ice for 30 min and centrifugation (1000 × *g*, 15 min, 4°C), 100-μL aliquots of the supernatant were used in the LPL assay. Liver (200–300 mg) was homogenized on ice in 10 volumes of Krebs-Ringer-HEPES buffer (pH 7.4) using a Potter-Elvehjem homogenizer. One-mL aliquots of the homogenates were centrifuged (6000 × *g*, 15 min, 4°C). The pellets obtained were then resuspended in 1.0 mL of 0.1 M Tris-HCl (pH 9.0) containing 15 U heparin/mL. After chilling on ice for 15 min, the samples were centrifuged again and 100-μL aliquots of the supernatants were used for the HL assay. Liver glucose-6-phosphate dehydrogenase (G6PDH) activity was assayed as previously described (9).

Other determinations. Plasma insulin levels were determined in duplicate using a standard insulin radioimmunoassay kit (Corning Medical and Scientific, Medfield, MA). Plasma TAG, CH, and glucose were measured using Baker kits with the Encore Chemistry System Centrifugal Analyzer (Baker Instruments Corp., Allentown, PA). The assay for free fatty acids (FFA) (ref. 17; Amano International Enzyme kit reagents, Troy, VA) was adapted for use on the Encore Chemistry System.

Statistical analysis. Statistical significance between groups was determined by the Tukey test using a 95% confidence level. Correlations between measured

TABLE 3

Body Weight, Liver Weight, and Fat Weight

	Corn oil diet	Lard diet	Menhaden oil diet
Body weight (g)	392.5 ± 9.26[c]	393.7 ± 6.1[c]	379.7 ± 7.2[c]
Liver weight (g)	11.7 ± 0.7[c]	12.1 ± 0.4[c]	11.8 ± 0.4[c]
Epididymal fat weight (g)	5.8 ± 0.7[c]	4.9 ± 0.5[c]	2.8 ± 0.2[d]
RLS[a] (%)	2.98 ± 0.36[c]	3.07 ± 0.14[c]	3.10 ± 0.22[c]
RFS[b] (%)	1.47 ± 0.38[c]	1.23 ± 0.30[c]	0.75 ± 0.09[d]

Values represent mean ± SEM, r = 6.

[a]RLS, relative liver size, *i.e.*, (liver weight/body weight) × 100.

[b]RFS, relative fat size, *i.e.*, (weight of fat pads/body weight) × 100.

[c,d]Values having different superscripts are significantly different, $p < 0.05$.

TABLE 4

Plasma Parameters

	Corn oil diet	Lard diet	Menhaden oil diet
Plasma glucose (mg/dL)	157.6 ± 12.1[a]	162.8 ± 10.6[a]	139.0 ± 5.2[a]
Plasma triacylglycerol (mg/dL)	121.67 ± 30.92[a]	105.67 ± 11.54[a]	40.20 ± 8.72[b]
Plasma free fatty acids (μM)	610 ± 46[a]	547 ± 25[a]	436 ± 33[b]
Plasma cholesterol (mg/dL)	61.0 ± 7.8[a]	73.5 ± 11.6[a]	29.0 ± 9.4[b]
Plasma insulin (μU/mL)	121.0 ± 23.4[a]	98.7 ± 5.0[a]	48.7 ± 11.6[b]

Values represent mean ± SEM, n = 6.

[a,b]Values having different superscripts are significantly different, $p < 0.05$.

TABLE 5

Lipoprotein Lipase, Hepatic Lipase and Glucose-6-Phosphate Dehydrogenase Activities

Tissue	Corn oil diet	Lard diet	Menhaden oil diet
Soleus LPL[a] (nmol[b]•min^{-1}•g^{-1})	318.2 ± 39.9[d]	209.0 ± 29.4[e]	444.2 ± 27.3[f]
Adipose LPL (nmol•min^{-1}•g^{-1})	97.4 ± 26.5[d]	64.5 ± 7.0[d]	89.0 ± 11.8[d]
Adipose LPL (nmol•min^{-1}•pr^{-1}[c])	593.7 ± 199.4[d]	326.0 ± 60.6[d]	260.5 ± 45.1[d]
Hepatic Lipase (nmol•min^{-1}•g^{-1})	6.74 ± 0.29[d]	10.43 ± 1.26[e]	7.07 ± 0.99[d]
Glucose-6-phosphate Dehydrogense (μmol•min^{-1}•liver^{-1})	54.75 ± 7.08[d]	76.93 ± 6.00[e]	18.55 ± 1.46[f]

Values represent mean ± SEM, n = 6.

[a]LPL, lipoprotein lipase.

[b]nmol, nmol fatty acid released.

[c]pr, Pair of epididymal fat pads.

[d,e,f]Values having different superscripts are significantly different, $p < 0.05$.

parameters were determined by least-squares regression analysis. All statistical analyses were performed using the SYSTAT statistical software package (Systat, Inc., Evanston, IL).

RESULTS

There were no differences in final body weight, liver weight, or relative liver size [(liver weight/body weight) × 100] between groups (Table 3). Plasma FFA, CH, and TAG were lower in the MO-fed rats (Table 4). Glucose did not differ significantly between groups but tended to be lower in the MO-fed *vs* the CO- and L-fed groups (Table 4).

Soleus muscle LPL activity differed significantly between diet groups (Table 5). MO-fed rats had the highest muscle LPL, followed by CO- and L-fed rats. Although epididymal adipose LPL activity per g (Table 5) did not differ significantly between groups, epididymal fat depot weight (Table 3) and relative fat size [(fat depot weight/body weight) × 100] (Table 3) were significantly reduced in the MO-fed rats as compared to CO- and L-fed rats. This resulted in a tendency for less total adipose LPL activity with MO-feeding (Table 5). HL activity, expressed per g (Table 5) or per total liver (data not shown), was significantly higher in the L-fed group compared to the CO- and MO-fed groups. Liver G6PDH activity differed significantly between groups with the lowest values in the MO-fed group and the highest in the L-fed rats (Table 5).

Plasma insulin levels were significantly lower in the MO-fed rats (Table 4). Insulin was significantly correlated with adipose LPL activity (Fig. 1). Plasma insulin also

showed significant positive correlations with plasma TAG and glucose (Figs. 2 and 3) and G6PDH (not shown, $r = 0.533$, $p < 0.05$). Soleus LPL activity was negatively correlated with TAG (Fig. 4), and exhibited a borderline significant relationship with insulin (Fig. 5).

DISCUSSION

This study stemmed from our interest in understanding the role of fish oil (ω3 fatty acids) in reducing plasma TAG. Both LPL and HL could be of potential importance if their activities were, in fact, influenced by dietary intake of marine oil. This potential contribution, combined with our finding of decreased plasma insulin levels and a significant correlation between plasma TAG and insulin in rats fed a 30% fat calorie MO diet (9) led to the present investigations.

The present results suggest that fish oil consumption, at the level used in this study, may exert either a direct or indirect (perhaps mediated *via* reduced insulin levels) influence on the activities of adipose and skeletal muscle LPL. Insulin is a key regulator of adipose LPL (18). Adipose tissue LPL is increased in situations where insulin levels are expected to be elevated, such as the fed state or with high carbohydrate-feeding (18). Adipose LPL is lower in untreated insulin-dependent diabetics, but normalizes or becomes elevated with insulin treatment (10). The significant positive correlation between adipose LPL activity and plasma insulin observed in the present study are consistent with the established role of insulin in the positive regulation of adipose LPL.

The soleus muscle LPL activity was significantly increased in the MO-fed rats in this study and demonstrated a borderline significant relationship with insulin (Fig. 5). This again may be related to decreased insulin levels, as skeletal muscle LPL activity increases in situations with reduced circulating insulin, such as starvation (19). Soleus muscle LPL was found by others to be the highest of several rat muscles sampled, but showed no

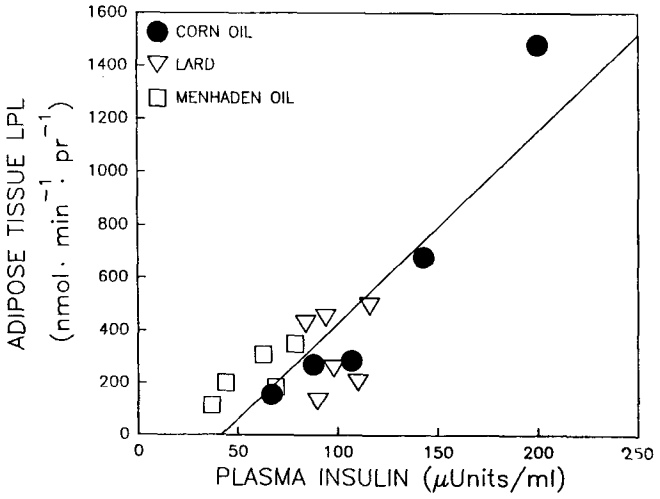


FIG. 1. Correlation between total epididymal adipose LPL and plasma insulin ($n = 16$, $r = 0.863$, $p < 0.05$).

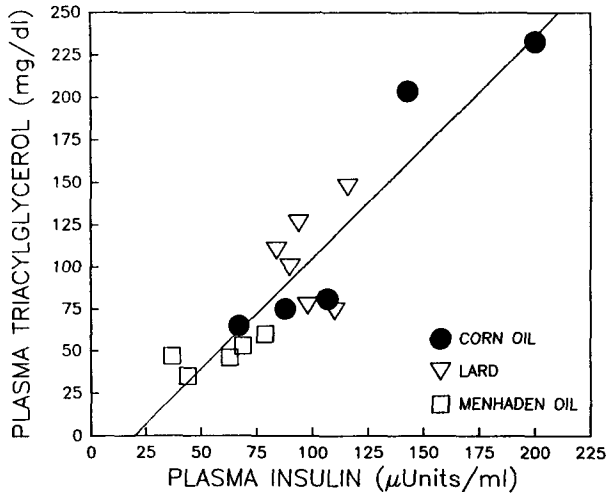


FIG. 3. Correlation between plasma glucose and plasma insulin ($n = 16$, $r = 0.517$, $p < 0.05$).

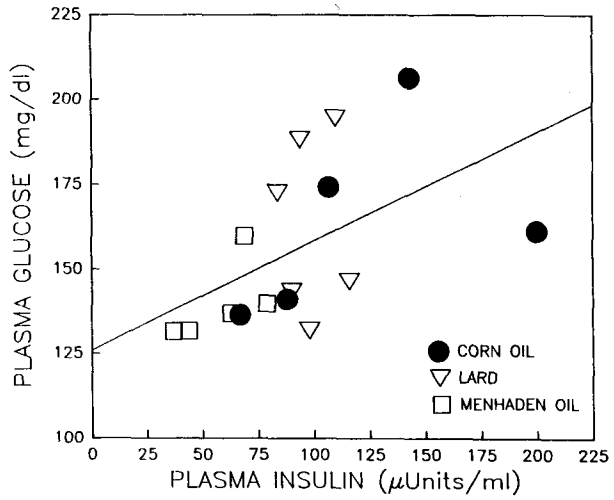


FIG. 2. Correlation between plasma triacylglycerol and plasma insulin ($n = 16$, $r = 0.903$, $p < 0.05$).

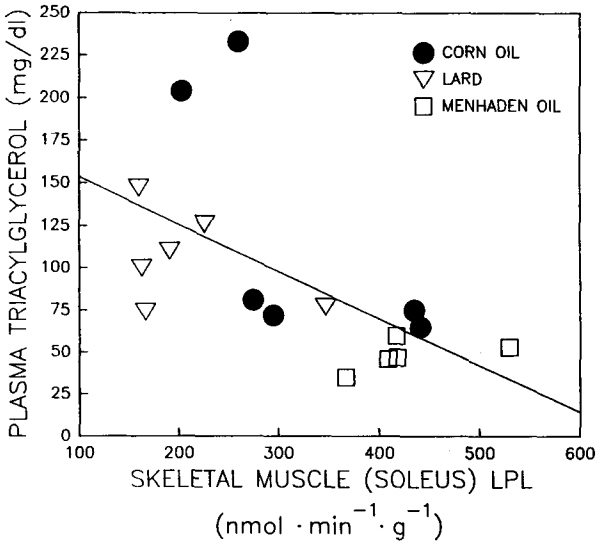


FIG. 4. Correlation between soleus LPL and plasma triacylglycerol ($n = 17$, $r = 0.590$, $p < 0.05$).

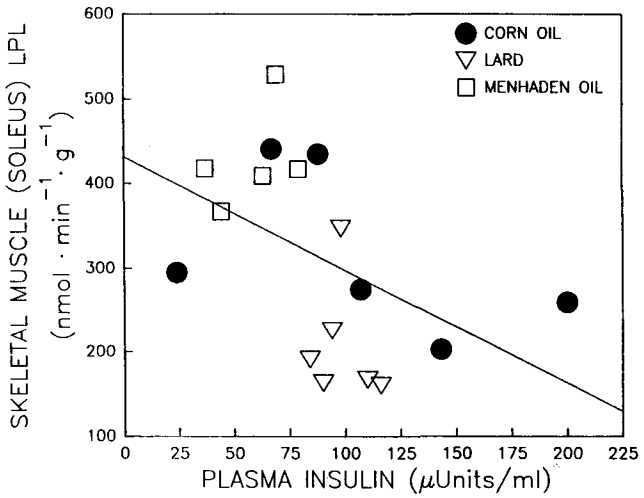


FIG. 5. Correlation between soleus LPL and plasma insulin (n = 17, r = 0.475, p = 0.059).

diet influenced difference between lard and glucose diets (67% of calories) (20). Skeletal muscle LPL activity was decreased with high-carbohydrate feeding and increased with fasting (21) and with high-fat feeding (21,22). The diets used in the present study provided 30% of total calories as fat, a percentage of dietary fat which is well above the recommended level for the rat (11), but not as high as the high-fat diets previously found to stimulate skeletal muscle LPL (21,22). Herzberg and Rogerson (6), in a recent report, demonstrated a stimulation of hind leg skeletal muscle LPL activity in rats fed fish oil (MaxEPA) at 10% by weight of diet (approximately 22% of calories). Insulin levels were not reported in this study and no effect of diet was observed on epididymal fat LPL.

In the present investigation, HL was increased in the L-fed group as compared to the MO-fed and CO-fed groups. This suggests that HL does not come under the same, possibly insulin related, regulatory system as skeletal muscle and adipose LPL. These data also provide evidence that HL does not contribute to the lowering of plasma TAG through hepatic reuptake of very low density lipoproteins (VLDL) or other TAG-rich lipoproteins.

Reaven and Chen (23) have reported that mature Sprague-Dawley rats are spontaneously insulin resistant and hyperinsulinemic. High-fat feeding may also promote this effect (24). Lower insulin and FFA levels after MO-feeding (Table 4) and the positive relationships between fasting plasma insulin and TAG, and between insulin and glucose (Figs. 2 and 3) are consistent with a protective effect of fish oil feeding against the development of an insulin resistant and hyperinsulinemic state. At first consideration, this interpretation appears in to be in contrast with reports from human diabetic studies, in which there is an apparent inhibition of insulin secretion and a worsening of glucose control with fish oil supplementation (25). However, the reduction of insulin in fish oil-fed animals in our present and previous studies (9) may be the result of decreased insulin secretion with a compensatory or independent improvement in insulin sensitivity. While this could be beneficial in normal individuals, it would be detrimental in a diabetic with an already compromised capacity for insulin secretion. Our results are consistent with a recent report showing that fish oil feeding prevents the insulin resistance induced by very high-fat feeding (59% of calories) in rats (24). Fish oil as compared to olive oil feeding has been shown to increase insulin action (glucose utilization) in adipose tissue (26). More recently, Zak *et al.* (27) observed increased glucose utilization (decreased blood glucose, decreased basal insulinemia and decreased urinary C-peptide excretion) following a glucose load in patients with primary hypertriglyceridemia treated with fish oil (10 g/day for three weeks). The authors suggested that the hypotriglyceridemic effects of fish oil were mediated by changes in insulin secretion and decreased tissue resistance to insulin. Similarly, Storlien *et al.* (28) recently reported increased insulin sensitivity (insulin-stimulated glucose metabolism) in red skeletal muscle (soleus, red gastrocnemius, red quadriceps) in rats fed ω3 fatty acids, and suggested a role for ω3 fatty acids in insulin action. Hepatic G6PDH activity (an insulin sensitive enzyme) was decreased in the MO-fed rats (Table 5) and correlated significantly with plasma insulin levels (not shown). This is in agreement with previous work in our laboratory (9) and suggests that the liver is not subject to the same fish oil-induced alterations in insulin sensitivity as observed in peripheral tissues. This is in apparent contrast to the recent report of Storlien *et al.* (28), who showed improved insulin sensitivity in both the liver and peripheral tissues. This difference may be related to the different fat concentrations used.

Soleus muscle represents a slow-twitch, primarily oxidative red muscle fiber (20). LPL activity results in the release of fatty acids from TAG. In the skeletal muscle, these fatty acids may be used as substrates for oxidation. Our finding of reduced epididymal fat depot size in the MO-fed rats is consistent with the recent report of Parrish *et al.* (29), who observed 35% less fat accumulation in the epididymal fat pads of fish oil-fed *vs* lard-fed rats, which was associated with a decreased cell volume, leading to speculation that this decrease in fat accumulation may represent reduced adipose LPL activity in the fish oil-fed animals. It is tempting to conjecture that the decreased epididymal pad weight and the increased skeletal muscle LPL activity we have observed in the MO-fed rats represent a metabolic shift from fat deposition to fat oxidation. This was similarly suggested in a preliminary report by Herzberg *et al.* (30), which demonstrated increased utilization of fatty acids by skeletal muscle from fish oil-fed rats. Since skeletal muscle contributes a substantial portion of total body weight (approximately 45% of body weight in the rat), increased fatty acid oxidation by this tissue could certainly contribute to the lowering of plasma TAG. Increased skeletal muscle fatty acid oxidation would be consistent with the proposal that fatty acid oxidation is involved in the TAG-lowering effects of fish oil (31) and the reports of increased peroxisomal oxidation in liver (32). From the present data, it is not possible to quantitate the total skeletal muscle LPL. We can only speculate that an increase in total skeletal muscle LPL in response to fish oil feeding, even with decreased adipose LPL, could result in increased catabolism of VLDL and contribute to the lowering of plasma TAG. This suggestion is strengthened by the strong correlation between soleus muscle LPL and TAG (Fig. 4). That VLDL catabolism could play a role is suggested by a recent report (33) demonstrating that a

reduction in VLDL pool size in miniature pigs fed fish oil was due, in part, to an increase in fractional catabolic rate. The possibility that insulin is involved in the TAG-lowering effect is supported by the highly significant relationship between insulin and TAG.

A further contribution to the lowering of plasma TAG could come from the significant decrease in FFA observed with the MO-fed rats (Table 4), resulting in reduced substrate for hepatic VLDL synthesis. Our observation of a positive correlation between FFA and relative fat size (not shown, $r = 0.510$, $p < 0.05$) suggests that this decrease in FFA could be due to reduced lipolysis as a result of smaller adipose TAG stores. An alternative explanation is that this results from the apparent improved peripheral insulin sensitivity caused by fish oil feeding.

This study sought to investigate the potential role of LPL and HL in the beneficial effects of fish oil feeding. The results do suggest that skeletal muscle LPL, together with other known effects on VLDL synthesis and secretion (9,31), could play a role in the reduction of plasma TAG by fish oil feeding. Our findings may also offer an explanation for the reduced postprandial TAG observed in humans following adaptation to fish oil-containing diets (34). The data further point toward changes in insulin levels and/or insulin sensitivity as a key regulator in the TAG-lowering effects of fish oil. Whether these effects will be seen at lower dietary fat levels is currently under investigation.

ACKNOWLEDGMENTS

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Methyl-Branched Octanoic Acids as Substrates for Lipase-Catalyzed Reactions[1]

Philip E. Sonnet* and **Mary Welch Baillargeon**
U.S. Department of Agriculture, ARS, Eastern Regional Research Center, Philadelphia, Pennsylvania 19118

Hydrolyses of racemic methyl-branched octanoic acid thiolesters are described using six commercial lipases as catalysts. Branching at positions 2, 4 and 5 greatly reduced activity; branching at the 3-position virtually eliminated activity. The reactivities of the racemic branched thiolesters relative to the unbranched ester were very similar for each lipase preparation examined. In reactions involving configurationally pure 2-methyloctanoic acids, the S-enantiomer reacted faster both in esterification of aliphatic alcohols and in hydrolyses of aliphatic alcohol esters with all of the lipases examined. Stereobiases in hydrolyses of the octanoic acid esters branched at other positions were low and variable. In sharp contrast to the hydrolyses of the thiolesters of 2-methyloctanoic acid, two aryl esters of 2-methyloctanoic acid catalyzed by *R. miehei* lipase hydrolyzed with a bias for the R-configuration. A view of the ester-enzyme complex is offered to explain the relative rates of reaction of the racemic esters.
***Lipids* 26, 295–300 (1991).**

Triacylglycerol hydrolases [EC 3.1.1.3], more often referred to as lipases, are targets of investigation for industries that employ animal fats and vegetable oils as feedstock. A recent symposium (1) highlighted this interest and indicated wide-ranging applications for these enzymes and a desire to better understand their structure and mechanism of action.

One way to probe the active site of a lipase is with chiral fatty acids, but few instances of stereoselection using acids bearing an asymmetric center have been reported thus far (ref. 2, and references therein). We felt that a series of configurationally pure methyl branched fatty acids would prove useful for this purpose and, therefore, have synthesized a set of methyl-branched octanoic acids (3,4). An initial survey of the reactivity of six commercial lipases with selected esters of these acids is reported here. The lipases employed were crude protein preparations, and are most likely mixtures of lipolytically active species. However, since few homogeneous lipases are readily available, we selected the commercial preparations for a preliminary study. Each lipase was employed to catalyze the hydrolysis of a series of esters, and comparisons of reactivities of the branched acid esters were made with the corresponding straight chain octanoic acid ester for each lipase.

MATERIALS AND METHODS

Infrared spectra (IR) were obtained on a Perkin-Elmer 1310 spectrophotometer (Norwalk, CT) using 3% solutions in CCl_4. ^1H and ^{13}C nuclear magnetic resonance (NMR) spectra were obtained with a Bruker 300 MHz spectrometer (Karlsruhe, Germany) using $CDCl_3$ solutions with tetramethylsilane as internal standard. Gas-liquid chromatography (GLC) was done using a Shimadzu GC-Mini 2 (Columbia, MD) with an SPB-1 column (0.25 mm i.d. × 30 m) fitted with a flame ionization detector. Thin-layer chromatography (TLC) was done on silica gel 60 plates obtained from Analabs (Foxboro, MA), and fractions were visualized with iodine. Spectrophotometric assays were conducted with a Hewlett-Packard (Palo Alto, CA) 8452A Diode Array Spectrophotometer interfaced with an HP89500 Chem-Station with kinetics software (HP89512A) supplied by the manufacturer. Titrations were performed with a Radiometer Titrilab II unit (Westlake, OH).

All organic solvents were reagent grade; hexamethylphosphoric triamide (HMPT), 99%, was purchased from Aldrich Chemical Co. (Milwaukee, WI) and was stored over 13Å molecular sieves. Ellman's Reagent, 5,5′-dithio-*bis*-(2-nitrobenzoic) acid (DTNB) was similarly obtained. All other chemicals were reagent grade and were obtained from commercial sources, or, in the case of the methyl branched octanoic acids, were synthesized and characterized in this laboratory (3,4). The enzyme preparations employed in this study, their suppliers, and their specific activities in hydrolysis of triolein in 0.05 M phosphate buffer (pH 7) (μmol/min/mg) are as follows: *Candida cylindracea* (Enzeco, New York, NY) 9.45; *Rhizomucor miehei* (Gist-Brocades, Charlotte, NC) 21.3; *Rhizopus delemar* (Seikagaku Kogyo, Tokyo, Japan) 140; *Pseudomonas fluorescence* (Amano, Troy, VA) 4.3; lipoprotein lipase (Amano) 2050; and porcine pancreas lipase (Sigma Chemical Co., St. Louis, MO) 2.0. The commercial preparations were assayed for protein content using the Bradford method (5) (Bio-Rad, Richmond, CA), and bovine serum albumin was used as the standard. The percent protein for each material was as follows: *C. cylindraea*, 1.25 ± 0.12; *R. miehei*, 7.78 ± 0.74; *R. delemar*, 6.04 ± 0.58; *P. fluorescence*, 0.53 ± 0.05; liprotein lipase, 4.28 ± 4.1; and porcine pancreas lipase, 3.32 ± 0.32. The lipases were also tested for protease activity using an agar diffusion method with casein as substrate (kit from Bio-

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

*To whom correspondence should be addressed at U.S. Department of Agriculture, ARS, Eastern Regional Research Center, 600 East Mermaid Lane, Philadelphia, PA 19118.

Abbreviations: DTNB, 5,5′-dithio-*bis*-(2-nitrobenzoic) acid, Ellman's reagent; E_R, enantiomeric ratio, ratio of specificity constants for enantiomers; GLC, gas-liquid chromatography; HMPT, hexamethylphosphoric triamide; IR, infrared; 2-MOA, 2-methyloctanoic acid; NMR, nuclear magnetic resonance; R and S, standard conventions for designating configuration; TLC, thin-layer chromatography.

Rad). Only the porcine pancreatic lipase showed some protease activity.

Thiolesters of 2-mercaptoethanol. Octanoic or methyl branched octanoic acid (1.40 mmol) and 2-mercaptoethanol (105 μL, 1.48 mmol) were dissolved in 5 mL of CH_2Cl_2 under dry nitrogen and cooled to 0-5°C. Dicyclohexylcarbodiimide (0.31 g, 1.47 mmol) and a few mg of *N,N*-dimethylaminopyridine were added. The resulting mixture was stirred at 25°C overnight. Hexane (15 mL) was added and the mixture was filtered into a separatory funnel; two washes of 10 mL each of hexane were added. The organic phase was shaken with 20 mL of H_2O for 3-5 min and the layers were separated. After re-extraction with another 20 mL of water, the organic phase was dried over anhydrous $MgSO_4$ and concentrated. The oily product was chromatographed over 5 g of silica gel, using mixtures of hexane and diethyl ether as solvent. Fifteen-mL fractions were collected and assayed by TLC using $CHCl_3$/acetone (90:10, v/v) as developing solvent. The thiol ester (R_f 0.33) was collected in the fractions containing 25-35% diethyl ether as eluant. The fractions showing a single spot in TLC were combined, evaporated to dryness, and finally dried *in vacuo* (0.1 mm Hg) for 1-2 hr. IR, 1680 cm^{-1}. Some of these compounds had been characterized previously as racemates (6); the new compounds also gave satisfactory chromatographic and spectral data.

p-Nitrophenyl esters. The racemic acid (0.95 g, 6.0 mmol) was dissolved in 3 mL of dry pyridine under nitrogen and cooled to 0-5°C. *p*-Nitrophenyl trifluoroacetate (1.18 g, 5.0 mmol) (7) was added and the resulting mixture was stirred without cooling for 15 min (30 min for the 2- and 3-isomers). The mixture was diluted with hexane (50 mL) and washed with 2N HCl (35 mL) and 2 × 50 mL H_2O. The organic phase was dried over anhydrous $MgSO_4$ and concentrated. The resultant oil was chromatographed on 25 g of silica gel using hexane and diethyl ether. Fractions of 100 mL each were collected, and the ester was eluted with 5-10% diethyl ether: IR, 1710, 1530, 1350 cm^{-1}; TLC (ethyl acetate/hexane, 15:85, v/v), R_f 0.40-0.44; GLC (220°C), k' = 3.93 (octanoic), 4.23-4.85 (methyl branched esters). For the octanoic acid ester: ^1H NMR, δ 0.90 (3H, CH_3, *t*,J = 6.8 Hz), 1.3-1.8 (10 H, *m*), 2.60 (2H, CH_2C =),*t*,J = 7.2 Hz), 7.28, 8.21 (4H, aryl H, *d*,J = 5.2 Hz) ppm; ^{13}C NMR, δ 13.9, 22.5, 24.9, 28.8, 29.1, 31.6, 34.4, 122.3, 125.1, 145.6, 155.8, 171.1 ppm.

Enzyme kinetics for hydrolysis of thiolesters. The general procedure of Renard *et al.* (8) was followed. Solutions of substrate in HMPT (up to 100 μL of stock, maintaining a volume of 100 μL with HMPT), 400 μL of DTNB in HMPT (25 mg/mL), and 2.45 mL of 0.05 M Tris buffer (pH 8) were placed in UV cells. The final solute concentrations ranged from 10^{-5} to 4 × 10^{-4}. The absorption at 412 nm (ε = 13,600 $mol^{-1}cm^{-1}$) (9) was immediately monitored for background hydrolysis (none observed) and corrected for the small amount of free thiol present in the synthesized esters. A solution of the enzyme (50 μL) was then added to initiate the reaction. For the unbranched octanoate esters it was possible to obtain a quadratic fit based on least squares of the time course data to an equation of the form: $A = at + bt_2 + c$. The absorbance at time t (since scanning began) is A, and a is the slope of the time trace at t = 0 and is employed as the initial rate. Kinetic parameters (K_m, V) were then obtained by a nonlinear regression curve (Marquardt analysis) (10). Many of the other substrates were much less reactive, and solubility limitations allowed only a best fit to the initial rate data to obtain a value for a pseudo first order rate constant (k_2/K_m) (Table 1). Initial velocities, v_o, were obtained with at least three initial substrate concentrations, S_o, having standard deviations ⩽ 10% using a quadratic fit to the kinetic runs. The slope of the plot of v_o *vs* S_o was divided by mg of protein used to give an apparent k_2/K_m for each substrate and each lipase preparation examined. The correlation coefficient, ϱ, for these plots was > 0.95. The values k_2/K_m for each branched acid ester relative to that of the unbranched octanoate ester for a particular lipase is given in Table 1.

Enzyme kinetics for hydrolysis of p-nitrophenyl esters. Solutions were prepared as described above for hydrolyzing the thiolesters, using the aryl esters and employing a molar extinction coefficient of 18,600 mol^{-1} cm^{-1} for the *p*-nitrophenate chromophore determined experimentally in this medium at this pH at 408 nm. Initial velocities for background hydrolysis of these esters (the rates were 1-2% of the enzymatic rates) were determined and then were subtracted from the rates determined in the presence of enzyme. Eight to sixteen initial substrate concentrations were used, and the time course data were then treated by nonlinear regression and fitted to a curve based on quadratic fit to obtain V_{max} and K_m. The value for V_{max} was divided by mg of protein to obtain an apparent k_2. Standard deviations for V and K_m were ⩽ 5% and 15%, respectively.

Confirmation of stereobias in kinetic studies by esterification of racemic 2-methyloctanoic acid. Reaction mixtures consisted of 2.5 mmol each of 2-methyloctanoic acid and tridecane (as an internal standard for GLC), 10 mmol of 1-butanol, and 0.25 g of enzyme powder in 10 mL of hexane saturated with H_2O. The mixures were stirred magnetically at 30°C for several days and were monitored by GLC for conversion. Analysis for configuration was performed when the conversion exceeded 20% as follows: the mixture was filtered and the clear hexane solution was extracted with cold 1 N NaOH (10 mL; to minimize racemization of the acid), and cold H_2O (5 mL). The aqueous solution was washed with diethyl ether (10 mL), and was then acidified with cold 2 N HCl (5 mL). The liberated acid was extracted into diethyl ether and converted *via* its acid chloride to an amide of S-α-phenylethylamine. Thionyl chloride (0.22 mL, 3.0 mmol) and dimethylformamide (15 μL) were added to the recovered acid in dry diethyl ether (5 mL). After standing at 25°C for 1 hr, the mixture was decanted, concentrated, dissolved in CH_2Cl_2 (5 mL), and added to a solution of S-α-phenylethylamine (0.38 mL, 3 mmol) and triethylamine (0.42 mL, 3 mmol) in CH_2Cl_2 (6 mL). After standing at 25°C for 15 min, the mixture was washed with 2N HCl, H_2O, dried over anhydrous $MgSO_4$, and concentrated. Analysis by GLC (SPB-1/210°C) gave relative amounts of the diastereomers that allowed configuration analysis of the unreacted acid. The capacity fac-

TABLE 1

k_2/K_m[a] for the Hydrolysis of Racemic Thiolesters Relative to the Octanoic Ester; Enantiomeric Ratios (Configuration of Faster Reacting Enantiomer)

		Methyl branched acid thiolesters: position of methyl group					
Enzyme source	C-8[b]	2	3	4	5	6	7
C. cylindracea	1.00[c]	0.04	0	0.09	0.05	0.47	0.45
		1.9(S)	—	4.3(S)	1.5(R)	6.2(R)	
R. miehei	1.00[c]	0.04	0.002	0.10	0.021	0.18	0.22
		2.7(S)	3.3(R)	2.0(R)	1.5(S)	1.8(R)	
R. delemar	1.00[c]	0.056	0.001	0.084	0.022	0.17	0.22
		4.6(S)	1.5(R)	1.5(R)	1	1.7(R)	
P. fluorescens	1.00[c]	0.28	0.005	0.022	0.14	0.13	0.73
		2.8(S)	9.3(R)	1.4(R)	4(R)	3.1(S)	
Porc. Pancreas	1.00[c]	0.055	0.01	0.58	0.19	0.40	1.5
		1	1	2.1(R)	3.1(R)	1.6(S)	
Lipoprotein	1.00[c]	0.001	0.003	0.008	0.11	0.16	0.92
		3.8(S)	1.9(R)	1.2(R)	6.3(R)	2.1(S)	

[a]Pseudofirst-order rate constant (min^{-1} mg^{-1}) divided by mg protein used.
[b]Octanoic acid thiolester.
[c]For each enzyme preparation: k_2/K_m from first order approximation, k_2/K_m from non-linear regression plots; *C. cylindracea:* 37.6, 45.6; *R. miehei:* 29.6, 60.5; *R. delemar:* 74.5, 146; *P. fluorescens:* 311, 173; *Porcine Pancreas:* 2.2, 1.7; *Lipoprotein:* 168, 119.

tor, k′, for the S,S-diastereomer was 3.38; and 3.66 for the R,S. Since the latter was always in excess, the S-acid reacted faster with these lipases (details in text).

Procedure for hydrolysis of racemic 2-methyloctanoic acid phenyl ester. Phenyl ester (1.0 g, 4.03 mmol) was suspended in distilled H_2O (50 mL) containing 1.0 g of NOVO "Lipozyme" which is *R. miehei* lipase from Gist-Brocades on an inert resin. The reaction mixture was agitated and kept at pH 7 using automatic titration with 0.1 N NaOH for a period of 12 hr, at which time 39.3% conversion had been achieved. The mixture was then suction filtered using CH_2Cl_2 as a rinse, and the organic phase was separated. The organic phase was washed with another portion of CH_2Cl_2, and the combined organic phase was then extracted with cold NaOH (20 mL, 1.23 N) and H_2O (10 mL). The aqueous phase was acidified with cold 2N HCl, and the product was extracted with CH_2Cl_2. The recovered acid was coverted to diastereomeric amides as above and characterized by GLC as previously described. The R,S-diastereomer predominated (72.5:27.5), which meant that the R-acid had hydrolyzed more rapidly.

RESULTS AND DISCUSSION

Octanoic acid, and the synthesized methyl branched octanoic acids, racemic as well as individual enantiomers, were converted to thiolesters of 2-mercaptoethanol using dicyclohexylcarbodiimide and catalytic amounts of N,N-dimethylaminopyridine (5). Such thiolesters can be employed in a homogeneous medium comprised of 17% hexamethylphosphoric triamide, HMPT, in aqueous buffer at pH 8 using Ellman's Reagent for a continuous spectrophotometric assay of lipase activity (8,11). Because the reaction medium is homogeneous, no information can be obtained about the particular events that mediate the special enzyme activity at an oil-water interface (12,13). On the other hand, homogeneity permits spectrophotometric analysis, gives results that are easily reproduced, and avoids ambiguities in measuring concentrations in emulsions.

The branched acid esters were much less reactive than octanoic acid ester itself and enzyme saturating conditions could not be reached without exceeding the solubilities of the substrates (ca. 5×10^{-4} M). Therefore kinetic data points for several low concentrations (10^{-5} to 10^{-4} M) were accumulated as described in Materials and Methods, and reaction velocities were calculated. At low concentrations the velocities were approximately proportional to substrate concentrations, and allowed an estimate of a pseudo-first-order rate constant (k_2/K_m) that was taken as a measure of relative activity of the substrate (Table 1). The tabulated data represent the value of (k_2/K_m) for each racemic ester relative to that of the (unbranched) octanoic ester taken as 1.00. The actual values of (k_2/K_m) obtained for the more reactive octanoic acid ester itself with each lipase, as well as the ratio that was obtained by calculating each variable independently by Marquardt analysis of nonlinear regression plots based on a wider range of substrate concentrations are footnoted. These values are dependent on enzyme purity, so the relative selectivities of a set of substrates using a single enzyme allows a better comparison of the way selectivity varies with substrate structure for a particular enzyme preparation. In addition, each enantiomer of each branched acid thiolester was also examined, and the ratios (k_2/K_m) for one enantiomer relative to the other, shown with the R,S-convention for designating configuration, are used to indicate the faster reacting enantiomer in parenthese. These values are equivalent to enantiomeric ratios, E_R, that can be obtained from kinetic resolution of racemic mixtures (14). In general, stereoselectivity was

TABLE 2

Kinetic Data for Hydrolysis of Racemic *p*-Nitrophenyl Esters

Enzyme source	Acid residue	$k_2 \times 10^3$[a]	$K_m \times 10^6$	k_2/K_m	Relative value[b]
R. miehei	Octanoic	41.8 ± 0.90	28.8 ± 3.7	1450	1.00
	2-MOA[c]	5.01 ± 0.26	47.4 ± 3.7	105	0.07
	3-MOA[c]	4.76 ± 0.13	61.2 ± 5.3	77.2	0.05
	4-MOA[c]	17.5 ± 0.39	33.0 ± 4.1	531	0.37
	5-MOA[c]	27.8 ± 0.78	78.7 ± 4.3	352	0.24
	6-MOA[c]	31.1 ± 0.78	182 ± 14	171	0.12
	7-MOA[c]	79.8 ± 3.21	121 ± 13	658	0.45
C. cylindracea	Octanoic	664 ± 21	28.3 ± 4.3	23400	1.00
	2-MOA[c]	253 ± 63	210 ± 17.8	1200	0.05
	3-MOA[c]	—	—	—	0
	4-MOA[c]	272 ± 12	77.3 ± 10.5	3650	0.16
	5-MOA[c]	87 ± 1	38.6 ± 3.9	2240	0.10
	6-MOA[c]	360 ± 12	97.7 ± 13.0	3690	0.16
	7-MOA[c]	260 ± 4	35.3 ± 3.8	7380	0.32

[a] k_2 is mol L^{-1} min^{-1} divided by mg of protein; K_m is mol/L.
[b] k_2/K_m relative to that of the unbranched ester.
[c] Methyloctanoic acid is abbreviated MOA.

TABLE 3

Kinetic Data for Hydrolysis of R- and S-2-Methyloctanoic Acid *p*-Nitrophenyl Esters

Enzyme source	Acid residue	$k_2 \times 10^3$[a]	$K_m \times 10^6$[a]	k_2/K_m	E_R[b]
R. miehei	R-2-MOA[c]	5.78 ± 0.13	40 ± 4.6	145	
	S-MOA	0.26 ± 0.06	54 ± 6.2	4.75	30.4(R)[d]
C. cylindracea	R-MOA	68.6 ± 4.8	28 ± 6.0	2650	
	S-MOA	217 ± 11	40 ± 8.0	5830	2.2(S)[d]

[a] Units as in Table 1.
[b] Ratio of k_2/K_m values for the enantiomers, also known as enantiomeric ratio.
[c] Methyloctanoic acid.
[d] Faster reacting enantiomer.

low, although the reactions of the 2-methyloctanoic acid esters were consistently selective for the S-enantiomer.

Experiments were conducted to confirm the uniform S-stereoselectivity for 2-methyloctanoic acid observed for the hydrolysis of the thiolesters. Several examples of preferential reaction of R-2-substituted acids, or acid derivatives, with lipase catalysis have been reported (2). However, in these cases the 2-substituent was always an electronegative atom, e.g., Cl, F, OC_6H_5, where nonbonded electron pairs offered electrostatic interactions that may alter stereoselection. Esterification of racemic 2-methyloctanoic acid with 1-butanol in wet hexane using lipases from *Pseudomonas fluorescence* and *Rhizomucor miehei* did indeed proceed at a faster rate for the S-2-enantiomer. For *R. miehei* at 57.0% conversion, the enantiomeric excess, ee, was 0.612, *i.e.*, the ratio of S to R in the recovered unreacted 2-methyloctanoic acid was 0.806:0.194, and therefore $E_R = 4.9$ (15). The selectivity of *P. fluorescence* was much greater and might even be synthetically useful; $E_R > 40$ (conversion: 24.8%, enantiomeric excess: 0.31). The selectivity for S-2-methyloctanoic acid, and likely 2-methyl substituted fatty acids, in hydrolysis of aliphatic esters and esterification of alcohols may be general.

The *p*-nitrophenyl esters were prepared from the trifluoroacetate of *p*-nitrophenol in pyridine (7). Lipase catalyzed hydrolysis was examined in the same medium as was used for the thiolesters, namely 17% HMPT buffered at pH 8. The maximum molar extinction coefficient for *p*-nitrophenate at that pH was determined to be 18,600 at 408 nm (at 3.33×10^{-4} M). Although the *p*-nitrophenyl esters were no more soluble than the thiolesters, their reactivity was considerably greater, and values of K_m and V were obtained using two of the lipases. *R. miehei* was chosen as a lipase characterized as positionally selective in its reactions with triglycerides, while *C. cylindracea* exemplified a lipase that is random in its action (16). The *p*-nitrophenyl esters underwent slow nonenzymatic hydrolysis at pH 8, and the kinetic data points for the enzymatic reaction were corrected by subtracting values obtained for the nonenzymatic hydrolysis rates. The background rate was 1-2% of the total rate and standard deviations for V and K_m were ⩽ 6 and 15%, respectively. Table 2 gives k_2, K_m, their ratio, and the ratio relative to that of the *p*-nitrophenyl octanoate. The enantiomeric 2-methyloctanoic acid *p*-nitrophenyl esters were treated with these

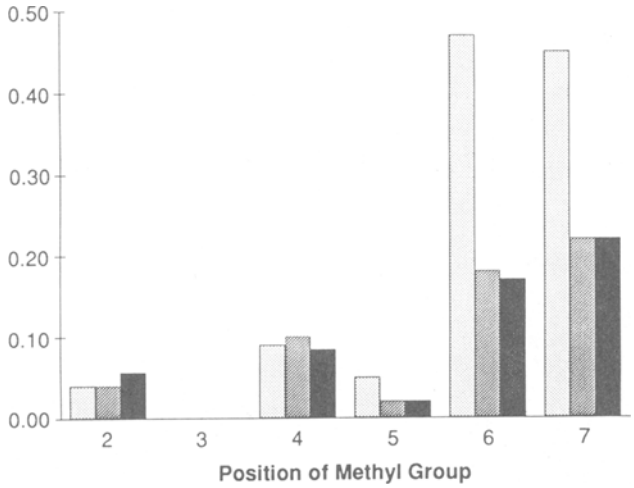


FIG. 1. Reactivity of racemic thiolesters relative to the octanoic acid ester with ⊡, *C. cylindracea;* ⊠, *R. miehei*; and ■, *R. delemar lipases.*

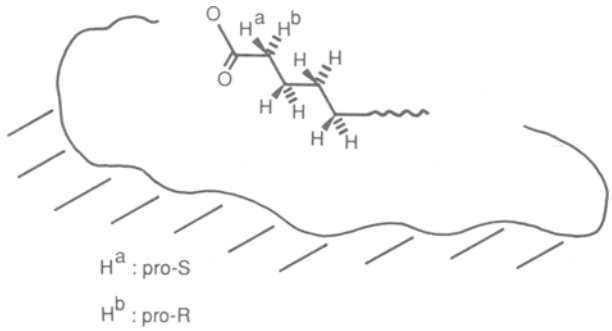


FIG. 2. Possible relationship of substrate to lipase showing carbonyl oxygen projecting into the binding pocket of the enzyme. H^a is the pro-S proton, and H^b is pro-R. The nucleophile may approach the carbonyl group from the side of H^b.

same two lipases, and the data are shown in Table 3. The S-ester hydrolyzed faster with *C. cylindracea* lipase, as had been the case for the thiolester. However, the lipase from *R. miehei* selected strongly for the R-enantiomer. This was confirmed by hydrolysis of the phenyl ester of racemic 2-methyloctanoic acid, which yielded predominantly the R-acid (E_R = 2.7). Changing the alcohol moiety from an aliphatic to an aryl alcohol had inverted stereoselection for the *R. miehei* lipase.

Although the enzymes employed were crude commercial preparations, there are features of the results that warrant discussion. Substitution at the 2-position by a methyl group markedly reduces reactivity for the esters examined. This observation has been made previously, albeit in a more limited study (17). Substitution at the 3-position generally causes a further reduction in reactivity for the lipases examined. The similarity among the lipases is quite striking, and the bar graph (Fig. 1) illustrates this using the three fungal lipases. We also noted that branching at the 5-position is often more deleterious to reactivity of the substrate than branching at positions 4 or 6. This variation in hydrolytic rate is consistent with a situation wherein the carbonyl oxygen of the ester is directed toward the enzyme in the complex (Fig. 2). Hydrophobically bound in this manner, carbons 3 and 5 of the chain would project similarly toward the enzyme, and branching at those positions would be predictably worse for binding. Branching further removed from the ester function could still affect binding but less severely; the substrate may still become hydrophobically associated with the enzyme, but use a shorter stretch of unbranched chain.

The relative rates of reaction of pairs of enantiomers of the branched acid esters are generally small. For the 2-methyl enantiomers, the K_m is similar, and the effect of the methyl branch is, perhaps predictably, on the catalytic event itself. Reaction of 2-methyloctanoic acid with these lipase preparations results in a faster reaction of the S-enantiomer when an aliphatic alcohol is involved as a nucleophile. Hydrolysis of an aryl ester, catalyzed by *R. miehei* lipase, however, displayed a reversed stereobias. This observation does not accord with a view of the enzyme binding site as a relatively static environment, but rather with one in which accommodation is possible with the stereochemical consequences reflecting the manner in which the alcohol (phenol) residue structures water in the immediate vicinity of the carbonyl group. Another possibility is the existence of a binding pocket that fortuitously accommodates an aryl ring and alters the favored direction of approach of the nucleophile to the carbonyl. The results emphasize the need for more specific protein structural information.

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We express our appreciation to Dr. Robert Dudley of our laboratory for obtaining NMR spectra, and to Dr. Harry Farrell for helpful discusions in the preparation of our manuscript. The lipases, excepting that of *R. delemar*, were generous gifts of the manufacturers.

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Preparation of Methyl 11,14,17-Eicosatrienoate-8,8,9,9-d_4 and Methyl 8,11,14,17-Eicosatetraenoate-8,9-d_2[1]

Henry Rakoff*
National Center for Agricultural Utilization Research, Agricultural Research Service, USDA, Peoria, Illinois 61604

Methyl 11,14,17-eicosatrienoate-8,8,9,9-d_4 was obtained by Wittig coupling of 3,6-nonadienyltriphenylphosphonium iodide with methyl 11-oxoundecanoate-8,8,9,9-d_4. For the synthesis of the phosphonium salt, 2-pentynol was converted to 1-bromo-2-pentyne with triphenylphosphine dibromide. Grignard coupling of 1-bromo-2-pentyne with 3-butynol in the presence of cuprous chloride gave 3,6-nonadiynol. The latter was hydrogenated in the presence of P-2 nickel catalyst to yield 3,6-nonadienol. The dienol was converted *via* the bromide and iodide to the phosphonium salt. The aldehyde ester was prepared starting with the coupling of 7-bromoheptanoic acid and 3-butynol with lithium amide in liquid ammonia. The hydroxyalkynoic acid obtained was converted to the methyl ester and the hydroxy group was converted to the tetrahydropyranyloxy group. Subsequent deuteration of the methyl 11-(2-tetrahydropyranyloxy)-8-undecynoate with deuterium gas and *tris*-(triphenylphosphine)chlorohodium gave the corresponding tetradeuterated product. The tetrahydropyranyl group was removed and the methyl 11-hydroxy-undecanoate-8,8,9,9-d_4 was oxidized to the aldehyde ester with pyridinium chlorochromate. Methyl 8,11,14,17-eicosatetraenoate-8,9-d_2 was obtained by a similar sequence of reactions. The methyl 11-oxo-8-undecenoate-8,9-d_2 required for the Wittig coupling was obtained by Lindlar deuteration of the protected alkynoic ester to methyl 11-(2-tetrahydropyranyloxy)-8-undecenoate-8,9-d_2. The unsaturated hydroxy ester was oxidized to the aldehyde ester by periodinane with negligible isomerization. The all *cis* isomers were isolated by silver resin chromatography.
***Lipids* 26, 301–305 (1991).**

For our study of the metabolism of fats in humans (1,2), we have synthesized various monoenoic (3), dienoic (4) and trienoic (5–7) fatty esters labeled with deuterium atoms in various positions. In this paper we describe the synthesis of deuterated analogs of methyl 11,14,17-eicosatrienate (11,14,17-20:3) and methyl 8,11,14,17-eicosatetraenoate (8,11,14,17-20:4), which are intermediates in the conversion of linolenate (9,12,15-18:3) to eicosapentaenoate (5,8,11,14,17-20:5). They have been prepared to study the path of this conversion.

RESULTS AND DISCUSSION

The syntheses of the two title compounds involve a combination of acetylenic, Grignard and Wittig couplings. The last coupling in both syntheses was a Wittig reaction between 3,6-nonadienyltriphenylphosphonium iodide and an appropriate aldehyde ester. With the compounds employed in these synthetic sequences, the Wittig Reaction usually yields the *cis* and *trans* isomers of the double-bond generated in the ratio of about 85:15. The phosphonium iodide was prepared by a sequence of reactions starting with 2-pentynol. The alkynol was converted to 1-bromo-2-pentyne with triphenylphosphine dibromide. Grignard coupling of this bromoalkyne with 3-butynol gave 3,6-nonadiynol, which was reduced with hydrogen and P-2 nickel catalyst to 3,6-nonadienol. The alcohol was converted to the bromide with triphenylphosphine dibromide, to the iodide with sodium iodide in acetone, and to the phosphonium iodide with triphenylphosphine in acetonitrile. Experimental details for all these steps have been published (6,7).

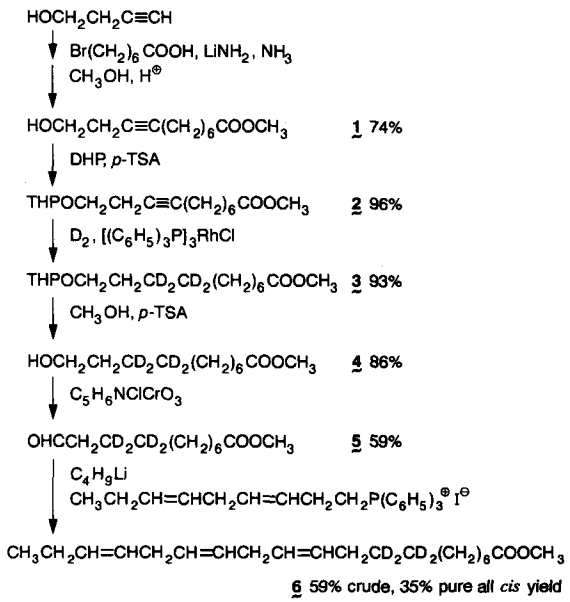
Methyl 11,14,17-eicosatrienoate-8,8,9,9-d_4 (6) was obtained by Wittig coupling of 3,6-nonadienyltriphenylphosphonium iodide with methyl 11-oxoundecanoate-8,8,9,9-d_4 (5). The synthesis of 5 is detailed in Scheme 1. 3-Butynol was condensed with 7-bromoheptanoic acid in the presence of lithium amide in liquid ammonia to give methyl 11-hydroxy-8-undecynoate 1 in 75% yield after esterification. The hydroxy group was protected as the tetrahydropyranyl ether (2) in 96% yield. Deuteration of 2 with deuterium gas in the presence of Wilkinson's catalyst, *tris*-(triphenylphosphine)chlororhodium, gave the tetradeuterated tetrahydropyranyl (THP) ester (3) in 94% yield. The THP group was removed with methanol and *p*-toluenesulfonic acid to give the tetradeuterated hydroxy ester (4) in 85% yield. Oxidation with pyridinium chlorochromate (6,8) gave methyl 11-oxoundecanoate-8,8,9,9-d_4 (5) in 59% yield. Reaction of 5 with the phosphonium iodide and butyl lithium gave 6 in 60% crude yield. The all *cis* isomer was separated by silver resin chromatography (9,10) with a 100% Ag/Na column using 3% acetronitrile in methanol as eluent. Nuclear magnetic resonance spectroscopy confirmed the structure and configuration assigned. Mass spectroscopy using the chemical ionization technique indicated 97% d_4.

Methyl 11-oxo-8-undecenoate-8,9-d_2 (9) was required for the synthesis of methyl 8,11,14,17-eicosatetraenoate-8,9-d_2 (10). Synthesis of this aldehyde ester is depicted in Scheme 2. Reaction of compound 2 with deuterium and Lindlar's catalyst, Pd on $CaCO_3$ poisoned with lead, gave the deuterated product (7) in 99% yield. The

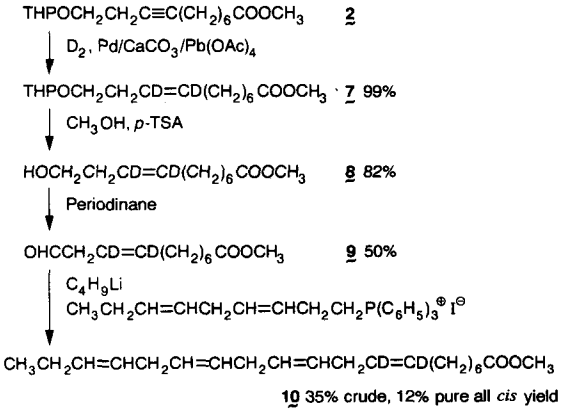
[1]The mention of firm names or trade products does not imply that they are endorsed or recommended by the U.S. Department of Agriculture over other firms or similar products not mentioned.

*To whom correspondence should be addressed at the National Center for Agricultural Utilization Research, 1815 N. University Street, Peoria, IL 61604.

Abbreviations: DHP, dihydropyran; EE, diethyl ether; PE, petroleum ether (35–60°C); *p*-TSA, *p*-toluenesulfonic acid; THF, tetrahydrofuran; THP, tetrahydropyranyl.



SCHEME 1



SCHEME 2

THP group was removed with methanol and *p*-toluenesulfonic acid to give the β,γ-unsaturated hydroxy ester (8) in 82% yield. Numerous oxidizing agents were investigated to find one that would oxidize the β,γ-unsaturated hydroxy ester to the β,γ-unsaturated aldehyde ester (9) without isomerization to the α,β-unsaturated aldehyde ester. It was found that periodinane, 1,1,1-*tris*-(acetoxy)-1,1-dihydro-1,2-benziodoxol-3(1H)-one (11,12) would accomplish this in about 50% yield.

The unsaturated aldester (9) was treated with butyl lithium and 3,6-nonadienyltriphenylphosphonium iodide to give compound (10) in about 35% crude yield. The all *cis* isomer was obtained from this mixture by silver resin chromatography on a 62% Ag/Na column using methanol as eluent. Mass spectroscopy, using the chemical ionization technique, indicated at least 92% d_2. Nuclear magnetic resonance (NMR) spectroscopy supported the structure and configuration assigned.

EXPERIMENTAL PROCEDURES

Reagents. Butyl lithium, triphenylphosphine, dihydropyran, sodium borohydride, ethyl magnesium bromide, 3-butynol. Lindlar's catalyst, pyridinium chlorochromate, ethylene diamine and periodinane were obtained from Aldrich Chemical Company (Milwaukee, WI). Aldrich no longer offers periodinane ostensibly due to an explosion hazard with an intermediate in its preparation. However, periodinane can be prepared fairly readily (11). A discussion of the safety aspects and an improved preparation of periodinane will be published in *Journal of the American Chemical Society* by Dess and Martin (personal communication). 2-Pentynol was purchased from Farchan Laboratories (Gainesville, FL), *tris*-(triphenylphosphine)chlororhodium from Strem Chemicals (Newburyport, MA) and 7-bromoheptanoic acid from Pfaltz and Bauer (Waterbury, CT). Silica gel (60–200 mesh) and Florisil (100–200 mesh) were obtained from J.T. Baker (Jackson, TN).

Gas chromatography. A 50 m × 0.25 mm CPS-2 capillary column (Quadrex Corp., New Haven, CT) was used for analyzing binary mixtures of geometric isomers. For other analyses, a 6 ft × 4 mm column packed with 3% EGSS-X on 100/120 GasChrom Q or a 6 ft × 4 mm column packed with 3% OV101 on 80/100 Supelcoport (Supelco, Bellefonte, PA) was employed.

^{13}C NMR. ^{13}C Nuclear magnetic resonance (NMR) spectra were recorded with a Bruker (Karlsruhe, Germany) WM 300 WB pulsed Fourier transform spectrometer operating at 75.5 MHz and ambient temperature. Typically, 2500 transients were collected on $CDCl_3$ solutions, which served as both the internal lock and secondary reference, using 5-mm tubes. Sweep widths of 200 ppm and 8 K real data points limited acquisition time to 0.54 seconds and were used to obtain chemical shift values within ± 1.85 Hz, *i.e.*, ± 0.05 ppm. A pulse width of 3 μs (40°) was employed with no delay between pulses. Decoupling power was held to *ca.* 1 W to provide adequate broadband decoupling power while minimizing sample heating. The signal from carbons bearing two deuterium atoms or one deuterium atom and a double bond is diminished to such an extent that it was usually not detected. Thus, no peaks for the ethylenic carbons were obtained from compounds 7, 8 and 9.

Mass spectroscopy. Mass spectra were determined on a Finnigan (Sunnyvale, CA) 4500 mass spectrometer using isobutane chemical ionization with data processing of the isotope distribution against standards (13).

Silver resin chromatography. The macroreticular resin used for the separations was Rohm and Hass XN1010 sulfonic acid resin ground to the mesh sizes indicated. For the Ag/H columns, the indicated percentage of hydrogen ions was displaced by Ag ions. For the Ag/Na columns, the hydrogen ions were displaced by sodium ions, and then the indicated percentage of sodium ions was displaced by Ag ions (9,10).

Methyl 11-hydroxy-8-undecynoate (1). Liquid am-

monia (*ca* 800 mL) was charged to a 2-L 3-necked flask equipped with a mechanical stirrer and a dry-ice cooled condenser and surrounded by Vermiculite. Ferric nitrate (1 g) was added, followed by lithium metal (11.05 g, 1580 mmol) which was added in small pieces over 45 min. 3-Butynol (55.26 g, 789 mmol) dissolved in tetrahydrofuran (THF) (150 mL) was added over 35 min. Then 7-bromoheptanoic acid (33 g, 158 mmol) dissolved in THF (100 mL) was added over 25 min. Ten hours later stirring was stopped, the Vermiculite was removed, and the ammonia was allowed to evaporate overnight. The next day the flask was immersed in a warm water bath and flushed with inert gas to remove residual ammonia. Water (100 mL) was added to ease stirring and the reaction vessel was immersed in an ice bath while the contents were acidified with 5N H_2SO_4 (450 mL). The tan liquid was extracted with diethyl ether (EE; 1 × 250 mL, 2 × 100 mL) and the combined EE extracts were washed with saturated NH_4Cl solution (100 mL) and saturated NaCl solution (100 mL) and dried over anhydrous Na_2SO_4. The drying agent was removed by filtration and the solvent was removed on a rotary evaporator. Excess 3-butynol was removed by distillation under reduced pressure. Methanol (130 mL) and conc. H_2SO_4 (1 mL) were added to the residue and the mixture was heated at the reflux temperature for one hr. The acid was neutralized with solid $NaHCO_3$ and solvent was removed on the rotary evaporator. The viscous liquid remaining was diluted with water (150 mL) and extracted with EE (2 × 50 mL). The combined EE extracts were washed with saturated NaCl solution (70 mL) and dried (Na_2SO_4). After removal of the drying agent and solvent, the residue was distilled to give compound 1 (24.88 g; 74.3% yield) b.p. 123–146°C (0.25 torr). ^{1}H NMR ($CDCl_3$): δ 3.52–3.56 (*m*,2H,CH_2OH), δ 3.54 (*s*,3H,OCH_3), δ 2.67(*s*,1H,O*H*), δ 2.26–2.31(*m*,2H,CH_2CH$_2$OH), δ 2.19(*t*,2H,CH_2COO), δ 2.02(*m*,2H,CH_2C≡C), δ 1.50(*m*,2H,CH_2CH$_2$COO), δ 1.20–1.39(*m*,6H,(CH_2)$_3$). ^{13}C NMR (ppm): C-1, 174.0; C-2, 33.7; C-3, 24.5; C-4, 28.3; C-5, 28.2; C-6, 28.5; C-7, 18.4; C-8, 81.7; C-9, 76.4; C-10, 22.8; C-11, 61.1; OCH_3, 51.2. Values for carbons numbered 4, 5, 6, 8, and 9 were established by correlation experiments. Proton assignments were verified with a COSY experiment and carbon assignments were determined by HETCOR and long-range HETCOR experiments.

Methyl 11-(2-tetrahydropyranyloxy)-8-undecynoate (2). Methyl 11-hydroxy-8-undecynoate (74 g, 349 mmol) in EE (150 mL) and *p*-TSA (0.5 g) were placed in a 500-mL, 3-necked flask equipped with a mechanical stirrer, a dropping funnel and a reflux condenser. Dihydropyran (38 g, 454 mmol) dissolved in EE (100 mL) was added dropwise over 30 min to maintain a gentle reflux. Two hr later, saturated Na_2CO_3 (10 mL) was added. The organic layer was separated and dried over Na_2SO_4. After removal of the drying agent and solvent, the liquid remaining was passed through a column (30 mm × 50 cm) containing silica gel (100 g) in hexane. A total of 99.72 g (96% yield) of product was obtained by elution with mixtures ranging from 0 to 15% EE in hexane. ^{13}C NMR (ppm): C-1, 174.0; C-2, 33.9; C-3, 24.7; C-4, 28.5; C-5, 28.3; C-6, 28.6; C-7, 18.6; C-8, 80.9; C-9, 76.8; C-10, 20.1; C-11, 66.1; OCH_3, 51.3; THP group: C-2, 98.6; C-3, 30.5; C-4, 19.3; C-5, 25.4; C-6, 62.0.

Methyl 11-(2-tetrahydropyranyloxy)-undecanoate-8,8,9,9,-d_4 (3). Methyl 11-(2-tetrahydropyranyloxy)-8-undecynoate (50 g, 169 mmol) in benzene (1 L) was treated with deuterium gas in the presence of *tris*-(triphenylphosphine)chlororhodium (5 g) in the manner described previously (3). Benzene was removed on the rotary evaporator and the red liquid remaining was diluted with hexane (300 mL), filtered and then passed through a column (30 mm × 50 cm) containing silica gel (100 g) in hexane. Elution with 10% EE in hexane gave compound 3 (47.85 g, 93% yield). ^{13}C NMR (ppm): C-1, 174.1; C-2, 34.0; C-3, 24.9; C-4 through C-7, 29.0–29.2; C-10, 29.4; C-11, 67.5; OCH_3, 51.3; THP group: C-2, 98.7; C-3, 30.7; C-4, 19.6 C-5,25.4; C-6, 62.2.

Methyl 11-hydroxyundecanoate-8,8,9,9-d_4 (4). Compound 3 (31.84 g, 105 mmol), *p*-TSA (0.8 g) and methanol (600 mL) were placed in a 1-L round bottom flask which was then flushed with nitrogen, capped, and stored for 16 hr. The acid was neutralized with solid Na_2CO_3. After filtration, methanol was removed on the rotary evaporator. The residue was diluted with saturated aqueous $NaHCO_3$ and extracted with EE (2 × 50 mL). The EE extracts were dried (Na_2SO_4) and after removal of the drying agent and solvent the residue was distilled to give compound 4 (19.66 g, 85.5% yield) b.p. 123–142°C (0.3 torr). ^{1}H NMR($CDCl_3$): δ 3.61(*s*,3H,OCH_3), δ 3.56(*t*,2H,CH_2OH), δ 2.25 (*t*,2H,CH_2COO), δ 1.90(*s*,1H,O*H*), δ 1.48–1.56 (*m*,4H, CH_2CH$_2$COO and CH_2CH$_2$OH), δ 1.22(*m*,8H,(CH_2)$_4$). ^{13} NMR (ppm): C-1, 174.2; C-2, 34.0; C-3, 24.8; C-4 through C-7, 29.0–29.2; C-10, 32.4; C-11, 62.8; OCH_3, 51.3.

Methyl 11-oxoundecanoate-8,8,9,9-d_4 (5). Pyridinium chlorochromate (28.8 g, 134 mmol) was slurried in CH_2Cl_2 (200 mL) in a 500 mL, 3-necked flask equipped with a mechanical stirrer, a low temperature thermometer, and a dropping funnel. While a stream of nitrogen was passed through the apparatus immersed in an ice bath, compound 4 (19.6 g, 89 mmol), dissolved in CH_2Cl_2 (50 mL), was added over 15 min. Two hr later, EE (100 mL) was stirred into the black liquid. A lump formed and the liquid was decanted. The tarry lump was stirred with EE (2 × 50 mL), and the combined EE decantates were passed through a column (15 mm × 25 cm) containing Baker Florisil (20 g). The dark green liquid which was obtained was concentrated on the rotary evaporator and the residue (23 g) was distilled through a short path still. The product solidifies in the condenser so it is best to not pass cooling water through the condenser. A total of 11.41 g (59% yield) of compound 5 was obtained, b.p. 112–120°C (0.2 torr). ^{1}H NMR($CDCl_3$): δ 9.69 (*t*1H,C*H*O), δ 3.59(*s*,3H,OCH_3), δ 2.33(*s*,2H,CH_2CHO), δ 2.23 (*t*,2H,CH_2COO), δ 1.54(*m*,2H,CH_2CH$_2$COO), δ 1.21(*m*,8H, (CH_2)$_4$). ^{13}C NMR (ppm): C-1, 174.0; C-2, 33.9; C-3, 24.8; C-4 through C-7, 28.9–29.0; C-10, 43.5; C-11, 202.6; OCH_3, 51.2.

Methyl 11,14,17-eicosatrienoate-8,8,9,9-d_4 (6). 3,6-Nonadienyltriphenylphosphonium iodide (30.8 g, 60 mmol) was slurried in THF (200 mL) in a 500-mL 3-necked flask equipped with a mechanical stirrer, a dropping funnel, a low temperature thermometer, and a $CaCl_2$ tube. While a stream of N_2 was passed through the apparatus immersed in an ice bath, butyl lithium

(2.5 M, 28 mL, 70 mmol) was added in portions over 10 min while the temperature was maintained below 10°C. The reaction mixture was very dark red in color. Compound 5 (11.4 g, 52.3 mmol) in THF (10 mL) was added over 5 min. The ice bath was removed and one hr later the reaction mixture was diluted with saturated NaCl solution (200 mL). The organic layer was separated, dried (Na_2SO_4), and concentrated on the rotary evaporator to give a solid (31.2 g). This was triturated several times with petroleum ether and the petroleum ether (PE) solution was passed through a column (30 mm × 50 cm) containing silica gel (100 g) in PE. Elution with PE and with 5% EE in PE gave compound 6 (10.08 g, 59.0% yield). To isolate the all *cis* isomer, the reaction mixture was separated on a silver resin column (5 × 82 cm) containing Amberlyst XN1010 (60/200 mesh) 100% Ag/Na using as eluents 100% methanol and 3% acetonitrile in methanol. The all *cis* isomer of compound 6 was obtained (5.93 g, 35% yield based on the Wittig reaction, 12% overall based on the 7-bromoheptanoic acid used). It was better than 98% pure by GC analysis on a 30-m SP 2330 column. Deuterium distribution: 0.19% d_1, 1.22% d_2, 97.04% d_4, 1.54% d_5. ^{1}H NMR ($CDCl_3$): δ 5.27-5.34(*m*,6H,C*H*=C*H*), δ 3.60(*s*,3H,OCH_3), δ 2.75(*t*,4H,C=CCH_2C=C), δ 2.24(*t*,2H,CH_2COO), δ 1.98-2.05(*m*,4H,CH_2C=C), δ 1.57(*m*,2H,CH_2CH$_2$COO), δ 1.23(*m*,8H,(CH_2)$_4$ δ 0.92(*t*,3H,CH_3). ^{13}C NMR (ppm): C-1, 173.9; C-2, 33.9; C-3, 24.8; C-4 through C-7, 29.0-29.3; C-10, 26.9; C-11, 130.1; C-12, 127.5; C-13,16, 25.4-25.5; C-14, C-15, 128.1; C-17, 127.0; C-18, 131.7; C-19, 20.4; C-20, 14.1.

Methyl 11-(2-tetrahydropyranyloxy)-8-undecenoate-8,9-d_2 (7). Methyl 11-(2-tetrahydropyranyloxy)-8-undecynoate (14.8 g, 50 mmol) and ethyl acetate (100 mL) were placed in a 250-mL round-bottom flask equipped with a magnetic stirring bar and connected to an apparatus for maintaining one atmosphere of pressure. The apparatus was evacuated and flushed three times with D_2. Then, Lindlar's catalyst (0.5 g) and quinoline (0.3 mL) were added and a pressure of one atmosphere of D_2 was maintained as the reaction mixture was stirred rapidly. After absorption of D_2 had ceased, the reaction mixture was filtered through a pad of Celite to remove the catalyst. Ethyl acetate was removed on the rotary evaporator to yield compound 7 (14.9 g, 99% crude yield). ^{13}C NMR (ppm): C-1, 174.1; C-2, 34.0; C-3, 24.9; C-4 through C-6, 28.8-29.4; C-7 or C-10, 27.12 or 27.8; C-11, 67.0; OCH_3, 51.4; THP group: C-2, 98.7; C-3, 30.7; C-4, 19.6; C-5, 25.4; C-6, 62.2.

Methyl 11-hydroxy-8-undecenoate-8,9-d_2 (8). In a manner similar to the conversion of 3 to 4, compound 7 was converted to 8 in 81.5% yield, b.p. 120-126°C (0.2 torr). ^{1}H NMR($CDCl_3$): δ 3.57(*s*,3H,OCH_3), δ 3.54(*m*,2H,CH_2OH), δ 2.40(*m*,1H,O*H*), δ 2.19-2.24(*m*,4H,CH_2COO and CH_2CH$_2$OH), δ 1.96 (*m*,2H,CH_2C=C), δ 1.53(*m*,2H,CH_2CH$_2$COO), δ 1.23(*m*,6H, (CH_2)$_3$). ^{13}C NMR (ppm): C-1, 174.1; C-2, 33.8; C-3, 24.7; C-4 through C-6, 28.6-29.2; C-7, 26.9; C-10, 30.5; C-11, 62.0; OCH_3, 51.2.

Methyl 11-oxo-8-undecenoate-8,9-d_2 (9). Periodinane (6.49 g, 15.3 mmol) in methylene chloride (90 mL, distilled from CaH_2 and stored over Molecular Sieve 4A) was placed into a 500-mL 3-necked flask equipped with a mechanical stirrer, a dropping funnel, a thermometer and a $CaCl_2$ drying tube. Compound 8 (3.g, 13.9 mmol) in methylene chloride (100 mL) was added dropwise over 20 min while a slow stream of N_2 was maintained through the apparatus. After 50 min, EE was added, followed 15 min later by a solution of $Na_2S_2O_3.5H_2O$ (27.54 g) in saturated $NaHCO_3$ solution (100 mL). The aqueous layer was separated and extracted with EE (50 mL). The combined EE layers were washed with saturated $NaHCO_3$ solution (50 mL), saturated NaCl solution (50 mL), and dried (Na_2SO_4). Removal of the drying agent and solvent gave a thick orange liquid. Passage of this liquid through a small silica gel column with hexane gave compound 9 (1.54 g, 52% yield). This compound isomerizes partially to the conjugated isomer on passage through a column of 3% EGSS-X in a gas chromatograph. Infrared analysis showed a carbonyl band at 1737 cm^{-1} and no trace of a band for an α,β-unsaturated aldehyde. In addition, there is no infrared band for a *trans* double bond which would be expected in the conjugated unsaturated aldehyde. In the proton NMR, the area under the doublet (conjugated aldehyde) is about 1% of the area under the triplet (nonconjugated aldehyde). ^{1}H NMR ($CDCl_3$): δ 9.59(*t*,1H, C=CCH$_2$C*H*O), δ 9.52(*d*,1H,C=CC*H*O), δ 3.60(*s*,3H,OCH_3), δ 3.12(*s*,2H,CH_2C*H*O), δ 2.24(*t*,2H,CH_2COO), δ 1.97(*m*,2H,CH_2C=C), δ 1.55(*m*,2H,CH_2CH$_2$COO), δ 1.25-1.31(*m*,6H, (CH_2)$_3$). ^{13}CNMR (ppm): C-1, 174.0; C-2, 33.9; C-3, 24.7; C-4 through C-6, 28.7-28.9; C-7, 27.3; C-10, 42.3; C-11, 199.4; OCH_3 51.3.

Methyl 8,11,14,17-eicosatetraenoate-8,9-d_2 (10). 3,6-Nonadienyltriphenylphosphonium iodide (4.13 g, 8 mmol) was slurried in THF (50 mL) in a 100-mL, 3-necked flask equipped with a mechanical stirrer, a dropping funnel, a thermometer and a $CaCl_2$ tube. While a slow stream of N_2 was passed through the apparatus immersed in an ice bath, butyl lithium (2.5M, 3.4 mL, 8 mmol) was added. The temperature rose from 5 to 18°C and the reaction mixture assumed a blood red color. The ice bath was removed and 20 min later a solution of compound 9 (1.5 g, 7 mmol) in THF (10 mL) was added over 7 min with intermittent use of an ice bath to keep the temperature below 15°C. The reaction mixture was pale orange in color. One hour later, saturated NaCl (50 mL) was added. The organic layer was dried over Na_2SO_4. Removal of the drying agent and solvent gave a thick orange oil (4.3 g). This material was passed through a column (15 mm × 25 cm) containing silica gel (12 g) in PE. Two fractions were collected: Fraction 1 (from PE), 0.23 g, 62% tetraene; Fraction 2 (from 5% EE in PE), 0.69 g, 91% tetraene by analysis on a 50-m CPS-2 gas chromatographic column. Yield of tetraene at this point was 35%. The all-*cis* isomer was separated from Fraction 2 on a column (0.76 × 70 cm) containing Amberlyst XN1010 (80/120 mesh) 62% Ag/Na using 2% acetonitrile in methanol as eluent. No attempt was made to separate the small amounts of other isomers present. Yield of the all-*cis* isomer from the Wittig reaction was 12%; overall yield from 7-bromoheptanoic acid was 6%. Deuterium distribution: 2.3% d_0, 92.2% d_2, 0.3% d_3, 5% d_4, 0.3% d_5. ^{1}H NMR ($CDCl_3$): δ 5.34(*m*,6H, C*H*=C*H*), δ 3.63(*s*,3H,OCH_3), δ 2.78(*m*,6H,C=CCH_2C=C), δ

2.27(t,2H,CH_2COO), δ 2.04(m,4H,CH_2C=C), δ 1.58(m,2H,CH_2CH_2COO), δ 1.30(m,6H, (CH_2)$_3$), δ 0.95(t,3H,CH_3). ^{13}C NMR (ppm): C-1, 174.0; C-2, 34.0; C-3, 24.8; C-4 through C-6, 28.8-29.3; C-7, 26.9; C-10, C-13, C-16, 25.5; C-11, C-14, C-15, 128.3-128.4; C-12, 127.9; C-17, 126.9; C-18, 131.8; C-19, 20.5; C-20, 14.2.

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Reactions of Dinitrogen Pentoxide and Nitrogen Dioxide with 1-Palmitoyl-2-Oleoyl-*sn*-Glycero-3-Phosphocholine

C.C. Lai and B.J. Finlayson-Pitts*
Department of Chemistry and Biochemistry, California State University, Fullerton, California 92634

The reactions of gaseous dinitrogen pentoxide (N_2O_5) and nitrogen dioxide (NO_2) with 1-palmitoyl-2-oleoyl-*sn*-glycero-3-phosphocholine (POPC) coated on the inside surface of a glass reaction cell were studied at 298 K. Unsaturated phosphatidylcholines are significant components of pulmonary surfactant in the alveolar region of the lung and hence serve as a simple model to examine reactions of pulmonary surfactant with these oxidant air pollutants. Using high-performance liquid chromatography (HPLC), Fourier transform infrared and fast atom bombardment mass spectroscopy, the major products of reactions of POPC with N_2O_5 and NO_2 were separated and identified. In the POPC-N_2O_5 reaction using either air or helium as a buffer gas, the nitronitrate, vinyl nitro and allylic nitro derivatives, as well as a small amount of the *trans*-isomer of the starting material, were obtained. The nature of the products obtained from the POPC-NO_2 reaction depends on the concentration of NO_2 as well as whether air is present. At low NO_2 concentrations ($P_{NO_2/N_2O_4} \leq$ 3.8 Torr) in air or in helium, the *trans*-isomer of POPC was formed almost exclusively. At higher NO_2 concentrations ($P_{NO_2/N_2O_4} \geq 20$ Torr) in helium, the dinitro, vinyl nitro and nitro alcohol derivatives were formed. In the presence of air (or 24% $^{18}O_2$ in helium), a nitronitrate and a dinitronitrate were additional products. Mechanisms for the formation of the observed products and implications for the inhalation of oxides of nitrogen are discussed.
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At the alveolar surface of the lung where gas exchange occurs, there is a liquid lining containing a soap-like substance which plays an important role in lung function. This substance, known as pulmonary surfactant (PS) (1,2), lowers the surface tension of the liquid layer and hence is essential for proper lung function (3–6).

The major surface-active components of pulmonary surfactant are phosphatidylcholines. They are believed to align themselves as a monolayer at the air-water interface where the hydrophobic fatty acid chains are projected into the gas phase (7). Since as much as 30% of the fatty acid components of the phosphatidylcholines are unsaturated (8), these double bonds may be susceptible to the attack of oxidant air pollutants such as ozone and nitrogen oxides.

Inhalation of oxidant air pollutants such as O_3, NO_2 or their combination are known to have a variety of effects on lung function and morphology (6,9–15). However, the chemical interactions of these reactive species with the unsaturated phosphatidylcholines in pulmonary surfactant have not been studied in detail, partly due to the difficulty of finding suitable analytical techniques to separate and identify the reaction products.

As part of our program to investigate the effects of oxidant air pollutants on the pulmonary surfactant system, we have used a wide range of analytical methodologies to investigate the reaction products of phosphatidylcholines with oxidant air pollutants. For example, we have demonstrated for the first time that isomeric ozonides are formed when 1-palmitoyl-2-oleoyl-*sn*-glycero-3-phosphocholine (POPC) is exposed to ozone (16). These isomeric ozonides, although sufficiently stable to be isolated, separated and characterized, can decompose at elevated temperatures or after sitting at room temperature for long periods of time, presumably *via* a free radical route similar to that proposed in the literature for the ozonides of alkenes and fatty acid methyl esters (17–20). Thus, their formation may initiate free radical processes leading to some of the observed health effects of ozone inhalation.

Since ozone and nitrogen dioxide are both present in polluted air (ref. 21, and references therein), the nitrate radical (NO_3) and dinitrogen pentoxide (N_2O_5) are also formed (Equations [1] and [2]);

$$O_3 + NO_2 \rightarrow NO_3 + O_2 \quad [1]$$

$$NO_3 + NO_2 \leftrightarrow N_2O_5 \quad [2]$$

NO_3 has been detected in both polluted and remote atmospheres (21) and the concentration of N_2O_5 has been estimated to be as high as 10–15 ppb (22). N_2O_5 is known to rapidly hydrolyze on surfaces to give nitric acid (21):

$$N_2O_5 + H_2O \xrightarrow{\text{surface}} 2\,HNO_3 \quad [3]$$

However, if significant concentrations of N_2O_5 do survive to reach the alveolar region, reaction between the hydrophobic unsaturated moieties of phosphatidylcholine and N_2O_5 may occur.

We reported earlier that dinitrogen pentoxide reacts with 1,2-dioleoyl-*sn*-glycero-3-phosphocholine (DOPC) and POPC to form nitronitrates that can be identified in the unseparated reaction product mixtures (23) using Fourier transform infrared (FTIR) spectrometry. In the case of DOPC, the structure of the

*To whom correspondence should be addressed.

Abbreviations: ATR, attenuated total reflectance; DOPC, 1,2-dioleoyl-*sn*-glycero-3-phosphocholine; FAB-MS, fast atom bombardment mass spectrometry; FTIR, Fourier transform infrared; GC, gas chromatography; HPLC, high performance liquid chromatography; KHP, potassium hydrogen phthalate; NO_x, oxides of nitrogen; PEPC, 1-palmitoyl-2-elaidoyl-*sn*-glycero-3-phosphocholine; POPC, 1-palmitoyl-2-oleoyl-*sn*-glycero-phosphocholine; PS, pulmonary surfactant.

nitronitrate was also confirmed by fast atom bombardment mass spectrometry (FAB-MS).

In the present, more extensive study, the major products of the reactions of N_2O_5 and NO_2 with a monounsaturated phosphatidylcholine, POPC have been separated using HPLC and identified using FTIR and FAB-MS. The products were studied as a function of the extent of reaction of the POPC and in the presence or absence of O_2, including isotopically labeled $^{18}O_2$. Possible mechanisms of formation of the observed products, as well as the implications for the inhalation of NO_x on pulmonary surfactant, are discussed.

MATERIALS AND METHODS

Materials. 1-Palmitoyl-2-oleoyl-*sn*-glycero-3-phosphocholine (99%) and the methyl esters of oleic, elaidic and stearic acid (all 99%) were obtained from Sigma (St. Louis, MO). Choline chloride (>99%) was obtained from Aldrich (Milwaukee, WI). High purity chloroform and methanol suitable for spectrophotometry and high-performance liquid chromatography were purchased from EM Science (Gibbstown, NJ). Nitric oxide (≥ 98.5%), and helium (>99.995%) were supplied by Union Carbide (Danbury, CT). Ultra-high purity grade air (>99.999%) was supplied by Scott-Marrin (Riverside, CA). Oxygen-18 (> 97 atom % ^{18}O) was obtained from Isotec (Miamisburg, OH). Reagent grade nitric acid was from Baker (Phillipsburg, NJ), and reagent grade sulfuric acid was from Fisher Scientific (Pittsburgh, PA).

Instrumentation. Nitro- and nitrated phosphatidylcholines were separated and isolated using high performance liquid chromatography (HPLC) with a Waters 600E multisolvent delivery system, a 30 cm × 0.39 cm Nova-Pak C18 4 μm reverse phase column (Waters, Milford, MA) preceded by a Waters Guard-Pak guard column module, and a Waters 900 photodiode array detector. The mobile phase was methanol/water (97:3, v/v), containing 40 mM choline chloride, at a flow rate of 1 mL/min.

Nitrate ion was analyzed using a Vydac 302-IC46 ion-exchange column (Hesperia, CA) with a Spectra Physics (St. Albans, U.K.) SP8700 solvent delivery system. A 2-mM aqueous solution of potassium hydrogen phthalate (KHP) was used as eluent, at a flow rate of 2 mL/min. The signal at 280 nm was followed using an UV detector; the mobile phase absorbs strongly at this wavelength so that nitrate appears as a negative peak on the continuous absorption due to KHP.

Gas chromatographic analyses were carried out on a Hewlett-Packard 5890 gas chromatograph (Palo Alto, CA) equipped with a flame ionization detector and an HP 3396A integrator. The *cis* and *trans* isomers of the monounsaturated C_{18} fatty acid methyl esters (*i.e.*, oleate and elaidate) were resolved using a high polarity cyanosilicone phase capillary column, SP-2330 (Supelco, Bellefonte, PA; 30 m × 0.25 nm i.d., 0.20 μm film). All GC samples were analyzed under isothermal conditions at 160°C for 12 min with helium as carrier gas at 5 mL/min. GC peak areas were converted to molar units using signal response factors referenced to an internal standard (methyl stearate) added just prior to analysis.

Samples for FTIR analysis were evaporated from chloroform onto the surface of a 25 × 10 × 2 mm 45° germanium crystal (Harrick Scientific, Ossining, NY) and spectra were recorded with a Mattson (Madison, WI) Cygnus FTIR spectrometer equipped with an optical accessory for attenuated total reflectance (ATR) measurements (24).

FAB mass spectrometric analysis was carried out in the positive ion mode using a 3-nitrobenzyl alcohol matrix, xenon gas and a VG-ZAB 1FHF (VG Analytical, Manchester, England) mass spectrometer.

Preparation of oxides of nitrogen. NO_2 and N_2O_5 were prepared using procedures described elsewhere (23,25). Gas-phase HNO_3 was prepared by distillation at 25°C of a 1:1 (v/v) mixture of fuming nitric and sulfuric acid into a trap at −196°C. The condensed HNO_3 was evaporated into a darkened 5-L bulb and was diluted with UHP grade air.

Exposure of POPC to nitrogen oxides. Approximately 2 mg of phospholipid was adsorbed from chloroform/methanol (2:1, v/v) onto the inner walls of 50-mL evacuable, tube-shaped borosilicate glass cells and, after evaporation of the solvent, exposed for 30 min in the dark to a gaseous mixture containing a known amount of nitrogen oxides (N_2O_5 or NO_2) in a buffer gas (helium or air) at one atm. The POPC reaction products were eluted from the walls of the reaction cells using 2 mL of chloroform or a chloroform solution containing a known amount of internal standard (cholesterol or methyl stearate). The extent of the reaction was calculated from the percentage recovery of the phospholipid using HPLC at a detector wavelength of 205 nm, or from the ratio of methyl oleate to methyl palmitate obtained by GC analysis of the sample which was esterified using acidified methanol and dimethoxypropane (26).

In experiments to isolate and characterize the reaction products, mixtures from at least three reaction runs were combined in order to obtain sufficient material for analysis. They were separated by HPLC and the peak fractions were collected from the outlet. The solvent from the HPLC fractions was evaporated on a water bath (~40°C) under a gentle stream of nitrogen. Most of the hydroscopic choline chloride salt, an additive to the HPLC mobile phase used to minimize peak tailing and facilitate peak separations of the phosphatidylcholine derivatives, was removed by extracting the moist residue from the fractions with chloroform, which was then concentrated and repeatedly filtered through a 0.45-micron Acrodisc filter (Gelman Sciences, Ann Arbor, MI).

RESULTS

Reaction of N_2O_5 with POPC. Table 1 summarizes the experiments carried out on the reaction of 2 mg of a solid film of POPC with N_2O_5. Since N_2O_5 also hydrolyzes on surfaces to form HNO_3 in competition with the POPC reaction, the ratios of the reactants shown in Table 1 are approximate upper limits.

The extent of the reaction as measured by the decrease in POPC increases with the ratio of N_2O_5 to POPC, and does not appear to be affected significantly by the presence of O_2 rather than He as the buffer gas.

TABLE 1

Percent Reaction of 1-Palmitoyl-2-Oleoyl-*sn*-Glycero-3-Phosphocholine (POPC) with N_2O_5[a]

Exp. no.	N_2O_5/POPC[b]	$P_{N_2O_5}$[c] (Torr)	% POPC consumed[d]	% PEPC observed[d]	% Other products[e]	Buffer gas
1	0.4	0.5	8	3	5	Air
2[f]	0.5	0.5	9	6	3	Air
3	0.5	0.5	11	6	5	He
4	0.8	0.5	20	6	14	Air
5	1.0	1.1	31	7	24	Air
6	1.5	1.7	40	7	33	Air
7[f]	2.0	2.0	50	10	40	Air
8	2.3	2.3	89	6	83	Air
9	3.3	3.2	98	2	96	He
10[f]	3.3	3.9	92	5	87	Air
11[f]	4.8	4.8	100	0	100	Air
12[f]	4.8	4.8	100	0	100	He
13[f]	6.1	5.6	100	0	100	18% $^{18}O_2$ in He

[a]All runs were carried out at 298 ± 3 K in the dark for 30 min.
[b]Molar ratios of N_2O_5 to POPC was calculated from the initial amount of POPC and N_2O_5 used; these were upper limits as discussed in the text.
[c]Initial pressure of N_2O_5 used.
[d]Mole % based on initial amount of POPC used; analytical error was estimated to be ±3%.
[e]% Other products = % POPC consumed − % PEPC observed.
[f]Experiments in which spectroscopic identification of products was carried out.

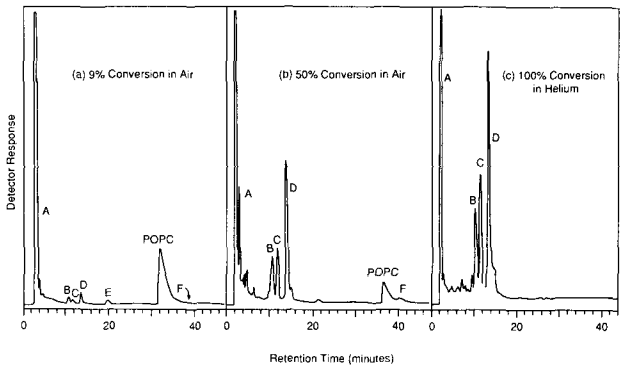


FIG. 1. HPLC chromatograms of the reaction mixture of N_2O_5 with POPC in air or in helium obtained at various percentage conversions of the POPC. See text for structural assignment of products.

The rather abrupt increase in the conversion of POPC from an N_2O_5/POPC ratio of 2.0 to 2.3 (Exp. 7 and 8) is probably due to the difficulty in reproducibly coating the solid POPC film on the surface of reaction cells, as well as the uncertainty in the amount of N_2O_5 available for the POPC reaction due to varying amounts of the competing N_2O_5 hydrolysis.

In order to make certain that the destruction of POPC was due to reaction with N_2O_5 (or its equilibrium mixture, containing NO_2 and NO_3) rather than the hydrolysis product HNO_3, the reaction of POPC with gaseous HNO_3 was also studied. No reaction products were observed by HPLC, and POPC was quantitatively recovered, indicating that the reaction of POPC with HNO_3, except to form the nitrate salt (23), can be discounted under our reaction conditions.

Figure 1 shows the HPLC chromatograms for experiments 2, 7 and 12, representing a low (9%), moderate (50%) and high (100%) conversion of POPC, respectively, either in air or in helium as the buffer gas. In all the reactions with less than 100% conversion, a small product peak, labeled F in Figures 1a and b, which eluted immediately after the POPC peak, was always observed. The UV, IR and FAB mass spectra of peak F are identical to those of POPC. However, esterification of peak fraction F shows that palmitic and elaidic (*trans* 18:1) acids are the component fatty acids. Peak F is therefore assigned to 1-palmitoyl-2-elaidoyl-*sn*-glycero-3-phosphocholine (PEPC), the *trans*-isomer of the starting material. The disappearance of PEAPC at higher extents of reaction suggests that, similar to POPC, PEPC can also react with N_2O_5.

The reaction of POPC with a large excess of N_2O_5 (N_2O_5/POPC ⩾ 3.3) in air or helium is relatively rapid. HPLC analysis of the reaction mixture indicated that POPC was completely consumed within the constant 30 min of reaction time used in these experiments.

At least four major reaction product fractions (labeled A, B, C and D in Fig. 1) were isolated from the reaction mixture by HPLC. The product fraction A, which eluted close to the solvent peak, consisted primarily of nitrate ion as determined by its characteristic UV spectrum, and by matching its retention time with that of sodium nitrate on an ion-exchange column.

The organic product fractions (B, C and D) were analyzed using FTIR (Fig. 2). The results of the mass spectral analysis are shown in Table 2 along with a summary of the product IR bands. For fraction B, the infrared spectrum shows that in addition to the C-H stretch (1475 cm^{-1}) and the C=O stretch (1750 cm^{-1}), which are also present in the starting material, two strong absorption bands at 1550 cm^{-1} and 1650 cm^{-1}, due to the asymmetric stretching mode of the $-NO_2$ (27,28) and $-NO_3$ (29) groups, respectively, are also

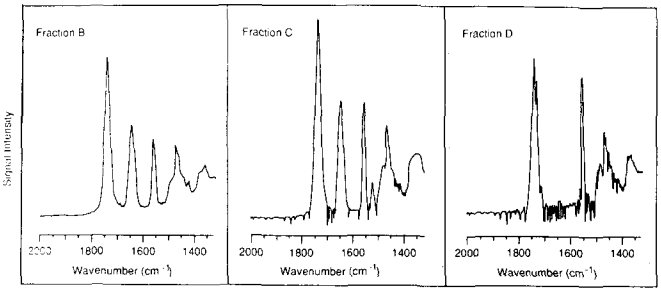


FIG. 2. FTIR spectra of the HPLC product peak fractions B, C and D obtained from the reaction of N_2O_5 with POPC in air or in helium (See Fig. 1).

observed. The FAB mass peak $[M+1]^+$ at *m/z* 869, suggests that both the $-NO_2$ and the $-NO_3$ groups, with a combined mass of 108 amu, have been introduced into the POPC molecule for which $[M+1]^+$ is found at *m/z* 761. The FTIR and FAB data are therefore consistent with a saturated nitronitrate phosphatidylcholine structure.

A new IR absorption band characteristic of a vinyl nitro group (27,28) at 1525 cm^{-1} was observed for product fraction C (Fig. 2). The mass spectrum of this fraction also showed an intense $[M+1]^+$ signal at *m/z* 806, consistent with a vinyl nitro phosphatidylcholine structure. The mass spectral [M+1] peak at *m/z* 869 and the IR bands due to the nitronitrate likely result from the overlapping of fractions B and C during chromatographic separation.

The infrared band at 1550 cm^{-1} (Fig. 2) and the mass peak $[M+1]^+$ at *m/z* 806 indicate that a nonconjugated nitro phosphatidylcholine is present in product fraction D. Based on previous work reported in the literature on the reaction of olefins with N_2O_5 (see Discussion section), these spectral peaks are tentatively assigned to an allylic nitro phosphatidylcholine structure.

Although the presence of O_2 did not appear to alter the nature or yields of the products (Table 1), the N_2O_5–POPC reaction was also carried out using 18% $^{18}O_2$ in helium as buffer gas (Exp. 13) in order to see if any of the observed reaction products incorporate oxygen from O_2. No incorporation of ^{18}O into the reaction products was found using FAB mass spectrometry.

A small peak due to a product labeled E in Figure 1a appears at low conversions of the POPC. This product showed an infrared band at 1650 cm^{-1} characteristic of an organic nitrate and a mass spectral peak $[M+1]^+$ at *m/z* 858, which corresponds to the addition of 97 amu to POPC. The peak at 858 was not observed during mass spectrometric analysis of an unseparated mixture of reactants and products, *i.e.*, a mixture that had never been chromatographed or in contact with methanol, water or choline chloride. The FAB peak at *m/z* 858 suggests that product E contains both a nitrate group (62 amu) and chlorine (35 amu), arising from the reaction of an initially formed product with choline chloride in the HPLC eluent. The formation of the product corresponding to peak E at low conversions suggests that the primary product which gives rise to peak E in the presence of choline chloride also reacts with N_2O_5 and/or NO_3.

Reaction of NO_2/N_2O_4 with POPC. The experiments carried out on the reaction of POPC with NO_2 are summarized in Table 3. Since nitrogen dioxide is always in rapid equilibrium with its dimer, that is,

$$N_2O_{4(g)} \leftrightarrow 2\ NO_{2(g)} \qquad K_{eq} \qquad [4]$$

the notation NO_2/N_2O_4 is used to signify the mixture

TABLE 2

Summary of FTIR and FAB-MS Analyses of HPLC Product Peak Fractions Obtained from the Reactions of POPC with Nitrogen Oxides

Nitrogen oxides	Buffer gas	Fraction[a]	Infrared bands[b] (cm^{-1})	Mass spectral $[M+1]^+$ peaks[b] (*m/z*)	Tentative product identification
N_2O_5	He or Air	B	1550,1650	869	Nitronitrate
		C[c]	1525,1550,1650	806,869	Vinyl nitro
		D	1550	806	Allylic nitro
		E	1650	858	Chloronitrate
		F	*d*	761	PEPC
NO_2/N_2O_4	He	B	1550	806,824,853	Dinitro, nitro alcohol
		C	1525	806	Vinyl nitro
		E	*d*	761	PEPC
NO_2/N_2O_4	Air	B	1550	806,824,853	Dinitro, nitro alcohol
		C	1525,1550,1650	806,869	Vinyl nitro, nitronitrate
		D[c]	1525,1550,1650	806,914	Vinyl nitro, dinitronitrate
NO_2/N_2O_4	$^{18}O_2$ in He	B	*e*	806,824,853	Dinitro, nitro alcohol
		C	*e*	806,873	Vinyl nitro, nitronitrate
		D[c]	*e*	806,916	Vinyl nitro, dinitronitrate

[a]HPLC product peak fraction designations are given in Figures 1 and 3.
[b]The signals common to POPC are not included.
[c]This HPLC fraction overlapped with the preceding fraction.
[d]The infrared bands were identical to those of the POPC (9,16).
[e]Not available.

TABLE 3

Percent Reaction of 1-Palmitoyl-2-Oleoyl-*sn*-Glycero-3-Phosphocholine (POPC) with NO_2/N_2O_4[a]

Exp. no.	$[NO_2/N_2O_4]$/POPC[b]	P_{NO_2/N_2O_4}[c] (Torr)	% N_2O_4 in NO_2/N_2O_4[d]	% POPC consumed[e]	% PEPC observed[e]	% Other products[f]	Buffer gas
1	1.2	3.8	3	4	4	0	Air
2	1.2	3.8	3	3	4	0	He
3	3.4	3.7	3	14	12	2	Air
4	3.4	3.7	3	13	11	2	He
5	3.7	3.8	3	14	12	2	He
6	8.1	12	9	50	48	2	He
7	11	12	9	60	58	2	He
8	13	11	8	76	74	2	He
9	18	20	13	87	65	22	Air
10[g]	28	29	18	91	72	19	He
11[g]	34	33	20	99	3	96	Air
12	45	50	25	92	45	47	He
13[g]	53	50	25	93	39	54	He
14[g]	53	50	25	100	0	100	Air
15[g]	59	54	26	100	0	100	24% $^{18}O_2$ in He
16[g]	79	90	35	100	0	100	He
17[g,h]	103	131	41	100	0	100	He

[a]Unless specified otherwise, all runs were carried out at 298 ± 3 K for 30 min.
[b]Molar ratios of NO_2/N_2O_4 to POPC was calculated based on initial total pressure of NO_2/N_2O_4.
[c]Total pressure of NO_2/N_2O_4 used.
[d]Mole % of N_2O_4 in the mixture of NO_2/N_2O_4.
[e]Mole % was calculated based on initial amount of POPC used; analytical error is estimated to be ± 3%.
[f]% Other products = % POPC consumed − % PEPC observed.
[g]Experiments in which spectroscopic identification of products was carried out.
[h]This run was carried out for 1 hr.

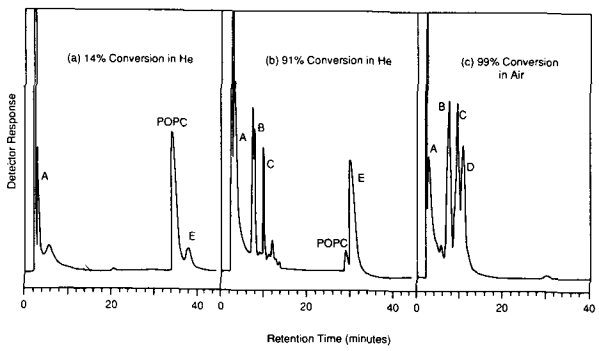


FIG. 3. HPLC chromatograms of the reaction mixture of NO_2/N_2O_4 with POPC in air or in helium obtained from experiments no. 5, 10 and 11 (Table 3). See text for structural assignment of products.

of the two throughout the text. The actual amount of N_2O_4 present in the equilibrium mixture was calculated using the known equilibrium constant, K_{eq} = 0.1461 atm at 298 K (30).

Figure 3 shows typical HPLC chromatograms at low (14%) and high (>90%) conversions of POPC, in either helium or in air as the buffer gas.

The reaction of POPC with NO_2/N_2O_4 is slower than that with N_2O_5. For example, in the reaction with N_2O_5, essentially all POPC was consumed at reactant ratios N_2O_5/POPC > 3.3 (Exp. 9, Table 1). However, in the reaction with NO_2/N_2O_4, complete destruction of the POPC in air within the 30 min reaction time required at least a 34-fold excess of NO_2/N_2O_4 (Exps. 10–16, Table 3).

At low molar ratios of NO_2/N_2O_4 to POPC with $P_{NO_2/N_2O_4} \leq 3.8$ Torr (Exps. 1–5), oxygen has no marked effect on the percent POPC consumed or on the amounts of PEPC and other products formed. The *trans*-isomer of the starting material, PEPC, labeled as peak E in Figures 3a and b, was the only major product.

For reactions that were carried out with a large excess of NO_2/N_2O_4 ($[(NO_2/N_2O_4)]$/POPC ≥ 18, P_{NO_2/N_2O_4} ≥ 20 Torr, Exps. 9–17), significant amounts of other products, in addition to PEPC, were also observed. The destruction of POPC, as well as the yield of these products, were enhanced by the increased concentration of NO_2/N_2O_4.

However, in contrast to the reactions of N_2O_5 with POPC, carrying out the reaction in air rather than in helium at these higher extents of reaction did decrease the yield of PEPC, as well as increase the yield of other products. In this case, the nature of the products observed was also affected by the presence of oxygen. For example, the major product peak fraction labeled D in the HPLC chromatogram of the product mixture obtained in air (Fig. 3c) is absent in the helium runs (Figs. 3a and b).

From ion-chromatography, fraction A in all NO_2 runs was found to be mainly nitrate ion present, at least in part, from trace amounts of HNO_3 in the NO_2 reactant.

The product peak fractions labeled B obtained from reaction in He or air were very similar, showing a strong IR absorption band due to a non-conjugated nitro group at 1550 cm^{-1}. Three $[M+1]^+$ mass spectral peaks were observed at *m/z* 806, 824 and 853, corre-

sponding to the addition of 45, 63 and 92 amu, respectively, to the POPC. Those at *m/z* 824 and 853 were attributed to the nitro alcohol and dinitro derivatives of the phosphatidylcholine, respectively. The peak at $[M+1]^+$ at *m/z* 806 was probably not due to a direct product because it corresponds to an unsaturated nitro derivative, yet it did not elute at the expected retention time for the vinyl or allylic nitro derivative. Thus it is attributed to fragmentation of one of the parent species in the mass spectrometer, *e.g.*, by elimination of HNO_2 from the dinitro derivative, or dehydration of the nitro alcohol.

In the runs with helium as a buffer gas, product fraction C mainly consisted of a vinyl nitro phosphatidylcholine, based on the infrared and FAB mass spectra. In the air runs, however, a small amount of nitronitrate was also detected. The infrared and mass spectral data of these products were similar to those obtained from the POPC-N_2O_5 reaction.

Product peak fraction D, which was only observed when air was present, showed infrared absorption bands at 1525, 1550 and 1650 cm^{-1} due to vinyl $-NO_2$, and unconjugated $-NO_2$ and $-NO_3$ groups, respectively. Two mass spectral peaks were observed. The infrared absorption at 1525 cm^{-1} and $[M+1]^+$ at *m/z* 806 in the mass spectrum are attributed to the vinyl nitro derivative resulting from the partial overlap of fractions C and D. The mass peak $[M+1]^+$ at *m/z* 914, which corresponds to the addition of 153 amu to the POPC, suggests a dinitronitrate structure, which is also consistent with the infrared spectrum.

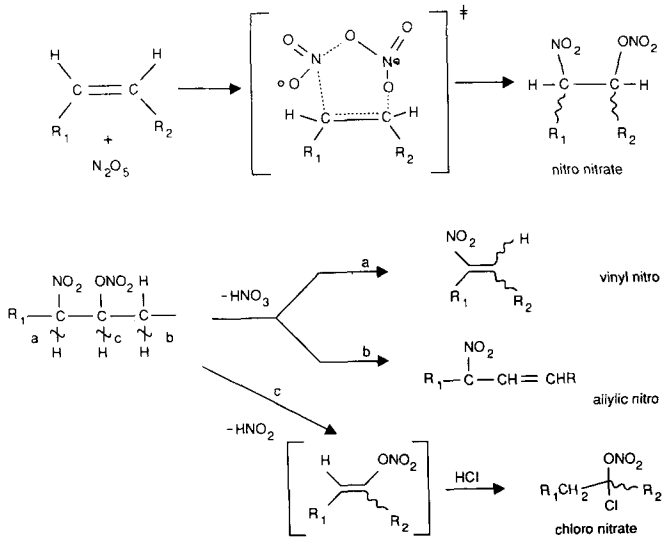
In order to see if oxygen is directly incorporated into the products when the reactions were carried out in air, the NO_2/N_2O_4-POPC reaction was carried out using 24% $^{18}O_2$ in helium. Exact mass spectral analysis on the reaction mixture, as well as on the separated HPLC product peak fractions, showed that only one ^{18}O label was incorporated into the dinitronitrate ($[M+1]^+$ at *m/z* 916) and two ^{18}O labels were found in the nitronitrate ($[M+1]^+$ at *m/z* 873).

DISCUSSION

Reaction of N_2O_5 with POPC. Using a combination of HPLC and spectroscopic techniques, we have shown that in addition to the nitronitrate product identified in earlier studies (23), vinyl and allylic nitro phosphatidylcholine derivatives are also formed in the reaction of N_2O_5 with POPC.

In the gas phase, N_2O_5 is in thermal equilibrium with the NO_2 and NO_3 radicals (Eqn. [2]). Thus, sequential addition of NO_3 and NO_2 to the olefin bond could yield the nitronitrate, as could a concerted addition of N_2O_5 to the POPC double bond. However, the lack of any observable effects of oxygen on the nature or the yields of the reaction products, the lack of incorporation of ^{18}O, and the absence of any detectable amount of dinitro or dinitrate products support the earlier hypothesis (31) that the addition of N_2O_5 to olefins is likely to occur in a concerted manner as shown in Scheme 1.

The relative yields of the nitronitrate and vinyl nitro derivatives change with the extent of reaction. Thus, as seen in Figure 1, the HPLC peak height of C



SCHEME 1

relative to peak B increases as the extent of conversion of the POPC increases (Fig. 1). This suggests that the vinyl nitro phosphatidylcholine is a secondary reaction product, perhaps due to further reaction of the nitronitrate. One possibility is elimination of nitric acid from the nitronitrate (path a in Scheme 1), involving either NO_3, NO_2 or NO_3^- as the hydrogen (or in the case of NO_3^-, the proton) acceptor. Since products due to trapping of an intermediate were not observed, such a bimolecular elimination process must occur in a concerted manner.

The allylic nitro derivative (peak D, Fig. 1) also appears to increase relative to peak B, at least up to 50% conversion of the POPC. Since it is not affected by the presence of O_2, its formation *via* a concerted bimolecular elimination of the nitronitrate is also a possibility (path b in Scheme 1).

The formation of vinyl and allylic nitro derivatives is consistent with the work of Stevens and Emmons (31), who observed such products in their studies of the reaction of N_2O_5 with propylene and 1-butene in an inert solvent at lower temperatures (243–273 K). Thus, 1-nitropropylene, 2-nitropropylene, 1-nitro-2-butene and 1-nitrobutene were obtained as minor products, in addition to the nitronitrates.

The chloronitrate (peak E, Fig. 1) observed at low conversions in the presence of methanol, water and choline chloride could arise from the ionic addition of an equivalent of HCl to an unsaturated nitrate during HPLC analysis, perhaps as shown in Scheme 1.

The formation of PEPC, the *trans*-isomer of the starting material, at low ratios of N_2O_5 to POPC is discussed below in conjunction with the isomerization induced by NO_2.

Reaction of NO_2/N_2O_4 with POPC. The reactions of NO_2/N_2O_4 with olefins are generally considered to be free radical in nature. For example, in the reaction of cyclohexene with NO_2/N_2O_4, the β-nitro alkyl radical intermediate can be trapped as β-nitrobromocyclohexane by abstraction of a bromine atom from a transfer

agent such as bromoform (32) or bromotrichloromethane (33). Thus, it is reasonable to assume that the initial step in the POPC-NO_2/N_2O_4 reactions is the addition of NO_2 to the double bond.

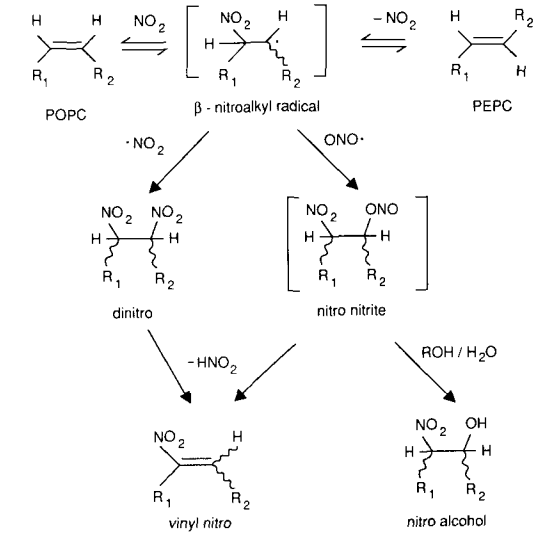
Thermal *cis-trans* isomerization of olefins normally requires a very high activation energy and can only occur at elevated temperatures (34). In the presence of free radicals, however, the isomerization can proceed smoothly at much lower temperatures. For example, in the presence of NO_2, a variety of alkenes have been observed to isomerize in solution, including *cis*-stilbene (35), unsaturated fatty acid esters (36), *cis*-2-butene and poly(*cis*-butadiene) (37). The same is true of NO_2 reactions with simple alkenes in the gas phase (38-40).

The mechanism of isomerization is believed to be as shown in Scheme 2 for POPC. The fact that oxygen does not change the PEPC yields at low pressures of NO_2/N_2O_4 (Table 3) suggests that both the C-C bond rotation and the loss of NO_2 in the β-nitro alkyl radical intermediate must be more rapid than the scavenging by molecular oxygen. Assuming a scavenging rate constant $k_{o_2} = 10^{-11}$ cm^3 molecule^{-1} s^{-1} for the addition of oxygen to a secondary radical (21,41,42) and $[O_2] = 4 \times 10^{18}$ molecule cm^{-3}, the first order rate constant for removal of the β-nitro akyl radical by O_2 is 4×10^7 s^{-1}. Thus, the rate of bond rotation and β-scission in the β-nitro alkyl radical would have to be faster than this for elimination of NO_2 to form PEPC to compete with scavenging of the radical by O_2. This is consistent with the results of Pitts and co-workers (38-40) who estimate that the rate constant for bond rotation of the NO_2 − *cis*-2-butene adduct in the gas phase is about 2×10^8 s^{-1} at 300 K.

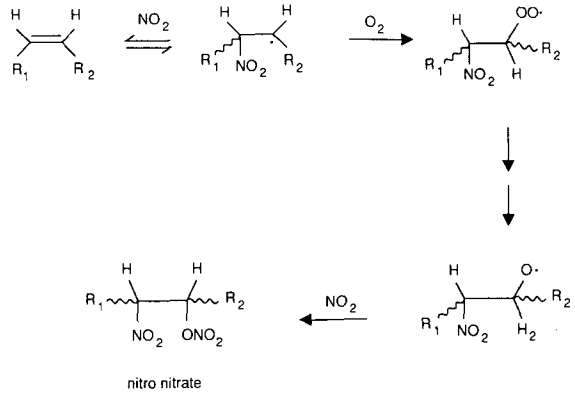
The yield of PEPC from the reaction of POPC with 0.5 Torr N_2O_5 is similar to those of the NO_2-POPC reactions at 3.8 Torr NO_2 (*i.e.*, Exps. 1-3 in Table 1 *vs* Exps. 1-2 in Table 3). The amount of NO_2 (and NO_3) in equilibrium with 0.5 Torr N_2O_5 can be calculated from the equilibrium constant (21) to be ~0.8 mTorr. Thus, NO_2 alone cannot be responsible for the PEPC formation in the N_2O_5-POPC system. This suggests that the nitrate radical must also isomerize POPC and, furthermore, is a more effective catalyst than NO_2 for this isomerization. Since there are no reports in the literature on the isomerization of alkenes by NO_3, this area warrants further study.

At high concentrations of NO_2 ($[NO_2/N_2O_4]$/POPC > 18, $P_{NO_2N_2O_4} \geq 20$ Torr, Table 3, Exps. 9-17), the β-nitro alkyl radical intermediate can be trapped by a second molecule of NO_2. Based on the literature (35,43-53), this can occur by either a C-N or a C-O attachment to give the dinitro or the nitronitrite (Scheme 2). Nitronitrites are unstable, and undergo elimination of nitrous acid to give the vinyl nitro compound (45,46), hydrolyze to the nitro alcohol in the presence of water or alcohol (43-53), or oxidize to the nitronitrate in the presence of excess oxygen (48-55). The results of this product study of POPC with NO_2/N_2O_4 are therefore consistent with earlier studies with alkenes.

In the NO_2/N_2O_4-POPC reaction, the nitronitrate and dinitronitrate can only be observed when air is present, indicating that these products likely result from oxygen scavenging of unstable intermediates. The



SCHEME 2

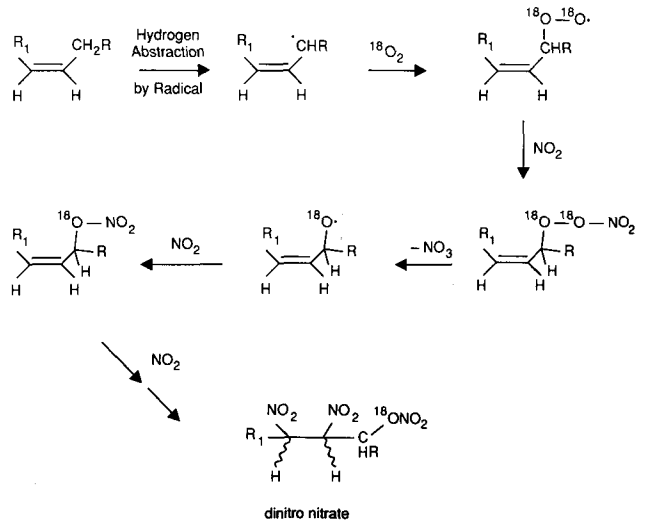


SCHEME 3

formation of nitronitrates in the NO_2/N_2O_4 reaction with simple alkenes in the presence of added O_2 has been suggested to proceed by a mechanism involving an unstable intermediate peroxynitrate (48,54-56), which decomposes to give an alkoxy radical. It has been proposed that this radical adds NO_2 to form the nitronitrate (Scheme 3). This mechanism, however, does not fully explain the formation of nitronitrate in the POPC reaction because the amount of oxygen in an atmosphere of air does not appear to efficiently scavenge the β-nitro alkyl radical as observed by the lack of effect of O_2 on the formation of PEPC at a low NO_x/POPC ratio (Exps. 1 and 2). Furthermore, this mechanism cannot explain the two ^{18}O labels observed in the nitronitrate by FAB-MS; as only one ^{18}O label would be expected, based on Scheme 3.

The mechanism of formation of nitronitrate is clearly complex. In solution, an unstable nitroso nitrate has been observed in the reaction of olefins with NO_2/N_2O_4 under anaerobic conditions at low temperatures (253 K); this species is slowly oxidized at room temperature to form a nitronitrate (55).

N_2O_3, which has been hypothesized to be an inter-



SCHEME 4

mediate in NO_2/N_2O_4/alkene reactions (49), is known to react with olefins to form nitroso nitrites (57). Two ^{18}O labels could be incorporated into the nitronitrate in the POPC-NO_2/N_2O_4 reaction by the formation and subsequent oxidation of such an intermediate:

$$N_2O_3 + R_1CH{=}CHR_2 \rightarrow R_1\overset{\overset{NO}{|}}{C}H-\overset{\overset{ONO}{|}}{C}HR_2$$

$$^{18}O_2/NO_x \quad \rightarrow\rightarrow \quad \overset{\overset{ON^{18}O}{|}}{R_1CH} - \overset{\overset{ONO^{18}O}{|}}{CHR_2} \qquad [5]$$

Clearly, the mechanism of the formation of the nitronitrate remains highly uncertain and warrants further investigation.

One mechanism for the formation of a dinitronitrate which contains only one ^{18}O label is shown in Scheme 4. In the studies of the reaction of NO_2/N_2O_4 with alkenes, Pryor and co-workers (58,59) have shown that at high NO_2 concentrations, direct allylic hydrogen abstraction by NO_2 is a minor process, consistent with our not finding allylic nitro phosphatidylcholine under anaerobic conditions. Thus, the allylic radical must result from hydrogen abstraction by species other than NO_2, such as peroxyalkyl radicals. Trapping of the allylic radical by molecular oxygen followed by scavenging by excess NO_2 would yield a peroxynitrate as proposed in earlier studies (48,54-56). Decomposition of the unstable peroxynitrate to an alkoxy radical followed by the reactions with NO_2 shown in Scheme 4 would form the ^{18}O labeled allylic nitrate.

The results of this study indicate that N_2O_5 reacts readily with the unsaturated fatty acid component of phosphatidylcholines, and hence if it reaches the alveolar region before hydrolyzing, it could form nitrated phosphatidylcholine derivatives. At the concentrations of NO_2 found in polluted air (<1 ppm), the formation of significant amounts of nitrogenated phosphatidylcholine derivatives from the NO_2 reactions is unlikely. However, a number of studies have shown that a change in configuration in the fatty acid chains of phosphatidylcholines can markedly affect the surface tension-reducing characteristics (60,61). Thus, isomerization of POPC to PEPC as we observed here, if it occurs in the lung, could alter the surface tension properties and hence the function of pulmonary surfactant. To investigate this possibility, the reactions of various nitrogen oxides with monolayers of phosphatidylcholines on a water surface in a film balance are currently being studied in this laboratory.

ACKNOWLEDGMENTS

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METHODS

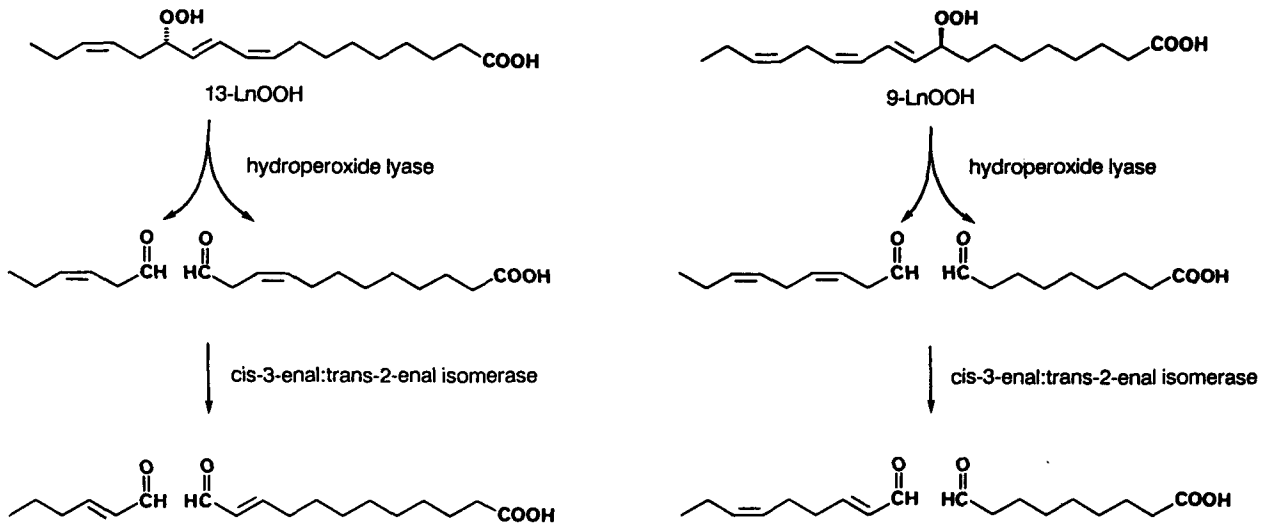
A Spectrophotometric Assay for Hydroperoxide Lyase[1]

Brady A. Vick*
U.S. Department of Agriculture, Agricultural Research Service, Northern Crop Science Laboratory, Fargo, North Dakota 58105

Spectrophotometric assays for hydroperoxide lyase have traditionally measured the loss in absorption at 234 nm due to the disruption of conjugated diene in the fatty acid hydroperoxide. However, that assay does not distinguish between hydroperoxide lyase and hydroperoxide dehydrase activities, both of which cause a loss of conjugation in the substrate hydroperoxide. A new assay has been developed which is specific for hydroperoxide lyase. It is based on the ability of hydroperoxide lyase products, aldehydes and ω-oxoacids, to serve as substrates for yeast alcohol dehydrogenase. Thus, the new hydroperoxide lyase assay is a coupled enzyme assay, which is conducted spectrophotometrically at 340 nm and measures the oxidation of nicotinamide adenenine dinucleotide, reduced form (NADH). In addition to its specificity for hydroperoxide lyase, the coupled assay allows higher concentrations of both fatty acid hydroperoxide substrate and crude enzyme extracts than the assay conducted at 234 nm.
Lipids 26, 315–320 (1991).

Hydroperoxide lyase is an enzyme of lipid metabolism which cleaves hydroperoxides of certain polyunsaturated fatty acids into aldehyde products. Fatty acid hydroperoxides are formed by oxygenation of linoleic or linolenic acids in a reaction catalyzed by the enzyme lipoxygenase. Depending upon the enzyme source, oxygen attack usually occurs at either carbon 9 or 13. Likewise, plant hydroperoxide lyases differ in their specificities, some catalyzing cleavage of only 9-hydroperoxides, others only 13-hydroperoxides, and still others with dual specificity (1,2). The reactions are shown in Scheme 1. Several assays have been used to measure hydroperoxide lyase activity. One common method employs gas chromatography for headspace analysis of the volatile aldehyde products (3). This procedure is time-consuming and usually results in a single data point to estimate the total amount of aldehyde produced within a specified time. A second method involves the derivatization of the aldehyde products with 2,4-dinitrophenylhydrazine, followed by extraction, chromatographic separation, and spectrophotometric measurement (4). Like headspace analysis, this is also a tedious process that results in one data point representing the total products formed up to that time. In a third method, the hydroperoxide lyase reaction is monitored spectrophotometrically at 234 nm to measure the decrease in absorbance caused by loss of the conjugated diene chromophore in the fatty acid hydroperoxide (5). This method is convenient, but is not specific for hydroperoxide lyase. It does not differentiate between hydroperoxide lyase and hydroperoxide dehydrase activity because both enzymes alter the conjugated diene chromophore in the fatty acid hydroperoxide.

In this paper, an alternative spectrophotometric assay is described for hydroperoxide lyase. This new method has a distinct advantage because it does not measure the presence of hydroperoxide dehydrase. The method is based on the ability of yeast alcohol dehydrogenase (ADH) to reduce aldehydes of various chain lengths (6).



SCHEME 1. Hydroperoxide lyase reaction with 9- or 13-hydroperoxylinolenic acid substrates.

[1]Names of products are included for the benefit of the reader and do not imply endorsement or preferential treatment by the United States Department of Agriculture.

*To whom correspondence should be addressed at USDA-ARS, P.O. Box 5677, Fargo, ND 58105.

Abbreviations: ADH, alcohol dehydrogenase; NADH, nicotinamide adenenine dinucleotide, reduced form.

Several of the aldehyde products of hydroperoxide lyase serve as substrates for yeast ADH and are converted to alcohols in the presence of the reduced form of nicotinamide adenine dinucleotide (NADH). Thus, hydroperoxide lyase can be assayed by coupling it with yeast ADH and measuring the loss in absorbance at 340 nm as NADH is oxidized.

MATERIALS AND METHODS

Chemicals. Linolenic and linoleic acid were purchased from NuChek Prep (Elysian, MN). Diethylaminoethyl (DEAE) cellulose, NADH, Triton X-100R, yeast ADH, soybean lipoxygenase type I-B, hexanal, and *trans*-2-hexenal were from Sigma Chemical Company (St. Louis, MO). Nonanal, *trans*-2-nonenal, and *trans*-2,*cis*-6-nonadienal were obtained from Aldrich (Milwaukee, WI); *cis*-3-hexenal was a product of Bedoukian Research Inc. (Danbury, CT) and was further purified by silver nitrate thin-layer chromatography to remove *trans*-2-hexenal. Bicinchoninic acid (BCA) protein assay reagent was purchased from Pierce (Rockford, IL).

Other substrates for ADH were prepared enzymically or chemically from fatty acids. 12-Oxo-*trans*-10-dodecenoic acid was prepared by cleavage of 13-hydroperoxylinolenic acid with cucumber hydroperoxide lyase. Cucumber flesh (1 g/mL of buffer) was homogenized at full speed with a Polytron homogenizer in 50 mM K-phosphate (pH 6.0) containing 0.1% Triton X-100R and centrifuged for 10 min at 12,000 × *g*. The reaction mixture contained 500 mL of 50 mM K-phosphate (pH 6.,0), 75 mL of 1.2 mM 13-hydroperoxylinolenic acid (prepared as described below), and 180 mL of cucumber extract. After 30 min, the mixture was adjusted to pH 3 with HCl and extracted with dichloromethane. The organic phase was dried with anhydrous sodium sulfate and evaporated under reduced pressure. 12-Oxo-*trans*-10-dodecenoic acid was purified from the extract by silica gel thin-layer chromatography in a solvent of hexane/diethyl ether/acetic acid (60:40:1, v/v/v). 12-Oxododecanoic acid was prepared by bubbling hydrogen through a methanolic solution of 12-oxo-*trans*-10-dodecenoic acid in the presence of PtO_2. α-Ketol, γ-ketol, and 12-oxophytodienoic acid were prepared from linolenic acid and a flaxseed extract as described for their 20-carbon homologs in an earlier report (7). 9-Oxononanoic acid was produced by reductive ozonolysis of oleic acid and purified by thin-layer chromatography in the solvent system described above.

Plant materials. Cucumbers (*Cucumis sativus* L.) were purchased from a local market. Sunflower (*Helianthus annuus* L., cv. NK 7111, Northrup King) seeds were germinated 2 to 3 days in moist paper towels in a germination chamber at 30°C. The seeds were then transferred to flats containing a sand/Perlite mixture (2:3, v/v) and placed in a growth chamber at 25°C under a 16 hr light, 8 hr dark cycle.

Preparation of partially purified enzymes. 9-Lipoxygenase was obtained from an acetone powder of harvest mature corn kernels (*Zea mays* L., var. NK PX443, Northrup King). The acetone powder (3 g) was extracted with 6 mL of 50 mM K-phosphate (pH 7.0) for 30 min at 4°C and centrifuged at 12,000 × *g* for 10 min. Lipoxygenase was partially purified from the supernatant on a small DEAE cellulose column containing about 2 mL bed volume. One mL of the supernatant was applied to a small column of DEAE cellulose equilibrated with the extraction buffer and allowed to drain into the matrix. Immediately following, the column was eluted with 3 mL of extraction buffer and three 1-mL fractions were collected and assayed for lipoxygenase. Fraction 2 or 3 usually contained the most lipoxygenase activity, which was free from both hydroperoxide dehydrase and hydroperoxide lyase activities.

Cucumber and sunflower hydroperoxide lyases were partially purified in a manner similar to that described for corn kernel lipoxygenase. Cucumber flesh (5 g) was diced and then homogenized at full speed with a Polytron homogenizer for 30 sec in 10 mL of 50 mM K-phosphate (pH 7.0) containing 0.1% Triton X-100R. The extract was centrifuged at 12,000 × *g* for 10 min and the supernatant saved. The first leaves of 8 to 12-day-old sunflowers were extracted similarly, except that the extraction buffer was at pH 6.0. One mL of each supernatant was applied to a small column of DEAE cellulose equilibrated with the appropriate extraction buffer containing 0.1% Triton X-100R. After the extract had eluted into the matrix, 3 mL of the extraction buffer was applied to the top of the column and three 1-mL fractions were collected. These fractions contained partially purified hydroperoxide lyase, with most of the activity usually in fraction 2. Protein concentrations of the partially purified enzyme solutions were determined using Pierce bicinchoninic acid protein assay reagent, with bovine serum albumin (BSA) as the standard.

Preparation of fatty acid hydroperoxides. The 13-hydroperoxide of linolenic or linoleic acid was prepared by the addition of 3 mL of soybean lipoxygenase solution (10 mg/mL in 10 mM borate buffer, pH 8.9) to 34.5 mL of oxygen-saturated distilled water. A sparge was used to bubble oxygen continuously through the enzyme solution during mixing with a magnetic stirrer. Then 7.5 mL of 8 mM linolenic or linoleic acid prepared according to Surrey (8) was added, and the reaction was allowed to proceed for at least 20 min. The final concentration of 13-hydroperoxide substrate was about 1.2 mM, estimated from a molar absorptivity of 25000 L/mol·cm at 234 nm.

The 9-hydroperoxide of linolenic or linoleic acid was prepared by an oxygenation reaction catalyzed with corn lipoxygenase. A 1.25-mL vol of 8 mM linolenic or linoleic acid (8) was added to 4.75 mL of oxygen-saturated 50 mM K-phosphate (pH 7.0). With oxygen bubbling through the solution, 1.5 mL of the partially purified corn kernel lipoxygenase solution was added and the reaction was allowed to proceed at least 30 min. The final concentrations were estimated by their absorbances at 234 nm to be 1.3 mM 9-hydroperoxylinolenic acid and 0.6 mM 9-hydroperoxylinoleic acid.

Enzyme assays. Except where noted, a typical hydroperoxide lyase assay mixture contained, in order of addition, 100 mM K-phosphate (pH 6.0), 0.1 mM NADH, 50 units/mL yeast ADH (one unit converts 1.0 μmol of ethanol to acetaldehyde per min at pH 8.8 at 25°C), 0.1 mM linolenic or linoleic acid hydroperoxide, and 0.05 to 0.2 mL of enzyme extract in a final volume of 1 mL. The pH of 6.0 was chosen because the optimum activity of many hydroperoxide lyases is in the range of pH 6 to 7. Although the pH optimum of yeast ADH is pH 8.8, the enzyme has sufficient activity at pH 6.0 that it is not rate-

limiting. In reactions conducted below pH 6.0, we sometimes observed protein precipitation which made the assay unsuitable. The above reactants were mixed in the absence of enzyme and the background oxidation rate of NADH was measured. The lyase reaction was then initiated by the addition of enzyme extract, and the initial rate of decrease in absorbance at 340 nm was measured. After subtraction of the background rate, the net enzyme rate was expressed as moles of NADH oxidized per min, based on a molar absorptivity of 6220 L/mol·cm for NADH (9).

Hydroperoxide dehydrase was assayed according to Zimmerman and Vick (5) by measuring the decrease in absorbance at 234 nm due to disruption of the conjugated diene chromophore of 13-hydroperoxylinolenic acid. An extract of flaxseed acetone powder was used as the source of hydroperoxide dehydrase. The powder (1 g) was extracted in 50 mM K-phosphate (pH 7.0) for 30 min and then centrifuged 10 min at 12,000 × *g*.

ADH activity was measured with several aldehyde substrates. The reaction mixture contained 0.1 M K-phosphate (pH 6.0), 0.1 mM NADH, 0.5 to 50 units yeast ADH (depending upon the reactivity of the aldehyde substrate), and 0.1 mM aldehyde substrate in a total volume of 1 mL. The reaction was initiated by the addition of aldehyde solution. All stock solutions of aldehydes, except for acetaldehyde, were at a concentration of 10 mM in acetonitrile solvent.

RESULTS

Comparison of two spectrophotometric hydroperoxide lyase assays. The spectrophotometric assay of hydroperoxide lyase at 234 nm (Fig. 1A) was compared with the coupled spectrophotometric assay at 340 nm (Fig. 1C). Initially, both assay mixtures contained 0.05 mM 13-hydroperoxylinolenic acid, but the coupled assay additionally contained 0.1 mM NADH and 50 units/mL ADH. After 2 min, a partially purified sunflower leaf extract with hydroperoxide lyase was added. Both assays showed a decrease in absorbance due to the hydroperoxide lyase reaction.

In contrast, hydroperoxide dehydrase activity was only detected by the assay at 234 nm (Fig. 1B). When an extract of flaxseed acetone powder containing hydroperoxide dehydrase was added to the mixture, there was a rapid decrease in absorbance at 234 nm due to the loss of conjugated diene. However, when the flaxseed extract was added to the coupled enzyme assay at 340 nm (Fig. 1D), no activity was detected. Thus, the coupled enzyme assay measured only hydroperoxide lyase, and not hydroperoxide dehydrase.

There was a slow rate of oxidation of NADH by 13-hydroperoxylinolenic acid in the presence of ADH (Fig. 1C) prior to the addition of sunflower hydroperoxide lyase. At high concentrations of 13-hydroperoxylinolenic acid, this background oxidation rate becomes significant and should be subtracted from the measured hydroperoxide lyase rate.

Relative efficiencies of yeast ADH substrates. Several carbonyl compounds were tested for their effectiveness as substrates for yeast ADH. Among the compounds tested were various metabolites of the hydroperoxide lyase and hydroperoxide dehydrase pathways. Table 1

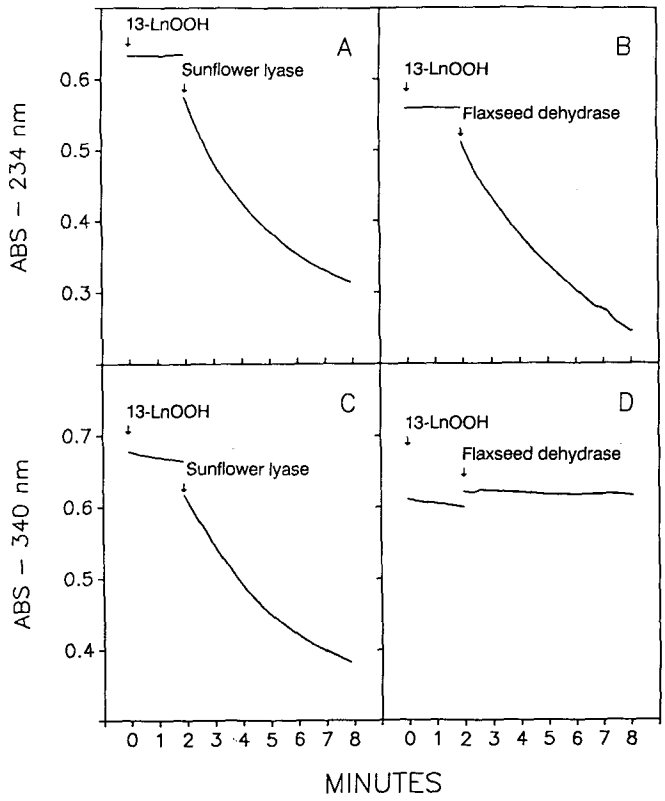


FIG. 1. Comparison of spectrophotometric assays for hydroperoxide lyase and hydroperoxide dehydrase monitored at 234 nm (hydroperoxide degradation) and at 340 nm (coupled with ADH). (A) Sunflower leaf lyase assay, 234 nm; (B) flaxseed acetone powder dehydrase assay, 234 nm; (C) sunflower leaf lyase assay coupled with ADH, 340 nm; (D) flaxseed acetone powder dehydrase assay coupled with ADH, 340 nm.

shows the relative rates of reduction of these compounds by NADH in the presence of ADH at pH 6.0. With the exception of nonanal, the NADH oxidation rates were quite low in comparison with acetaldehyde, the physiological substrate. At high concentrations of ADH (>50 units/mL), however, easily measurable rates were achieved.

Hexanal was a good substrate for ADH. Therefore, the coupled enzyme assay should always be applicable when 13-hydroperoxylinoleic acid is an effective substrate for hydroperoxide lyase, because hexanal is a product of that reaction. While *trans*-2-hexenal was also an effective substrate, *cis*-3-hexenal was not. Apparently, insertion of a *cis* double bond into the aldehyde chain remarkably reduces substrate efficiency. Consequently, one might predict that 13-hydroperoxylinolenic acid would be ineffective as a substrate for the ADH-coupled assay of hydroperoxide lyase, because the initial products of that reaction are generally thought to be *cis*-3-hexenal and 12-oxo-*cis*-9-dodecenoic acid (Scheme 1) (10). Both products possess *cis*-3 double bonds and neither would be expected to be a good substrate for ADH. Nevertheless, for reasons that are unclear, 13-hydroperoxylinolenic acid worked well as a substrate for all of the plant lyases we tested with this assay. For example, hydroperoxide lyase from tomato fruit was conveniently measured by the coupled assay, despite the fact that *cis*-3 aldehydes were reported by Galliard *et al.* (10) to be the major tomato

TABLE 1

Effectiveness of Various Aldehydes as Substrates for Yeast ADH[a]

Substrate	Rate of alcohol dehydrogenase
	nmol/min/unit
Acetaldehyde	200
Hexanal[b]	4.3
trans-2-Hexenal[b]	1.8
cis-3-Hexenal[b]	0
12-Oxo-*trans*-10-dodecenoic acid[b]	2.4
12-Oxododecanoic acid	0.5
Nonanal	51
trans-2-Nonenal[b]	7.4
trans-2,*cis*-6-Nonadienal[b]	3.9
9-Oxononanoic acid[b]	0.7
12-Oxophytodienoic acid	0
α-Ketol	0.05
γ-Ketol	0

[a]The assay mixture contained 0.1 M K-phosphate (pH 6.0), 0.1 mM NADH, 0.5 to 50 units alcohol dehydrogenase, and 0.1 mM aldehyde substrate.

[b]Metabolites of the hydroperoxide lyase pathway.

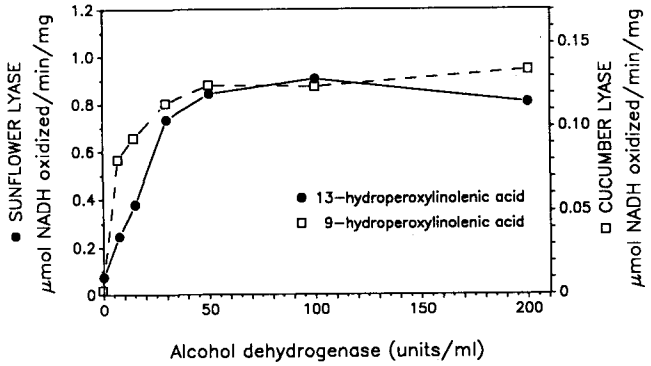


FIG. 2. Effect of yeast ADH concentration on the rate of the hydroperoxide lyase coupled enzyme assay.

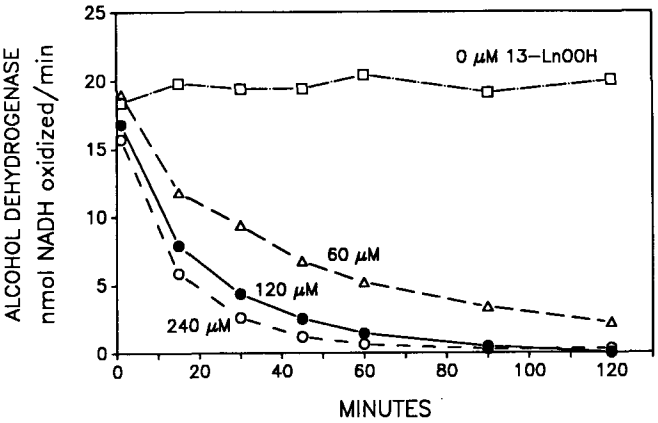


FIG. 3. Effect of 13-hydroperoxylinolenic acid concentration on the activity of yeast ADH. The concentration of ADH in each reaction was 50 units/mL.

lyase products. Our investigations of tomato hydroperoxide lyase have shown that both *cis*-3 and *trans*-2-hexenal are products in the ratio of approximately 2:1, respectively. It is possible that reduction of the *trans*-2-enal products to alcohols shifts the *cis*-3-enal/*trans*-2-enal equilibrium to favor production of the *trans*-2 isomer. This may explain the satisfactory results for the measurement of tomato lyase by the coupled assay.

Most plant extracts catalyze the *cis*-3 to *trans*-2 isomerization, albeit with various capacities. Evidence suggests that this isomerization is catalyzed by a separate isomerase enzyme (11), but the possibility that it may be catalyzed by the hydroperoxide lyase enzyme itself in some species cannot be excluded. If no isomerization to the *trans*-2 isomer occurs, then the coupled enzyme assay with ADH would not be expected to be as sensitive.

Three products of the hydroperoxide dehydrase reaction were also tested as substrates for ADH. All three compounds, 12-oxophytodienoic acid, α-ketol (13-hydroxy-12-oxo-*cis*-9,15-octadecadienoic acid), and γ-ketol (9-hydroxy-12-oxo-*trans*-10,*cis*-15-octadecadienoic acid), possess ketone functional groups rather than aldehydes. None were effective ADH substrates.

Effect of ADH concentration. To ensure that ADH was not rate-limiting in the coupled hydroperoxide lyase assay, reactions were conducted at several concentrations of ADH (Fig. 2). Substrates for the reaction were 9- or 13-hydroperoxylinolenic acid and the source of hydroperoxide lyase was partially purified cucumber flesh or sunflower leaf, respectively. The results showed that, at 50 units/mL, the ADH concentration was not rate-limiting in the measurement of hydroperoxide lyase from these typical plant extracts.

Inactivation of ADH by fatty acid hydroperoxides. Yeast ADH is affected by the presence of fatty acid hydroperoxides. Figure 3 shows the loss in activity of ADH over time at various concentrations of 13-hydroperoxylinolenic acid. Even at a hydroperoxide concentration of only 60 μM, over 36% of the ADH activity was lost after 15 min. To reduce this problem in the coupled assay, it is important to add the fatty acid hydroperoxide substrate immediately before the assay. The usual procedure was to first combine buffer, NADH, and ADH. Next the hydroperoxide substrate was added and the background NADH oxidation rate was measured for 1 to 3 min. Finally, the extract containing hydroperoxide lyase activity was added and the initial rate of decrease in absorbance at 340 nm was measured.

Stoichiometry. For every mole of fatty acid hydroperoxide cleaved by hydroperoxide lyase, two moles of aldehyde products are formed (Scheme 1). Accordingly, the coupled reaction with ADH should result in the oxidation of 2 mol of NADH for each mole of fatty acid hydroperoxide cleaved. Experimentally, spectrophotometric measurements of NADH oxidation showed that the aldehyde product yields were somewhat less than the expected 2 mol. The amount of NADH oxidized per mole of substrate was 1.5 mol for 13-hydroperoxylinoleic acid, 1.7 mol for 13-hydroperoxylinolenic acid, and 1.4 mol for 9-hydroperoxylinolenic acid. It is likely that some fatty acid hydroperoxide was converted non-enzymically to other products that were not substrates for ADH.

Effect of hydroperoxide dehydrase. Although the coupled hydroperoxide lyase-ADH assay does not measure

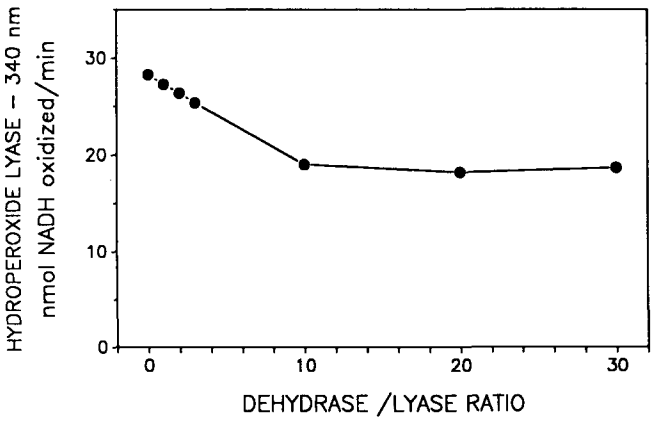


FIG. 4. Effect of various ratios of hydroperoxide dehydrase/hydroperoxide lyase activities (HD/HL) on the measurement of initial rates of the coupled hydroperoxide lyase/ADH reaction. Lyase activity was held constant and the HD/HL ratio was changed by adjusting the amount of dehydrase activity.

hydroperoxide dehydrase activity, the assay may be adversely affected by the presence of high levels of dehydrase. Hydroperoxide dehydrase competes for the same substrate as hydroperoxide lyase, and when dehydrase activity is high relative to lyase, the concentration of fatty acid hydroperoxide substrate available to hydroperoxide lyase is reduced. Consequently, the rate of the hydroperoxide lyase reaction declines. Figure 4 shows the effect of increasing the proportion of hydroperoxide dehydrase activity on the measurement of hydroperoxide lyase. Hydroperoxide dehydrase from a flaxseed acetone powder extract and hydroperoxide lyase from sunflower leaves were combined in various ratios based on their activities in the enzyme assay at 234 nm. As the proportion of hydroperoxide dehydrase increased, the initial rate of hydroperoxide lyase measured by the coupled assay at 340 nm decreased. When the ratio of dehydrase to lyase was greater than 10, the initial lyase rate was still about 66% of the rate observed when dehydrase was absent, but the total amount of NADH oxidized was reduced substantially.

DISCUSSION

The objective of this research was to develop a convenient spectrophotometric assay specific for hydroperoxide lyase. One common method to assay enzymes spectrophotometrically is to couple the product of the reaction with NADH and an appropriate dehydrogenase enzyme, and then measure the rate of oxidation of NADH by the decrease in absorbance at 340 nm. Two of the products of the hydroperoxide lyase pathway, hexanal and *trans*-2-hexenal, have been reported by Pietruszko *et al.* (6) to be good substrates for yeast ADH. Thus, the lyase reaction appeared to be a good candidate for a coupled enzyme assay, and this was confirmed by the present investigation.

The traditional spectrophotometric assay at 234 nm which measures the decomposition of fatty acid hydroperoxide is a convenient assay, but it does not discriminate between hydroperoxide lyase and hydroperoxide dehydrase. The coupled assay described here is specific for hydroperoxide lyase and is particularly useful in chromatographic separations to identify fractions containing lyase. Some plant tissues contain both lyase and dehydrase activities. Chromatographic separations of proteins from these tissues may yield two or more peaks with hydroperoxide degradation activity when the fractions are measured by the assay at 234 nm. Measurement of NADH oxidation by the coupled assay with ADH is a convenient method to establish which of the peaks correspond to hydroperoxide lyase.

Another disadvantage of the spectrophotometric assay at 234 nm is that proteins and other compounds present in crude and partially purified plant extracts often contribute to strong absorption in this region of the ultraviolet spectrum. This property often limits the amount of enzyme that can be added to the assay reaction mixture, and extracts with low enzyme activity often cannot be measured adequately. The assay at 340 nm allows five to ten times higher concentrations of enzyme extracts in the assay mixture because proteins do not absorb as strongly in this region. The same limitations are true for hydroperoxide concentration in the direct assay at 234 nm. This wavelength is used because the conjugated diene portion of the fatty acid hydroperoxide absorbs most strongly at 234 nm. Therefore, the concentration of hydroperoxide in the 234 nm assay is usually limited to less than 40 μM ($A \sim 1$).

Fatty acid hydroperoxides do not absorb significantly at 340 nm, and consequently higher concentrations of hydroperoxide can be used in the coupled assays conducted at this wavelength. The hydroperoxide concentration is limited only by its contribution to the background NADH oxidation rate and its adverse effect on ADH. Typically, the upper limit of hydroperoxide concentration is approximately 200 μM for the coupled lyase assay. The expanded concentration range permits acquisition of better kinetic data for enzyme properties such as K_m.

A few drawbacks to the coupled assay at 340 nm were noted. One disadvantage is that not all aldehyde products of the lyase reaction are effective substrates for ADH. Hexanal and *trans*-2 aldehydes are good substrates, but *cis*-3 aldehydes apparently are not. Thus, a situation could exist in which a plant hydroperoxide lyase is substrate specific for 13-hydroperoxylinolenic acid, resulting in *cis*-3-hexenal as a product. If the sample is devoid of *cis*-3-enal:*trans*-2-enal isomerase to convert the product to the *trans*-2-isomer, then the coupled assay may not be sensitive to the lyase activity. Such a situation could also occur during lyase purification procedures if the lyase became separated from the isomerase. Another consideration is the apparent reduction in the initial rate of hydroperoxide lyase when hydroperoxide dehydrase is present and competing for substrate (Fig. 4). However, all lyase assay methods in which hydroperoxide concentrations are in the region that displays first order kinetics are subject to the same competition by dehydrase.

In the process of developing this assay method, several plant tissues have been extracted and assayed, including young watermelon cotyledons, cucumber fruit, young soybean cotyledons, sunflower leaves, and tomato fruit. Hydroperoxide lyase activity was successfully assayed in each of these tissues by the coupled oxidation of NADH described here. In each tissue, the oxidation only occurred in the presence of a hydroperoxide substrate. There were

no endogenous substrates or NADH-requiring enzymes that interfered with the assay. Thus, the assay should be applicable to a wide range of plant sources. Despite minor limitations of the coupled assay, problems can be minimized or eliminated by following the precautions outlined here. The method should prove to be a useful alternative to investigators interested in the measurement of hydroperoxide lyase.

ACKNOWLEDGMENT

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Determination of Phospholipase D-Mediated Hydrolysis of Phosphatidylethanolamine

Zoltan Kiss
The Hormel Institute, University of Minnesota, Austin, Minnesota 55912

While phospholipase D-mediated hydrolysis of phosphatidylcholine is well documented, we have recently shown that phospholipase D-mediated hydrolysis of phosphatidylethanolamine (PtdEtn) [Kiss, Z., and Anderson, W.B., *J. Biol. Chem. 264,* 1483-1487 (1989); *J. Biol. Chem. 265,* 7345-7350 (1990)] is equally prominent. This made it necessary to define in detail the conditions required for the detection of agonist-stimulated PtdEtn hydrolysis. Using the [^{14}C]ethanolamine-prelabeled rat-1 fibroblast model and 12-*O*-tetradecanoylphorbol 13-acetate (TPA) as a model compound with the known ability to stimulate phospholipase D, we demonstrated that optimal detection of TPA-induced ethanolamine release requires i) fractionation of water-soluble ethanolamine products; ii) addition of unlabeled ethanolamine to quench the phosphorylation of newly formed [^{14}C]ethanolamine; and/or iii) prolonged preincubation of prelabeled cells in an isotope-free medium before the addition of TPA. This preincubation step reduces the cellular content of unincorporated ^{14}C-labeled ethanolamine metabolites and improves the signal-to-noise ratio.
***Lipids 26,* 321-323 (1991).**

Phospholipase D has recently emerged as an important enzyme component of the *trans*-plasma membrane signaling system. A number of agonists, including several growth factors, have been shown to stimulate its activity (reviewed in refs. 1 and 2). Phosphatidylcholine (PtdCho) has been widely regarded as the predominant substrate for this enzyme (1,2), with little consideration given to phosphatidylethanolamine (PtdEtn), usually the second most abundant phospholipid in mammalian membranes. Apart from our studies showing that 12-*O*-tetradecanoylphorbol 13-acetate (TPA) (3), adenosine triphosphate, guanosine triphosphate, and sphingosine (4) each stimulate PtdEtn hydrolysis, TPA has been shown only in one study to similarly stimulate the hydrolysis of PtdEtn and PtdCho (5).

The issue whether PtdEtn is also a substrate for phospholipase D activity (not necessarily identical with phospholipase D involved in PtdCho hydrolysis) is important to understanding the biological role of enhanced phospholipid metabolism. This requires utilization of a technique suitable for the detection of PtdEtn hydrolysis. Such a technique has not yet been fully defined. In this paper, we describe in detail optimal conditions which must be met in order to be able to demonstrate agonist-induced PtdEtn hydrolysis.

EXPERIMENTAL PROCEDURES

Materials. TPA, sphingosine, Dowex 50X8-400-WH$^+$ cation exchange resin, phospholipase C (Type IX; from *C. perfringens*), phospholipase A_2 (from bee venom) and phospholipase B (from vibrio species) were purchased from Sigma Chemical Company (St. Louis, MO). [2-^{14}C]-Ethanolamine (20 mCi/mmol) was from Amersham (Arlington Heights, IL), tissue-culture reagents were bought from GIBCO (Grand Island, NY), and fetal calf serum was bought from Flow Laboratories (McLean, VA).

Cell culture. Rat-1 fibroblasts were cultured continuously in Dulbecco's modified Eagle medium (DMEM) supplemented with 10% fetal calf serum, penicillin-streptomycin (50 U/mL and 50 μg/mL, respectively), and glutamine (2 mM). Cells used in the experiments were seeded (1-2 × 10^5/dish) in 100-mm plastic dishes, and growing (60-80% confluent) cell populations were harvested after two days in culture.

Determination of water-soluble products of phosphatidylethanolamine degradation in [^{14}C]ethanolamine-prelabeled cells. Rat-1 fibroblasts were incubated with [2-^{14}C]ethanolamine (0.20 μCi/mL) for 48 hr in DMEM containing 10% fetal calf serum. Cells were then washed and incubated in fresh medium (containing 10% serum) for an additional 3 hr or used without preincubation, as described. Cells were harvested by gentle scraping from the dish. Cells collected from 3-5 dishes (150 cm^2) were washed with fresh DMEM. At this step, 95-98% of cells were viable, determined by the trypan blue dye exclusion assay. Aliquots (0.3 mL) of the intact cell suspension (1.2-1.6 × 10^6 cells in 1 mL DMEM) were incubated (final volume 0.30 mL) in polypropylene tubes in an incubator at 37°C in the absence or presence of 2 mM unlabeled ethanolamine, as indicated, along with 100 nM TPA, as specified. Incubations were terminated by the addition of 4 mL of an ice-cold mixture of chloroform/methanol (1:1, v/v). Cells were extracted in this mixture for 60 min, followed by the addition of 3 mL of water. Complete phase separation was achieved by centrifuging the mixtures at 2,000 × *g* for 5 min. Five-milliliter aliquots of the water/methanol phase were used for the separation of ethanolamine metabolites using the method of Cook and Wakelam (6), with some modifications. Dowex-50-WH$^+$ resin was treated with two volumes of 1 M HCl for 2 hr. The Dowex-50-WH$^+$ packed columns (1 mL bed volume) were washed with water until the washings were approximately pH 5.5. The initial flow-through (5 mL), along with a following 3-mL wash, contained [^{14}C]glycerophosphoethanolamine. [^{14}C]Ethanolamine phosphate was eluted by 15 mL water, while [^{14}C]ethanolamine was eluted by 8 mL 1 M HCl. For the preparation of appropriate ^{14}C-labeled standards, the ^{14}C-prelabeled cells were treated with either phospholipase A_2 (1 U/mL) + phospholipase B (1 U/mL) or phospholipase C (1 U/mL), respectively, for 60 min, and extracts were prepared as above. Two-mL fractions were collected for the determination of the elution profiles of standards (determined in each experiment). The metabolites of [^{14}C]ethanolamine present in the peak fractions were further identified by thin-layer chromatography (3). Phospholipids were separated and their ^{14}C content was determined as previously described (7).

Abbreviations: DMEM, Dulbecco's modified Eagle medium; PtdCho, phosphatidylcholine; PtdEtn, phosphatidylethanolamine; TPA, 12-*O*-tetradecanolylphorbol 13-acetate.

TABLE 1

Effect on TPA on the Formation of Ethanolamine Products in Rat-1 Fibroblasts[a]

Preincubation (3 hr)	Ethanolamine (2 mM)	[^{14}C] Total water-soluble fraction (dpm/10^5 cells) None (0 min)	None (30 min)	TPA (30 min)
−	−	26,125 ± 670	24,130 ± 360	27,540 ± 690
−	+	26,930 ± 1000	26,760 ± 440	33,690 ± 400
+	−	4,576 ± 250	4,650 ± 540	7,690 ± 240
+	+	4,720 ± 350	5,500 ± 200	9,910 ± 450

[a][^{14}C]Ethanolamine-prelabeled rat-1 cells were incubated for 30 min under the conditions shown in the absence or presence of 100 nM TPA. After phase separation, aliquots were taken to determine ^{14}C activity in the total released water-soluble fraction. The ^{14}C content of PtdEtn at the beginning of incubation was 115,000 dpm/10^5 cells. Data are the mean ± S.E. of four determinations and are representative of four experiments.

TABLE 2

Effect of TPA on the Formation of Ethanolamine[a]

Preincubation (3 hr)	Ethanolamine (2 mM)	[^{14}C]Ethanolamine (dpm/10^5 cells) None (0 min)	None (30 min)	TPA (30 min)
−	−	1,880 ± 100	1,990 ± 90	5,180 ± 520
−	+	2,160 ± 120	3,930 ± 720	10,700 ± 430
+	−	520 ± 30	690 ± 320	3,570 ± 240
+	+	720 ± 120	1,460 ± 110	6,580 ± 470

[a]In the same experiment described in the legend to Table 1, aliquots of the upper phase were separated on Dowex-50-WH$^+$ columns as described in Experimental Procedures. Data are the mean ± S.E. of four determinations and are representative of four experiments.

RESULTS AND DISCUSSION

The purpose of this study was to establish conditions best suited for the determination of phospholipase D-mediated PtdEtn hydrolysis. Because TPA is one of the best activators of phospholipase D, this agent was used to enhance PtdEtn metabolism. Rat-1 fibroblasts were chosen for this work as these cells exhibit an average response to TPA compared to well-responding (such as NIH 3T3 fibroblasts) and less well-responding cells (such as the HL60 leukemic cell line). Thus, it is likely that data presented here will be applicable for many other cell lines. Data presented in Tables 1–4 are derived from the same experiment performed with [^{14}C]ethanolamine-prelabeled cells.

Effect of TPA on the release of total water-soluble [^{14}C]ethanolamine products. Some investigators who found no agonist-stimulated PtdEtn hydrolysis (*e.g.*, ref. 8) added the agonist to cells immediately after the removal of radiolabeled material, with no unlabeled ethanolamine added to the incubation medium. As shown in Table 1, under these conditions cells contain large amounts of water-soluble [^{14}C]ethanolamine metabolites, and TPA appears to have only minor effects, if any at all, on the release of [^{14}C]ethanolamine. Even under these conditions, addition of 2 mM ethanolamine to the incubation medium was beneficial in demonstrating some [^{14}C]ethanolamine release in response to TPA. The relatively small experimental errors in these experiments were due to the ability to control cell number by using scraped cells. With attached cells, the experimental error was much higher (in the range of 20%), which made it impossible to demonstrate any significant TPA effect.

Incubation of prelabeled cells for a 3-hr period in the absence of [^{14}C]ethanolamine before the addition of TPA resulted in a large reduction of the cellular pool of ^{14}C-labeled water-soluble ethanolamine metabolites. This significantly increased the signal-to-noise ratio, particularly in the presence of 2 mM unlabeled ethanolamine (Table 1). However, even under the latter condition, the background ^{14}C activity (present in untreated cells) was still relatively high.

Effect of TPA on the formation of [^{14}C]ethanolamine. After separation of ethanolamine from the other water-soluble products it becomes clear that ethanolamine is only a relatively minor component of the total water-soluble fraction in these fibroblasts (Table 2). Accordingly, the chromatographic step employed made the stimulatory effect of TPA more apparent on a percentage basis. Addition of 2 mM ethanolamine to cells preincubated for 3 hr appeared to be optimal for demonstrating the effect of TPA on [^{14}C]ethanolamine release (Table 2). Under these latter conditions, in four experiments 100 nM TPA stimulated [^{14}C]ethanolamine formation four–six-fold.

Effect of TPA on the formation of ethanolamine phosphate. As shown in Table 3, ethanolamine phosphate was the predominant water-soluble derivative of ethanolamine

TABLE 3

Effect of TPA on the Formation of Ethanolamine Phosphate[a]

Preincubation (3 hr)	Ethanolamine (2 mM)	[^{14}C]Ethanolamine phosphate (dpm/10^5 cells) None (0 min)	None (30 min)	TPA (30 min)
−	−	15,110 ± 230	16,150 ± 250	13,960 ± 810
−	+	17,280 ± 860	11,380 ± 650	11,760 ± 690
+	−	2,240 ± 150	2,230 ± 120	3,120 ± 250
+	+	2,410 ± 370	2,860 ± 180	2,530 ± 320

[a]Data are the mean ± S.E. of four determinations and are representative of four experiments.

TABLE 4

Effect of TPA on the Formation of Glycerophosphoethanolamine[a]

Preincubation (3 hr)	Ethanolamine (2 mM)	[^{14}C]Glycerophosphoethanolamine (dpm/10^5 cells) None (0 min)	None (30 min)	TPA (30 min)
−	−	4,690 ± 900	5,110 ± 560	3,960 ± 640
−	+	5,680 ± 440	4,350 ± 310	4,850 ± 630
+	−	1,420 ± 40	1,460 ± 90	1,410 ± 70
+	+	1,490 ± 170	2,130 ± 240	1,780 ± 180

[a]Data are the mean ± S.E. of four determinations and are representative of four experiments.

present in cells. Additional incubation of prelabeled cells for 3 hr resulted in a dramatic decrease in the cellular content of ethanolamine phosphate. In preincubated cells, TPA slightly enhanced the level of [^{14}C]ethanolamine phosphate in the absence of unlabeled ethanolamine (Table 3); this effect of TPA was prevented by the addition of 2 mM ethanolamine to the medium. These data indicate that in the absence of unlabeled ethanolamine a portion of newly formed [^{14}C]ethanolamine is further phosphorylated. In certain cell lines, such as HL60 cells (3), TPA stimulates PtdEtn resynthesis, which results in rapid elimination of the newly formed [^{14}C]ethanolamine pool. For this reason, in HL60 cells addition of unlabeled ethanolamine to the medium was important to demonstrate ethanolamine release (3). By comparison, in rat-1 fibroblasts TPA does not stimulate PtdEtn synthesis (data not shown). Thus, addition of unlabeled ethanolamine, while beneficial, is not essential for demonstrating the TPA effect on [^{14}C]ethanolamine release (Table 2).

Effect of TPA on the formation of glycerophosphoethanolamine. Glycerophosphoethanolamine represents the second largest pool of water-soluble ethanolamine derivatives (Table 4). Its intracellular level was also decreased during a 3-hr preincubation period; however, TPA did not significantly affect its metabolism.

In conclusion, the results establish the conditions required for the optimal detection of the TPA effect on PtdEtn hydrolysis in [^{14}C]ethanolamine-prelabeled cells. It is hoped that these data will stimulate further work on the regulation of PtdEtn hydrolysis.

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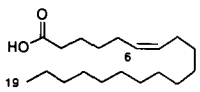
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Two Novel Phospholipid Fatty Acids from the Caribbean Sponge *Geodia gibberosa*

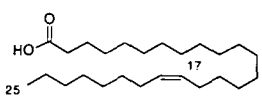
Néstor M. Carballeira* and **Jaime Rodriguez**
Department of Chemistry, University of Puerto Rico, Rio Piedras, Puerto Rico 00931

The novel fatty acids (Z)-6-nonadecenoic acid (1) and (Z)-17-pentacosenoic acid (2) were characterized in the sponge *Geodia gibberosa*. These fatty acids were mainly found in phosphatidylethanolamine and phosphatidylcholine.
***Lipids* 26, 324-326 (1991).**

The myriad of novel secondary metabolites arising from marine organisms have attracted the attention of an active group of marine natural products chemists (1). One particular area of interest has been that of novel lipids from marine sponges such as sterols and fatty acids (2). Fatty acids from sponges have recently provided numerous examples of structures of unusual biosynthetic origin, which are mostly without counterpart in the terrestrial environment (3). Most of the early research by Jeffert's group (4) indicated that these unusual acids were non-methylene interrupted polyenes, rather than the more commonly occurring methylene interrupted polyenes. Two rather interesting biosynthetic routes are thought to be operative in these invertebrates, namely the even sequences 18:1n-9 to either 24:1n-9 or 26:1n-9, and the route 18:1n-7 to either 24:1n-7 or 26:1n-7. There is no evidence that the biosynthetic route 19:1n-8 to 25:1n-8 is operative in nature. In fact, few nonadecenoic acids are known to exist, the most unusual examples being the 19:1n-11 and 19:1n-9 acids from the dolphin *Tursiops truncatus* (5). In the present communication, we report the identification of two novel odd-chain fatty acids, namely (Z)-6-nonadecenoic acid (1) and (Z)-17-pentacosenoic acid (2) from the phospholipids of the sponge *Geodia gibberosa*.



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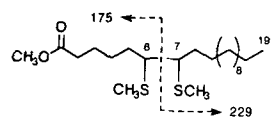
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*To whom correspondence should be addressed.

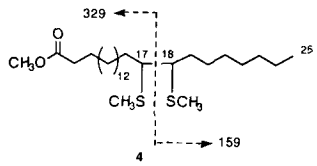
Abbreviations: ECL, equivalent chain length; FT-IR, Fourier transform-infrared; GC/MS, gas chromatography/mass spectrometry; TLC, thin-layer chromatography.

EXPERIMENTAL PROCEDURES

Geodia gibberosa was collected in July 1989 near the shelf edge of La Parguera, Puerto Rico, at a depth of 10 m. The sponge (500 g) was washed in sea water, carefully cleaned of all nonsponge debris and cut into small pieces. Immediate extraction with 600 mL of chloroform/methanol (1:1, v/v) yielded the total lipids (350 mg). The neutral lipids, glycolipids and phospholipids (100 mg) were separated by column chromatography on Silica Gel (60-200 mesh) using a procedure similar to that described by Privett *et al.* (6). The phospholipid classes were fractionated by preparative thin-layer chromatography (TLC) using Silica Gel G and chloroform/methanol/water (25:10:1, v/v/v) as solvent, affording mainly phosphatidylethanolamine and phosphatidylcholine. The fatty acyl components of these phospholipids were obtained as their methyl esters by reaction with methanolic hydrogen chloride (7) followed by column chromatographic purification and elution with hexane/diethyl ether (9:1, v/v). The resulting methyl esters were analyzed by gas chromatography/mass spectrometry using either a Hewlett-Packard 5995 A gas chromatograph/mass spectrometer (Hewlett-Packard Company, Palo Alto, CA) or a Hewlett-Packard 59970 MS ChemStation equipped with a 30-m × 0.25-mm nonpolar fused silica column coated with DB-1. Gas chromatographed/Fourier transform-infrared (GC/FT-IR) spectra were recorded on a Nicolet (Madison, WI) 740 FT-IR spectrometer. For the localization of double bonds, dimethyldisulfide derivatives were prepared by dissolving the esters (2 mg) in dimethyldisulfide (0.2 mL) and adding a solution (0.05 mL) of iodine in diethyl ether (60 mg/mL). The solution was heated at 50°C for 24 hr and the product was purified (8). Hydrogenations were carried out in 10 mL of absolute methanol in the presence of catalytic amounts of platinum oxide (PtO_2). Mass spectral data for the key fatty acids described are presented here:



3



4

Methyl 6,7-bis(methylthio)nonadecanoate **(3)** MS(70 eV) *m/z* (relative intensity), 404 (M^+, 12), 230(13), 229(68),

TABLE 1

The Major Phospholipid Fatty Acids from *Geodia gibberosa*[a]

Fatty acid	ECL	Abundance (%)
12-Methyltridecanoic (*i*-14:0)	13.60	1
Tetradecanoic (14:0)	14.00	1
4,8,12-Trimethyltridecanoic (16:0)	14.48	2
13-Methyltetradecanoic (*i*-15:0)	14.62	9
12-Methyltetradecanoic (*ai*-15:0)	14.70	10
Pentadecanoic (15:0)	15.00	2
9-Hexadecenoic (16:1)	15.72	4
5-Hexadecenoic (16:1)	15.76	1
14-Methylpentadecanoic (*i*-16:0)	15.60	4
Hexadecanoic (16:0)	16.00	10
9-Heptadecenoic (17:1)	16.78	4
15-Methylhexadecanoic (*i*-17:0)	16.60	8
14-Methylhexadecanoic (*ai*-17:0)	16.69	5
Heptadecanoic (17:0)	17.00	2
11-Octadecenoic (18:1)	17.74	3
Octadecanoic (18:0)	18.00	5
6-Nonadecenoic (19:1)	18.84	4
17-Methyloctadecanoic (*i*-19:0)	18.60	6
17-Tetracosenoic (24:1)	23.74	1
17-Pentacosenoic (25:1)	24.80	1
24-Methyl-5,9-pentacosadienoic (26:2)	25.08	1
5,9-Hexacosadienoic (26:2)	25.42	2
25-Methyl-5,9-hexacosadienoic (27:2)	26.15	3
24-Methyl-5,9-hexacosadienoic (27:2)	26.37	1

[a]Some minor acids are not included in this table.

213(7), 175(55), 174(34), 149(10), 145(14), 144(10), 143(100), 127(26), 117(11), 115(12), 111(15), 109(13), 101(9), 97(28), 95(23), 93(13), 87(23), 85(29), 83(23), 81(25), 79(13), 75(13), 74(17), 73(12), 71(21), 69(41), 67(34), 61(37), 55(46).

Methyl 17,18-bis(methylthio)pentacosanoate (4) MS(70 eV) *m/z* (relative intensity), 488(M^+, 7), 330(20), 329(100), 298(14), 297(55), 280(6), 249(8), 169(6), 163(7), 161(10), 160(10), 159(80), 158(15), 149(8), 123(13), 111(18), 109(20), 107(18), 97(24), 96(13), 95(35), 93(14), 87(29), 84(15), 83(28), 81(38), 79(16), 74(25), 69(82), 67(32), 61(44), 55(53).

RESULTS AND DISCUSSION

Capillary GC and gas chromatography/mass spectrometry (GC/MS) analysis of the fatty acid methyl esters from *Geodia gibberosa* gave the data presented in Table 1. As can be seen, the mixture contained large amounts of odd-chain fatty acids (60% of the total composition). The longest acids found belong to the *iso-anteiso* $\Delta^{5,9}$-27:2 series (3), while several aldehydes (arising from plasmalogens) with chain lengths between 22 and 24 carbons were also observed, including the common sponge aldehyde 17-tetracosenal (9). The compounds were characterized by comparison with authentic standards, based on equivalent chain length (ECL) values and mass spectral data. Two of the more abundant compounds arising from phosphatidylethanolamine and phosphatidylcholine were characterized as (Z)-6-nonadecenoic acid (1) and (Z)-17-pentacosenoic acid (2). Capillary GC on a DB-1 column and GC/MS were the principal tools of characterization. The (Z)-6-nonadecenoic acid (1) methyl ester gave a molecular ion peak at *m/z* 310 (M^+, 1%), a base peak at *m/z* 55 (100%), and typical fragmentation ions at *m/z* 278 (M^+-MeOH, 10%), *m/z* 236 (M^+-74, 4%), *m/z* 194 (M^+-116, 5%), *m/z* 87 (40%), and *m/z* 74 (62%). The data support the nonadecenoic acid methyl ester structure. Upon catalytic hydrogenation (PtO_2), the compound was transformed to nonadecanoic acid methyl ester as was confirmed by GC upon coinjection with an authentic standard. The bound bond position was determined on the corresponding dimethyldisulfide adduct (8,10). The mass spectrum of the adduct afforded a molecular ion peak at *m/z* 404 (M^+, 12%). The double bond was localized between carbons 6 and 7 based on the abundant fragmentations at *m/z* 175 ($C_8H_{15}SO_2^+$, 55%) and *m/z* 229 ($C_{14}H_{29}S^+$, 68%). These fragments reflect the cleavage exemplified in 3. We could also observe a characteristic peak at *m/z* 143 ($C_7H_{11}SO^+$, 100%) which is due to *m/z* 175 and the loss of methanol. The double bond was assigned to be *cis* because there was no absorption in the 968–980 cm^{-1} region. The experimental data thus support structure 1.

The other fatty acid, namely (Z)-17-pentacosenoic acid (2), as methyl ester, gave a molecular peak at *m/z* 394 (M^+, 1%), a base peak at *m/z* 55 (100%), and typical fragmentation ions at *m/z* 362 (M^+-MeOH, 11%), *m/z* 320 (M^+-74, 3%), *m/z* 87 (38%), and *m/z* 74 (56%). All the data are consistent with the structure of a pentacosenoic acid methyl ester. Upon catalytic hydrogenation (PtO_2), the compound yielded pentacosanoic acid methyl ester. The double bond position was determined by preparing the dimethyldisulfide adduct, which afforded a molecular ion peak at *m/z* 488 (M^+, 1%). The

double bond was determined to be between carbons 17 and 18 based on the abundant fragments at *m/z* 329 ($C_{19}H_{37}SO_2^+$, 100%) and *m/z* 159 ($C_9H_{19}S^+$, 80%) as exemplified in 4. We also observed a typical peak at *m/z* 297 ($C_{18}H_{33}SO^+$, 100%) which is due to *m/z* 329 plus loss of methanol. The double bond was identified as *cis* because of the absence of absorption in the 968–980 cm^{-1} region.

Over one hundred naturally occurring monoenoic acids have been identified in nature (11). In general, these acids have an even number of carbon atoms and the double bond has *cis* or Z configuration. As examples of Δ6 fatty acids we could mention 16:1n-10 from the seed oil of *Picramnia sellowii,* from sea anemones, and from other marine animals, or petroselinic acid 18:1n-12 which is common in umbelliferous seed oils (11). Our present report of the novel 19:1n-13 acid from *G. gibberosa* describes one of the first naturally occurring odd-chain Δ6 acids. *G. gibberosa* seems to possess a Δ6 desaturase system capable of utilizing long, odd-chain fatty acids as substrates. The second acid, (Z)-17-pentacosenoic acid (2), seems to have originated from a 19:1n-8 acid. The even-chain 24:1n-7 and 26:1n-9 acids have been identified before in other sponges (9), but the 25:1n-8 acid reported here had not been found in nature before. Research is in progress to delineate the biosynthetic routes that are operative in these invertebrates.

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Leukotriene B_4 Promotes Phospholipid Acylation in Human Neutrophils

Jen-sie Tou* and **Sean Healey**
Department of Biochemistry, Tulane University School of Medicine, New Orleans, Louisiana 70112

The present study was undertaken to test the hypothesis that leukotriene B_4 (LTB_4) may promote extracellular fatty acid incorporation into neutrophil choline glycerophospholipids (PC) to replenish phospholipids after deacylation. Incubation of human neutrophils with LTB_4 (1.5 to 150 nM) for 1 to 5 min resulted in increased fatty acid incorporation into phosphatidylinositol (PI), diacyl-*sn*-glycero-3-phosphocholine (diacyl-GPC) and alkylacyl-GPC. The magnitude of stimulation (percentage of control) of fatty acid incorporation appears to reflect increased activity of the acyltransferases catalyzing acylation of the respective lysophospholipids. LTB_4 stimulation of arachidonic acid incorporation into PI was greater than into PC, whereas the stimulation of palmitic acid incorporation into PC was greater than into PI. LTB_4 stimulated phosphatidic acid labeling by palmitic acid but not by arachidonic acid. LTB_4 and 1-*O*-alkyl-2-*N*-methylcarbamyl-*sn*-glycero-3-phosphocholine (cPAF) exhibited a similar stimulatory effect on fatty acid incorporation into the PC fraction. Phosphate analysis could not detect changes in the mass of PI or of PC in neutrophils exposed to LTB_4 or cPAF. The results suggest that increased fatty acid incorporation into phospholipids in LTB_4-activated neutrophils reflects activation of phospholipase A_2 and acyltransferases as well as of *de novo* phospholipid synthesis.
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Human neutrophils synthesize leukotriene B_4 (LTB_4) in response to chemotactic peptide (1–4). The precursor fatty acid of LTB_4, arachidonic acid, is liberated from cellular phospholipids by phospholipase A_2 (1,2) or can be provided in the extracellular medium (3,4). Neutrophils also synthesize LTB_4 in response to platelet-activating factor (PAF;5,6), and LTB_4 in turn promotes PAF formation (7,8). Both LTB_4 (9,10) and PAF (11) are agonists for neutrophil chemotaxis and aggregation, thereby amplifying inflammatory reactions. Increased PAF synthesis in LTB_4-stimulated neutrophils (7,8) suggests an activation of alkylacyl-*sn*-glycero-3-phosphocholine (alkylacyl-GPC) deacylation. The present study was undertaken to test the hypothesis that LTB_4 may promote extracellular fatty acid incorporation into both diacyl-GPC and alkylacyl-GPC to replenish phospholipids after deacylation. We demonstrate that LTB_4 promotes the incorporation of extracellular arachidonic acid and palmitic acid into phosphatidylinositol (PI), diacyl-GPC and alkylacyl-GPC. We also demonstrate that LTB_4 and a non-metabolizable platelet-activating factor analog, 1-*O*-alkyl-2-*N*-methylcarbamyl-*sn*-glycero-3-phosphocholine (cPAF), (12), exhibit a similar stimulatory effect on fatty acid incorporation into choline glycerophospholipids (PC).

MATERIALS AND METHODS

Materials. [1-^{14}C]Arachidonic acid (52.0 Ci/mol) and [1-^{14}C]palmitic acid (56.0 Ci/mol) were purchased from DuPont-New England Nuclear (Boston, MA). LTB_4, *Bacillus cereus* phospholipase C and fatty acid-free bovine serum albumin (BSA) were obtained from Sigma Chemical Co. (St. Louis, MO). cPAF was obtained from Calbiochem (San Diego, CA). Silica gel H plates were from Analtech (Newark, DE).

Preparation of human neutrophils. Human neutrophils were prepared as previously described (13). Cells were suspended in Dulbecco's balanced salt solution (14) containing 5 mM glucose at a concentration of 20 $\times$ 10^6 cells/mL. Cell counts were made in a hemocytometer, and cell viability was measured by trypan blue exclusion. Cell preparations contained more than 95% neutrophils.

Incubation of cells with LTB_4 or with cPAF. Each radiolabeled fatty acid was suspended in 0.9% NaCl containing BSA (4 mg/mL) at a concentration of 38–39 μM. LTB_4 and cPAF were each suspended in 0.9% NaCl containing BSA (2.5 mg/mL) at a concentration of 30 μM. Each incubation tube in a final volume of 2 mL contained 0.97 μM (0.1 μCi) [1-^{14}C]arachidonic acid or 0.95 μM (0.1 μCi) [1-^{14}C]palmitic acid, 0.15 to 150 nM LTB_4 or 150 nM cPAF, and 20 $\times$ 10^6 cells. Control tubes contained an equivalent volume of 0.9% NaCl containing BSA (2.5 mg/mL). Incubations were done at 37°C and started by adding 20 $\times$ 10^6 cells per tube.

Lipid analysis. Incubations were terminated by adding 5 mL methanol to each tube. Total lipids were extracted according to the method of Bligh and Dyer (15) and dissolved in chloroform/methanol (2:1, v/v) containing 0.01% butylated hydroxytoluene. Individual phospholipids were resolved by thin-layer chromatography (TLC) on Silica gel H plates (16). Phospholipid phosphorus and radioactivity were measured as previously described (16). Since phosphatidic acid (PA) levels could not be accurately measured by phosphate assay, the radioactivity of individual phospholipids was expressed as dpm/6 $\times$ 10^6 cells.

The distribution of labeled fatty acids in diacyl-GPC and alkylacyl-GPC was analyzed as previously described (16). Purified PC fractions from the control and LTB_4-activated neutrophils were each treated with

*To whom correspondence should be addressed at the Department of Biochemistry, Tulane Medical School, 1430 Tulane Avenue, New Orleans, LA 70112

Abbreviations: BSA, fatty acid-free bovine serum albumin; cPAF, 1-*O*-alkyl-2-*N*-methylcarbamyl-*sn*-glycero-3-phosphocholine; GPC, *sn*-glycero-3-phosphocholine; LTB_4, leukotriene B_4, 5S,12R-dihydroxy-6,14-*cis*-8,10-*trans*-eicosatetraenoic acid; PA, phosphatidic acid; PAF, platelet-activating factor, 1-*O*-alkyl-2-acetyl-*sn*-glycero-3-phosphocholine; TLC, thin-layer chromatography.

Bacillus cereus phospholipase C, and the resulting 1,2-diradylglycerols were acetylated in the presence of acetic anhydride/pyridine (10:1,v/v). The resulting 1-radyl-2-acyl-3-acetylglycerols were resolved into 1-alkenyl-2-acyl-3-acetylglycerol, 1-alkyl-2-acyl-3-acetylglycerol, and 1,2-diacyl-3-acetylglycerol fractions by TLC (16), and the radioactivity in each fraction was measured by liquid scintillation counting. Mild alkaline hydrolysis of the palmitic acid-labeled PC fraction was performed as previously described (13).

RESULTS AND DISCUSSION

LTB_4 promoted [1-^{14}C]arachidonic acid incorporation into PC and PI in a concentration-dependent manner. Figure 1 illustrates that LTB_4 at 0.15 to 150 nM did not significantly change the radioactivity of PA after a 2-min incubation. In contrast, LTB_4 enhanced the incorporation of labeled arachidonic acid into the PI and PC fractions from 1.5 to 150 nM. In the presence of 150 nM LTB_4 after a 2-min incubation, the radioactivity of PI and PC reached 400% and 250% of control cells, respectively. LTB_4 at 0.15 nM showed no stimulatory effect on arachidonic acid incorporation into PI or PC. Under the experimental conditions, there were no changes in the labeling of phosphatidylethanolamine and phosphatidylserine.

LTB_4 (150 nM) enhanced [1-^{14}C]arachidonic acid incorporation into PC and PI at all time points (1 to 5 min) examined (Fig. 2). Figure 3 shows that LTB_4 enhanced the incorporation of [1-^{14}C]palmitic acid into PA, PI and PC. The magnitude of stimulation (percentage of control) of fatty acid incorporation into phospholipids appears to reflect the specificity of the acyltransferases catalyzing the acylation of the respective lysophospholipids. LTB_4 stimulation of arachidonic acid incorporation into PI was greater than into PC, whereas stimulation of palmitic acid incorporation into PC was greater than into PI. Phospholipid phosphorus assays did not detect changes in individual phospholipid mass. The LTB_4 effect on labeled fatty acid distribution in diacyl-GPC and alkylacyl-GPC was examined as described previously (16). Figure 4 shows that LTB_4 stimulated the incorporation of labeled arachidonic acid and palmitic acid into both diacyl-GPC and alkylacyl-GPC. While more radioactivity appeared in the diacyl species, stimulation was more pronounced in the alkylacyl species than in the diacyl species (Fig. 4). In the presence of 150 nM LTB_4, the radioactivities of [^{14}C]arachidonic acid-labeled diacyl-GPC and alkylacyl-GPC reached 159% and 308% of control cells, respectively. Similarly, the radioactivities of [^{14}C]palmitic acid-labeled diacyl-GPC and alkylacyl-GPC reached 177% and 222% of control cells, respectively. The positional distribution of the [^{14}C]palmitoyl moiety in alkylacyl-GPC was examined by mild alkaline hydrolysis of the palmitic acid-labeled PC fraction (13). The radioactivity of the palmitic acid-labeled PC fraction was completely recovered in the fatty acid fraction; the resulting "alkaline-stable lysoPC" fraction contained no radioactivity.

The effects of a non-metabolizable PAF (12), cPAF and LTB_4 on fatty acid incorporation into diacyl-GPC and alkylacyl-GPC were compared. Figure 5 shows that

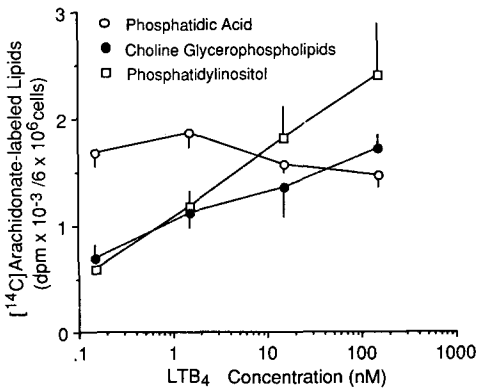


FIG. 1. The effect of LTB_4 concentrations on [1-^{14}C]arachidonic acid incorporation into phospholipids. Neutrophils (20 × 10^6 in 2 mL), 0.1 μCi [1-^{14}C]arachidonic acid (0.97 μM) and different LTB_4 concentrations (0.15 nM to 150 nM) were incubated at 37°C for 2 min. Each point represents the mean value ± standard deviation from three separate experiments.

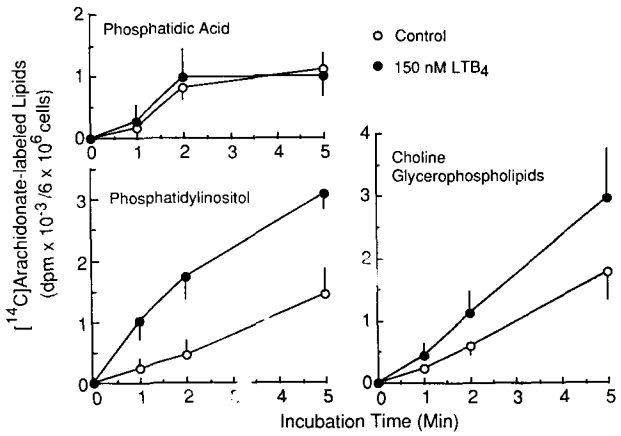


FIG. 2. Time course of LTB_4 stimulation of [1-^{14}C]arachidonic acid incorporation into neutrophil phospholipids. Neutrophils (20 × 10^6 in 2 mL) were incubated at the indicated time (1-5 min) with 0.1 μCi [1-^{14}C]arachidonic acid (0.97 μM) in the absence or presence of 150 nM LTB_4. Each point represents the mean value ± standard deviation from three separate neutrophil preparations.

cPAF promoted the incorporation of both arachidonic acid and palmitic acid into diacyl-GPC and alkylacyl-GPC. It appears that cPAF and LTB_4 exhibited a similar stimulatory effect on fatty acid incorporation into the PC fraction. In the presence of 150 nM cPAF after a 5-min incubation, the radioactivities of [^{14}C]arachidonic acid-labeled diacyl-GPC and alkylacyl-GPC reached 144% and 338% of control cells, respectively. Under identical conditions, the radioactivites of [^{14}C]palmitic acid-labeled diacyl-GPC and alkylacyl-GPC increased to 157% and 236% of control cells, respectively.

LTB_4 has been shown to enhance phospholipase A_2 activity in neutrophils (7,8). LTB_4 stimulation of phospholipid acylation as demonstrated in the present study may be brought about by a combined activation of phospholipase A_2 and acyltransferases. Activation

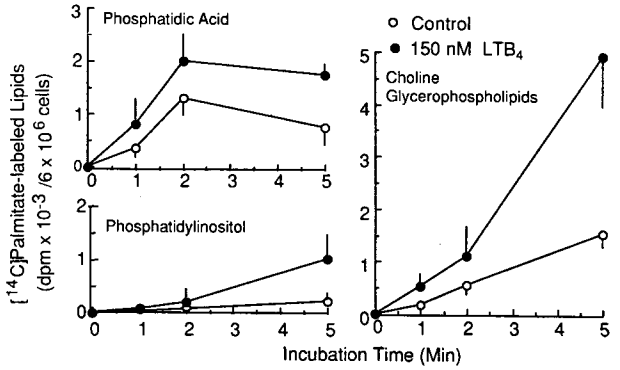


FIG. 3. Time course of LTB_4 stimulation of [1-^{14}C]palmitic acid incorporation into neutrophil phospholipids. Neutrophils (20×10^6 in 2 mL) were incubated at the indicated time (1–5 min) with 0.1 μCi [1-^{14}C]palmitic acid (0.95 μM) in the absence or presence of 150 nM LTB_4. Each point represents the mean value ± standard deviation from three separate neutrophil preparations.

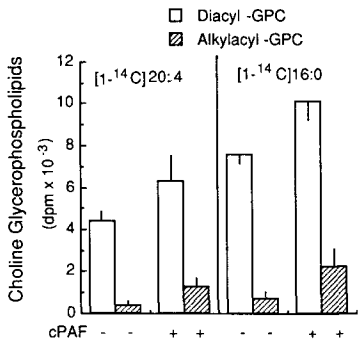


FIG. 5. cPAF promotes the incorporation of [1-^{14}C]arachidonic acid and [1-^{14}]palmitic acid into diacyl-GPC and alkylacyl-GPC. The incubation condition and method of lipid analysis were identical to those described in Figure 4, except that 150 nM cPAF was included in the incubation medium. The data presented are mean values ± standard deviation from three separate experiments.

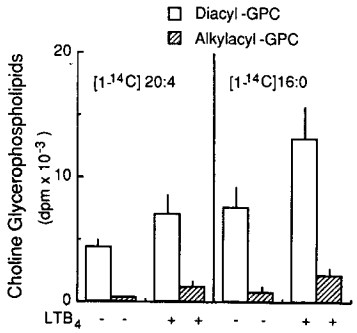


FIG. 4. LTB_4 promotes the incorporation of [1-^{14}C]arachidonic acid and [1-^{14}C]palmitic acid into diacyl-GPC and alkylacyl GPC. Neutrophils (20×10^6 in 2 mL) were incubated for 5 min with 0.1 μCi [1-^{14}C]arachidonic acid (0.97 μM) or 0.1 μCi [1-^{14}C]palmitic acid (0.95 μM) in the absence or presence of 150 nM LTB_4. An aliquot of PC (3 μg phosphorus) isolated from three incubation tubes was resolved into diacyl-GPC and alkylacyl-GPC as described in Materials and Methods. The radioactivity in each fraction was counted by liquid scintillation. The data presented are mean values ± standard deviation from three separate experiments.

of oleoyl-CoA acyltransferase but not arachidonoyl-CoA acyltransferase has been demonstrated in ionophore A23187-stimulated neutrophils (17). However, increased *de novo* synthesis of phospholipids may also contribute to increased fatty acid incorporation in view of the observed rapid incorporation of fatty acids into PA (Figs. 2 and 3) and increased labeling of PA by palmitic acid (Fig. 3). Increased glycerol incorporation into phospholipids has been shown in neutrophils activated by chemotactic peptide (17) and by ionophore A23187 (17,18). Increased incorporation of extracellular fatty acids into PI and PC in LTB_4-activated neutrophils may serve to replenish these phospholipids following deacylation. This may also divert extracellular arachidonic acid from the 5-lipoxygenase pathway, thereby attenuating the formation of LTB_4 and 5-hydroxy-eicosatetraenoic acid both of which are mediators of inflammation (9,10). Furthermore, increased acylation of 1-*O*-alkyl-*sn*-glycero-3-phosphocholine in LTB_4-activated cells may allow rapid termination of further PAF synthesis.

Increased incorporation of extracellular fatty acids into diacyl-GPC and alkylacyl-GPC by neutrophils also occurred in neutrophils challenged with PAF (13). The present study demonstrates that cPAF enhances fatty acid incorporation into both species of PC, indicating that the increased incorporation of extracellular fatty acids into alkylacyl-GPC in PAF-activated neutrophils (13) did not solely result from exogenous PAF after deacetylation. Since both LTB_4 (7,8) and PAF (7,19) promote PAF synthesis, the possibility remains that LTB_4 may induce its own synthesis from arachidonic acid. Furthermore, extracellular arachidonic acid may serve as a potential source for LTB_4 synthesis in neutrophils activated by LTB_4 or by PAF.

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Plasma Lipoproteins and Liver Lipids in Two Breeds of Geese with Different Susceptibility to Hepatic Steatosis: Changes Induced by Development and Force-Feeding

Dominique Hermier[a,b]*, Ali Saadoun[b], Marie-Rose Salichon[b], Nadine Sellier[c], Daniel Rousselot-Paillet[c] and M. John Chapman[a]

[a]INSERM Unite 321, Hopital de la Pitié, 75651 Paris Cedex, [b]INRA, Station de Recherches Avicoles, Nouzilly, 37380 Monnaie, and [c]INRA, Station experimentale de l'Oie, Artigueres, 40002 Mont de Marsan Cedex, France

Susceptibility to fatty liver in the force-fed goose is partly under genetic control. However, the mechanisms leading to liver steatosis in this avian model are poorly understood, but may involve perturbation in hepatic lipoprotein synthesis. Plasma lipoproteins were fractionated by density gradient ultracentrifugation from plasma of geese differing in their susceptibility to liver steatosis (Landes breed, highly susceptible; Rhine breed, partly resistant). The concentrations and chemical compositions of the major lipoprotein classes (VLDL, IDL, LDL and HDL) were characterized at 8, 22 and 27 wk of age and compared to the lipid composition of the corresponding liver. In non-force-fed geese, the lipoprotein profile was typical of birds, with high-density lipoprotein (HDL) predominating (4–5 g/L). However, at 22 and 27 wk of age, very low-density lipoprotein (VLDL) levels were significantly lower in Landes geese suggesting that this breed may possess a lower ability to export liver lipids, which would explain its susceptibility to liver steatosis when overfed. The livers of force-fed geese were specifically enriched in triglyceride, and to a lesser extent, in cholesteryl esters and non-esterified fatty acids as compared to those of control geese of the same age (27 wk). This accumulation of lipids was more pronounced in the Landes breed and was responsible for the higher liver weight in that breed. In both breeds, liver steatosis was accompanied by an increase in plasma levels of HDL (11 g/L), whereas low-density lipoproteins were essentially absent. An increase in VLDL plasma levels occurred in the Landes breed only (2.51 g/L *vs* 1.85 g/L in the Rhine breed), and was positively correlated with liver weight. However, VLDL in force-fed geese in both breeds were deficient in triglyceride (28–29% by wt) but enriched in cholesterol (41% by wt). These results indicate that a defect in the incorporation of triglyceride into nascent hepatic VLDL may result in liver steatosis in this species.

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Geese which have been force-fed with a carbohydrate-rich diet develop a fatty liver or "foie gras". This "foie gras" involves the development of a dramatic liver steatosis, in which the weight of the liver may increase ten-fold in two weeks and may account for up to 10% of the weight of the animal. Liver steatosis in the goose represents a quasi-pure form of acquired steatosis of nutritional origin, resulting in a specific accumulation of triglyceride by the parenchymal cells (1).

The induction of a marked fatty liver by force-feeding occurs only in specific breeds of geese, and more specifically in those derived from the French Landes grey geese. In other breeds, such as Rhine white geese, the resulting steatosis from force-feeding remains moderate. Moreover, even in Landes geese, there is considerable variability between individual birds in the response of the liver to force-feeding. Thus, the nutritional induction of fatty liver is modulated by an intrinsic susceptibility of genetic origin, for which no metabolic explanation is presently available. Despite the fact that this avian model constitutes a useful tool for the further understanding of the metabolic processes which underlie development of liver steatosis, the mechanisms which lead to specific accumulation of hepatic triglyceride in the goose remain poorly understood. While some studies have described the histological accumulation of intracellular lipids (2,3), and modifications in plasma lipids during force-feeding (4,5), others have attempted to document alterations in hepatic enzymes in both liver and plasma, and have related changes in these parameters to the development of liver steatosis (1,6).

Since very low-density lipoproteins (VLDL) are the predominant form in which triglyceride is secreted by the liver, we hypothesize that an increase in hepatic lipogenesis in response to force-feeding, associated with deficient synthesis or secretion of VLDL, may explain the occurrence of liver steatosis in the goose. However, there is a paucity of data on the qualitative and quantitative modifications of plasma VLDL and of other lipoproteins which may occur in association with force-feeding. Nevertheless, we have previously demonstrated that the plasma lipoprotein profile of young Landes geese did not reveal any specific feature which might explain the high susceptibility of this breed to liver steatosis (7).

For these reasons, we were led to investigate changes in plasma lipoproteins which occur during growth and force-feeding in two breeds of geese differing in their genetic susceptibility to liver steatosis. Inter-and intra-breed variations in lipoprotein profile were then related to the lipid composition of the livers.

MATERIALS AND METHODS

Animals and diets. The geese were males of the Landes

*To whom correspondence should be addressed at INSERM U.321, Pavillon B. Delessert, Hopital de la Pitié, 83 Bd de l'Hopital, 75651 Paris Cedex 13, France.

Abbreviations: IDL, intermediate density lipoproteins; HDL, high density lipoprotein; LDL, low density lipoprotein; mRNA, messenger ribonucleic acid; Na_2EDTA, disodium ethylenediamine tetraacetate; TG, triglyceride; VLDL, very low density lipoprotein.

breed (highly susceptible to liver steatosis and used for the production of fatty liver) and of the Rhine strain (less susceptible to liver steatosis and used for the production of meat). Twenty birds of each breed, hatched on the same day, were bred under natural conditions of light and temperature at the Experimental Station for Goose Breeding (INRA, Artiguères, France). From hatching to 8 wk, young animals were fed *ad libitum* a starting and growing diet (2780 Kcal and 150 g protein/kg) with added grass. From 8 to 22 wk, they were restricted to 150 g/day of a diet containing 2601 Kcal and 133 g protein/kg and to 500 g/day of grass. At 22 wk of age, 15 birds of each strain received the same diet *ad libitum* for 18 days in order to stimulate liver cell proliferation and to increase the stability of parenchymal cell membranes before force-feeding. They were then force-fed for 2 wk up to sacrifice with a carbohydrate-rich diet consisting mainly of boiled, salted maize with 0.5% lard added (3368 Kcal, 90 g protein and 4.5 g fat/kg). The five remaining birds of each strain were used as non-force-fed controls and continued to receive the same diet *ad libitum* from 22 wk to the time of sacrifice.

Blood and liver samples. At 8 wk, 22 wk (one day before the removal of dietary restriction) and 27 wk (one day before slaughter), all birds were fasted overnight for 18 hr, with water provided *ad libitum*. Blood (20 mL) was then withdrawn by puncture of the occipital venous sinus, collected on 0.12% (w/v) disodium ethylenediamine tetraacetate (Na_2EDTA) in a vacuum tube and kept at 2–4°C during the subsequent procedures. Individual plasma samples were separated by low-speed centrifugation. Antibacterial agents (sodium azide and sodium merthiolate, at final concentrations of 0.01% and 0.001% (w/v)) and a chelator of metal cations (EDTA, 0.04% w/v) were then added to each plasma sample. Plasmas were frozen at −80°C until further analyses.

After slaughter, the livers were removed, weighed and refrigerated at 4°C; a 10-g sample was taken from the ventromedial portion of the main lobe (right lobe) of each liver and stored at −20°C for subsequent analyses.

Lipoprotein isolation. Lipoproteins were isolated from plasma on the basis of their hydrated density in a single step according to the ultracentrifugal gradient procedure described by Chapman *et al.* (8) and modified by Hermier *et al.* (9). Ultracentrifugation was performed in a Beckman L8-55 ultracentrifuge using a Beckman SW41 rotor (Beckman Instruments, Palo Alto, CA) at 40,000 rpm (19.4×10^5 g) for 48 hr at 15°C. The gradients were fractionated by collecting fractions from the meniscus of each tube by aspiration with a narrow bore Pasteur pipette. The fractionation procedure took into account the alternance of pigmented and non-pigmented bands corresponding to the successive lipoprotein classes, as described previously in man (8) and chicken (10). Four lipoprotein classes were thus separated from the top to the bottom of the gradient, with the following respective densities: VLDL (d <1.013), intermediate density lipoproteins (IDL, d 1.013–1.020), low-density lipoproteins (LDL, d 1.020–1.040) and high-density lipoproteins (HDL, d 1.065–1.165) (7).

Lipoproteins were dialysed in Spectrapor tubing (Spectrum Medical Industries, Los Angeles, CA; mol wt cut-off 12,000) for 48 hr at 4°C against a solution containing 0.05 M NaCl, 0.005 M NH_4HCO_3, 0.04% EDTA and 0.01% sodium azide at pH 7.4.

Chemical analyses. The following components were quantified in each lipoprotein fraction and in whole plasma as follows: protein by the Lowry method (11) using bovine serum albumin (Sigma Chemical Co., St. Louis, MO) as a standard; triglyceride, free cholesterol, total cholesterol and phospholipids by enzymatic methods (12–14), using the kits provided by Bio-Merieux (Charbonnière-les-Bains, France). The amount of plasma free glycerol was determined using the kit provided by Biotrol (Paris, France) and was subtracted from the values obtained for plasma triglyceride. The amount of cholesteryl esters was calculated using the formula:

$$\text{cholesteryl ester} = (\text{total cholesterol} - \text{free cholesterol}) \times 1.67$$

In this calculation, it was assumed that the factor for the ratio of the average molecular weight of goose plasma cholesteryl esters to that of the molecular weight of free cholesterol (*i.e.* 1.67), was the same in man and the goose. The precision of these analyses was estimated by calculation of the technical error, the latter being defined as $\sqrt{\Sigma d^2/2N}$, where d is the difference between duplicate estimations and N the number of duplicates. The technical errors were: triglyceride 3.8%, cholesterol 1.2%, phospholipid 2.0% and protein 5.7%. Lipoprotein concentrations have been calculated by addition of the concentrations of the individual protein and lipid components.

Lipids were extracted from liver samples by the method of Folch *et al.* (15), and then quantified by thin-layer chromatography and flame ionization detection, using an Iatroscan TH 10 (Iatron Laboratories, Tokyo, Japan), as previously described (16). Eight liver samples from force-fed geese of each breed and all samples from control geese (5 in each breed) were submitted to such analysis.

Statistical analysis. Results were expressed as mean ± SD and their significance was tested using a one-way analysis of variance after verification of the homogeneity of variance by Barlett's test (17).

RESULTS

Effects of growth on plasma lipids and lipoproteins. Changes in plasma lipid and lipoprotein concentrations occurred independently of force-feeding and were documented at 8, 22 and 27 wk of age (Table 1). The plasma levels of free cholesterol (0.30–0.40 g/L) and cholesteryl esters (1.80–2.00 g/L) were not modified by growth and development, whereas phospholipid concentrations increased slightly from 2 g/L at 8 and 22 wk to 2.7–2.9 g/L in 27 week-old birds. In contrast, plasma triglyceride levels increased progressively with age from 0.40–0.45 at 8 wk to 1.3–1.6 g/L at 27 wk. However, no significant differences were detected between the Landes and the Rhine breed, irrespective of the age and class of lipid studied.

TABLE 1

Plasma Lipid Concentrations During Growth (age in wk) and Force-Feeding in Geese of the Rhine and Landes Strains[a]

	Growing (8 wk)[b]		Immature (22 wk)[b]		Control (27 wk)[b]		Force-fed (27 wk)[b]	
Concentration (g/L)	R[c] (20)[c]	L[c] (20)	R (20)	L (20)	R (5)	L (5)	R (15)	L (15)
Free cholesterol	0.34 ± 0.04	0.36 ± 0.05	0.31 ± 0.05	0.29 ± 0.04	0.38 ± 0.09	0.37 ± 0.09	0.84 ± 0.20	0.83 ± 0.15
Cholesteryl esters	1.84 ± 0.21	1.90 ± 0.32	1.84 ± 0.20	1.77 ± 0.20	1.79 ± 0.09	1.97 ± 0.30	4.31 ± 0.86	4.51 ± 0.84
Triglyceride	0.45 ± 0.09	0.41 ± 0.07	0.90 ± 0.20	0.81 ± 0.22	1.64 ± 1.06	1.25 ± 0.77	1.39 ± 0.93	1.95 ± 0.71
Phospholipids	2.04 ± 0.28	2.11 ± 0.24	1.99 ± 0.30	1.98 ± 0.21	2.86 ± 0.64	2.67 ± 0.64	5.15 ± 0.82	5.20 ± 1.28

[a]Values for lipid concentrations are mean ± SD.
[b]Details of diets for physiological status—growing, immature, control and force-fed birds— are provided in the Methods section.
[c]The strain is R, Rhine geese, L, Landes geese; the number of geese in each group is given in parentheses.

The progressive increase in plasma triglyceride levels was paralleled by a concomitant increase in plasma VLDL, whose concentrations were 7- to 10-fold higher in the oldest birds as compared to the younger ones (Table 2). On the other hand, the chemical composition of VLDL was grossly similar to that observed in other species, and varied but little with aging. Proportions of free cholesterol (6.5–7.0%) and phospholipids (17–18%) remained remarkedly constant, whereas the percentages of cholesteryl esters and triglycerides fluctuated between 15 and 20%, and 42 and 51%, respectively. The proportion of protein was higher (20%) in the VLDL of younger birds than in that of older animals (11–15%). At 22 and 27 wk of age, VLDL level was significantly higher in Rhine geese. At the latter age, the breed-related differences in VLDL were not only quantitative, but also qualitative, since VLDL in Landes geese contained significantly less triglyceride and more cholesteryl esters and protein than in the Rhine geese.

The denser classes of lipoproteins, *i.e.* IDL, LDL and HDL, were distinct from VLDL in that their concentrations and compositions exhibited little variation as a function of age or genetic origin. IDL concentrations (Table 3) fluctuated between 0.25 and 0.38 g/L; these lipoprotein particles exhibited similar contents of cholesteryl esters, triglyceride, phospholipid and protein, with lesser proportions of free cholesterol. The circulating concentrations of LDL (Table 4) and HDL (Table 5) (approximately 1 g/mL and 5 g/L, respectively) were typical of avian lipoproteins irrespective of the age or breed of geese. The major chemical constituent of LDL was cholesterol (8–10% free cholesterol and 28–34% cholesteryl esters) whereas that of HDL was dominated by protein (40–48%). No significant quantitative or qualitative difference could be detected between the relative concentrations and chemical compositions of corresponding lipoprotein classes in Rhine and Landes geese, whatever their age.

Effects of force-feeding on plasma lipids and lipoproteins. The effects of force-feeding on plasma lipids and lipoproteins were then evaluated by comparison with corresponding data in control birds of the same age (27 wk). Plasma cholesterol and phospholipid concentrations were approximately two-fold higher in force-fed birds than in controls, while differences in triglyceride levels were not significant (Table 1). Indeed, force-feeding induced considerable alterations in lipoprotein profile (Fig. 1), which were associated with dramatic modification in the lipid composition of individual lipoprotein classes. VLDL levels (Table 2) were markedly increased in Landes geese in which they attained a value (2.51 g/L) higher than that in Rhine geese (1.85 g/L) and significantly different from their nonforce-fed counterparts (0.76 g/L). By contrast, force-feeding did not modify the VLDL concentration in Rhine geese. However, the major effect of force-feeding on goose VLDL concerned the elevated proportion of cholesteryl esters (32–33%), and diminished proportion of triglyceride (28–29%), the contents of phospholipid and protein remaining comparable in both force-fed and control birds.

The denser classes of lipoprotein were also modified considerably by force-feeding (Fig. 1). The pig-

TABLE 2

Plasma Concentration and Chemical Composition (as mean wt %) of VLDL[a] in Geese of the Rhine and Landes Breeds During Growth (age in wk) and Force-Feeding

	Growing (8 wk)[b]		Immature (22 wk)[b]		Control (27 wk)[b]			Force-fed (27 wk)[b]	
	R[c] (20)[c]	L[c] (20)	R (20)	L (20)	R (5)		L (5)	R (15)	L (15)
Concentration (g/L)	0.13 ± 0.04	0.11 ± 0.03	0.44 ± 0.15*	0.30 ± 0.10	1.82 ± 0.86*		0.76 ± 0.47	1.85 ± 1.0*	2.51 ± 1.26
Chemical composition[d] (mean wt %)									
FC	6.5 ± 0.4	6.5 ± 0.9	7.2 ± 0.9	7.0 ± 1.1	6.7 ± 0.8		6.4 ± 0.4	8.9 ± 1.9	8.5 ± 1.3
CE	14.9 ± 2.7	15.3 ± 2.4	20.9 ± 3.8	19.9 ± 1.7	14.0 ± 3.4	*	19.3 ± 1.6	33.2 ± 8.0	31.8 ± 4.3
TG	42.3 ± 3.8	37.5 ± 10.9	43.5 ± 6.8	44.0 ± 4.6	51.2 ± 6.9	*	42.7 ± 8.7	28.0 ± 8.1	29.1 ± 7.9
PL	17.2 ± 1.4	17.0 ± 0.8	18.0 ± 1.4	17.2 ± 1.6	16.6 ± 2.0		17.9 ± 0.5	19.2 ± 1.8	18.4 ± 2.4
PR	19.2 ± 2.4	20.6 ± 2.4	10.5 ± 1.9	12.2 ± 2.1	11.5 ± 5.9	*	13.7 ± 6.5	10.8 ± 2.7	12.2 ± 2.5

[a]Values are mean ± SD of the number of geese given in parentheses. VLDL were isolated from the gradient at d<1.013 g/mL (7). Difference between breeds at a given age: *, $p<0.05$. For details of the legend, see Table 1.

[b]Physiological status.

[c]See footnote c, Table 1.

[d]FC, free cholesterol; CE, cholesteryl esters; TG, triglycerides; PL, phospholipids; PR, protein.

mented band corresponding to LDL (d 1.020–1.040) in control geese had disappeared, with the consequence that fractions corresponding to IDL, LDL and the fraction intermediate between LDL and HDL (d 1.013–1.065) could not be distinguished visually and were therefore isolated together. However, the concentrations of lipoprotein particles which float in the density range 1.006–1.065 and which correspond to both IDL and LDL were increased when compared to those in the other groups (Table 6). This increase is probably due either to a major contribution of IDL which accompanies the increase of VLDL during force-feeding, or to a contamination of this fraction with residual VLDL. Moreover, the chemical composition of the d 1.006–1.065 lipoproteins resembled that of VLDL from force-fed animals, presenting elevated proportions of free and esterified cholesterol and a reduced content of triglyceride.

Finally, the most striking feature of the lipoprotein pattern in the force-fed geese concerned the considerable increase in HDL concentrations (approx. 11 g/L as compared to 5 g/L in control birds of the same age) (Table 5). In contrast to VLDL, the chemical composition of these HDL was modified but slightly; thus the increase in HDL level was a consequence of parallel increases in each of its lipid and protein components. It is noteworthy that the amount of triglyceride secreted as HDL is far from negligible. However, in HDL particles from force-fed geese, 50% of triglyceride in weight is replaced by a similar amount of cholesteryl esters, resembling our findings for VLDL.

It must be emphasized that, with the notable exception of VLDL concentration, the response to force-feeding of the two strains regarding either the plasma level or the chemical composition of the various lipoprotein classes was not significantly different.

Effects of force-feeding on body weight and liver composition. Force-feeding modified not only the liver, but also the whole body weight (Table 7). Thus, in both breeds, body weight remained constant from 8 to 22 wk of age, *i.e.* as long as the birds were submitted to food restriction. The release of dietary restriction and the subsequent force-feeding led to a two-fold increase in body weight in only 5 wk. However, and despite the fact that mean liver weight was considerably higher in the Landes (469 g) than in the Rhine geese (285 g), there was no parallel breed-related difference in body weight at the day of sacrifice. It is noteworthy that net weight gain during the two weeks of force-feeding was exactly the same (1.8 kg) in the two strains, and as such corresponds primarily to an overdevelopment of adipose tissue.

Control, non-force-fed animals exhibited no breed differences in their liver weight or composition. In both breeds, the liver contained only 4–6 g of lipid, among which phospholipids were the major component (Table 8). After force-feeding, liver composition was dramatically modified in both breeds, but the phenomenon was much more pronounced in the Landes geese which are susceptible to fatty liver. Indeed, the liver weight of force-fed geese is significantly higher ($p<0.05$) in the Landes breed, although each of the hepatic components was not modified by the same proportion. Indeed, the non-lipid weight of the liver increased two-

TABLE 3

Plasma Concentration and Chemical Composition (as mean wt %) of IDL[a] in Geese of the Rhine and Landes Breeds (age in wk)

	Growing (8 wk)[b]		Immature (22 wk)[b]		Control (27 wk)[b]	
	R[c] (20)[c]	L[c] (20)	R (20)	L (20)	R (5)	L (5)
Concentration g/L	0.28 ± 0.10	0.26 ± 0.07	0.38 ± 0.11	0.36 ± 0.06	0.25 ± 0.06	0.34 ± 0.08
Chemical composition (mean wt%)[d]						
FC	8.3 ± 0.8	8.8 ± 1.8	8.6 ± 0.9	8.5 ± 0.9	7.6 ± 0.3	8.1 ± 1.0
CE	25.8 ± 2.4	26.6 ± 3.3	28.7 ± 3.0	27.9 ± 3.1	26.9 ± 3.2	27.2 ± 3.2
TG	24.1 ± 3.5	22.9 ± 4.1	21.9 ± 3.5	22.0 ± 3.5	21.3 ± 3.8	21.2 ± 4.4
PL	19.7 ± 1.7	18.3 ± 1.2	20.0 ± 1.5	19.1 ± 1.8	21.2 ± 1.0	18.8 ± 2.0
PR	22.1 ± 1.5	23.3 ± 1.8	20.8 ± 3.6	23.1 ± 3.1	23.1 ± 0.8	21.9 ± 2.1

[a]IDL were isolated as lipoproteins of d 1.013–1.020 g/mL. For details of the legend, see Tables 1 and 2.
[b]Physiological status.
[c]See footnote c, Table 1.
[d]See footnote d, Table 2.

TABLE 4

Plasma Concentration and Chemical Composition (as mean wt %) of LDL[a] in Geese of the Rhine and Landes Breeds (age in wk)

	Growing (8 wk)[b]		Immature (22 wk)[b]		Control (27 wk)[b]	
	R[c]	L[c]	R	L	R	L
Concentration g/L	0.91 ± 0.17	0.93 ± 0.16	0.84 ± 0.27	0.92 ± 0.22	1.09 ± 0.34	1.06 ± 0.37
Chemical composition (mean wt%)[d]						
FC	9.3 ± 0.7	10.1 ± 1.7	8.9 ± 0.9	8.8 ± 0.7	8.0 ± 0.4	7.9 ± 1.0
CE	33.5 ± 1.5	34.3 ± 1.6	30.6 ± 2.8	30.9 ± 3.7	28.4 ± 5.3	29.9 ± 2.7
TG	8.3 ± 1.6	8.2 ± 1.2	14.4 ± 2.3	13.5 ± 3.0	16.9 ± 4.3	16.6 ± 3.6
PL	21.6 ± 1.8	21.0 ± 1.3	21.3 ± 1.3	21.0 ± 1.0	22.3 ± 1.0	20.8 ± 1.4
PR	27.3 ± 2.0	26.4 ± 1.7	24.6 ± 2.7	25.8 ± 1.9	24.4 ± 1.3	24.9 ± 1.4

[a]IDL were isolated as lipoproteins of d 1.020–1.040 g/mL. For details of the legend, see Tables 1 and 2.
[b]Physiological status.
[c]See footnote c, Table 1. The number of geese is the same.
[d]See footnote d, Table 2.

fold during force-feeding to reach approximately 200 g in both strains, as compared to 115 g in control birds of the same age. Thus the difference in liver weight between the two strains arose from a specific accumulation of lipids, which was higher in geese of the Landes breed (190 g) than of the Rhine breed (81 g).

Moreover, such lipid accumulation was mainly due to triglyceride rather than cholesteryl esters and non-esterified fatty acids. The elevation in the amounts of hepatic free cholesterol and phospholipids was less pronounced and similar in both breeds. Thus, the genetic difference in the susceptibility to liver steatosis is associated with a higher accumulation of triglyceride, and to a lesser extent of cholesteryl esters and non-esterified fatty acids, in the Landes breed.

Since force-feeding is associated with an increase in the circulating concentrations of lipoproteins of hepatic origin, *i.e.* VLDL and HDL, we have tested the correlation between VLDL or VLDL-triglyceride on the one hand, with liver weight or liver triglyceride content on the other (Table 9). In both Landes and Rhine geese, there was a significant positive correlation between the plasma level of VLDL or VLDL-triglyceride (TG) during force-feeding and whole liver weight. However, the triglyceride content of the liver is positively associated with VLDL or VLDL-TG concentration in the force-fed state only in the Landes strain. Despite the fact that plasma VLDL levels were significantly higher in the Rhine strain at 22 wk of age, there was no significant correlation beween VLDL levels before force-feeding and liver weight or composition. Finally, we could not demonstrate any relationship between the plasma levels or chemical composition of HDL and the different hepatic parameters (data not shown).

DISCUSSION

The production of "foie gras" relies on the induction of liver steatosis in specialized breeds of ducks and geese. In order to enhance *de novo* fatty acid synthesis in the liver (16,18), these birds are force-fed with a

TABLE 5

Plasma Concentration and Chemical Composition (as mean wt %) of HDL[a] in Geese of the Rhine and Landes Breeds (age in wk)

	Growing (8 wk)[b]		Immature (22 wk)[b]		Control (27 wk)[b]		Force-fed (26 wk)[b]	
	R	L[c]	R	L	R	L	R	L
Concentration (g/L)	4.66 ± 0.56	5.00 ± 0.42	4.46 ± 0.53	4.47 ± 0.48	5.30 ± 0.54	4.89 ± 0.53	11.00 ± 1.22	11.31 ± 1.46
Chemical composition (mean wt %)[d]								
FC	4.2 ± 0.2	4.2 ± 0.2	3.6 ± 0.4	3.7 ± 0.3	3.4 ± 0.2	3.4 ± 0.2	4.5 ± 0.5	4.4 ± 0.5
CE	19.5 ± 1.9	19.9 ± 1.3	18.3 ± 2.4	18.2 ± 1.8	16.2 ± 2.1	17.9 ± 1.5	20.2 ± 2.3	19.3 ± 2.7
TG	3.8 ± 1.1	3.7 ± 0.6	7.1 ± 1.1	6.6 ± 2.2	8.9 ± 2.0	6.9 ± 1.4	3.4 ± 1.6	4.4 ± 1.5
PL	25.6 ± 2.2	24.9 ± 2.1	25.0 ± 2.1	25.6 ± 1.6	30.9 ± 1.5	29.0 ± 0.8	29.9 ± 4.2	30.4 ± 2.0
PR	46.9 ± 2.4	47.7 ± 2.5	45.8 ± 2.0	46.3 ± 2.8	40.6 ± 1.9	42.8 ± 1.6	42.5 ± 5.3	42.5 ± 2.2

[a]HDL was isolated as lipoproteins of d 1.065–1.165 g/mL. For details of the legend, see Tables 1 and 2.
[b]Physiological status.
[c]See footnote c, Table 4.
[d]See footnote d, Table 2.

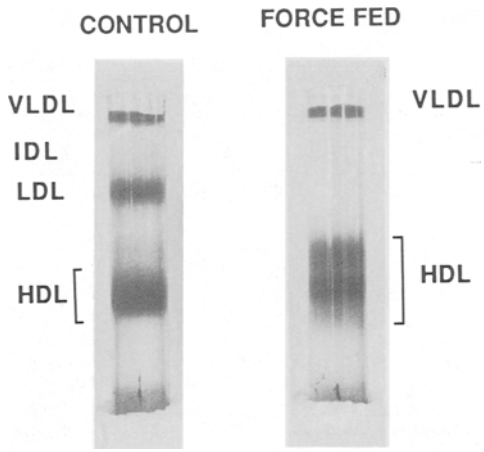


FIG. 1. Separation of plasma lipoproteins from control and force-fed geese (at 27 wk of age) by density gradient ultracentrifugation.

carbohydrate-rich feed essentially consisting of maize. In the present study, we have confirmed that fatty liver in the goose is due mainly to the accumulation of triglyceride (Table 8) (2,3). Moreover, other chemical components of the liver were modified by force-feeding. Thus, the hepatic non-lipid moiety and phospholipid content were increased two-fold, whereas free cholesterol was increased 6–10 fold, in both breeds of geese. Thus, it may be postulated that, since force-feeding has been shown to induce cellular proliferation (2), the increase in phospholipid and free cholesterol reflects the need for newly synthesized cell membranes, and is a feature independent of genetic susceptibility to liver steatosis. Furthermore, the increase in hepatic triglyceride and cholesteryl ester content, which was significantly higher in the Landes breed, may reflect the intracytoplasmic accumulation of lipids, whose level clearly depended upon the genetic origin of the geese.

The greater accumulation of triglyceride and cholesteryl esters in the liver of Landes geese is clearly related to genetic differences in lipid metabolism between the two breeds. Indeed, in non-force-fed Landes geese of 22 and 27 wk, the plasma concentration of VLDL was lower than in Rhine geese (Table 2). Moreover, when control birds were allowed to refeed *ad libitum* at 27 wk of age, VLDL particles contained significantly less triglyceride in the Landes breed. Consequently, the plasma level of VLDL triglyceride was only 0.345 g/L in the latter breed as compared to 0.959 g/L in Rhine geese. Thus, when considering birds before force-feeding, the genetic difference in the basal level of VLDL could be related to the relative ability of the liver to secrete VLDL-triglyceride, and thus to the variable susceptibility to further liver steatosis. This hypothesis is supported by the fact that, in bird species, VLDL level is more indicative of the ability of liver to secrete triglyceride than of the rate of VLDL catabolism (19). Nevertheless, even if this finding could explain the difference between the two breeds, it can-

TABLE 6

Plasma Concentration and Chemical Composition (mean wt %) of the Lipoprotein Population of d 1.013–1.065 g/mL in Control and Force-Fed Geese of the Landes and Rhine Breeds (at 27 wk)[a]

	Control		Force-fed	
	R (5)[b]	L (5)	R (15)	L (15)
Concentration g/L	1.71 ± 0.32	1.82 ± 0.34	2.36 ± 0.73	2.31 ± 1.02
Chemical composition (mean wt %)				
FC	7.5 ± 0.4	8.8 ± 4.8	9.4 ± 1.4	9.2 ± 1.5
CE	25.5 ± 4.8	28.4 ± 2.4	33.2 ± 4.7	30.2 ± 5.1
TG	15.6 ± 2.6	15.4 ± 2.9	6.5 ± 3.4	8.4 ± 3.3
PL	25.9 ± 2.9	22.6 ± 1.6	25.9 ± 3.6	28.8 ± 4.8
PR	24.6 ± 1.0	25.0 ± 1.5	24.2 ± 3.7	24.4 ± 2.1

[a]Since no pigmented band corresponding to LDL was visible on plasma density gradients from force-fed geese, the lipoprotein fractions in the density range between that of VLDL and that of HDL, *i.e.* d 1.013–1.065 were analyzed together. In control geese, these three fractions which corresponded to IDL (d 1.013–1.020), LDL (1.020–1.040) and some particles intermediate between LDL and HDL (1.040–1.065) (7) were added after their chemical characterization (see Results) and the mean chemical composition calculated. For details of the legend, see Tables 1 and 2.
[b]Values are mean ± SD of the number of geese given in parentheses.

TABLE 7

Body and Liver Weight During Growth and Force-Feeding in Geese of the Rhine and Landes Breeds (age in wk)

	Body weight (kg)						
	8 wk growing (20)[a]	22 wk immature (20)	27 wk control (5)	27 wk force-fed (15)	Weight gain during force-feeding (kg)	Liver weight (g)	
Rhine	4.4 ± 0.3	4.2 ± 0.2	6.4 ± 0.7	8.2 ± 0.5	1.8 ± 0.5	285 ± 81	**[b]
Landes	4.4 ± 0.4	4.4 ± 0.3	6.0 ± 0.8	7.9 ± 0.8	1.8 ± 0.5	469 ± 124	

[a]Values are mean ± SD of the number of geese given in parentheses.
[b]Difference between breeds: **, $p<0.01$.

TABLE 8

Liver Weight (g) and Composition (g/total liver) in Control and Force-Fed Geese of the Landes and Rhine Breeds (at 27 wk of age)

	Control		Force-fed		
Dietary condition Parameter	Rhine (5)[a]	Landes (5)	Rhine (8)		Landes (8)
Whole liver	133 ± 36	113 ± 25	298 ± 89	*[b]	388 ± 90
Non-lipid mass	126 ± 32	108 ± 25	217 ± 39		198 ± 35
Lipid mass	7.5 ± 5.5	4.7 ± 0.9	81 ± 56	**[c]	190 ± 66
Triglycerides	0.99 ± 0.91	0.49 ± 0.27	64 ± 50	**	168 ± 60
Cholesteryl esters	0.24 ± 0.14	0.21 ± 0.06	3.3 ± 2.6	**	7.1 ± 2.8
Free cholesterol	0.48 ± 0.28	0.32 ± 0.05	2.8 ± 0.5		3.2 ± 1.5
Phospholipids	3.7 ± 1.0	3.5 ± 0.6	8.0 ± 1.9		7.0 ± 1.5
Non-esterified fatty acids	0.36 ± 0.26	0.23 ± 0.06	2.6 ± 1.3	**	4.7 ± 1.7

[a]Values are mean ± SD of the number of geese given in parentheses.
[b]Difference between breeds: *$p<0.05$.
[c]Difference between breeds: **$p<0.01$.

TABLE 9

Correlations Between the Plasma Levels of VLDL or VLDL-TG at Different Ages (in wk) and Liver Weight or Liver-TG After Force-Feeding[a]

		8 wk (n=15)		22 wk (n=15)		27 wk (n=10)	
		VLDL[b]	VLDL-TG[b]	VLDL	VLDL-TG	VLDL	VLDL-TG
Liver (n=15)	Landes	−0.385	−0.343	+0.225	+0.247	+0.543*	+0.613*
	Rhine	−0.577*	−0.601*	+0.146	+0.108	+0.769**	+0.947**
Liver-TG (n=8)	Landes	−0.025	−0.016	+0.594	+0.287	+0.667	+0.715*
	Rhine	+0.247	+0.431	−0.403	−0.220	+0.313	+0.389

[a]*, $p<0.05$; **, $p<0.01$ — TG, triglyceride. Correlations at 27 wk of age concerned force-fed geese only.
[b]Parameter.

not account for the intrabreed variability in the Landes geese, since there was no significant correlation between VLDL or VLDL-triglyceride concentration in non-force-fed geese and liver weight or triglyceride content in the same birds after force-feeding (Table 9).

Moreover, the relationship between circulating VLDL levels and liver triglyceride content is not so simple when one considers the changes occurring in lipoprotein profile after force-feeding. In this latter state, VLDL levels did not differ in control and force-fed Rhine geese (1.82 and 1.85 g/L) (Table 2). However, since VLDL from force-fed Rhine geese contained only 28% triglyceride, the amount of plasma VLDL-triglyceride decreased to 0.501 g/L (as compared to 0.959 g/L in controls). By contrast, VLDL from the Landes geese increased from 0.76 g/L in controls to 2.51 g/L in force-fed birds; consequently, plasma VLDL-triglyceride reached 0.982 g/L as compared to 0.345 g/L in controls, and this despite the fact that VLDL contained only 29% triglyceride.

It appears then that Landes geese may respond to the increase in liver lipogenesis induced by force-feeding by an increase in VLDL production, in accordance with a number of previous studies showing that feeding birds with a carbohydrate-rich diet enhances hepatic lipogenesis and increases plasma VLDL levels (16,18). Nevertheless, this process is not sufficient to prevent liver steatosis, probably because of a specific retention of triglyceride by the liver. This latter hypothesis derives from the observation that VLDL from force-fed geese contained considerably less triglyceride (29%) than that from controls (Table 2). A defect in VLDL assembly may therefore occur, leading to the secretion of VLDL deficient in triglyceride, the latter remaining in the liver where they accumulate. The reason why force-fed geese of the Rhine breed do not develop a full fatty liver is unclear. It may be hypothesized that hepatic lipogenesis is lower in the Rhine breed, with the consequence that VLDL, even though deficient in triglyceride, are secreted at a rate sufficient to prevent marked accumulation of liver triglyceride. Clearly, this question requires further study. It is however noteworthy that, despite similar chemical compositions, VLDL in these geese are probably different from β-VLDL found in animals fed cholesterol, since their diet was essentially devoid of this component.

The biochemical basis of the accumulation of triglyceride in the liver remains indeterminate. Under normal feeding conditions, triglycerides are synthesized in the liver on the cytosolic surface of the endoplasmic reticulum, and are then transferred into the lumen of the endoplasmic reticulum to be assembled with phospholipid and apo B (20). In the chicken (21), as in the rat (22), triglycerides synthesized in excess are normally stored by the liver in temporary cytoplasmic droplets when their production exceeds the capacity of their secretion as VLDL. In the rat, stimulation of triglyceride synthesis leads to a saturation of the process of triglyceride assembly into nascent VLDL and enhances their transfer to lipid droplets (23). However, it remains doubtful that triglyceride assembly into VLDL is saturated in geese, since VLDL in force-fed geese contain less than 30% triglyceride. It is more likely that there is an imbalance in the channeling of triglyceride between that destined for storage and that destined for incorporation into nascent VLDL, or alternatively that triglyceride stored in cytoplasmic vesicles is no longer available for further VLDL assembly and secretion and remains sequestered in the liver, leading to steatosis.

A further alteration observed in the goose lipoprotein profile after force-feeding concerns the disappearance of LDL (Table 4), which may be related to the abnormal composition of VLDL. Indeed, VLDL particles containing only 28–29% triglyceride and 40–42% cholesterol may be less susceptible to be hydrolyzed by lipoprotein lipase, which preferentially attacks triglyceride rather than phospholipids or cholesteryl esters (24). However, some of these VLDL have to be catabolized, since the fattening of birds during force-feeding is considerable.

As for HDL, they represent the second major form of lipid secretion from the liver, in birds as in mammals (19). In force-fed geese, plasma HDL concentrations were two-fold higher than those in controls, but did not show any difference between the two breeds (Table 5). This phenomenon is accompanied by an increase during force-feeding in the amount of liver messenger ribonucleic acid (mRNA) coding for apo A-I, the major protein component of HDL (Forgez, P. and Hermier, D., unpublished results). These results suggest that the increase in plasma HDL level is due to an increase in HDL synthesis by the liver rather than to a decrease of their catabolism.

In conclusion, we propose the following putative

mechanism to explain both the occurrence of fatty liver in the goose and the difference in susceptibility between the Landes and the Rhine breeds. Initially, force-feeding is responsible for the stimulation of hepatic lipogenesis, which is reflected in an increase in circulating VLDL and HDL concentrations. However, for reasons which remain to be elucidated, newly synthesized triglycerides tend to be stored in the cytoplasm rather than incorporated into nascent VLDL, finally leading to steatosis. This phenomenon is associated with the preferential enrichment of VLDL in cholesteryl ester. The resulting steatosis is more pronounced in the Landes geese, probably as the result of (i) a higher rate of lipogenesis, this hypothesis being supported by the higher VLDL concentration when force-fed; and (ii) a reduced ability to transport triglyceride as VLDL, as demonstrated in non-force-fed birds. These two parameters may explain the difference in susceptibility to fatty liver between the two breeds. However, the regulation of the synthesis and secretion of VLDL in the goose must now be investigated, and particularly as concerns hepatic lipogenesis and intracytoplasmic storage of triglyceride. Finally, the defect in VLDL assembly which leads to particles that lack triglyceride is apparently common to the two breeds. Such an uncoupling of triglyceride synthesis and secretion represents a useful model in which to improve our knowledge of the regulation of hepatic lipoprotein production.

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The Hypercholesterolemic Effect of Dietary Coconut Fat *versus* Corn Oil in Hypo- or Hyperresponsive Rabbits Is Not Exerted Through Influencing Cholesterol Absorption

G.W. Meijer*, A.G. Lemmens, A. Versluis, L.F.M. Van Zutphen and **A.C. Beynen**
Department of Laboratory Animal Science, State University, 3508 TD Utrecht, The Netherlands

In two inbred strains of rabbits with high or low response of plasma cholesterol to dietary saturated *versus* polyunsaturated fatty acids, the efficiency of intestinal cholesterol absorption was measured. The feeding of a cholesterol-free purified diet containing saturated fatty acids in the form of coconut fat, when compared with a diet containing corn oil as polyunsaturated fatty acids, did not influence the efficiency of cholesterol absorption in the two rabbit strains. Irrespective of the dietary fat source, the hyperresponsive rabbits absorbed cholesterol more efficiently. It is concluded that the hypercholesterolemic effect of dietary coconut fat *versus* corn oil is not exerted by influencing cholesterol absorption.
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Replacement of dietary polyunsaturated fatty acids by saturated fatty acids generally causes an increase in plasma cholesterol levels, but the mechanism by which this occurs is unclear (1). The hypercholesterolemic effect of saturated fatty acids is diminished by a background diet rich in soybean protein instead of casein (2,3). Since soybean protein decreases the efficiency of cholesterol absorption (4–6), dietary saturated fatty acids may stimulate cholesterol absorption from the intestinal lumen.

The objective of the present study was to investigate the effect of polyunsaturated *versus* saturated fatty acids on cholesterol absorption. In order to increase the sensitivity of measuring such a dietary effect, if any, we used two inbred strains of rabbits which differ markedly in their cholesterolemic response to dietary saturated fatty acids (7). It was anticipated that any effect of the type of dietary fat would be more pronounced in the hyperresponsive than in the hyporesponsive rabbits. To have better control on the within-animal variation, cholesterol absorption measurements were performed according to a repeated measures design.

MATERIALS AND METHODS

Animals and housing. Male rabbits of two inbred strains were used for two experiments in which cholesterol absorption was determined. The animals were 0.5–5 years old, the average age being 2.7 years for both strains in the two experiments. For a third experiment, male and female rabbits were used. They were aged 1.2–4 years; the average age of each strain was 2.3 years. The strains were IIIVO/JU and AX/JU, and originated from the Jackson Laboratory colony, Bar Harbor, ME (8). The IIIVO/JU strain has previously been shown to be hyporesponsive and the AX/JU strain to be hyperresponsive to dietary cholesterol (7,9), saturated *versus* polyunsaturated fatty acids and casein *versus* soybean protein (7). The animals were maintained at the Department of Laboratory Animal Science, State University (Utrecht, The Netherlands) as described (9). They were fed 100 g daily of commercial pellets (LKK-20^{R}, Hope Farms, Woerden, The Netherlands).

Experimental designs. Two experiments were performed to study cholesterol absorption. Rabbits of both strains underwent all treatments simultaneously. In the first experiment (expt. 1), the effect of dietary casein *versus* soy protein was studied in order to validate the method for measurement of cholesterol absorption and to demonstrate in our inbred strains of rabbits that casein enhances cholesterol absorption, as would be expected on the basis of literature data concerning outbred rabbits (4). It is well-known (10) that casein, when compared with soybean protein, produces hypercholesterolemia in rabbits. In the second experiment (expt. 2), we studied the effect on cholesterol absorption of saturated fatty acids in the form of coconut fat *versus* polyunsaturated fatty acids as corn oil. In the third experiment (expt. 3), the diet compositions of those in expt. 2 were used again, and liver cholesterol concentrations were measured. Cholesterol-free diets were used throughout. The rabbits were allowed to practice cecotrophy.

On day 0 of expt. 1, six hypo- and nine hyperresponsive rabbits were transferred from the commercial diet to the purified diet containing soy protein and coconut fat (Table 1). From day 55, they received the

TABLE 1

Composition of the Cholesterol-Free, Experimental Diets

Ingredient	Dietary variables		
	Casein coconut fat	Soy protein coconut fat	Casein corn oil
Casein	21	—	21
Soy isolate	—	20.8	—
Methionine	—	0.2	—
Coconut fat	9	9	—
Corn oil	1	1	10
Constant components[a]	69	69	69

[a]The constant components consisted of (g/100 g diet): corn starch, 17; dextrose, 21; molasses, 5; sawdust, 18; dicalcium phosphate, 2.9; sodium chloride, 0.6; potassium bicarbonate, 1.8; magnesium carbonate, 0.3; magnesium oxide, 0.2; vitamin premix, 1.2; mineral premix, 1. The compositions of the vitamin and mineral premixes have been described elsewhere (7).

*To whom correspondence should be addressed at Department of Laboratory Animal Science, State University, P.O. Box 80.166, 3508 TD Utrecht, The Netherlands.

Abbreviations: apo, apolipoprotein; FNS, fecal neutral steroids; OD; oral dose.

casein/coconut fat diet and from day 132 they were fed on the soy protein diet again. Body weights were measured on days 0, 14, 28, 35, 105, 112, 126, 133, 154, 168, 196 and 210; the results were averaged per rabbit per dietary period. On days 35, 118 and 209, after an 18 hr fast, each rabbit ingested a mixture of [1α, 2α(n)-^{3}H]cholesterol (255-511 kBq) and β-[4-^{14}C]sitosterol (39-79 kBq) which had been added to a cube of whole wheat bread. The feces of individual rabbits were collected daily as described previously (9) on five consecutive days after each administration of both labels. Percentage cholesterol absorption was calculated as described (9):

$$\text{Cholesterol absorption (\%)} = \left\{1 - \frac{(^3\text{H in FNS})}{(^{14}\text{C in FNS})} \times \frac{(^{14}\text{C in OD})}{(^3\text{H in OD})}\right\} \times 100\%,$$

where FNS stands for fecal neutral steroids, and OD for oral dose. Radioactivities are expressed in dpm.

On day 0 of expt. 2, six rabbits of each strain were transferred from the commercial diet to the purified diet containing corn oil and casein (Table 1). On day 46, the animals were switched to the coconut fat/casein diet, and from day 121 they received the corn oil diet again. Body weights were measured on days 0, 14, 28, 49, 63, 77, 98, 112, 147, 161, 182 and 196; the results were averaged per rabbit per dietary period. Radioactive cholesterol and β-sitosterol were administered on days 35, 112 and 196. The feces of individual rabbits were collected daily during three periods: days 36-40, 113-117 and 197-201. Two rabbits of the hyperresponsive strain refused to eat the purified diets, and had to be excluded. Thus, data analyses on this experiment were performed using four hyperresponders.

From day 0 of expt. 3, six male and six female rabbits of each of the two strains received the corn oil diet for 21 days. Then, the animals were divided into two dietary groups, each consisting of three female and three male rabbits per strain. For another 42 days, one group remained on the corn oil diet and the other received the diet containing coconut fat. Body weights were measured on days 21 and 63. On day 63, the animals were killed and liver cholesterol concentrations determined.

The rabbits were fed 75 g (expt. 1 and 2) or 100 g (expt. 3) of the purified diets per day and had free access to tap water. The daily feed intake was recorded throughout all experiments. Plasma total cholesterol and body weights were determined regularly.

Analytical methods. Samples of blood were taken from the marginal ear vein of the rabbits into heparinized tubes. Sampling and body weight measurements were performed between 07:00 and 10:00 hr after the removal of any remaining food at 16:00 hr the previous day. Total cholesterol in the plasma was measured enzymatically using a test-combination (Monotest^R) supplied by Boehringer-Mannheim GmbH, Mannheim (Germany). The feces samples were extracted and radioactivity in neutral steroid fractions was determined as previously described (9,11). Liver samples were homogenized in 2 vol of distilled water, and cholesterol was extracted and analyzed according to Abell *et al.* (12).

Statistics. The effects of the type of dietary protein and the type of dietary fat (as "within-rabbits" factors) and the degree of responsiveness (as "between-rabbits" factors) were evaluated using a repeated measures analysis of variance, using orthonormalized contrasts. In expt. 3, comparisons between the hypo- and hyperresponsive strains and the effects of the diets within one strain were evaluated by Student's two-tailed t-test. The level of statistical significance was pre-set at $p<0.05$.

RESULTS

The feed intake was consistently lower in the hyperresponsive rabbits than in the hyporesponsive rabbits (Tables 2 and 5; $p<0.05$ in each experiment). The rabbits tended to reduce feed intake while on the coconut fat/casein diet, this effect reaching statistical significance ($p<0.05$) in the hyperresponders in expt. 1 and expt. 2. Tables 2 and 5 also show that group mean body weights of the hyporesponders were higher than those of their hyperresponsive counterparts ($p<0.05$ in each experiment), which probably explains the strain difference in feed intake. In the course of experiments 1 and 2, body

TABLE 2

Feed Intake and Body Weight in Hypo- and Hyperresponsive Rabbits Fed Purified Diets Differing in the Type of Dietary Fat or Protein[a]

	Feed intake (g/day)		Body weight (kg)	
Diet	Hypo	Hyper	Hypo	Hyper
Expt. 1				
Soy protein	75 ± 0.5 (6)	65 ± 7.7 (9)	3.17 ± 0.19	2.88 ± 0.21
Casein	71 ± 3.0 (6)	58 ± 8.0 (9)	3.25 ± 0.20	2.83 ± 0.20
Soy protein	72 ± 2.0 (6)	66 ± 7.9 (9)	3.35 ± 0.16	2.90 ± 0.24
Expt. 2				
Corn oil	75 ± 0.4 (6)	61 ± 9.7 (4)	3.12 ± 0.25	2.80 ± 0.17
Coconut fat	71 ± 4.0 (6)	51 ± 8.4 (4)	3.20 ± 0.20	2.78 ± 0.21
Corn oil	74 ± 1.5 (6)	57 ± 11.5 (4)	3.40 ± 0.21	2.87 ± 0.25

[a]Results are expressed as means ± SD; the number of rabbits is indicated in parentheses. Statistically significant differences are indicated in the text.

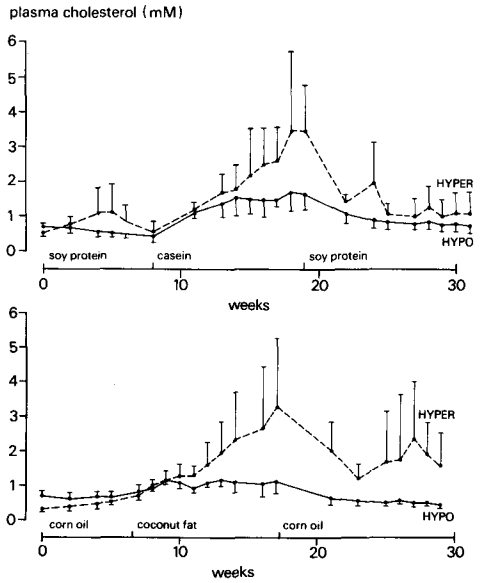


FIG. 1. Plasma cholesterol concentrations in hypo- and hyperresponsive rabbits fed purified diets differing in the type of dietary protein (upper panel) or fat (lower panel). Results expressed as means ± SD for 4, 6 or 9 rabbits per strain.

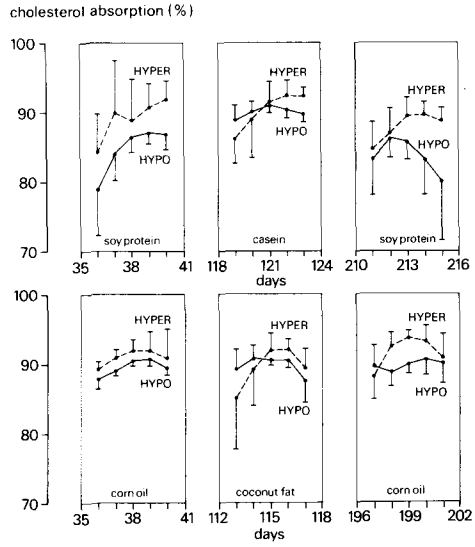


FIG. 2. Calculated percentages of cholesterol absorption after oral administration of [^{3}H]cholesterol and β-[^{14}C]sitosterol in hypo- and hyperresponsive rabbits fed purified diets differing in the type of dietary protein (upper panel) or fat (lower panel). Results, expressed as means ± SD for 4, 6 or 9 rabbits per strain.

weight of the hyporesponsive rabbits increased significantly ($p<0.05$).

The replacement of soy protein in the diet by casein caused a more pronounced increase of plasma cholesterol in the hyperresponsive rabbits than in the hyporesponders (Fig. 1). After the rabbits had been switched back to the soy protein diet, plasma cholesterol concentrations dropped, but did not reach baseline levels.

Figure 2 illustrates the calculated percentages of cholesterol absorption during 5 consecutive days after oral administration of [^{3}H]cholesterol and β-[^{14}C]sitosterol. Calculated efficiencies of cholesterol absorption varied during the course of fecal collection after administration of the 2 labels. Nevertheless, cholesterol absorption was almost always more effective in the hyper- than in the hyporesponders. The maximum value of cholesterol absorption is not biased by any error introduced by re-excretion of absorbed [^{3}H]cholesterol (13). Thus, these values were calculated and presented in Table 3. The hyperresponders absorbed higher percentages of cholesterol than did the hyporesponders ($p<0.005$), but in both rabbit strains dietary casein, when compared with soy protein, increased cholesterol absorption ($p<0.001$). Table 4 shows the recoveries of radioactive β-sitosterol in feces. Recoveries on the soy protein diet were lower in the hyper- than in the hyporesponsive rabbits ($p<0.01$). After feeding the casein diet, recoveries were similar in the two strains.

When rabbits were transferred from the corn oil to the coconut fat diet, plasma cholesterol concentrations rose significantly (Fig. 1). The increase in plasma

TABLE 3

Calculated Percentage Cholesterol Absorption ([^{3}H]cholesterol/β-[^{14}C]sitosterol method) in Hypo- and Hyperresponsive Rabbits Fed Purified Diets Differing in the Type of Dietary Protein or Fat[a]

		Cholesterol absorption (%)	
Diet	Period	Hypo	Hyper
Expt. 1			
Soy protein	days 36 to 40	87.0 ± 1.88	91.2 ± 3.18
Casein	days 119 to 123	90.7 ± 1.11	92.3 ± 1.76
Soy protein	days 211 to 215	86.0 ± 2.67	89.5 ± 2.36
Expt. 2			
Corn oil	days 36 to 40	90.6 ± 0.78	91.5 ± 2.68
Coconut fat	days 67 to 71	90.5 ± 0.64	92.0 ± 1.95
Corn oil	days 197 to 201	90.2 ± 2.54	93.7 ± 1.43

[a]See legend to Table 2.

TABLE 4

Effect of Dietary Protein or Fat on Cumulative Recovery of β-[^{14}C]sitosterol in the Feces of Hypo- and Hyperresponsive Rabbits[a]

Diet	β-Sitosterol recovery (%)[b]	
	Hypo	Hyper
Expt. 1		
Soy protein	53 ± 6.7	40 ± 2.7
Casein	41 ± 5.8	41 ± 7.4
Soy protein	48 ± 5.9	36 ± 8.9
Expt. 2		
Corn oil	39 ± 2.7	40 ± 7.7
Coconut fat	38 ± 3.8	31 ± 3.3
Corn oil	44 ± 8.3	36 ± 11.3

[a]See legend to Table 2.
[b]Cumulative recovery in fecal neutral steroids up to 5 days after the radioactive dose was administered.

cholesterol was more pronounced in the hyper- than in the hyporesponders. After feeding the corn oil diet again, plasma cholesterol concentrations fell, but did not reach baseline values in the hyperresponders. The type of fat in the diet did not influence the efficiency of cholesterol absorption (Fig. 2, Table 3). The hyperresponders systematically absorbed cholesterol more efficiently than the hyporesponders ($p<0.05$), irrespective of the type of fat in the diet. β-Sitosterol recoveries in feces were similar in both strains (Table 4).

In keeping with expt. 2, expt. 3 also showed that plasma cholesterol levels were significantly increased in rabbits fed the coconut fat diet, when compared with rabbits fed the corn oil diet (Table 5). The coconut-fat-induced increase in plasma cholesterol concentration in the hyperresponders ($p<0.01$) was greater than in the hyporesponsive rabbits ($p<0.025$); the difference between the two strains was statistically significant ($p<0.01$). In hyporesponsive rabbits, liver cholesterol concentrations tended to be higher on the coconut fat than on the corn oil diet. In hyperresponders the fat type effect was seen more clearly ($p<0.025$). Irrespective of diet composition, hyperresponders had significantly higher liver cholesterol concentrations than hyporesponders ($p<0.005$).

DISCUSSION

As would be expected (4–6), dietary casein increased cholesterol absorption, when compared with dietary soy protein. This "casein effect" was seen in both strains. Irrespective of the type of dietary protein, the hyperresponsive rabbits absorbed cholesterol more efficiently than the hyporesponders. This observation is in agreement with our earlier work (9) and suggests that the increased efficiency of cholesterol absorption in the hyperresponsive rabbits is an important determinant of their increased susceptibility of cholesterol-induced, "exogenous" hypercholesterolemia (9) as well as casein-induced, "endogenous" hypercholesterolemia. Although the difference in percentage cholesterol absorption between hypo- and hyperresponders is small, it may have a significant impact on cholesterol metabolism. There is an enterohepatic cycle of cholesterol through which this sterol may circulate up to seven times per day (14). Cholesterol absorption as calculated in the present study does not include the contribution of previously absorbed and re-excreted radiolabelled cholesterol (13).

Dietary coconut fat, when compared with corn oil, caused a more pronounced increase in plasma cholesterol concentrations in hyper- than in hyporesponsive rabbits. This confirms previous studies (7). The type of dietary fat did not influence the efficiency of intestinal cholesterol absorption in either strain. However, it could be argued that true diet-induced differences in the percentage of cholesterol absorption are obscured by differences in recovery in the feces of orally administered labelled β-sitosterol, which could be related to diet-induced differences in colonic bacterial activity. However, β-sitosterol recovery was not systematically

TABLE 5

Effect of Dietary Fat Type on Feed Intake, Body Weight, Plasma and Liver Cholesterol Concentrations of Hypo- and Hyperresponsive Rabbits in Expt. 3[a]

Measure	Hyporesponders		Hyperresponders	
	Corn oil	Coconut fat	Corn oil	Coconut fat
Feed intake (g/day)	87 ± 9.3	76 ± 5.1	68 ± 12.4[b]	62 ± 13.4[b]
Body weight (kg)				
Initial	3.46 ± 0.38	3.48 ± 0.31	3.02 ± 0.35[b]	3.05 ± 0.42[b]
Final	3.62 ± 0.30	3.57 ± 0.25	3.17 ± 0.35[b]	3.09 ± 0.42[b]
Plasma cholesterol (mM)				
Initial	1.06 ± 0.26	1.02 ± 0.40	0.81 ± 0.41	0.85 ± 0.49
Change	−0.16 ± 0.21	0.40 ± 0.16[c]	0.30 ± 0.51[b]	1.19 ± 0.45[b,c]
Liver cholesterol (μmol/g)	6.9 ± 0.2	9.2 ± 2.4	16.8 ± 3.7[b]	25.9 ± 6.7[b,c]

[a]Results are expressed as means ± SD; (n=6).
[b]Significant difference between hypo- and hyperresponsive strain fed the same diet.
[c]Significantly different from rabbits of the same strain but fed the corn oil diet.

influenced by the type of fat in the diet. In hyperresponders fed the coconut fat diet, β-sitosterol recovery was lower than after feeding the corn oil diet. Thus, the efficiency of cholesterol absorption in hyperresponders fed the diet containing coconut fat would be over-, rather than underestimated. The apparent lack of an effect of dietary fat type on cholesterol absorption in the two inbred strains of rabbits has also been observed in other animal species. In rats, either no effect of fat saturation on cholesterol absorption (15) or increased cholesterol absorption after feeding corn oil, when compared with coconut fat (16), has been found. Likewise, in baboons, cholesterol absorption was not affected by the type of dietary fat (17).

Dietary coconut fat, when compared with corn oil, caused increased liver cholesterol concentrations, this effect being most pronounced in the hyperresponders. The effect of coconut fat on liver cholesterol may be caused by a higher rate of hepatic cholesterol synthesis as has been shown in hamsters and rats (18,19). This would imply that hyperresponsive rabbits are more sensitive than hyporesponders to stimulation of cholesterol synthesis as induced by coconut fat. Since increased liver cholesterol pools will reduce the number of hepatic apoB/E receptors (20), these receptors may be more depressed in hyper- than in hyporesponsive rabbits when fed diets containing coconut fat. This could explain the increased sensitivity of plasma cholesterol concentration to dietary coconut fat in hyperresponders.

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Protective Effect of Vitamin E on Linoleic Acid Hydroperoxide-Induced Injury to Human Endothelial Cells[1]

Takao Kaneko, Shun-ichiro Nakano and Mitsuyoshi Matsuo*
Tokyo Metropolitan Institute of Gerontology, 35-2 Sakaecho, Itabashiku, Tokyo 173, Japan

The protective effects of vitamin E (α-tocopherol) and its model compounds, which act as antioxidants, on linoleic acid hydroperoxide-induced injury to human umbilical vein endothelial cells were examined. When incubated at 50 μM with endothelial cells at 37°C for 24 hr, α-tocopherol protected the cells from injury, and 2,2,5,7,8-pentamethylchroman-6-ol showed a similar protective effect. Trolox C, a water-soluble vitamin E model compound, had no protective effect. Tocol, a poor antioxidant, proved toxic. During preincubation, α-tocopherol was incorporated into the cells at 16.6 nmol/mg protein, while the pentamethylchromanol was incorporated at 0.5 nmol/mg protein; Trolox C was not incorporated at all. The results suggest that agents having both high antioxidant activity and lipophilicity can protect endothelial cells from linoleic acid hydroperoxide-induced injury.
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Injury to endothelial cells, which line the blood vessels, appears to be a prerequisite for the progression of atherosclerosis (1–3). One cause of endothelial cell injury may be oxidative stress, such as a rise in lipid peroxidation and exposure to active oxygen species (4). In fact, lipid peroxides and secondary products of lipid peroxidation are toxic toward cells. Recently, we reported that linoleic acid hydroperoxide and its degradation products, unsaturated aliphatic aldehydes, are toxic toward cultured human endothelial cells (5) and human diploid fibroblasts (6). Lipid peroxide-induced injury to endothelial cells is considered a risk factor of cardiovascular disease. Hence, agents to protect cells from such injury are of interest for the prevention and treatment of the disease.

In the present study, we investigated the protective effects of α-tocopherol and its model compounds on linoleic acid hydroperoxide-induced injury to human umbilical vein endothelial cells.

MATERIALS AND METHODS

Materials. α-Tocopherol, gelatin, and heparin were purchased from Sigma Chemical Co. (St. Louis, MO). α-Tocopherol was purified by high-performance liquid chromatography (HPLC) before use. Epidermal growth factor (EGF) was obtained from Collaborative Research (Waltham, MA), MCDB-104 medium from GIBCO Laboratories (Grand Island, NY), and fetal bovine serum (FBS) from Biocell Laboratories (Carson, CA). Endothelial cell growth factor (ECGF) was isolated from newborn-bovine brains by the method of Lobb and Fett (7). 2,2,5,7,8-Pentamethylchroman-6-ol was synthesized by the method of Nilsson *et al.* (8). Linoleic acid hydroperoxide (13-hydroperoxy-9,11-octadecadienoic acid) was prepared as described previously (9). Trolox C (6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid) and tocol were kindly provided by Dr. K.U. Ingold and Dr. S. Urano, respectively (see Acknowledgments). Trolox C was purified by HPLC before use. Falcon 24-well culture plates and 60-mm culture dishes were purchased from Becton Dickinson Labware (Oxnard, CA).

Cell culture. Endothelial cells were isolated from the veins of human umbilical cords as described previously (5), and identified by an indirect immunofluorescence technique using an antibody to coagulant factor VIII. Cells were grown in gelatin-coated 24-well plates or 60-mm dishes at 37°C under an atmosphere containing 5% carbon dioxide and 95% air. Culture medium used was MCDB-104 medium supplemented with 10% FBS, 30 ng/mL ECGF, 10 ng/mL EGF, and 10 ng/mL heparin (hereafter, referred to as the MCDB-104 system). Cells at passage 6 to 14 were used for the experiments.

Estimation of the extent of cell injury. Endothelial cells at confluency were incubated for 24 hr in multiwells containing the MCDB-104 system and either each antioxidant solubilized with ethanol or ethanol alone. The final ethanol concentration was less than 0.1%, which had no effect on the cells. Cells were rinsed with phosphate buffered saline (PBS) and then incubated for 3 hr in Earle's solution with LOOH. After removal of the medium, the cells were rinsed with PBS. Cell viability was examined by the trypan blue exclusion test of adhering cells as described previously (6).

Quantification of α-tocopherol and its model compounds in cells. α-Tocopherol and its model compounds were extracted from cells and quantified as described below (10,11). Cells grown on a 60-mm dish were washed twice with PBS, treated with 0.025% trypsin, suspended in MCDB-104 medium (2 mL) supplemented with 10% FBS, and then sedimented at 150 × *g* for 5 min. The cells in a pellet were resuspended in distilled water (1 mL) and homogenized at 4°C for 30 sec with a Model W-10 sonicator (Heat System Ultrasonics Inc., Plainview, NY). After the addition of 80 mM sodium dodecyl sulfate (1 mL), ethanol (0.5 mL), and an ethanolic solution (2 μL) of 2,2,5,7,8-pentamethylchroman-6-ol (PMC) or tocol (1 μg/mL), the homogenate was mixed by shaking, and to it was added a hexane/ethyl acetate mixture (9:1, v/v; 2 mL). PMC and tocol were added as internal standards for the HPLC quantitative analysis of cell α-tocopherol and PMC, respectively; the peak intensity of α-tocopherol was calibrated relative to that

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*To whom correspondence should be addressed.

Abbreviations: ECGF, endothelial cell growth factor; EGF, epidermal growth factor; FBS, fetal bovine serum; HPLC, high-performance liquid chromatography; LOOH, linoleic acid hydroperoxide; PBS, phosphate buffered saline; PMC, 2,2,5,7,8-pentamethylchroman-6-ol.

of PMC and the peak intensity of PMC was calibrated relative to that of tocol. The mixture obtained was vigorously mixed by vortex-stirring for 1 min and then centrifuged at 1,200 × *g* for 10 min. A portion of the organic layer (1.5 mL) was pipetted into a flask and the solvent was evaporated under reduced pressure. The residue was redissolved in 100 μL of hexane/isopropyl alcohol (99.25:0.75, v/v) and the solution (50 μL) was subjected to HPLC analysis. HPLC was conducted on a Model 5560 high-performance liquid chromatograph (Varian, Walnut Creek, CA) equipped with a MicroPak Si-5 column (Varian) and a RF-535 fluorometric detector (Shimadzu, Kyoto, Japan; Ex. 295 nm and Em. 330 nm). The same hexane/isopropyl alcohol mixture was used as mobile phase at a flow rate of 2 mL/min. The extraction recoveries of α-tocopherol, 2,2,5,7,8-pentamethylchroman-6-ol, and Trolox C from cell homogenates were 66.6, 90.2, and 9.4%, respectively. Protein content was measured as described by Bradford (12).

RESULTS AND DISCUSSION

Figure 1 depicts the molecular structures of α-tocopherol and its model compounds, the protective effects of which were examined.

Figure 2 shows that preincubation with α-tocopherol protects human umbilical vein endothelial cells from injury induced by linoleic acid hydroperoxide (LOOH). We have already shown that LOOH, with a 50% lethal concentration of 20 μM, is injurious toward endothelial cells (5). α-Tocopherol acts as a protective agent against LOOH-induced injury to the cells, because more than 80% of the cells treated with 50 μM α-tocopherol survived after exposure to 50 μM LOOH for 3 hr.

Hennig *et al.* (13,14) reported that α-tocopherol protected porcine pulmonary artery endothelial cells from LOOH-induced injury. Our results are consistent with their observations. This suggests that irrespective of animal species, vitamin E plays an important role in the protection of endothelial cells against oxidative injury.

2,2,5,7,8-Pentamethylchroman-6-ol (PMC), a vitamin E model compound, also protected the cells from LOOH-induced injury, although the effect was slightly less pronounced than that of α-tocopherol (Fig. 2). Trolox C, a water-soluble vitamin E model compound, showed no effect (Fig. 2). However, tocol was toxic toward endothelial cells (data are not shown). When incubated with tocol for 24 hr, cell viability was reduced to less than 40%. Tocol is a vitamin E model compound corresponding to α-tocopherol without the three methyl groups attached to the aromatic ring (Fig. 1): *i.e.*, it has an unhindered, phenolic hydroxyl group and hence shows a poor antioxidant activity (15). Although the mechanism of tocol toxicity is unclear, tocol appears to exert its toxic effect as an unhindered phenol (16).

α-Tocopherol, PMC, and Trolox C are homologs of 2,5,7,8-tetramethylchroman-6-ol and each has a very lipophilic isoprenoid side-chain, a lipophilic methyl group, or a hydrophilic carboxyl group, respectively, at the C-2 position (Fig. 1). Although their structures are identical except for having these different substituents at the C-2 positions, there are large differences in their protective effects on LOOH-induced injury to endothelial cells. The differences may at least in part be due to their different lipophilicities, as the lipophilic agents are more readily incorporated into cells.

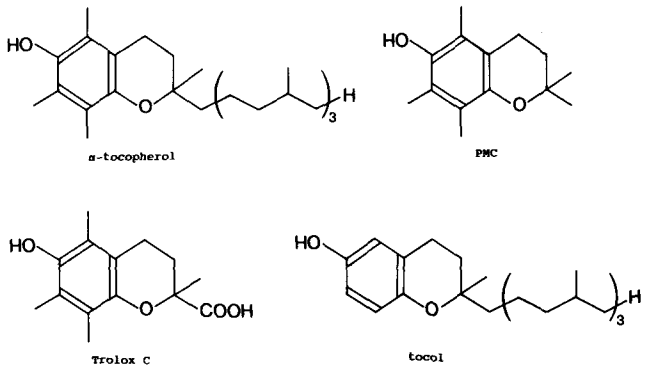


FIG. 1. Molecular structures of α-tocopherol, 2,2,5,7,8-pentamethylchroman-6-ol, Trolox C, and tocol.

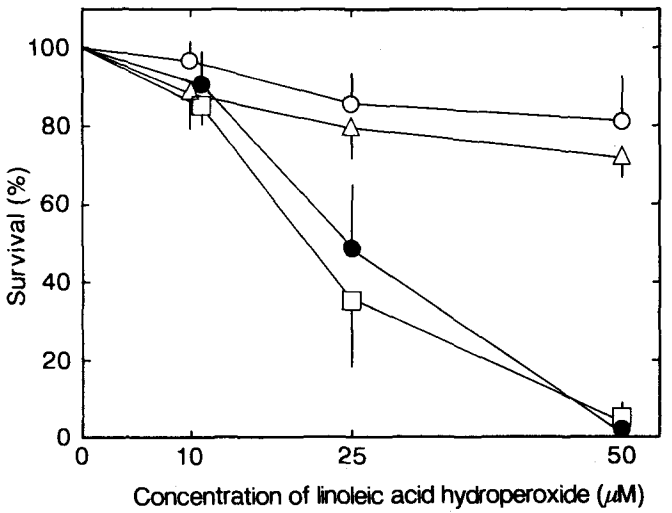


FIG. 2. Effect of preincubation with α-tocopherol, 2,2,5,7,8-pentamethylchroman-6-ol, or Trolox C on the cytotoxicity of linoleic acid hydroperoxide toward human umbilical vein endothelial cells. Percent survivals of cells exposed to the hydroperoxide for 3 hr are given. Cells were preincubated in culture medium with α-tocopherol (open circles), PMC (triangles), Trolox C (squares), each of which was solubilized in ethanol, or ethanol alone (closed circles). The concentration of each antioxidant was 50 μM. Each point represents the mean ± the standard deviation of three separate experiments performed in duplicate.

Table 1 shows the amounts of antioxidants incorporated into endothelial cells during 24-hr preincubation at 37°C. When incubated at 50 μM with endothelial cells, α-tocopherol was incorporated into the cells at 16.6 nmol/mg protein, while PMC was incorporated at 0.5 nmol/mg protein; Trolox C, however, was not incorporated at all. This means that endothelial cells take up α-tocopherol better than PMC and do not take up Trolox C. The ineffectiveness of Trolox C appears

TABLE 1

Incorporation of Antioxidants into Human Umbilical Vein Endothelial Cells (nmol/mg protein)[a]

Antioxidants	Concentration of antioxidants in medium[b] (μM)				
	0	5	10	25	50
α-Tocopherol	0.2 ± 0.1	0.8 ± 0.1	1.9 ± 0.6	4.6 ± 1.8	16.6 ± 2.7
PMC		n.d.[c]	< 0.1	0.1 ± 0.1	0.5 ± 0.1
Trolox C					n.d.[c]

[a]Each value represents the mean ± the standard deviation of three separate experiments.
[b]Concentration of antioxidants in medium; cells were incubated with each antioxidant in the MCDB-104 system at 37°C for 24 hr.
[c]Not detected.

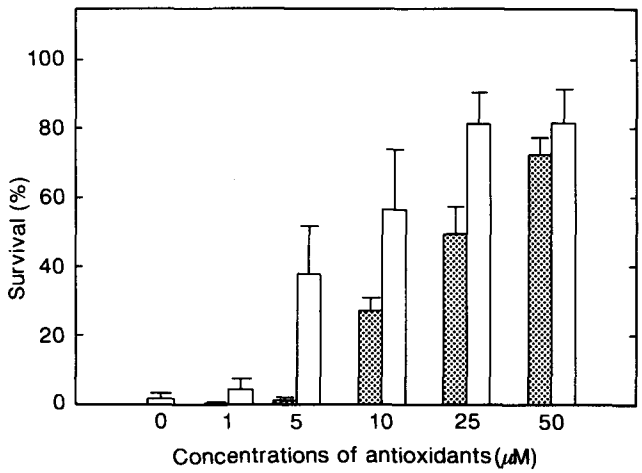


FIG. 3. Effect of α-tocopherol or 2,2,5,7,8-pentamethylchroman-6-ol on the cytotoxicity of 50 μM linoleic acid hydroperoxide toward human umbilical vein endothelial cells as a function of concentration. Percent survivals of cells exposed to the hydroperoxide for 3 hr are given. Cells were preincubated in culture medium with α-tocopherol (open bars) or PMC (shaded bars), each solubilized in ethanol. Each value represents the mean ± the standard deviation of three separate experiments performed in duplicate.

to be due to its hydrophilicity. The results suggest that agents having both high antioxidant activity and lipophilicity can protect endothelial cells from LOOH-induced injury.

α-Tocopherol bears both an isoprenoid side-chain with high affinity for the lipophilic interior region of cell membranes and a hydroxyl group with a high affinity for the hydrophilic exterior region. Thus, α-tocopherol incorporated into membranes is probably oriented in such a manner that its isoprenoid side-chain is located in the interior region and its hydroxyl group is projected toward the exterior region. This orientation would anchor α-tocopherol in the membrane. In contrast, PMC having no isoprenoid side-chain cannot be anchored and may be moveable without orientation in the membrane (17).

Figure 3 shows the effects of various concentrations of α-tocopherol and PMC in the preincubation medium on the survival of endothelial cells exposed to 50 μM LOOH. At 25 μM or lower concentrations, the protective effect of α-tocopherol was greater than that of PMC; *e.g.*, at 10 μM, the former was twice as effective as the latter.

As presented in Table 1, α-tocopherol was incorporated at 0.8 nmol/mg protein into endothelial cells in 5 μM α-tocopherol-containing medium, and PMC was incorporated at 0.5 nmol/mg protein into the cells in 50 μM PMC-containing medium. Under these conditions, the cellular concentrations of α-tocopherol and PMC were similar. As shown in Figure 3, however, α-tocopherol protected about 38% of endothelial cells from 50 μM LOOH-induced injury, while PMC protected about 73% of the cells. When the antioxidants are incorporated at a relatively low concentration into endothelial cells, PMC appears to protect the cells more efficiently than does α-tocopherol.

The reason for this is unclear. It has been reported though that the activity of phenolic antioxidants correlates with the reactivity of their phenolic hydrogens (18); the greater the rate constant of the hydroxyl group of a phenolic antioxidant for hydrogen abstraction by the peroxyl radical, the more effective the phenolic antioxidant. It has been further reported that the reactivities of phenolic hydrogens in PMC and α-tocopherol are similar (18); the rate constants of PMC and α-tocopherol in homogeneous solution are 3.8×10^6 and 3.2×10^6 M^{-1} s^{-1}, respectively. As mentioned before, PMC may be moveable in cell membranes, and hence its phenolic hydrogen may be both in the exterior and interior regions of cell membranes, while the phenolic hydrogen of α-tocopherol may be mainly in the exterior region. If LOOH is incorporated into cell membranes containing a small amount of α-tocopherol or PMC and then decomposed to produce radicals, the PMC might scavenge the radicals more efficiently than would α-tocopherol.

The underlying mechanism of LOOH-induced injury to endothelial cells is still unknown. We do know, however, that the typical radical scavengers α-tocopherol and PMC have protective effects on LOOH-induced injury to the cells. Thus, we presume that some LOOH-induced radicals, such as the alkoxyl radical (19) and the αβ-epoxy carbon-centered radical (20), are causative factors of cell injury.

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Age-Related Changes in the Metabolism of Cholesterol in Rat Liver Microsomes

Dagny Ståhlberg*, Bo Angelin and Kurt Einarsson
Department of Medicine, Karolinska Institutet at Huddinge University Hospital, Stockholm, Sweden

The effects of aging on the hepatic metabolism of cholesterol were studied in 1-, 6- and 24-month-old male Sprague-Dawley rats. Microsomal 3-hydroxy-3-methylglutaryl coenzyme A (HMG-CoA) reductase activity, which regulates cholesterol biosynthesis, decreased from 835 ± 144 (SEM) pmol/min/mg protein in the youngest group to 219 ± 34 and 205 ± 53 pmol/min/mg protein ($p<0.001$) in the 6- and 24-month-old groups, respectively. Cholesterol 7α-hydroxylase activity, which governs bile acid synthesis, was gradually reduced from 70 ± 14 pmol/min/mg protein in the 1-month-old group to 32 ± 7 and 16 ± 3 pmol/min/mg protein ($p<0.05$) in the 6- and 24-month-old groups, respectively. Acyl coenzyme A:cholesterol acyltransferase activity, which catalyzes the esterification of cholesterol, averaged 431 ± 47 and 452 ± 48 pmol/min/mg protein in the 1- and 6-month-old groups, respectively, and was increased to 585 ± 55 pmol/min/mg protein ($p<0.05$) in the 24-month-old group. The level of total cholesterol showed an age-related increase from 1.56 ± 0.16 mg/g liver in the 1-month-old group to 1.70 ± 0.15 and 2.20 ± 0.19 mg/g liver ($p<0.05$) in the 6- and 24-month-old groups, respectively. The increase was mainly caused by an accumulation of esterified cholesterol. We conclude that a marked decrease in HMG-CoA reductase occurs between 1 and 6 months of age; thereafter the enzyme activity stays unchanged. The activity of cholesterol 7α-hydroxylase decreases progressively and drastically with age, whereas the capacity for esterifying cholesterol increases slightly. We speculate that the reduced conversion of cholesterol to bile acids may be one explanation of the age-related increase of plasma cholesterol seen in rats.
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Hypercholesterolemia is considered one of the main causes of atherosclerosis in humans and it is well known that serum cholesterol levels rise with advancing age. Also, age-related increases of plasma lipids are observed in rats (1–3). The mechanism of the rise in serum cholesterol with age is not well understood. Since the liver has a central role in cholesterol metabolism, some studies on the influence of age on hepatic metabolism of cholesterol in rats have been reported. Using radio-labeled acetate, Yamamoto and Yamamura (4) found a decreasing hepatic cholesterogenesis with age in Wistar rats 2- to 18-months-old. The activity of the rate-determining enzyme in hepatic cholesterol biosynthesis, 3-hydroxy-3-methylglutaryl coenzyme A (HMG-CoA) reductase was studied by Story *et al.* (2) in Fisher rats. The activity decreased from 2 to 9 months and then stabilized at that level through 24 months of age. These authors also determined the activity of cholesterol 7α-hydroxylase, the regulatory enzyme in the formation of bile acids, and found that it was unchanged from 2 to 24 months of age (2). In a later study, Story *et al.* (3) did not find any changes with age, between 2 and 24 months, for the two enzymes in either Fisher 344 or Sprague-Dawley strains of rats. Choi *et al.* (5) reported lower activities of HMG-CoA reductase and cholesterol 7α-hydroxylase in adult (8-month-old) than in young (5-week-old) Sprague-Dawley rats. Stange and Dietschy (6) studied cholesterol synthesis, using ^{3}H-labeled water, in immature (4-week-old) and mature (15-week-old) Sprague-Dawley rats, and they found a marked decrease in the rate of cholesterol synthesis in the mature rats. Previous studies of acyl-CoA:cholesterol acyltransferase (ACAT), which catalyzes the esterification of cholesterol, have not shown any age-related changes in enzyme activity (7,8).

We considered it important to more thoroughly study the influence of age on the three key enzymes of cholesterol metabolism in rat liver. In the present work we have simultaneously determined the activities of HMG-CoA reductase, cholesterol 7α-hydroxylase, and ACAT in three different age groups of male Sprague-Dawley rats. In addition, the concentrations of free and esterified cholesterol in liver tissue and microsomes were analyzed.

MATERIALS AND METHODS

Materials. Deuterium-labeled cholesterol was obtained from Larodan Fine Chemicals (Malmo, Sweden). Deuterium-labeled 7α-hydroxycholesterol was synthesized as described previously (9). [3-^{14}C]HMG-CoA (sp. act. 19 mCi/mmol), [1-^{14}C]oleoyl coenzyme A (sp. act. 57.8 mCi/mmol), and [1,2,6,7-^{3}H]cholesteryl oleate (sp. act. 82.7 Ci/mmol) were obtained from New England Nuclear Corp. (Boston, MA). DL-[2-^{3}H]mevalonic acid (sp. act. 125 mCi/mmol) was obtained from Radiochemical Centre (Amersham, England). Unlabeled mevalonic acid lactone, cholesteryl oleate, NADP, NADPH, EDTA, dithiothreitol (DTT), glucose-6-phosphate, glucose-6-phosphate dehydrogenase, and human serum albumin were purchased from Sigma Chemicals Co. (St. Louis, MO).

Experimental procedure. Male Sprague-Dawley rats of three different ages, 1 month, 6 months and 24 months, were used in the experiment. Each age group was comprised of 12 rats, and the mean weights of the rats at the different ages were 78 g, 465 g and 600 g, respectively. The 1-month-old group was not sexually mature. All groups had free access to water and rat chow (R3; Ewos AB, Södertälje, Sweden), a commercial pellet diet. The 1-month-old group had been fed chow for at least 10 days after weaning.

The animals were killed in the morning by a blow on the head after a 12-hr fast. The livers were immediately

*To whom correspondence should be addressed at the Department of Medicine, Huddinge University Hospital, S-141 86 Huddinge, Sweden.

Abbreviations: ACAT, acyl-coenzyme A:cholesterol acyltransferase; DTT, dithiothreitol; EDTA, ethylenediaminetetraacetic acid; HMG-CoA, 3-hydroxy-3-methylglutaryl coenzyme A.

removed, weighted, cut into two pieces and put into cold buffer solutions.

Preparation of liver microsomes. One aliquot of the liver was minced and homogenized with a loose-fitting Teflon pestle in nine volumes of 50 mM Tris-HCl buffer, pH 7.4, containing 0.3 M sucrose, 10 mM DTT, 10 mM EDTA and 50 mM NaCl. The homogenate was centrifuged at 20,000 × *g* for 15 min. The supernatant was centrifuged at 100,000 × *g* for 60 min. Half of the microsomal fraction was resuspended in homogenizing medium and recentrifuged at 100,000 × *g* for 60 min. This fraction was then suspended in 20 mM imidazole buffer, pH 7.4, containing 10 mM DTT in a volume corresponding to that of the 20,000 × *g* supernatant, and used for the assay of HMG-CoA reductase. Half the original microsomal fraction was recentrifuged at 100,000 × *g* in a homogenizing medium lacking DTT. The resulting fraction was suspended in 0.1 M phosphate buffer, pH 7.4, containing 1 mM EDTA and used for the assay of cholesterol 7α-hydroxylase activity.

The remaining piece of the liver was minced and homogenized in 50 mM Tris-HCl buffer, pH 7.4, containing 0.3 M sucrose and 1 mM EDTA as described above. The homogenate was centrifuged at 20,000 × *g* for 15 min. The supernatant solution was centrifuged at 100,000 × *g* for 60 min. The pellet obtained was resuspended in 0.1 M phosphate buffer, pH 7.4, containing 1 mM EDTA and used for the assay of ACAT activity.

Microsomal and liver homogenate protein were determined by the method of Lowry *et al.* (10).

Assay of HMG-CoA reductase activity. The HMG-CoA reductase activity was assayed according to Brown *et al.* (11), essentially as described recently (12). An aliquot of the microsomal fraction, 40 μL, was preincubated for 15 min at 37°C in a total volume of 0.2 mL containing 100 mM phosphate buffer, pH 7.4, 10 mM imidazole buffer, pH 7.4, 5 mM DTT, 10 mM EDTA, 3 mM NADP, 12 mM glucose-6-phosphate and 1 unit of glucose-6-phosphate dehydrogenase. The HMG-CoA reductase assay was then initiated by the addition of 90 nmoles of [3-^{14}C] HMG-CoA (0.5 μCi) dissolved in 25 μL distilled water. The incubation was run for 15 min and was stopped by the addition of 25 μL 6M HCl. Tritium-labeled mevalonic acid (0.01 μCi) was added as internal standard together with 3 mg of unlabeled mevalonic acid lactone, and the incubation mixture was allowed to stand overnight at room temperature for complete lactonization. The incubation mixture was then subjected to thin-layer chromatography with toluene/acetone (1:1, v/v) as the developing solvent. The mevalonic acid lactone band was located, scraped off and analyzed for radioactivity. Corrections for losses were made based on use of an internal standard.

Assay of cholesterol 7α-hydroxylase activity. The standard assay system consisted of 1.0 mL of the microsomal fraction, 0.1 M phosphate buffer, pH 7.4, containing 1 mM EDTA, and 1 mM NADPH. The enzyme assay was carried out in duplicate for 15 min at 37°C according to the method of Björkhem and Kallner (9) with the product 7α-hydroxycholesterol being analyzed by gas-liquid chromatography/mass spectrometry. Each set of experiments also included two assays in which the mass of 7α-hydroxycholesterol was determined in the incubation mixture extracted at zero-time. The amount of 7α-hydroxycholesterol formed during an incubation was estimated by subtracting the amount present in the zero-time assay from the amount present at the end of the incubation and was expressed as pmol/min/mg protein.

Assay of ACAT activity. ACAT activity was assayed both in the absence and presence of exogenous cholesterol according to Billheimer *et al.* (13), as described recently (14). The assay system contained 0.1 mL of the microsomal preparation. 0.1 M phosphate buffer, pH 7.4, 1 mM EDTA, and 1 mg of fatty acid-free bovine serum albumin in a total volume of 1.0 mL. The mixture was preincubated for 5 min at 37°C, or for 30 min at 37°C after the addition of 50 nmol of unlabeled cholesterol dissolved in 600 μg of Triton. The reaction was initiated by the addition of 25 nmol (1.45 μCi) of [1-^{14}C]oleoyl coenzyme A. After 4 min the assay was stopped by the addition of 10 mL chloroform/methanol (2:1, v/v). Tritium-labeled cholesteryl oleate (0.01 μCi) was added as an internal standard to estimate recovery followed by 1 mL 0.9% (w/v) NaCl. The chloroform phase was collected and evaporated to dryness under N_2. The residue was resuspended in chloroform/methanol (2:1, v/v) and subjected to thin-layer chromatography. Unlabeled cholesteryl oleate was added as marker. The chromatogram was developed in hexane/ethyl acetate (95:5, v/v). The cholesteryl oleate band was made visible with iodine vapor, scraped off into a counting vial, and analyzed for radioactivity.

Determination of liver cholesterol. The concentrations of total cholesterol in liver homogenates and microsomal fractions were determined by isotope dilution-mass spectrometry after the addition of deuterium-labeled cholesterol as an internal standard, as described previously (15) with the modifications described by Schaffer *et al.* (16). Free cholesterol was determined by the same method except that the hydrolysis step was omitted. The concentration of esterified cholesterol was calculated as the difference between total and free cholesterol in the same sample. The cholesterol levels in the homogenates are given as mg cholesterol/g liver.

Statistical analysis. Data are presented as means ± SEM (standard error of the mean). The significance of differences was evaluated by Student's *t*-test.

RESULTS

The relative liver weights, expressed as % of total body weight, were 4.3 ± 0.2% for the 1-month-old group, 3.6 ± 0.1% for the 6-month-old group, and 3.3 ± 0.1% for the 24-month-old group (Table 1). The amount of protein in liver homogenates and microsomes was the same in the three groups.

HMG-CoA reductase activity decreased by about 75% from 1–6 months of age (835 ± 144 and 219 ± 34 pmol/min/mg protein, respectively; $p<0.001$), and remained essentially unchanged from 6–24 months of age (Table 2).

The cholesterol 7α-hydroxylase activity showed a successive decrease with age (Table 2). The activity declined by 55% from 1 to 6 months of age, and by another 50% between 6 and 24 months of age.

ACAT activity increased gradually with age (Table 2). The activity for the 1-month-old group was 431 ± 47, for the 6-month-old group 452 ± 49, and for the 24-month-old group 585 ± 55 mol/min/mg protein. The increase was significant between the 1- and 24-month-old groups ($p<0.05$). In eight of the rats from each group, ACAT activity was also determined when exogenous cholesterol

TABLE 1

Body and Liver Weight, and Protein Concentration in Liver Homogenates and Microsomes from Male Sprague-Dawley Rats of Various Ages[a]

	1Month	6 Months	24 Months
Body weight (g)	78 ± 2	465 ± 25	600 ± 50
Liver weight (g)	3.4 ± 0.2	16.6 ± 1.0	19.5 ± 0.9
Liver weight (% body weight)	4.3 ± 0.2	3.6 ± 0.1	3.3 ± 0.1
Protein concentration in liver homogenate (mg/mL)	19.7 ± 6.3	20.7 ± 8.5	24.9 ± 9.4
Protein concentration in microsomal fraction (mg/mL)	1.4 ± 0.3	1.5 ± 0.2	1.6 ± 0.3

[a]Data represent means ± SEM from 12 rats in each group.

TABLE 2

Enzyme Activities in Hepatic Microsomes from Rats 1-, 6- and 24-Month Old[a]

Enzyme activity (number of animals)	1 Month	6 Months	24 Months
	pmol/min/mg protein		
HMG-CoA reductase (n=12)	835 ± 144	219 ± 3[b]	205 ± 53[b]
Cholesterol 7α-hydroxylase (n=58)	70 ± 14	32 ± 7[c]	16 ± 3[d,e]
ACAT (n=512)	431 ± 47	452 ± 49	585 ± 55[c]
ACAT; exogenous cholesterol added (n=8)	3081 ± 527	3173 ± 466	4565 ± 774

[a]Data represent means ± SEM.
[b]Significantly different from 1-month-old rats, p<0.001.
[c]Significantly different from 1-month-old rats, p<0.05.
[d]Significantly different from 1-month-old rats, p<0.005.
[e]Significantly different from 6-month-old rats, p<0.05.

TABLE 3

Cholesterol Concentration in Liver Microsomes and Liver Tissue of 1-, 6- and 24-Month-Old Rats[a]

	Total cholesterol	Free cholesterol	Esterified cholesterol
Liver microsomes (nmol/mg protein)			
1 Month	59.4 ± 5.7	50.0 ± 5.1	9.3 ± 1.1
6 Months	62.5 ± 5.2	51.4 ± 4.0	11.2 ± 1.9
24 Months	63.7 ± 4.4	49.9 ± 3.6	11.8 ± 2.1
Liver tissue (mg/g liver)			
1 Month	1.56 ± 0.16	1.37 ± 0.2	0.19 ± 0.02
6 Months	1.70 ± 0.2	1.38 ± 0.2	0.32 ± 0.04[b]
24 Months	2.20 ± 0.19[b,d]	1.75 ± 0.16	0.45 ± 0.05[c,d]

[a]Data represent means ± SEM.
[b]Significantly different from the 1-month-old group; p<0.05.
[c]Significantly different from 1-month-old group; p<0.001.
[d]Significantly different from 6-month-old rats; p<0.05.

was added to the assay system. The activity was stimulated about seven-fold in all groups (Table 2).

The level of total cholesterol in the liver increased with age from 1.56 ± 0.16 mg/g liver in the 1-month-old group to 1.70 ± 0.15 and 2.20 ± 0.19 mg/g liver (p<0.05) in the 6- and 24-month-old groups, respectively. The amount of esterified cholesterol showed a corresponding increase from 0.19 ± 0.02 mg/g liver in the 1-month-old group to 0.32 ± 0.04 (p<0.05) and 0.45 ± 0.05 mg/g liver (p<0.001) in the 6- and 24-month-old groups, while the amount of free cholesterol did not significantly differ between the age groups. No significant changes were observed in microsomal cholesterol levels (Table 3).

DISCUSSION

In the present study, the HMG-CoA reductase activity, which regulates the synthesis of cholesterol in the liver, decreased by about 75% from 1 month to 6 months of age, and then remained unchanged up to 24 months of age. Previously Yamamoto and Yamamura (4) reported reduced incorporation of acetate into hepatic cholesterol from 2 to 18 months of age in Wistar strain rats. Similar observations were made by Story *et al.* in the Fischer 344 strain, while in Sprague-Dawley strain rats acetate incorporation into cholesterol was unchanged from 2 to 18 months of age, and was significantly reduced at 24 months of age. The activity of HMG-CoA reductase, however, was unchanged with advancing age (2-24 months of age) in both rat strains. Choi *et al.* (5) reported reduced HMG-CoA reductase activity in 8-month-old as compared to 5-week-old Sprague-Dawley rats. Our results indicate a considerably higher activity of HMG-CoA reductase in immature (1-month-old) rats compared to mature (6- and 24-month-old) rats. The activity of HMG-CoA reductase seems to decrease drastically during sexual maturation, a process that starts at about 40 days and ceases at about 3 months of age. This interpretation is also consistent with the previous results mentioned above (2-5). One explanation for the increased synthesis of cholesterol in immature rats would be a higher demand for cholesterol during the phase of rapid growth. Stange and Dietschy (6) had previously arrived at similar conclusions upon measuring the rate of cholesterol synthesis in different organs of immature (4-week-old) and mature (15-week-old) Sprague-Dawley rats. Suggested malabsorption of bile acids during the first weeks of life and increased activity of cholesterol 7α-hydroxylase (see below), may also contribute to stimulated cholesterol synthesis in immature rats. Other factors, such as hormonal changes at maturity, may contribute to the decline in HMG-CoA reductase activity.

The activity of the cholesterol 7α-hydroxylase gradually decreased with advancing age. The activity was reduced by about 55% from 1 to 6 months of age, and by another 50% from 6 to 24 months of age. Previously, Choi *et al.* reported a reduction of cholesterol 7α-hydroxylase activity in Sprague-Dawley rats between 5 weeks and 8 months of age. This decrease in cholesterol 7α-hydroxylase activity can at least in part explain the reduced excretion of bile acids with age observed in previous studies (4). Fetal and neonatal animals show only a passive transport of bile acid absorption in jejunum and ileum. Lester *et al.* (17) found that the ileal mechanism

for active bile salt transport in dogs was not activated until at least 2 weeks after birth, and adult levels were reached between 2 and 5 weeks of age. Similar results have been reported for an *in vivo* study with Wistar rats (18). Cholesterol 7α-hydroxylase activity is considered to be regulated by the portal inflow of bile acids (19). A decreased capacity to absorb bile acids may cause malabsorption of bile acids and thereby an upregulation of bile acid synthesis and of cholesterol 7α-hydroxylase activity. This change in intestinal absorption of bile acids may be of importance to explain the higher activity of cholesterol 7α-hydroxylase in the 1-month-old compared to the 6-month-old rats.

ACAT activity increased slightly with advancing age. When exogenous cholesterol was added, the activity increased about seven-fold in all groups, which indicates that differences in activity cannot be explained solely by differences in access to free cholesterol as substrate. Our results are at variance with two previous studies, which showed that ACAT activity did not change with aging (7,8). However, in accordance with our finding of increased ACAT activity with aging, we observed a concomitant rise in esterified cholesterol in liver tissue. Thus, our results could be interpreted as demonstrating the importance of ACAT activity as a modulator of intracellular cholesterol balance, which would contribute to the maintenance of a critical concentration of free cholesterol. The reason for the accumulation of liver cholesterol, which occurs with age, can only be speculated upon. One interesting possibility would be that the imbalance between cholesterol synthesis and bile acid production would progress with age, as is reflected in the increased ratio between HMG-CoA reductase and cholesterol 7α-hydroxylase activities for 6-month-old and 24-month-old rats (Table 2). The relatively stable HMG-CoA reductase activity, which is maintained in spite of the increased hepatic cholesterol levels, suggests that regulation of cholesterol synthesis becomes more sluggish with age.

In conclusion, the present study has shown that a marked decrease in hepatic HMG-CoA reductase activity occurs in rats between 1 and 6 months of age, whereas the activity remains essentially unchanged during adult life. Cholesterol 7α-hydroxylase activity gradually decreases with age, while the capacity to esterify cholesterol increases slightly. We suggest that the declining cholesterol 7α-hydroxylase activity, which reflects a decreased capacity to metabolize cholesterol to bile acids, may contribute to the age-related increase of plasma cholesterol in rats. If so, the well-known age-related rise in serum cholesterol levels in humans could at least in part be due to the decrease in bile acid synthesis with age reported previously (20).

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ω3 Fatty Acids Increase Spontaneous Release of Cytosolic Components from Tumor Cells

Laura J. Jenski*, Laura Kathleen Sturdevant, William D. Ehringer and **William Stillwell**
Department of Biology, Indiana University-Purdue University at Indianapolis, Indianapolis, Indiana 46205

Mice fed menhaden (fish) oil or coconut oil-rich diets were inoculated intraperitoneally with a rapidly growing leukemia, T27A. After one week, the tumor cells were harvested, and ^{51}Cr was used to label intracellular molecules. Spontaneous release of ^{51}Cr was used as a measure of plasma membrane permeability. Compared to cells from mice fed coconut oil (rich in saturated fatty acids), tumor cells from mice fed menhaden oil (rich in long chain polyunsaturated ω3 fatty acids) showed an increased level of spontaneous ^{51}Cr release, which was exacerbated by increased temperature and reduced by extracellular protein. At physiological salt concentrations, the released ^{51}Cr was detected in particles of ~2700 daltons. Enhanced permeability correlated with the incorporation of dietary (fish oil) ω3 polyunsaturated fatty acids docosahexaenoic and eicosapentaenoic acid into the tumor cells. The results demonstrate that ω3 fatty acids are incorporated into cellular constituents of tumor cells and change properties associated with the plasma membrane. This result suggests that dietary manipulation may be used to enhance tumor cell permeability and contribute to tumor eradication.
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Diets rich in cold water fish are associated with a reduced incidence of certain cancers (1). Beyond the epidemiologic evidence, however, we understand very little about how fish oil-rich diets may prevent cancer. The unique components of fish oil, ω3 polyunsaturated fatty acids (PUFA) such as docosahexaenoic acid (DHA, 22:6) and eicosapentaenoic acid (EPA, 20:5), may play critical roles hormonally by altering prostaglandin and leukotriene synthesis; and structurally, by altering the composition of lipid bilayers in cellular membranes (2).

There is evidence that dietary ω3 PUFA become incorporated into membranes of various tissues (3-5); furthermore, rapidly growing tumors also utilize dietary fats (6). It is not clear, however, whether structural alterations of tumor cell membranes, resulting from incorporation of highly unsaturated fatty acids into phospholipids of the bilayer, affect human health. The unique shape of ω3 PUFA (7-9) suggests that phospholipid bilayers containing DHA and EPA are likely to display packing, domain, and fluidity characteristics distinct from bilayers with saturated fatty acyl chains. Thus, events that involve the cell surface may be affected by the PUFA content of the membrane, as suggested by altered methotrexate uptake and melphalan and phenylalanine transport (10), enhanced protein kinase C activity (11), and increased membrane fluidity (12).

*To whom correspondence should be addressed at the Department of Biology, IUPUI, 1125 E. 38th Street, Indianapolis, IN 46205.

Abbreviations: ANOVA, analysis of variance; BSA, bovine serum albumin; CCCP, carbonyl cyanide *m*-chlorophenylhydrazone; DHA, docosahexaenoic acid; 2,4-DNP, 2,4-dinitrophenol; EPA, eicosapentaenoic acid; FBS, fetal bovine serum; PUFA, polyunsaturated fatty acid; RBC, red blood cell; SFA, saturated fatty acid.

Immunologic effector mechanisms directed against tumor cells (*e.g.*, cell-mediated cytolysis) are among the many biological phenomena occurring at the cell surface. Cytolysis occurs when a killer cell, in intimate contact with a tumor cell, delivers a lethal hit to the tumor cell membrane. Historically, the definitive assay for killer cell activity has been the release of ^{51}Cr-labeled intracellular components from prelabeled targets after delivery of the lethal hit. Tumor cell destruction is accompanied by the release of intracellular components to the environment; under certain conditions, intracellular components are released spontaneously without killer cell activity or tumor death (13). In this study we have compared spontaneous release of ^{51}Cr-labeled molecules from a transplantable leukemia grown in mice fed diets rich in ω3 PUFA or saturated fatty acids (SFA). We have shown here that a diet containing 10-20% (w/w) menhaden (fish) oil produces tumor cells with increased permeability to certain molecules.

MATERIALS AND METHODS

Mice and experimental diets. BALB/c mice, purchased from Harlan Sprague Dawley (Indianapolis, IN), were bred and maintained in the animal facilities at the Purdue University School of Science at Indianapolis. Prior to enrollment on experimental diets, the animals were given Chow #5001 (Purina Mills, St. Louis, MO) and water *ad libitum*. Experimental food pellets were prepared by soaking menhaden oil (Zapata Haynie Corp., Reedville, VA) or hydrogenated coconut oil (ICN Nutritional Biochemicals, Cleveland, OH) to a final concentration of 10% (w/w) into Chow #5001 (which contains fish meal). Experimental powdered diet was prepared with 20% (w/w) menhaden oil or hydrogenated coconut oil blended into a fat-free basal diet (ICN Nutritional Biochemicals) containing 21.10% casein, 16.45% alphacel, 58.45% sucrose, 4.00% salt mixture USP XIV, and ICN vitamin diet fortification mix with choline chloride (6.008 g/kg). The major fatty acids in coconut oil are 12:0 and 14:0 (14), the major saturated and monounsaturated fatty acids in menhaden oil are 14:0, 16:0, 16:1, and 18:1, and the major polyunsaturated fatty acids are 20:5 and 22:6 (15). The diets were protected from fluorescent lights and stored frozen at −20°C. In the afternoon, mice were given an amount of experimental diet twice their expected consumption. The food was removed the following morning, and fresh food was again provided in the afternoon; tap water was available at all times. Age and sex matched mice, four mice per group per experiment (three experiments), were fed the experimental diet for five weeks, inoculated with tumor cells, and then maintained on the experimental diet for one additional week.

Tumor line. T27A is a transplantable non-T, non-B leukemia that originated in a BALB/c mouse infected with Friend murine leukemia virus (16). These cells were obtained from the American Type Culture Collection (Rockville, MD). The subline we are maintaining readily adapts to either ascitic growth or culture *in vitro;* an

intraperitoneal inoculum between 1 and 5 × 10^5 cells will generally kill a normal syngeneic host in less than nine days. Ascites T27A were passed weekly in normal syngeneic (BALB/c) mice fed Purina chow #5001.

Radiolabeling and radiolabel release assay. T27A cells were harvested from mice by peritoneal lavage with Hanks' balanced salt solution (Gibco Biologicals, Grand Island, NY) plus 5 mM HEPES buffer (Aldrich Chemical Co., Milwaukee, WI). The tumor cells were depleted of red blood cells (RBC) on Ficoll-Hypaque (Histopaque,Sigma Chemical Co., St. Louis, MO) and diluted in medium composed of equal volumes of RPMI 1640 and Dulbecco's modified Eagle medium plus antibiotics, 25 mM HEPES buffer, 2 mM glutamine, and 5% fetal bovine serum (FBS). ^{51}Cr (100 μCi) as sodium chromate (New England Nuclear, Boston, MA) was added to 5 × 10^6 cells in 0.3 mL of medium, and the cells were incubated at 37°C for 1 hr, with occasional agitation. The ^{51}Cr-labeled cells were washed three times in phosphate-buffered saline plus 5% FBS, counted, and diluted in medium.

^{51}Cr release assays were performed in 96-well plates with 5 × 10^3 T27A cells per well in a total volume of 200 μL of medium supplemented with FBS or bovine serum albumin (BSA, 98–99% pure, essentially fatty acid free, Sigma). To determine the maximum releasable cpm of ^{51}Cr, cells were similarly incubated in detergent (4% cetrimide, Sigma). Unless otherwise stated, the plates were centrifuged at 200 × *g* for 5 min to pellet the cells, and then incubated for 4 hr at 37°C in a humidified incubator containing 7.5% CO_2. A 100-μL aliquot of supernatant was removed from each well, and the released ^{51}Cr present in the supernatant was measured in a Beckman 5500 gamma counter (Arlington, IL). The statistical difference between groups was assessed by one-way analysis of variance (ANOVA) with the program ABSTAT (Anderson Bell, Parker, CO).

Gel filtration. ^{51}Cr-labeled molecules released from T27A cells spontaneously or after two freeze-thaw cycles were chromatographed on Sephadex G-25-80 (Sigma) in a 1 × 60 cm column. Chromatography was performed at room temperature in 10 mM Tris buffer (pH 7.2) containing 0, 0.15, or 0.5 M NaCl. The ionic conditions were varied to assess aggregation and the minimal size of the released material. The flow rate was ~7 mL/hr; 0.7-mL fractions were collected and the radioactivity present in each fraction was measured in a gamma counter.

Analysis of fatty acids by gas chromatography. Tumor cells devoid of RBC were suspended in 20 mL of distilled water and extracted three times with 80 mL of $CHCl_3/CH_3OH$, 2:1 (v/v). Extracts were then rotovaporated to a small volume and saponified with 0.5 N methanolic NaOH. The fatty acids were then methylated with BF_3 in methanol (17). Methyl esters of the fatty acids were quantified by gas-liquid chromatography on a Varian 1400 Gas Chromatograph (Varian Associates, Sunnyvale, CA) equipped with a flame ionizing detector and interfaced to a Hewlett-Packard 3394-A integrator (Palo Alto, CA). The 1/8 × 36 inch glass column was packed with 10% SP-2330 on 100/120 Chromosorb WAW. The column conditions were as follows: oven temperature, 225°C; injector temperature, 210°C; detector temperature, 250°C; carrier gas flow rate, 40 cm^3/min; sample size, 1.5 μL. Retention times of the fatty acids were compared to standards of the methyl esters purchased from Sigma.

RESULTS

Tumor cells from menhaden oil fed mice demonstrate significantly more spontaneous release of intracellular components than do cells from coconut oil fed mice. Transplanted tumor cells (T27A) were harvested from mice fed menhaden oil or hydrogenated coconut oil-rich diets, ^{51}Cr-labeled, and then incubated for 4 hr in medium plus 5% FBS. As shown in a representative experiment in Figure 1, spontaneous release of ^{51}Cr-labeled molecules was significantly higher in tumor cells exposed to ω3 PUFA in the menhaden oil diet compared to cells exposed to dietary SFA ($P<0.01$, ANOVA). Trypan blue exclusion, an accepted measure of cell viability, performed hourly during the 4 hr incubation indicated that the cells remained greater than 97% viable. The maximal releasable radioactivity from PUFA and SFA cells was similar, and thus the difference in spontaneous ^{51}Cr release was not due to disparate levels of labeling.

RBC in ascites fluid take up and release ^{51}Cr, but spontaneous ^{51}Cr release from RBC alone did not account for the results observed. If RBC were not removed from the T27A preparation, we observed a higher level of spontaneous release; importantly, however, even partial RBC-depletion reduced overall spontaneous release to the level of RBC-free preparations. T27A cells, harvested from chow-fed mice and freed of RBC, gradually released ^{51}Cr at ~2–3% per hour over a 4-hr period, depending upon the incubation medium (Fig. 2).

Gel filtration at low ionic strength revealed that the ^{51}Cr-labeled molecules released from T27A ranged from ⩾ 5000 (excluded from the gel) to ~2700 daltons. At higher ionic strengths (0.15 and 0.5 M NaCl), a minority of the ^{51}Cr-labeled material remained in the void volume, whereas most of the label was found associated with a 2700 dalton species (Fig. 3A). A similar profile was

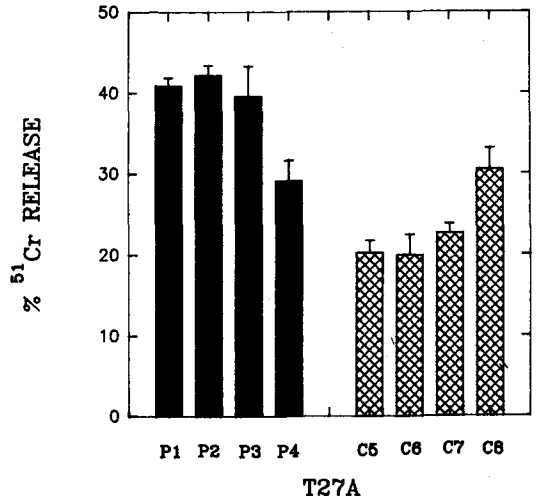


FIG. 1. Compared to cells from coconut oil-fed mice, tumor cells from menhaden oil-fed mice are more permeable to ^{51}Cr-labeled molecules. T27A cells were grown in mice fed chow diets supplemented with 10% menhaden oil (P1–P4, solid bars) or coconut oil (C5–C8, hatched bars), labeled with ^{51}Cr, and incubated at 37°C in wells with medium plus 5% FBS. After 4 hr, supernatant was removed from each well and the radioactivity present in the supernatant was measured. The error bars are SD of triplicate determinations.

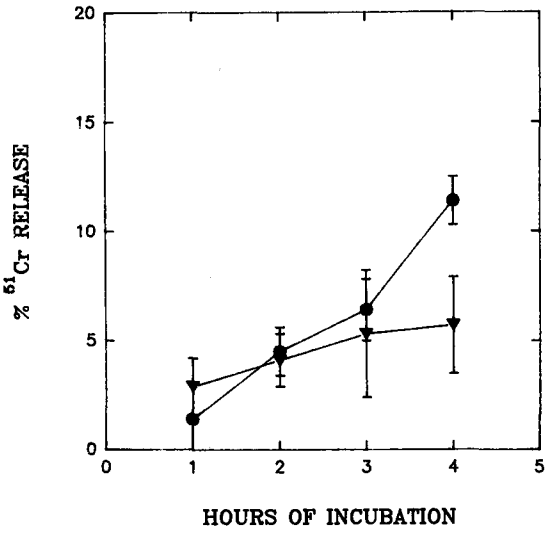


FIG. 2. Spontaneous ^{51}Cr release from tumor cells is approximately linear over a 4-hr period. T27A cells from a mouse fed standard rodent chow were ^{51}Cr-labeled and incubated at 37°C in many replicate wells in medium plus 5% (circles) or 20% (inverted triangles) FBS. At various time points, supernatant was removed from different wells, and the radioactivity present in the supernatant was measured. The error bars are SD of triplicate determinations.

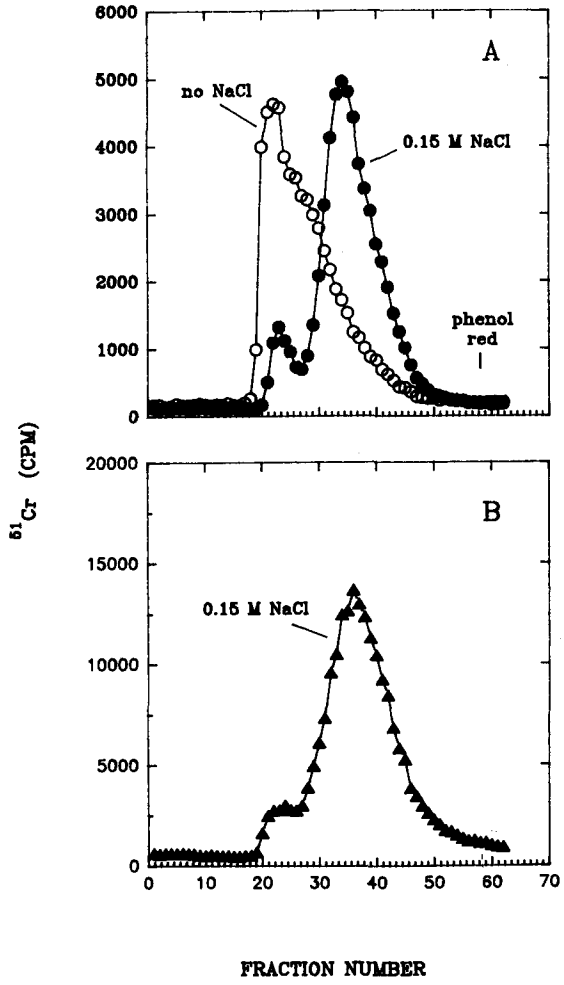


FIG. 3. Sephadex G-25 chromatography of spontaneously ^{51}Cr-labeled molecules. A, spontaneously released, ^{51}Cr-labeled molecules were subjected to gel filtration in 10 mM Tris buffer (pH 7.2) without (open circles) or with (closed circles) 0.15 M NaCl. The void volume was fraction 19–20; phenol red, smaller than the fractionation range, eluted at fraction 58. B, chromatography of intracellular ^{51}Cr-labeled molecules obtained from T27A cells by freezing and thawing.

obtained with material spontaneously released from a cultured human leukemia line, K562 (data not shown). ^{51}Cr-labeled molecules released spontaneously and those retained intracellularly had similar elution profiles on Sephadex G-25 (Fig. 3B), suggesting that a representative portion, not a select subset of ^{51}Cr-labeled molecules permeated the plasma membrane.

The dramatic spontaneous release of ^{51}Cr is reduced by supplementation of the incubation medium with serum or serum albumin. In vivo, serum proteins play an important role in maintaining osmotic balance between cells and their milieu. FBS (fetal bovine serum) is often added to cells *in vitro* to preserve osmotic balance and to supply growth factors. To test their response to osmotic pressures, PUFA and SFA cells were incubated for 4 hr at 37°C in culture medium supplemented with 2.5–20% FBS (Fig. 4) or 0.1–10% (1–100 mg/mL) purified BSA (Fig. 5). In both media, PUFA cells and, to a lesser extent, SFA cells displayed reduced spontaneous ^{51}Cr release as increased serum or protein was added. Because ≥5% albumin reduced spontaneous ^{51}Cr release as effectively as 20% FBS, it would appear that protein (*i.e.*, albumin) is the vital component in the serum-dependent osmotic gradient. These results suggest that PUFA cells are more permeable to ^{51}Cr-labeled molecules, having responded dynamically to the osmotic gradient applied by the reduction in serum protein. Importantly, augmented permeability of PUFA cells implies that *in vivo*, under conditions of moderate osmolarity, tumor cells exposed to dietary fish oil would be highly permeable.

Spontaneous ^{51}Cr release is exacerbated by increased temperature. We anticipate that several temperature dependent processes may affect spontaneous ^{51}Cr release, including increase in energy of the transported solute, increase in the number and size of the statistical "membrane pores", and change in the nature and size of domains and domain boundaries. Because of disparity in the response of saturated and unsaturated fatty acids to temperature changes, we predicted that PUFA and SFA cells would be differentially affected by changes in temperature. To test this prediction, PUFA and SFA cells were ^{51}Cr-labeled and incubated in medium plus 5% FBS for 4 hr at room temperature (22°C), 30°C, or 37°C. As shown in Figure 6, PUFA cells released significantly more ^{51}Cr at 30 and 37°C when compared with the SFA cells. At 22°C, there was little spontaneous release, and there was no difference between the two groups.

Dietary PUFA are incorporated into ascites cells. RBC-depleted T27A from mice fed menhaden oil contained significant amounts of DHA (22:6) and EPA (20:5), 5–10% of the total fatty acids (Fig. 7). Importantly, tumor cells from coconut oil fed mice did not contain detectable levels of these ω3 PUFA unless the diet also contained fish meal. The analyses suggested a direct correlation between the amount of EPA and DHA in the diet and the amount of these fatty acids in the tumor cells (Spearman rank correlation, $P<0.001$). In all cases, the incorporation

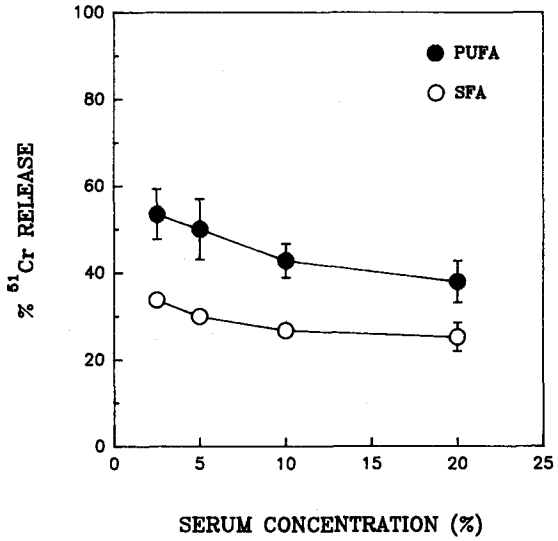


FIG. 4. Increased serum in the incubation medium reduces the spontaneous release of ^{51}Cr-labeled molecules. T27A cells from mice fed fat-free basal diets supplemented with 20% menhaden oil (closed symbols) and coconut oil (open symbols) were ^{51}Cr-labeled and incubated for 4 hr in medium containing 2.5 to 20% FBS. Radioactivity present in the medium was then measured. Mean ± SD (four mice/group).

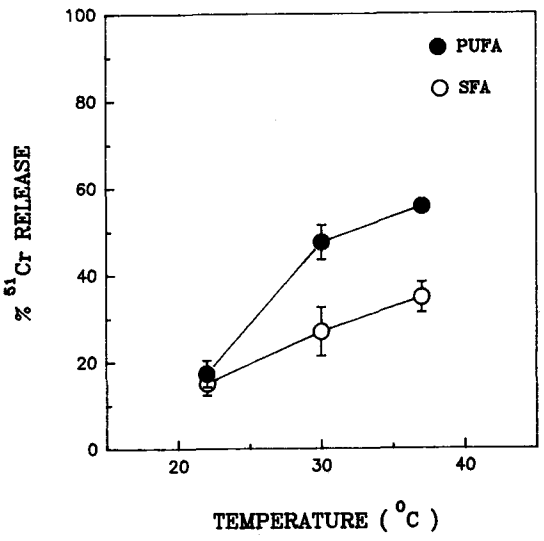


FIG. 6. Spontaneous ^{51}Cr release from tumor cells of menhaden oil-fed mice is particularly sensitive to increased incubation temperature. T27A cells from mice fed fat-free basal diets supplemented with 20% menhaden oil (closed symbols) and coconut oil (open symbols) were ^{51}Cr-labeled and incubated at room temperature (22°C), 30°C, and 37°C for 4 hr. Radioactivity present in the medium was then measured. Mean ± SD (four mice/group).

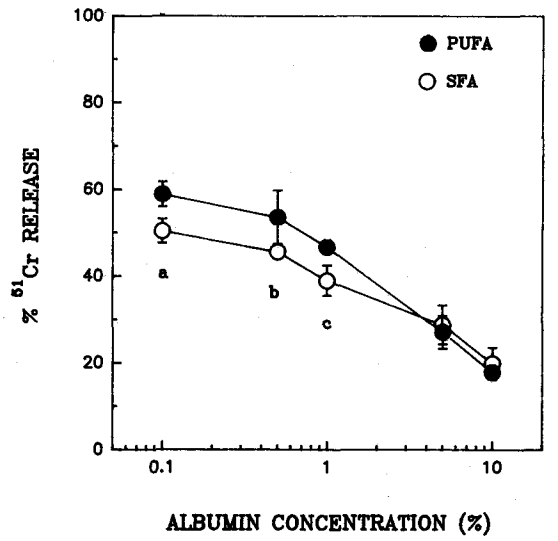


FIG. 5. Purified serum albumin will reduce spontaneous ^{51}Cr release. T27A cells from mice fed chow supplemented with 10% menhaden oil (closed symbols) and coconut oil (open symbols) were ^{51}Cr-labeled and incubated for 4 hr in medium containing 0.1 to 10% BSA. Radioactivity present in the medium was then measured. Mean ± SD (four mice/group). Statistically significant differences between diets were tested by ANOVA: a, P=0.0069; b, P=0.0467; c, P=0.0056.

of DHA into tumor cell lipids was greater than for EPA.

Plasma membranes were isolated by the method of Kaduce *et al.* (6), and their fatty acid compositions were determined. The percent of total fatty acids represented by nine fatty acids (14:0, 16:0, 18:0, 18:1, 18:2, 18:3, 20:4, 20:5, 22:6) was calculated for PUFA and SFA membranes, respectively: 2.7, 25.7; 32.2, 23.9; 6.2, 7.8; 13.1, 15.6; 5.7, 7.5; 1.4, 16.3; 7.1, 0; 2.1, 0; 4.8, 0 (each value is an average of duplicate analyses). As was shown for whole cells, isolated membranes contained EPA and DHA only if these fatty acids were present in the diet.

Tumor growth in mice on a menhaden oil diet is less than in mice fed coconut oil. Table 1 shows that mice on the two diets maintained comparable body weights. The difference in weight between menhaden oil and coconut oil-fed mice observed on day 35 in experiment 1 was not seen on day 33 or earlier, and therefore was likely to be coincidental. On day 42, after tumor transplant and before the onset of cachexia, mice on the coconut oil diet had significantly greater body weights as compared to menhaden oil fed mice, suggesting greater tumor burdens. The relative change in body weight (weight on day of tumor harvest minus weight on day of tumor inoculation) was 2.37 ± 0.98 g for menhaden oil-fed mice and 4.13 ± 0.60 g for hydrogenated coconut oil-fed mice (ANOVA, P=0.0566). The term "tumor burden" refers to the tumor cell mass and the peritoneal fluid that accumulates during exponential tumor growth. Because menhaden oil-fed mice ate as much food as the hydrogenated coconut oil-fed mice (data not shown), and showed no signs of premature cachexia, lower body weight was likely to result from a smaller tumor burden.

DISCUSSION

In this report we have shown that dietary menhaden oil leads to the incorporation of ω3 PUFA into a rapidly growing ascites tumor, and that tumor cells modified in this way spontaneously release more ^{51}Cr-labeled intracellular molecules than do tumor cells lacking ω3 PUFA. We do not, at this time, know the mechanism for PUFA-augmented spontaneous release; however, heightened spontaneous release may be due to increased

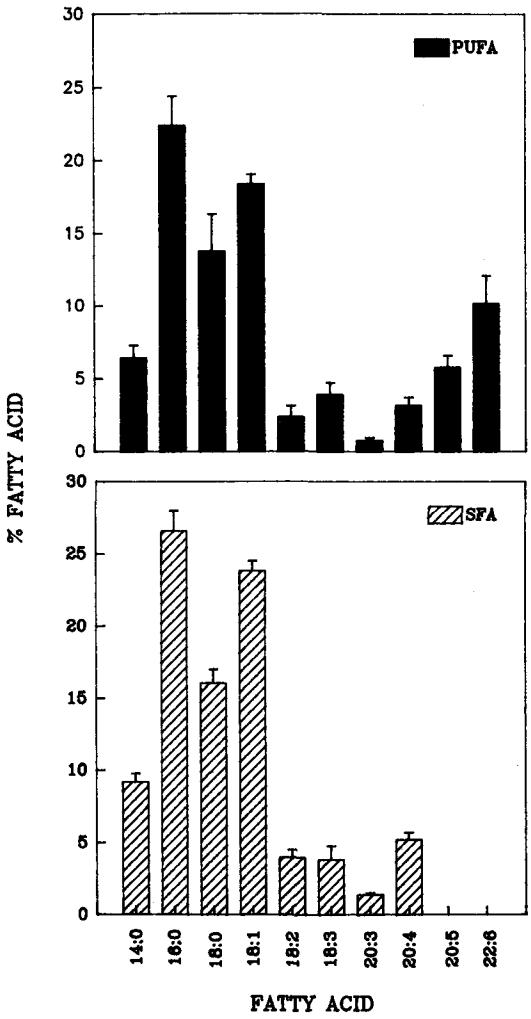


FIG. 7. Dietary ω3 PUFA are incorporated into ascites tumor cells. T27A cells were harvested from mice fed fat-free basal diets supplemented with 10% menhaden oil or hydrogenated coconut oil. After RBC depletion, the fatty acid content of the cells was analyzed by gas-liquid chromatography. The tumors shown in the top panel (solid bars) are from menhaden oil-fed mice; the tumors shown in the bottom panel (hatched bars) are from coconut oil-fed mice. EPA and DHA are 20:5 and 22:6 fatty acids, respectively.

permeability of the surface membrane. Permeability does not correlate directly with cell disintegration, as ω3 PUFA modified cells remained viable during the spontaneous release process. Increased permeability, particularly at elevated temperatures, is consistent with the effect of DHA and EPA incorporation into membranes as predicted from experiments on phospholipid bilayers (8). DHA exhibits a profound effect on bilayer membrane permeability and fusion while not altering membrane fluidity from that of a linolenic acid (18:3)-containing membrane. This implies DHA is not universally affecting membrane properties but instead is acting at specific membrane regions or domains.

It is generally accepted that lipid oxidation products have deleterious effects on cells. Introduction of additional polyunsaturated fatty acids to the diet dilutes the antioxidant vitamin E and other components of the diet, increasing susceptibility of the lipids to oxidation. To minimize oxidation-related problems, the diets were stored frozen, protected from light, and removed promptly after feeding. Furthermore, because the mice were provided with food essentially *ad libitum* during the dark period, small reductions in vitamin and mineral concentration were abrogated by increased food consumption. Essential fatty acid deficiency was unlikely to play a role in increased permeability because the chow-based diets contained essential fatty acids as well as the menhaden oil or hydrogenated coconut oil supplementation.

It is remarkable that the substantial loss of ^{51}Cr-labeled molecules ensues without concomitant loss of cell viability. At this time we cannot unequivocally rule out the possibility that cellular components chelated with chromium are subject to active transport, but very preliminary data from our laboratory suggest that metabolic inhibitors, carbonyl cyanide *m*-chlorophenylhydrazone, 2,4-dinitrophenol, and sodium azide, do not significantly decrease ^{51}Cr release (Table 2).

Sodium chromate is thought to enter the cell by an energy-independent mechanism, and be reduced to chromic ion (18). Although some data indicate that ^{51}Cr released from lysed target cells is bound to macromolecules (19), numerous reports suggest (as we have observed) that ^{51}Cr-labeled molecules have molecular weights less than 4000 (13,20). Ronai (21) reported that released ^{51}Cr displayed electrophoretic behavior similar to ^{51}Cr-labeled peptides and distinct from ^{51}Cr-lysine and ^{51}Cr-arginine. The theory advanced by Ronai is that ^{51}Cr-labeled peptides lost from the cell are the result of protein turnover. Thus, increased protein turnover in the PUFA cells may account for increased ^{51}Cr-labeled peptide available for release. Because internal and released ^{51}Cr-labeled molecules did not differ in molecular size, and PUFA and SFA cells attained comparable levels of ^{51}Cr-labeling, we doubt that augmented protein turnover in PUFA cells induces high spontaneous ^{51}Cr release in an environment of moderate osmolarity.

We have also entertained the possibility that ^{51}Cr was adsorbed onto the external tumor cell surface, and was maintained in this fashion during the washing procedures, only to be released during incubation at 30° or 37°C. Similar radioactivity incorporation by PUFA and SFA cells implies that adsorption, if it occurred, was the same for both cell types, whereas release was more extensive for PUFA cells. We do not favor this interpretation because, typically, membrane-associated ^{51}Cr is only a minor component of the total incorporated ^{51}Cr (18). Additionally, the released ^{51}Cr-labeled material did not cochromatograph with sodium chromate.

Tumor cell membrane function, whether it is bilayer permeability or membrane protein activity, is important to immunologic surveillance for cancer because cytolytic cells and antibodies act initially at the tumor cell membrane. ω3 PUFA may alter the tumor cell membrane by affecting phospholipid packing and domain formation, rotational movement, or fluidity of distinct domains. In turn, these structural changes may affect the conformation and motility of critical membrane antigens, the membrane insertion of pore forming substances such as perforin or complement, and the temporal kinetics of irreparable membrane damage. Furthermore, when cell-mediated tumor cell cytolysis is viewed as an active process of the

TABLE 1

Hydrogenated Coconut Oil Fed Mice Have a Greater Tumor Burden than Menhaden Oil Fed Mice

Exp.	Diet[a]	Total body weight (g) Day 0	Day 35	Day 37	Day 40	Day 42
1	MO	26.8 ± 1.0[b]	27.8 ± 1.0	28.5 ± 0.6	ND[c]	31.3 ± 1.0
	HCO	25.8 ± 1.0	29.3 ± 0.5	30.0 ± 1.4	ND	33.5 ± 1.3
		ns[d]	P=0.0321	ns		P=0.0312
2	MO	25.5 ± 0.6	26.0 ± 0.8	26.8 ± 0.5	26.3 ± 1.0	27.8 ± 1.3
	HCO	26.8 ± 1.0	27.0 ± 1.8	27.8 ± 1.7	28.0 ± 1.6	30.5 ± 1.9
		ns	ns	ns	ns	P=0.0533
3	MO	23.5 ± 1.0	23.0 ± 0.8	23.8 ± 1.0	24.5 ± 1.3	24.8 ± 1.0
	HCO	24.0 ± 0.8	23.3 ± 0.5	23.5 ± 0.6	23.8 ± 0.5	28.0 ± 1.4
		ns	ns	ns	ns	P=0.0089

[a]MO, menhaden oil; HCO, hydrogenated coconut oil.
[b]Mean ± SD, n=4.
[c]ND, not done.
[d]P values for ANOVA; ns, not significant (P>0.05).

TABLE 2

Spontaneous Release Is Independent of Metabolic Activity

Inhibitor	Concentration	% ^{51}Cr release (mean ± SD)
None	—	5.6 ± 0.4
ethanol	0.5%	5.9 ± 0.9
CCCP[a]	5 μM	11.1 ± 0.9
	1 μM	6.6 ± 0.1
	0.2 μM	5.4 ± 0.6
	0.04 μM	5.7 ± 0.6
2,4-DNP[a]	700 μM	10.0 ± 0.8
	150 μM	6.3 ± 0.4
	30 μM	5.4 ± 0.3
	6 μM	4.9 ± 0.6
NaN_3	2%	14.5 ± 0.8
	0.4%	7.8 ± 0.6
	0.08%	6.1 ± 0.2
	0.016%	6.2 ± 0.5

[a]CCCP, carbonyl cyanide *m*-chlorophenylhydrazone; 2,4-DNP, 2,4-dinitrophenol.

tumor cell itself (22), fatty acid composition of the tumor cell membrane may influence the activity of membrane-associated enzymes involved in cellular activation, such as protein kinase C (11). Thus, there is potential for ω3 PUFA-enriched diets to modify immune surveillance at the level of the tumor cells, regardless of effects on cells of the immune system. The temperature dependence of ^{51}Cr permeability may provide another means to selectively eradicate tumor cells. When combined with the fish oil diet, increasing local temperature of tumor cells relative to adjacent healthy cells (local hyperthermia) may make the tumors even more susceptible to immunologic or pharmacologic destruction. We have observed increased permeability of tumors in mice fed experimental diets during adulthood, not as weanlings. Thus, dietary modification may be useful in the aged, when cancer incidence is increased.

ACKNOWLEDGMENT

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Do Rat Kidney Cortex Microsomes Possess the Enzymatic Machinery to Desaturate and Chain Elongate Fatty Acyl-CoA Derivatives?

Sanoj K. Suneja, Mahmoud N. Nagi, Lynda Cook, Peter Osei and Dominick L. Cinti*
Department of Pharmacology, University of Connecticut Health Center, Farmington, Connecticut 06030

Rat kidney cortex microsomal preparations were unable to catalyze Δ9, Δ6 and Δ5 desaturation of stearoyl-coenzyme A (CoA), linoleoyl-CoA and dihomo-γ-linolenoyl-CoA, respectively. The kidney cortex microsomal fraction, however, did catalyze the malonyl-CoA dependent fatty acyl-CoA elongation. The biochemical properties of palmitoyl-CoA elongation were studied as a function of protein concentration, time, reduced nicotinamide adenine dinucleotide phosphate (NADPH), malonyl-CoA and substrate concentrations; of the substrates investigated, Δ6,9,12-18:3 was the most active. Unlike what was observed in the hepatic system, a high-carbohydrate, fat-free diet did not induce kidney fatty acid chain elongation. All intermediate kidney cortex microsomal reactions, *i.e.*, β-ketoacyl-CoA reductase, β-hydroxyacyl-CoA dehydrase and *trans*-2-enoyl-CoA reductase activities, were significantly higher (greater than one order of magnitude) than the condensing enzyme activity, suggesting that the rate-limiting step in total elongation is the initial condensation reaction. Contrary to other reports, the results suggest that the kidney cannot synthesize arachidonic acid needed for eicosanoid production.
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The general catalytic mechanism of the microsomal fatty acid chain elongation system (FACES) in liver involves the sequential activity of condensing enzyme, β-ketoacyl-coenzyme A (CoA) reductase, β-hydroxyacyl-CoA dehydrase and *trans*-2-enoyl-CoA reductase (1-5). Although the fatty acid chain elongation system has been studied extensively in liver and brain (1,5,6), the biosynthetic pathway responsible for the synthesis of such physiologically important fatty acids as arachidonic acid has received less attention in other tissues, *e.g.*, kidney (7).

Another fatty acid metabolic pathway which appears to be coupled to or interact sequentially with the elongation system is the desaturation pathway. The liver contains, at least, three separate desaturase enzymes—Δ9, Δ6 and Δ5, all of which receive electrons from the microsomal hemoprotein, cytochrome b_5. The latter has also been implicated in providing reducing equivalents to the first reduction step of FACES (8).

More than ten years ago, Cinti and Montgomery (9) reported the inability of rat kidney cortex microsomes to catalyze the Δ9 desaturation of either palmitoyl-CoA or stearoyl-CoA. Oshino and Sato (10) also observed no measurable Δ9 desaturase activity in kidney microsomes. These results were unexpected because the microsomes contained significant amounts of cytochrome b_5 and its flavoprotein, reduced nicotinamide adenine dinucleotide (NADH) cytochrome b_5 reductase (9,11). By contrast, Chern and Kinsella (12) reported the presence of radiolabeled arachidonic acid in cultured rat kidney cells which had been incubated with [1-^{14}C]linoleic acid, suggesting that these cells possess Δ5 and Δ6 desaturase activities. Clark and Queener (13) reported "little or no activity" in rat kidney cortex microsomal fractions assayed for Δ6 and Δ9 desaturase, but found detectable activities when "fraction 3 homogenate" replaced the microsomal fraction. More recently, Mandon *et al.* (14) compared Δ6 desaturase activities in liver, kidney and lung microsomes and found that the renal microsomal activity was very low ranging from 5-9 pmol/min/mg protein. Similar values were reported for kidney microsomal Δ6 desaturase by Kawata *et al.* (15).

The present study was designed to fully characterize the biochemical properties of the rat kidney cortex microsomal fatty acid chain elongation system and to re-evaluate the presence of Δ9, Δ6 and Δ5 desaturase activities in the renal cortex microsomal fraction.

MATERIALS AND METHODS

Chemicals. All biochemicals were obtained from Sigma Chemical Co. (St. Louis, MO); general chemicals were purchased from Baker (Phillipsburg, NJ) and fatty acid methyl ester standards were obtained from Nu Chek Prep Inc. (Elysian, MN). Radioactive compounds, [2-^{14}C]malonyl-CoA, [1-^{14}C]linoleic acid, [1-^{14}C]dihomo-γ-linolenic acid and [1-^{14}C]palmitic acid or [1-^{14}C]stearic acid were obtained from DuPont NEN Company (Wilmington, DE). Fatty acyl-CoA derivatives were prepared by the acid chloride method (16).

Isolation of kidney cortex microsomes. Male Sprague-Dawley rats weighing 175-200 g were given access to Purina rat chow or deprived of food for 24 hr and then fed a high-carbohydrate diet (fat-free diet, United States Biochemical Corporation, Cleveland, OH) and tap water for 2 days. Forty-eight hours after feeding, the animals were killed by decapitation between 8:00 and 9:00 A.M. Kidneys were removed and placed into ice-cold saline, followed by decapsulation and excision of the medulla. The kidney cortex microsomes were prepared as described previously (17). Protein was determined by the method of Bradford (18).

Enzyme assays. The assay conditions for measuring total elongation activity, unless otherwise noted, were as described earlier (19). The reaction mixture for measuring the condensing enzyme activity was the same as for total elongation, except that 500 μM reduced nicotinamide adenine dinucleotide phosphate (NADPH) was omitted from the mixture.

Thin-layer and gas-liquid chromatographic separation of elongated products. The pooled hexane extracts of fatty acid chain elongation products were evaporated under N_2. Either the residues were redissolved in 50 μL $CHCl_3$, spotted on 0.25mm-thick, activated silica gel G plates and

*To whom correspondence should be addressed.

Abbreviations: ATP, adenosine triphosphate; BHT, butylated hydroxytoluene; BSA, bovine serum albumin; CoA, coenzyme A; FACES, fatty acid chain elongation system; NADH, reduced nicotinamide adenine dinucleotide; NADPH, reduced nicotinamide adenine dinucleotide phosphate.

developed in petroleum ether/diethyl ether/formic acid (75:25:1 v/v/v) or converted to their methyl esters by diazomethane for radio gas-liquid chromatography as described previously (20,21).

Chemical synthesis of CoA esters. [3-^{14}C]β-Ketostearoyl-CoA was synthesized according to the procedures of Stoffel and Pruss (22) and Al-Arif and Blecher (23). The β-keto fatty acid methyl esters were reduced with sodium borohydride to form the β-hydroxy derivatives. The coenzyme A esters of *trans*-2-enoate and β-hydroxy acid were synthesized by the mixed anhydride procedure as described by Fong and Schulz (24). The concentrations of the synthesized CoA esters of β-keto acid, β-OH acid, or *trans*-2-enoate were measured by the method of Ellman (25) after cleavage of the thioester bond with hydroxylamine.

β-Ketoacyl-CoA reductase. The β-ketoacyl-CoA reductase activity was measured and products identified as described earlier (20).

β-Hydroxyacyl-CoA dehydrase. The activity of β-hydroxyacyl-CoA dehydrase was assayed in the forward direction, *i.e.,* by following the conversion of the β-hydroxy derivative to the *trans*-2-enoyl-CoA (26).

Trans-*2-enoyl-CoA reductase. Trans*-2-enoyl-CoA reductase activity was followed by measuring the rate of oxidation of NADPH at 340 nm–400 nm with the Shimadzu UV-3000 spectrophotometer (Kyoto, Japan) as described earlier (17).

Kidney cortex microsomal desaturases. Δ9 Desaturation of stearoyl-CoA was assayed in kidney cortex microsomes as described previously (9). Δ6 Desaturation of linoleoyl-CoA (18:2ω6) was assayed by measuring the formation of γ-[1-^{14}C]linolenoyl-CoA. The reaction medium (1.0 mL) contained: 100 mM Tris-HCl, pH 7.4, 1.0 to 3.0 mg kidney cortex microsomal protein and 1.0 mM NADH. Following 1 min preincubation at 37°C, the reaction was initiated with 40 μM [1-^{14}C]linoleoyl-CoA, incubated for 20 min, and terminated by addition of 1.0 mL 15% KOH in methanol. The reaction products were processed as described earlier (19) and methylated using diazomethane in diethyl ether and redissolved in 50 μL of $CHCl_3$ and spotted on activated silica gel G plates impregnated with 10% $AgNO_3$. Carrier methyl esters of linoleic and γ-linolenic acid were included with the labeled acids. Plates were developed in a solvent system of toluene/acetone (95:5 v/v, containing 0.05% butylated hydroxytoluene (BHT) as an antioxidant). The spots were visualized and counted with betafluor as described earlier (20). The reaction products were also identified by radio gas-liquid chromatography.

Δ5 Desaturation of eicosatrienoic acid (20:3ω6) was estimated in kidney cortex microsomes by measuring the conversion to arachidonic acid (5,8,11,14-eicosatetraenoic acid) (27). The 1.0-mL reaction medium contained: 100 mM Tris-HCl, pH 7.4, 2.0 mg microsomal protein, 5 mM adenosine triphosphate (ATP), 200 μM CoA, 5 mM $MgCl_2$ and 1 mM NADH. After 1 min preincubation at 37°C, the reaction was started by adding 40 μM [1-^{14}C]-dihomo-γ-linolenic acid, and the mixture was incubated for 20 min at 37°C. The product processing and identification was done as above (see also 20,21). The oven temperature was 160°C. The post-mitochondrial supernatant (4.0 mg protein/mL) was also used to assay Δ6 and Δ5 desaturase activities in kidney cortex.

RESULTS

Desaturase activities. Microsomes isolated from kidney cortex of rats fed either a Purina rat chow (controls) or a high carbohydrate, fat-free diet were shown to be devoid of stearoyl-CoA desaturase activity, confirming our earlier results (9,10).

When [1-^{14}C]linoleoyl-CoA was employed as a substrate, hepatic microsomal Δ6 desaturase activity was 136 and 180 pmol γ-linolenate formed/min/mg protein in control and fat-free diet rats, respectively. However, kidney cortex microsomes from either group had no detectable Δ6 desaturase activity even after a one-hour incubation period and a microsomal protein concentration of 4 mg/mL in the assay.

When Δ5 desaturase activity was measured following incubation of [1-^{14}C]dihomo-γ-linolenoyl-CoA with hepatic microsomes, 250 and 480 pmol arachidonate formed/min/mg protein in control and fat-free diet rats, respectively. By comparison, under identical assay conditions, kidney cortex microsomes from either diet were unable to catalyze Δ5 desaturation of dihomo-γ-linolenoyl-CoA. Varying assay conditions, such as extending the incubation up to 6 hr in the presence of a NADPH-generating system and increasing the microsomal protein to 4 mg/mL, did not result in any demonstrable Δ5 desaturase activity. Substitution of the post-mitochondrial supernate or total kidney cortex homogenate for the microsomal fraction still resulted in no desaturase activity.

Renal cortex microsomal fatty acid chain elongation. Although the microsomal fraction of the renal cortex did not possess any detectable Δ9, Δ6 and Δ5 desaturase activities using our assay conditions, the same fraction did possess fatty acid chain elongation activity. The elongation of palmitoyl-CoA as a function of microsomal protein is shown in Figure 1 and was linear to approximately 1.0 mg per mL. Total elongation activity was calculated as 160 pmol (range 150–300 pmol) per min per mg microsomal protein. Renal cortex microsomes from rats on a fat-free diet did not have induced elongation activity, contrary to microsomes obtained from liver. Condensation activity was linear only to 400–500 μg microsomal protein (Fig. 1), and the diminished activity in contrast

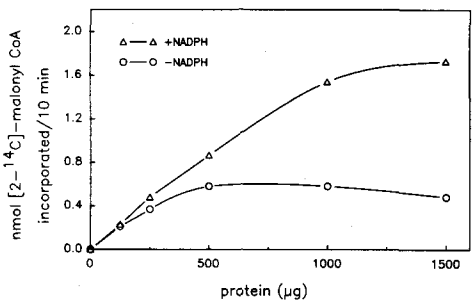


FIG. 1. Effect of varying amounts of rat kidney cortex microsomal protein on the condensation and elongation of palmitoyl-CoA. The incubation mixture contained microsomal protein, 20 μM palmitoyl-CoA, 10 μM BSA, 50 μM [2-^{14}C]malonyl-CoA (50,000 cpm) and 500 μM NADPH (for elongation assay only) in 0.1 M Tris-HCl buffer, pH 7.4. The incubation was carried out at 37°C for 10 min. Values are means from two different experiments done in duplicate.

to total elongation can be attributed to product inhibition, *i.e.*, the β-ketoacyl-CoA product of the condensation reaction is a potent inhibitor of the condensing enzyme (Cinti, D.L., unpublished observations).

Condensation of palmitoyl-CoA with malonyl-CoA was linear up to 10 min, whereas total chain elongation was linear for 20 min. Both condensation and elongation activities increased as a function of malonyl-CoA concentration, plateauing at approximately 40 μM, with apparent K_m values of approximately 6 μM for both activities.

As shown in Figure 2, total elongation activity increased linearly with increasing concentration of palmitoyl-CoA to approximately 5 μM, plateauing at 20 μM palmitoyl-CoA; condensation activity followed a similar pattern. The apparent K_m values for palmitoyl-CoA were 3.3 μM and 6.3 μM for the elongation and condensation reactions, respectively. For NADPH, the apparent K_m value for total elongation approximated 24 μM (data not shown).

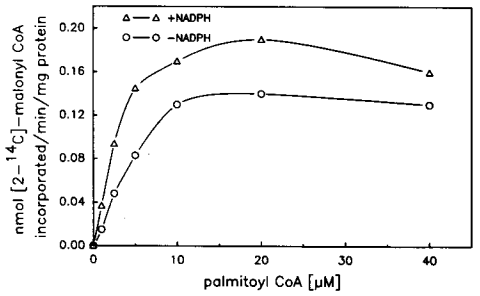


FIG. 2. Rat kidney cortex microsomal palmitoyl-CoA condensation and elongation as a function of substrate concentration. The assay mixture, which was incubated for 10 min at 37°C, contained 50 μM [2-^{14}C]malonyl-CoA, 500 μM NADPH (in the elongation reaction only), 1 μM to 40 μM palmitoyl-CoA and BSA at 2:1 ratio of substrate/BSA in 0.1 M Tris-HCl buffer, pH 7.4; microsomal protein concentration was 250 μg/mL.

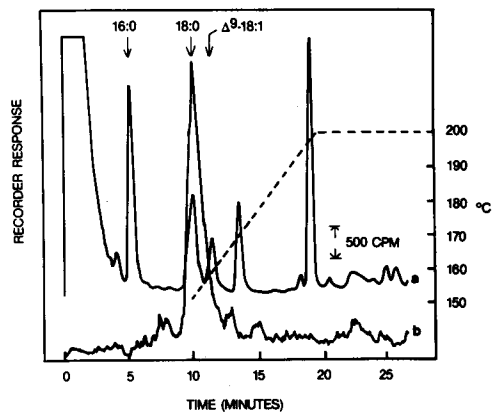


FIG. 3. Determination of the products of rat kidney cortex microsomal NADPH-dependent palmitoyl-CoA elongation by radio gas-liquid chromatography. Radio-chromatogram depicts the reaction products formed after incubation of 20 μM palmitoyl-CoA, 10 μM BSA, 500 μM NADPH, 50 μM [2-^{14}C]malonyl-CoA with 250 μg/mL kidney cortex microsomes in 0.1 M Tris-HCl buffer, pH 7.4 for 10 min at 37°C; a, mass trace; b, radioactive trace.

The palmitoyl-CoA elongation products were identified by both thin-layer and radio gas-liquid chromatography (Fig. 3). Using 20 μM palmitoyl-CoA, greater than 95% of the radioactivity was associated with a single peak identified as stearate (tracing b). There was no identifiable radiolabeled oleate peak (28) reconfirming the absence of Δ9 desaturase activity in kidney cortex microsomes.

Similar to what was observed with liver microsomes (5,29), both condensation and elongation activities were stimulated by the presence of bovine serum albumin (BSA) (fraction IV, Sigma) with maximal activity occurring at a substrate/BSA ratio of 1.5:1 or 2.0:1. When several CoA derivatives were tested as substrates for kidney cortex microsomal FACES, γ-linolenoyl-CoA was found to be the most active substrate, with a specific activity of approximately 700 pmol 8,11,14-20:3 formed per min per mg protein (Fig. 4); least activity was noted with stearoyl-CoA and oleoyl-CoA. In one experiment utilizing 25 μM 9,12-18:2-CoA and 5,8,11,14-20:4-CoA as substrates, kidney cortex microsomal elongation activities were 45 and 44 pmol/min/mg protein, respectively.

β-Ketoacyl-CoA reductase activity. Employing chemically synthesized [3-^{14}C]β-ketostearoyl-CoA, the reductase activity reached a maximum in the presence of 50

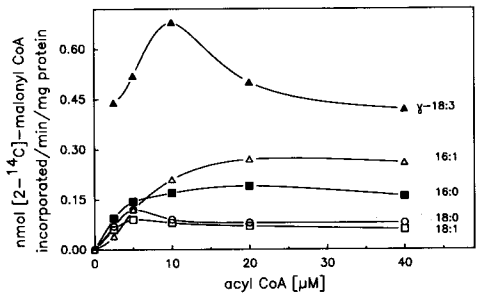


FIG. 4. Fatty acyl-CoA substrates for the kidney cortex microsomal elongation system. The various acyl-CoA substrates used were: ■—■, palmitoyl-CoA (16:0); △—△, palmitoleoyl-CoA (Δ9-16:1); O—O, stearoyl-CoA (18:0); □—□, oleoyl-CoA (Δ9-18:1), and ▲—▲, γ-linolenoyl-CoA (6,9,12-18:3). These substrates were chain elongated in an assay mixture containing 2:1 ratio of substrate/BSA, 500 μM NADPH, 50 μM [2-^{14}C]malonyl-CoA and 250 μg/mL of microsomal protein in 0.1 M Tris-HCl buffer, pH 7.4.

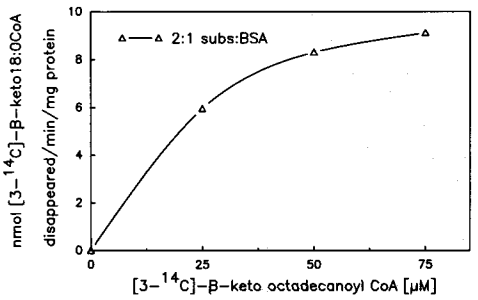


FIG. 5. The effect of increasing concentrations of β-keto octadecanoyl-CoA on rat kidney cortex microsomal β-keto reductase activity. The assay medium contained [3-^{14}C]β-keto 18:0-CoA with a substrate/BSA ratio of 2:1, 500 μM NADPH and 200 μg microsomal protein/mL in 0.1 M Tris-HCl buffer, pH 7.4. The incubation was carried out at 37°C for 5 min. Thin-layer chromatography (TLC) was employed to separate the reaction products.

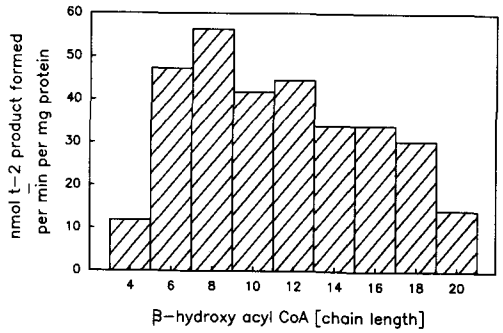


FIG. 6. β-Hydroxyacyl-CoA substrates for rat kidney cortex microsomal dehydrase. The assay mixture contained optimal amounts of β-hydroxyacyl-CoA derivatives (C_4, 200μM; C_6, C_8, C_{10}, 100μM; C_{12}, 75μM; C_{14}, C_{16}, C_{18}, C_{20}, 50μM) and 100 μg microsomal protein/mL in 0.1 M Tris-HCl, pH 7.4; dehydrase activity was recorded by the increase in absorbance at 37°C at 280 nm minus 350 nm (dual wavelength) on a Shimadzu UV-3000 spectrophotometer.

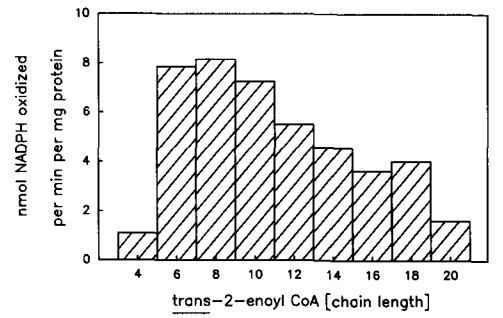


FIG. 7. *Trans*-2-enoyl-CoA substrates for rat kidney cortex microsomal *trans*-2-enoyl-CoA reductase activity. The spectrophotometric cuvette contained various substrates (*t*-2-4:1, 50μM; *t*-2-6:1, 8:1, 10:1, 12:1, 30 μM; *t*-2-14:1, 16:1, 18:1, 20 μM; *t*-2-20:1, 10 μM), 150 μM NADPH, substrate to BSA ratio of 1:2 except for *t*-2-4:1 (1:1) and *t*-2-20:1 (1:4) and 100 μg microsomal protein/mL in 0.1 M Tris-HCl buffer, pH 7.4; decrease in absorbance was recorded at 37°C at 340 nm - 400 nm (dual wavelength) using a Shimadzu UV-3000 spectrophotometer.

to 75 μM substrate (Fig. 5); the apparent K_m value was 20 μM. The β-ketoacyl-CoA reductase activity was linear i) with protein to at least 200 μg/mL, ii) with time for at least 5 min and iii) with NADPH to about 200 μM (apparent K_m: 130 μM). The specific activity of this reduction step was markedly greater than that of either the condensing enzyme step or the overall elongation reaction (8 nmol *versus* 200 pmol/min/mg microsomal protein). Due to the unavailability of various β-keto derivatives and the difficulty in their chemical synthesis and purification, substrate specificity analysis was not performed.

β-Hydroxyacyl-CoA dehydrase activity. The dehydrase activity in the presence of β-hydroxyoctadecanoyl-CoA (βOH18) was linear to about 200 μg microsomal protein/mL and was maximal at 40–50 μM substrate; the apparent K_m for the βOH18 was 10 μM. Highest activity was observed with β-hydroxyoctanoyl-CoA (about 55 nmol/min/mg protein) (Fig. 6). Chain lengths from C_4 to C_{20} were tested and provided activities ranging from 10 to 55 nmol. These specific dehydrase activities were 100–200-fold greater than the overall chain elongation activity.

Trans-2-enoyl-CoA reductase activity. The terminal reaction was studied with *trans*-2-octadecenoyl-CoA (*t*-2-18:1) and its conversion to stearoyl-CoA was followed by monitoring NADPH oxidation and verified by both gas-liquid chromatography and thin-layer chromatography. The reductase activity was linear to at least 150 μg/mL protein and 50 μM NADPH and reached a maximum of 4 nmol/min/mg protein in the presence of 20 μM substrate; higher substrate concentrations showed a slightly reduced activity. Lineweaver-Burk analysis yielded apparent K_m values of 12 μM and 45 μM for the substrate and NADPH, respectively. Substrates, varying in chain length from C_4 to C_{20}, generated *trans*-2-enoyl-CoA reductase activities ranging from 1 to 8 nmol NADPH oxidized/min/mg microsomal protein (Fig. 7); these values were 5- to 20-fold higher than the overall elongation rate.

DISCUSSION

It is now well established that several 20-carbon atom polyunsaturated fatty acids are substrates for lipoxygenation, cyclooxygenation and epoxygenation (30,31). The products of these reactions possess a variety of important biological activities (30). The precursors for these products are generated in the cell by a series of sequential desaturation-elongation reactions.

In recent years, it has been shown that the kidney also contains the enzymatic machinery to carry out lipoxygenation, cyclooxygenation and epoxygenation (32–34). Recent evidence suggests that some of the renal eicosanoid products (*e.g.*, PGE_2, PGI_2) of these reactions play a major role in the regulation of renal hemodynamics (34). Although the kidney can synthesize various physiologically important eicosanoid products, does it also possess the enzyme systems to synthesize the precursor substrates, *i.e.*, arachidonic acid? To respond affirmatively, the kidney must have the capability to catalyze both desaturation and elongation reactions. However, Oshino and Sato (10) and Montgomery and Cinti (11) reported the lack of Δ9 desaturation activity in rat kidney cortex microsomes. By contrast, Clark and Queener (13) noted Δ6 and Δ9 desaturase activities in a non-microsomal fraction of rat kidney.

Brenner (35) reported measurable Δ6 desaturase activity in liver, adrenals and testicles, but none in heart, brain and kidney. More recently, Mandon *et al.* (14) reported a Δ6 desaturase activity of 5–8 pmols per min per mg protein in kidney microsomes. However, this low activity could be due to as little as a 1% contamination of the highly radioactive substrate employed in the incubation.

In the present study, under conditions in which liver Δ9, Δ6 and Δ5 desaturase activities were optimally expressed, we could not detect any desaturase activity in kidney microsomes. Variations in protein, substrate and co-factor concentrations and incubation period resulted in no detectable renal activities. Furthermore, no measurable desaturase activity was observed following replacement of the microsomal fraction with either the post-mitochondrial supernate (cytosol plus microsomes) or total homogenate. At the present time, we have no reasonable explanation for the discrepancy between our

data and those of Chern and Kinsella (12) who reported radiolabeled arachidonic acid in cultured renal cells incubated with [1-^{14}C]linoleic acid.

Kidney cortex microsomes did, however, have an active malonyl-CoA dependent fatty acid chain elongation system. The biochemical characteristics of the kidney FACES are similar to that of liver. It requires an activated acyl moiety (CoA derivative), and malonyl-CoA acts as the 2-carbon elongating unit. Both condensation and total elongation activities are enhanced by BSA, with an optimal substrate to BSA ratio of 2:1. The analysis of each of the four reactions suggests that condensation is the slowest and therefore a rate-limiting step since the β-keto reductase, dehydrase and enoyl reductase had catalytic activities at least one order of magnitude greater than did the condensation step. Total elongation activity was highest with γ-18:3-CoA.

A difference between kidney and liver FACES is the inability of a high-carbohydrate diet (fat-free) to induce the kidney system.

Recently, Hagve and Sprecher (36) employing radio gas-liquid chromatography reported that rat heart myocytes were not capable of either elongation or desaturation. Furthermore, Lefkowith *et al.* (37) described similar uptake mechanisms for arachidonate in both heart and kidney. These results suggest that both heart and kidney obtain arachidonate from the circulation possibly from lipoproteins derived from liver. Such a mechanism would circumvent the need for kidney cells to synthesize arachidonic acid.

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Thermotropic Phase Properties of the Hydroxyceramide/ Cholesterol System

Timothy S. Wiedmann* and **Amir Salmon**[1]
University of Minnesota, College of Pharmacy, Department of Pharmaceutics, Minneapolis, Minnesota 55455

The interaction of cholesterol with ceramides containing α-hydroxy fatty acyl chains (hydroxyceramides) has been studied as a foundation for characterizing the lipid bilayers of the *stratum corneum*. A relatively large quantity of cerebrosides was obtained from bovine brain and converted to ceramides through removal of the carbohydrate side chain. The ceramides were separated based on the absence or presence of hydroxy fatty acyl chains. The lyophilized hydroxyceramides showed a broad melting region at 92°C. Hydroxyceramides dispersed in water produced a relatively narrow, thermotropic transition at 75°C. The effect of cholesterol on this thermotropic phase transition of hydroxyceramides was determined by differential scanning calorimetry. With respect to the main transition, cholesterol caused a broadening of the phase transition at relatively low levels as well as a decrease in the peak transition temperature. The presence of cholesterol at levels in excess of 7 wt% gave rise to an additional low-temperature transition at 55°C. Upon immediate rescanning, this transition was exothermic, but with increasing incubation time the area under the excess heat capacity curve as a function of temperature became smaller. After two days or more, the transition observed was endothermic. At cholesterol levels between 40 and 50 wt%, multiple peaks were observed. From comparisons with related systems, the cooperative thermal transitions of hydroxyceramides with cholesterol are suggested to result from changes in hydrogen bonding or be due to phase separation. The composition of isolated brain ceramides is being compared with that reported for the *stratum corneum*.
***Lipids* 26,** 364–368 (1991).

Ceramides are *N*-acyl linked bases that act as precursors for cerebrosides (1). While cerebrosides are primarily found in brain and epidermal tissue, ceramides occur in abundance only in the *stratum corneum* (2) where they have been implicated to contribute to the water impermeability of this tissue (3–5). The relatively small polar head group consisting of two hydroxy groups at the 1 and 3 positions of the sphingosine base and an amide-linked acyl chain are unique, structural features which appear to contribute to an effective permeability barrier. Ceramides have also been implicated in the control of epidermal turnover (2). Furthermore, the asymmetric chain lengths together with the terminal hydroxyl groups could form an interdigitated multilamellar arrangement and thus may provide cohesive links within the intercellular region of the *stratum corneum* (5,6).

Cholesterol is also a relatively abundant component of *stratum corneum* and has been proposed to modulate the properties of the lipid bilayers in this system (2,7). In addition, it appears likely that cholesterol affects permeability properties and the control of epidermal turnover. Changes in the relative amounts of cholesterol present occur in certain disease states which results in perturbations of normal *stratum corneum* function (3,8,9).

Although the interactions of cholesterol with various polar lipids have attracted considerable attention (10), cholesterol/ceramide interactions have not been studied. It would appear that investigations on the physiological role of ceramides in the *stratum corneum* would be greatly facilitated by understanding their interaction with other lipid components. In this study, the effect of cholesterol on the thermotropic phase behavior of hydroxyceramides has been investigated.

MATERIALS AND METHODS

Isolation of ceramides. Cerebrosides were isolated from bovine brain following the procedure of Radin (11). The cerebrosides were converted to ceramides by cleaving off the carbohydrate moieties using the procedure of Carter *et al.* (12). The resulting ceramides were separated into two subclasses, containing either fatty acids or hydroxy fatty acids, by silica (Bio-Sil A, 100–200 mesh, Bio-Rad, Richmond, CA) column chromatography using a chloroform/methanol gradient.

Thermal analysis. Ceramides were lyophilized from cyclohexane to obtain a dry powder that readily dispersed in water. Pure hydroxyceramide, 1–2 mg, was weighed and placed in a coated aluminum differential scanning calorimeter pan (DuPont, Wilmington, DE). Mixtures of ceramide and cholesterol were prepared by dissolving both in chloroform and adding the appropriate volumes to preweighed pans. The solution was dried overnight under high vacuum and excess distilled water was added to bring the solid content to less than 33 wt%. Uniform hydration was achieved by heating in a steam bath (85°C) for 20 min. Due to the metastability of mixtures of hydroxyceramides and cholesterol, variable incubation conditions were used. Thin-layer chromatography (TLC) ascertained the chemical stability of the compounds throughout these procedures (13).

Calorimetric scans were usually performed at 5°/min on a DuPont 910 differential scanning calorimeter. The melting point was determined by extrapolation to the baseline of the most rapid rise in the excess heat capacity curve as a function of temperature. This value is more reproducible than the maximum value of the excess heat capacity as a function of temperature curve and compensates for the rapid scan rate. The enthalpy was determined by integrating the area under the curve from a plot of the excess heat capacity as a function of temperature.

RESULTS AND DISCUSSION

In Figure 1, the differential scanning calorimetry traces of lyophilized and hydrated multilamellar dispersions of

*To whom correspondence should be addressed.

[1] Present address: Department of Biophysics, Boston University School of Medicine, Boston, MA.

Abbreviations: FTIR, Fourier transform infrared spectroscopy; TLC, thin-layer chromatography.

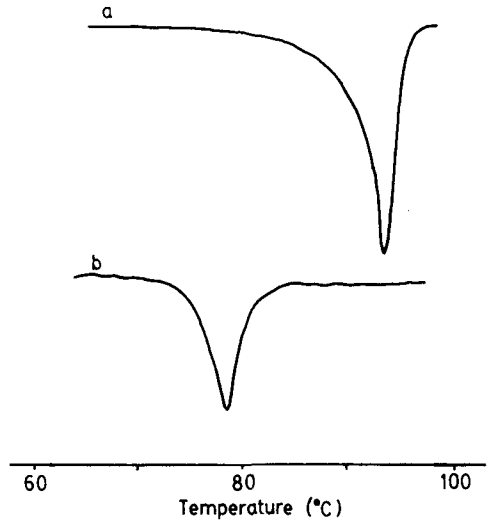


FIG. 1. Calorimetric scans at 5°C/min of (a) lyophilized hydroxyceramide and (b) hydrated hydroxyceramide (66 aqueous wt%).

hydroxyceramide are given. The lyophilized material undergoes an endothermic transition at 92°C (Fig. 1a). This involves conversion from a dry, solid form to an isotropic liquid as observed in a capillary tube. The transition is reversible and reproducible in repeated scans.

In Figure 1b, the endothermic transition of hydrated hydroxyceramide is illustrated. The transition occurs at 75.3 ± 0.2°C; the transition is reproducible and reversible. The width of the transition is reduced in comparison with the anhydrous material as can be expected because hydrating the lipid often enhances the cooperative nature of the phase transition. The opaque hydroxyceramide became slightly translucent when undergoing the transition as observed with a hot stage microscope or in a melting point apparatus. This is also a characteristic of the transition that occurs with phospholipids undergoing the gel-to-liquid crystalline phase transition. Thus, the endothermic transition observed with the hydroxyceramides may be analogous.

Previous investigators had indicated that difficulties exist in dispersing ceramides in water (14). The problems that were encountered may in part be due to the use of mixtures of hydroxyceramides and ceramides without hydroxy fatty acids, because the latter do not appear to undergo well-defined transitions and to form a lamellar phase. Although a detailed study was not undertaken, hydration of the sample could be accomplished by maintaining the temperature between 75 and 92°C, *i.e.* above the main endothermic transition of the hydrated material, but below the melting point of the lyophilized material. Another interesting observation is the lack of any decomposition, as judged by TLC, while maintaining the samples at these elevated temperatures for up to 30 min. This apparent stability may be significant in light of the presence of ceramides in the *stratum corneum.*

The enthalpy of hydrated hydroxyceramide was found to be 57 ± 8 J/g. Using an average molecular weight of 650, this corresponds to an enthalpy of 8.8 kcal/mol. Enthalpies of transition have been reported to be about 16 kcal/mol for kerasin and 7.4 kcal/mol for phrenosin, which are the corresponding fractions of nonhydroxy and hydroxy fatty acid galactosylceramides, respectively (15,16). Maggio *et al.* (14) reported enthalpies of kerasin and phrenosin of 6.2 and 5.0 kcal/mol. However, a later report revealed that the value given for phrenosin corresponded to a metastable phase change (17). The temperature of the main transition was apparently not altered, but upon long-term storage there was an increase in the enthalpy. The value obtained, 15.9 cal/g, is thus comparable to that observed with kerasin. In addition, fractionated hydroxy-containing galactosylceramides displayed a similar metastable behavior (17).

Based on Raman spectroscopic data, the differences in the enthalpies of transition were attributed to a higher disorder in the aliphatic chains in the gel state of phrenosin as compared to kerasin (15). In light of more recent results, phrenosin appears to also have highly ordered acyl chains in the gel state, but the presence of the hydroxyl group interferes with the rate of formation of the stable state (17). For both hydroxy and nonhydroxygalactosylceramides, the phase transition temperature appears to be relatively independent of acyl chain length which is in sharp contrast to the marked dependence seen with polar lipids such as phosphatidylcholines (17). This observation suggests that head group interactions are very important in the gel-to-liquid crystalline phase transition. Overall, the transition no doubt reflects a contribution from disruption of the acyl chain order as well as of intermolecular hydrogen bonding that exists in the gel state.

At this time it is not known whether the hydroxyceramides undergo a similar metastable-to-stable transition upon long-term storage as was found with phrenosin. Clearly the presence of the hydroxyl group would be expected to interfere with the formation of a highly ordered gel state. On the other hand, ceramides do not possess a galactosyl head group which contributes to the strong intermolecular interactions of cerebrosides in the gel state.

Another major lipid component found in *stratum corneum,* as in other membranes, is cholesterol; thus the effect of cholesterol on the thermal properties of hydroxyceramides was examined. Preliminary investigations revealed that above 7 wt%, but below 30 wt% cholesterol, a second transition was observed at a lower temperature. This was further followed with a sample of 25 wt% cholesterol in hydroxyceramide, and the results are shown in Figure 2.

The sample was prepared by drying a solution of cholesterol and ceramide under high vacuum overnight. Water was introduced and hydration was achieved by incubating at 85–90°C for 20 min. The sample was stored at room temperature for 2 days. The scan which appears in Figure 2a shows that the main transition is broadened and lowered. The interesting feature in this scan is the lower temperature transition seen between 55 and 60°C. After obtaining this scan, the sample was cooled rapidly to 20°C in an ice bath, and another scan was obtained immediately (Fig. 2b). As can be seen, the lower temperature transition occurs at about the same temperature, but is no longer endothermic but exothermic. Again the sample was cooled and thereafter stored in the refrigerator for 2 days. The calorimetric scan obtained is shown in Figure 2c which fails to reveal any low-temperature transition; however the low-temperature endotherm is reproduced

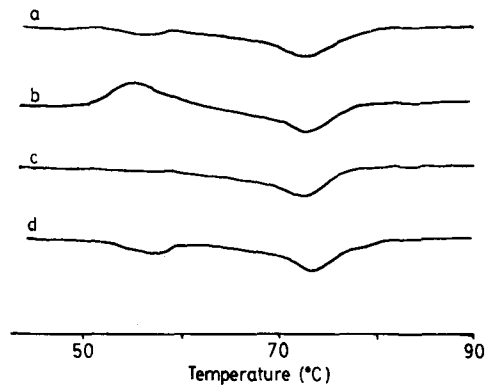


FIG. 2. Calorimetric scans at 5°C/min of hydrated 25 wt% cholesterol in hydroxyceramide after the following sequential storage conditions of (a) hydrated at 85°C for 20 min and cooled to room temperature and stored for 2 days, (b) immediate scan after quenching the sample used above to 20°C with ice, (c) stored in refrigerator for 2 days, and (d) cooled to room temperature and stored for 2 days.

(Fig. 2d) after holding the sample at room temperature for 2 days. Storage of samples for longer periods of time at room temperature, 5 days and 14 days, led to scans which had larger areas under the curve for the low-temperature endotherm suggesting that equilibrium still had not been obtained.

It should be noted that the temperature scan began at 30°C with a flat baseline obtained at 33–34°C. No transitions between 35 and 40°C were observed which would correspond to the polymorphic change of pure cholesterol hydrate (39°C) (18).

Since the temperature of each transition was apparently not affected by the length of incubation time, a somewhat arbitrary minimum time of 2 days at room temperature was used for all cholesterol/ceramide mixtures. Thereafter, thermal analyses of mixtures of cholesterol and hydroxyceramides were performed, and the results are shown in Figure 3. The high-temperature transition was broadened and lowered with increasing cholesterol content. The transition was still visible up to 40% cholesterol, although the transition was broadened to the extent that resolution from the baseline and the other endotherms became difficult. As mentioned, above 7 wt% a second endothermic transition was observed which occurred at a lower temperature (Fig. 3c and 3d). The temperature of this transition did not appear to vary with cholesterol content over the range of 15 to 30 wt% cholesterol. At 40 wt% and above, an additional transition was observed (Fig. 3h). The position of each of the two lower peaks varied in different samples and on occasion almost merged into one peak.

All calorimetric scans were analyzed in order to obtain an estimate of the peak transition temperature. The results are plotted in Figure 4 as a function of weight fraction cholesterol. The region of the diagram corresponding to temperatures above the high-temperature transition in all likelihood arises from miscible mixtures of hydroxyceramides with cholesterol in the liquid crystalline state. The other regions require further study for complete characterization, but it is of interest to consider several possibilities in the light of findings obtained with related lipid systems.

Thermal transitions with hydroxyceramides and cholesterol mixtures may arise from a geometric reorientation, rearrangement of hydrogen bonds, or phase separation. With pure *N*-palmitoylgalactosylsphingosine (nonhydroxy fatty acid galactosylceramide), the endothermic transition is believed to be the result of geometric reorientation associated with the hydration of the head group (19).

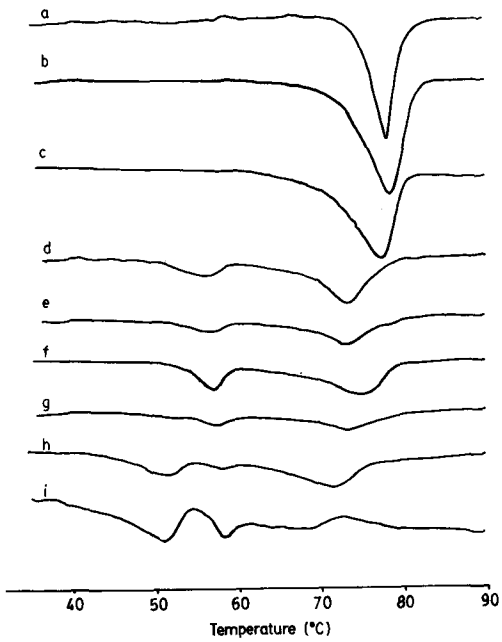


FIG. 3. Calorimetric scans at 5°C/min of hydrated mixtures of cholesterol and hydroxyceramide with percentage of cholesterol being (a) 0, (b) 3.9, (c) 7.3, (d) 15, (e) 20.8, (f) 25.2, (g) 31, (h) 40.4, and (i) 50%.

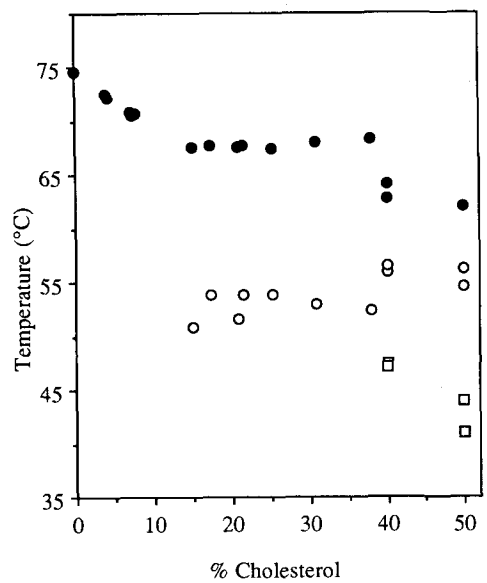


FIG. 4. Transition temperatures, which were determined by extrapolation of the most rapid rise of the calorimetric trace to the baseline, of cholesterol and hydroxyceramide mixtures as a function of percent cholesterol. Symbols indicate the (●) high-temperature, (○) intermediate-temperature, or (□) low-temperature thermal events. Most samples had only two thermal transitions.

Specifically, the low-temperature metastable state appears to have characteristics similar to those of the anhydrous lipid; however, as time-dependent hydration of the head group occurs, the increase in effective area causes a tilt in the lipids within the bilayer. For hydroxyceramides, the cross sectional area of the head group has been estimated by analysis of the surface pressure/surface area isotherm (20,21). The limiting area was found to be 40.5 $Å^2$/molecule which is roughly the same area as would be expected from the two aliphatic chains. Thus, there does not appear to be an energetic advantage for a change in the tilt angle.

Since the head groups of hydroxyceramides and cholesterol consist of amide and hydroxyl moieties, explanation for the observed thermal behavior is likely to account for changes in hydrogen bonding. Pascher and Sundell (22) have determined the crystal structure of β-D-galactosyl-*N*-(2-D-hydroxyoctadecanoyl)-D-dihydrosphingosine. They found that the hydrogen of the amide nitrogen was involved in intramolecular hydrogen bonding with the oxygens of the glycosidic linkage as well as the fatty acid hydroxyl group. This aspect played a pivotal role in determining the molecular conformation; and since this hydrogen bonding arrangement can also occur in hydroxyceramides, it may also be of importance in the interaction of hydroxyceramides with cholesterol.

It has been suggested that thermal transitions are a consequence of changes in the hydrogen bonding network. Specifically, bovine brain galactocerebrosides (type II, nonhydroxy fatty acid) shows metastable behavior which has been characterized by Fourier transform infrared spectroscopy (FTIR) (23). Initially, formation of hydrogen bonds with the carbonyl oxygen occurs; however, hydrogen bonds involving N-H groups in a plane perpendicular to N-H bending are formed with conversion to the stable form. Incorporation of cholesterol into galactosylceramide causes a disruption of the hydrogen bonds involving either the galactosyl or the sphingosine hydroxyl group; and since only one of the vibrational modes of the head group is affected, a very specific interaction between the cerebroside and cholesterol is indicated (23).

The importance of hydrogen bonding in the formation of the stable gel states has also been demonstrated in mixtures of phrenosin (bovine brain hydroxy fatty acid galactosylceramide) and cholesterol. In this study, low concentrations of cholesterol (9%) were observed to disrupt the cerebroside-cerebroside interaction (24). Thermal transitions were interpreted to be a result of two phases formed consisting of pure cerebroside and a mixture of cerebroside and cholesterol. As the cholesterol content was increased, four domains were noted. This was believed to be the result of a phase separation of the heterogeneous cerebroside sample. Thus, the studies indicate the intrinsic relationship between hydrogen bonding and phase separation in cerebrosides.

Finally, the thermal analysis shown in Figure 4 bears a striking similarity to that obtained with *N*-palmitoylgalactosylsphingosine and cholesterol mixtures where phase separation accounted for thermal transitions. In this study, Ruocco and Shipley (25) identified the region near the high-temperature transition as coexistence of a mixture of palmitoylgalactosylceramide and cholesterol in the liquid-crystalline state in combination with a cholesterol-free region of palmitoylgalactosylceramide in the gel state. The region about the low-temperature transition involved the transformation of cholesterol hydrate crystals and gel state palmitoylgalactosylceramide. Several possibilities were suggested for the region between the low- and high-temperature transitions.

In the present work, the use of samples containing a mixture of hydroxyceramides did not result in complicated phase separation as was seen with the phrenosin/cholesterol system. This may be due to weaker intermolecular attractions between ceramides which do not possess the galactosyl head group. The similarity between the thermal transitions observed with galactosylceramide/cholesterol and the hydroxyceramide/cholesterol mixtures makes the conclusion, that thermal behavior is a result of phase separation, tempting. If this is the case, then the region below the low-temperature transition would consist of an immiscible mixture of hydroxyceramide and cholesterol crystals. Furthermore, the low-temperature transition would correspond to the formation of a hydroxyceramide/cholesterol mixture coexisting with pure gel-state hydroxyceramide. Finally, the upper transition region would be a result of the melting of the gel-state hydroxyceramide with the eventual formation of a uniform liquid-crystalline mixture. Obviously, further studies are needed to characterize each phase with special emphasis on inter- and intramolecular hydrogen bonding occurring in the hydroxyceramide/cholesterol system.

For the study of the lipid interactions in the *stratum corneum*, use of the abundant ceramides isolated from bovine brain offers advantages (26,27). However, a careful examination of the composition of both the *stratum corneum* as well as the model system is necessary in order to identify potential pitfalls. With respect to bovine brain ceramides, the sphingosine base consists of a 88.5:8.5:3 ratio of C_{18}-sphingenine/C_{18}-sphinganine/C_{20}-sphingosine of which the sphinganine does not have a double bond in the 4,5 position (28,29). In the *stratum corneum*, the composition varies somewhat with the species under study (30–33). The amount of sphingolipids ranges from 15 to 35% of the total lipid weight. Upon further fractionation into glycosylated and non-glycosylated ceramides, the latter appear to become more abundant in the outer layers of the *stratum corneum*. Only about 22% of the pig epidermal ceramides have structural similarities to the hydroxyceramides used in the present study (32). By comparison, other laboratories have found in the pig (29), neonatal mouse (29), and human (28,30,31) that about one-third of the sphingolipids contain the sphingenine base, 15 to 20% 16:1, 15% 20:1, and about 3% representing phytosphingosine. Thus the *stratum corneum* would appear to have greater variability with respect to the sphingosine base than is observed with bovine ceramides.

With consideration of the amide-linked acyl chain, the bovine brain lipids consist of primarily 18:0, 24:0, and 24:1 with a smaller amount of 22:0 and trace amounts of the other fatty acids (34). In the present study, all the fatty acids contain an α-hydroxy group. In comparison, the skin contains primarily chains of 20, 24, 26 and to a lesser extent 28 carbons (28–32). In addition, only 10 to 15% of the ceramides contain α-hydroxy groups, and in one case, only negligible amounts were reported (29). Thus the bovine lipids have on average shorter acyl chains. The phase transition of galactosylceramides does not appear to be strongly dependent on chain length; however,

unsaturation does appreciably decrease the transition temperature (17). In addition, the metastability can be greatly affected by both chain length as well as unsaturation with galactosylceramides.

In summary, hydroxyceramides appear to form hydrated lamellar arrays which undergo endothermic phase transition. In the presence of cholesterol, there is a lowering and broadening of this transition. At cholesterol levels greater than 7%, a second transition is observed at a lower temperature which exhibits metastability. Finally, although mixtures of ceramides isolated from brain have a more uniform sphingosine base composition and relatively shorter *N*-acyl chains than found in *stratum corneum*, they should be useful for analysis of the lipid interactions that occur in *stratum corneum*.

ACKNOWLEDGMENTS

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Lymphatic Absorption of Structured Glycerolipids Containing Medium-Chain Fatty Acids and Linoleic Acid, and Their Effect on Cholesterol Absorption in Rats

Ikuo Ikeda[a,*], Yoshiaki Tomari[a], Michihiro Sugano[a], Soichiro Watanabe[b] and Junichi Nagata[b]

[a]Laboratory of Nutrition Chemistry, Kyushu University School of Agriculture 46-09, Hakozaki, Fukuoka 812 and [b]Nisshin Oil Mills Co., Nihonbashi, Tokyo, 103 Japan

The effects of various structured triglycerides containing medium-chain (caprylic or capric acids) and long-chain (linoleic acid) fatty acids on fatty acid and cholesterol absorption were studied in lymph-cannulated rats. A considerable portion of capric and caprylic acid was absorbed through the lymph duct, although to a lesser extent than was linoleic acid. Capric and linoleic acid located at the 2-position of 2-decanoyl-1,3-dilinoleoyl-glycerol (18:2/10:0/18:2) and 2-linoleoyl-1,3-didecanoyl-glycerol (10:0/18:2/10:0), respectively, tended to be absorbed more efficiently than those located at the 1- and 3-position or those from tricaprin (10:0/10:0/10:0) or trilinolein (18:2/18:2/18:2). A similar trend was observed when the medium-chain fatty acid was caprylic acid instead of capric acid. Caprylic acid absorption from 2-octanoyl-1,3-dilinoleoyl-glycerol (18:2/8:0/18:2) was significantly greater ($p<0.05$) than from 2-linoleoyl-1,3-dioctanoyl-glycerol (8:0/18:2/8:0) or tricaprylin (8:0/8:0/8:0). Preferential absorption of caprylic and linoleic acid was not observed when the 1 to 2 and the 2 to 1 mixtures of 8:0/8:0/8:0 and 18:2/18:2/18:2, respectively, were administered. The structured lipids did not affect the lymphatic absorption of cholesterol. The results suggest that structured triglycerides composed of medium-chain fatty acids and linoleic acid may be more useful for the treatment of lipid malabsorption than are mixtures of medium-chain triglyceride (MCT) and long-chain triglyceride (LCT).

Lipids 26, 369–373 (1991).

The absorption of medium-chain triglycerides (MCT) in the intestine is different from that of long-chain triglycerides (LCT). LCT is hydrolyzed to 2-monoglyceride and free fatty acids by pancreatic lipase in the upper intestine, and the hydrolysis products are absorbed as components of mixed micellar solutions into the intestinal mucosa (1–3). 2-Monoglycerides are micellarized and absorbed more readily than are free fatty acids. The hydrolysis products are reutilized to form triglycerides in the mucosal cells and are transported to the lymph in chylomicrons. In chylomicrons, approximately 75% of the fatty acids located in the 2-position of the triglyceride are conserved in the original position, whereas fatty acids released from the 1- and 3-positions bind to glycerol at random (4).

*To whom correspondence should be addressed.

Abbreviations: 18:2/10:0/18:2, 2-decanoyl-1,3-dilinoleoyl-glycerol; 10:0/18:2/10:0, 2-linoleoyl-1,3-didecanoyl-glycerol; 8:0/18:2/8:0, 2-linoleoyl-1,3-dioctanoyl-glycerol; EDTA, ethylenediaminetetraacetic acid; HPLC, high-performance liquid chromatography; LCT, long-chain triglyceride; MCT, medium-chain triglyceride; TLC, thin-layer chromatography; 18:2/8:0/18:2, 2-octanoyl-1,3-dilinoleoyl-glycerol; 10:0/10:0/10:0, 1,2,3-tridecanoyl-glycerol (tricaprin); 18:2/18:2/18:2, 1,2,3-trilinoleoyl-glycerol (trilinolein); 8:0/8:0/8:0, 1,2,3-trioctanoyl-glycerol (tricaprylin).

MCT are hydrolyzed readily and completely to free fatty acids and glycerol *via* 2-monoglyceride not only by pancreatic lipase but also by lingual or gastric lipase, and are then incorporated into mucosal cells. Medium-chain fatty acids are more hydrophilic than are their long-chain counterparts, and hence solubilization as micelles is not a prerequisite for absorption. MCT can also be incorporated into mucosal cells without hydrolysis (5) and can readily be hydrolyzed in the cell. The medium-chain fatty acids that are liberated are directly transported into the portal vein and readily oxidized in the liver to serve as an energy source. Thus, MCT have been used as a preferable fat source in the treatment of lipid malabsorption, such as pancreatic insufficiency and intestinal resection (6–10). Since MCT do not provide essential fatty acids, they are for enteral alimentation usually supplemented with polyunsaturated fats. However, there is a strong possibility that supplemented polyunsaturated fat may not be sufficiently absorbed by the intestine of patients with malabsorption disorders.

Recently, structured triglycerides which contain both medium-chain and polyunsaturated fatty acids in the same molecule have become available, and they are expected to be an efficient source for both medium-chain and essential fatty acids (7,11). However, the role of these structured lipids in intestinal absorption and fatty acid transport is not fully understood.

In the present study, the characteristic absorption patterns of structured glycerolipids containing medium-chain fatty acids, *i.e.* caprylic or capric acid, and linoleic acid, have been investigated in lymph-cannulated rats. The effect of these structured lipids on cholesterol absorption was also investigated.

MATERIALS AND METHODS

[4-^{14}C]Cholesterol (60 mCi/mmole) was purchased from NEN Research Products (Boston, MA) and purified prior to use by thin-layer chromatography (TLC) with benzene/ethyl acetate (7:3, v/v) as developing solvent. Fatty acid-free bovine serum albumin was obtained from Miles Inc. (Kankakee, IL). Sodium taurocholate (purity >98%) and cholesterol were purchased from Nacalai Tesque (Kyoto, Japan). Caprylic, capric and linoleic acid (purity>99%) were purchased from Wako Pure Chemicals Co. (Osaka, Japan). Tricaprylin (8:0/8:0/8:0), tricaprin (10:0/10:0/10:0) and trilinolein (18:2/18:2/18:2) were synthesized by direct esterification of glycerol with fatty acid (12). 2-Decanoyl-1,3-dilinoleoyl-glycerol (18:2/10:0/18:2), 2-linoleoyl-1,3-didecanoyl-glycerol (10:0/18:2/10:0), 2-octanoyl-1,3-dilinoleoyl-glycerol (18:2/8:0/18:2) and 2-linoleoyl-1,3-dioctanoyl-glycerol (8:0/18:2/8:0) were prepared by transesterification of 8:0/8:0/8:0, 10:0/10:0/10:0 or 18:2/18:2/18:2 with caprylic, capric or linoleic acid using 1,3-specific lipase (13). The crude preparations of LCT, MCT and structured

triglycerides were purified with preparative high-performance liquid chromatography (HPLC) (ODS column, 20 cm × 1 m) using ethanol as eluent. The purity of the structured lipids obtained was estimated by the IUPAC method (14). In short, after hydrolysis of the triglycerides with pancreatic lipase, the free fatty acids and 2-monoglycerides formed were separated by TLC using hexane/diethyl ether/formic acid (70:30:1, v/v/v) as developing solvent. Lipids were transmethylated with BF_3/methanol, and fatty acid methyl esters were subjected to gas-liquid chromatography on a 10% SILAR 10C column (15). Purities of the triglycerides were as follows: 18:2/18:2/18:2, 95.3%; 10:0/10:0/10:0, 98.2%; 8:0/8:0/8:0, 99.5%; 18:2/10:0/18:2, 92.2%; 10:0/18:2/10:0, 90.4%; 18:2/8:0/18:2, 91.0%, and 8:0/18:2/8:0, 91.2%. The major impurities of the structured lipids were the corresponding positional isomers. For example, 18:2/10:0/18:2 contained 1,2-dilinoleoyl-3-decanoyl-glycerol as major impurity.

Male Sprague-Dawley rats (Seiwa Experimental Animals, Fukuoka, Japan) weighing 290–350 g were given *ad libitum* a commercial non-purified diet (type NMF, Oriental Yeast Co., Tokyo, Japan) and drinking water. Rats were subjected to cannulation of the left thoracic lymphatic channel, and an indwelling catheter was placed in the stomach. Physiological saline/5% glucose solution was infused continuously, at a rate of 3 mL/hr until the end of lymph collection (15). After surgery, animals were placed in restraining cages in a warm recovery room and allowed free access to drinking water containing physiological saline/5% glucose. Each animal was administered 2 mL of a test emulsion in experiments 1 to 3, and 3 mL in experiment 4, through the gastric tube; lymph was collected for 24 hr beginning at 10:00 a.m. The composition of the test emulsion is shown in Table 1. In experiment 4, the composition of test emulsion was changed to emulsify cholesterol more completely. The mole amount of triglyceride used was adjusted to be the same for each type of triglyceride in each experiment. A control emulsion that did not contain lipid was administered to a control group in each experiment.

The emulsions were prepared by sonication and contained 18:2/18:2/18:2, 10:0/10:0/10:0, 18:2/10:0/18:2 or 10:0/18:2/10:0 in experiment 1, and 18:2/18:2/18:2, 8:0/8:0/8:0, 18:2/8:0/18:2 or 8:0/18:2/8:0 in experiment 2. Although the mole amount of triglyceride administered to the rats was the same in experiments 1 and 2, the number of moles of linoleic acid administered differed. In experiment 3, to adjust the number of moles of both triglyceride and linoleic acid, the absorption of fatty acids from structured lipids was compared with the absorption of mixtures of LCT and MCT which resembled the fatty acid composition of the structured lipids. Thus, the test emulsions contained 8:0/18:2/8:0, 18:2/8:0/18:2 or the 1 to 2 or the 2 to 1 mixtures of 18:2/18:2/18:2 and 8:0/8:0/8:0. In experiment 4, radioactive cholesterol was added to the emulsion to determine the effect of the structured lipids, LCT and MCT on cholesterol absorption. The lymph was collected in a tube containing ethylenediaminetetraacetic acid (EDTA) at 3 hr-intervals during the first 9 hr after administering the test or control emulsion and as a single collection from 9–24 hr thereafter.

Chylomicrons from the lymph were collected by ultracentrifugation (16). Lipids were extracted with 20 vol of chloroform/methanol (2:1, v/v) according to the method of Folch *et al.* (17). Pentadecanoic acid (Aldrich, Milwaukee, WI) was added as an internal standard. After the chloroform extract was dried under nitrogen, lipids were analyzed by gas-liquid chromatography as described above. The fatty acid absorption rate was calculated by subtracting the fatty acid amount in blank lymph of the control group from that of the groups administered a test emulsion.

In experiment 4, 1-mL aliquots of the lymph were transferred into scintillation vials and 10 mL of Aquasol II (NEN Research Products) was added. Radioactivity was measured using an Aloka liquid scintillation counter (LSC-900, Aloka, Tokyo, Japan).

Statistical analysis. One-way analysis of variance was followed by Duncan's new multiple-range test to determine the exact nature of the difference between groups (18).

TABLE 1

Composition of Test Emulsion

	Experiments 1, 2 and 3	Experiment 4
Sodium taurocholate	47 mg	192 mg
Albumin	8.3 mg	33.3 mg
Cholesterol	—	10 mg
[4-^{14}C]Cholesterol	—	1 μCi
Structured lipid	0.2 mmole	0.3 mmole
Water	to 2 mL	to 3 mL

RESULTS

The lymph flow rates are summarized in Table 2. Although there were significant differences in the lymph flow rates in different experiments, no consistent pattern of change was observed.

Experiment 1. The lymphtic absorption of capric and linoleic acid is shown in Figure 1. The absorption of capric acid was considerably lower than that of linoleic acid. Absorption of linoleic acid in the case of 10:0/18:2/10:0 (81.4 ± 11.0%) for 24 hr tended to be higher than for 18:2/18:2/18:2 (66.5 ± 8.3%) and 18:2/10:0/18:2 (58.8 ± 13.0%). Similarly, more capric acid was absorbed from 18:2/10:0/18:2 (34.3 ± 4.9%) as compared to 10:0/10:0/10:0 (25.9 ± 7.8%) and 10:0/18:2/10:0 (26.4 ± 2.9%). However, the portion of these fatty acids carried as chylomicrons tended to decrease in the structured lipids when compared to 18:2/18:2/18:2 and 10:0/10:0/10:0, although the differences were not significant (Table 3).

Experiment 2. The lymphatic absorption of caprylic and linoleic acid is shown in Figure 2. The results are similar to those for capric and linoleic acid which were studied in experiment 1. Lymphatic absorption of caprylic acid was much lower than that of linoleic acid, and was also lower than that of capric acid (compare Figs. 1 and 2). Linoleic acid absorption from 8:0/18:2/8:0 (92.4 ± 9.7%) for 24 hr tended to be higher than from 18:2/18:2/18:2 (77.2 ± 7.2%) and 18:2/8:0/18:2 (75.3 ± 9.1%). The trend toward efficient absorption of medium-chain fatty acids located at the 2-position of the triglyceride was more pronounced when caprylic acid rather than capric acid was used.

Caprylic acid absorption from 18:2/8:0/18:2 (15.0 ± 2.9%) was significantly higher ($p<0.05$) than from 8:0/8:0/8:0 (4.3 ± 1.0%) or 8:0/18:2/8:0 (6.5 ± 1.7%). The portion of absorbed caprylic and linoleic acid carried in chylomicrons was significantly lower when these fatty acids were given as 8:0/18:2/8:0 rather than as 18:2/18:2/18:2, 18:2/8:0/18:2 or 8:0/8:0/8:0 (Table 4).

Experiment 3. Absorption of linoleic and caprylic acid as structured lipids (8:0/18:2/8:0 and 18:2/8:0/18:2) and as the 1 to 2 and the 2 to 1 mixtures of 18:2/18:2/18:2 and

TABLE 2

Lymph Flow (mL/24 hr)

Groups	Lymph flow
Experiment 1	
18:2/18:2/18:2	223 ± 36[a]
10:0/10:0/10:0	187 ± 41[a]
18:2/10:0/18:2	244 ± 29[a]
10:0/18:2/10:0	187 ± 18[a]
Experiment 2	
18:2/18:2/18:2	201 ± 30[a]
8:0/8:0/8:0	212 ± 14[a]
18:2/8:0/18:2	239 ± 6[a]
8:0/18:2/8:0	219 ± 12[a]
Experiment 3	
Mixture A[c]	224 ± 17[a]
Mixture B[d]	234 ± 12[a]
18:2/8:0/18:2	244 ± 8[a]
8:0/18:2/8:0	251 ± 14[a]
Experiment 4	
18:2/18:2/18:2	176 ± 15[a]
10:0/10:0/10:0	202 ± 11[a,b]
8:0/8:0/8:0	198 ± 9[a,b]
18:2/10:0/18:2	218 ± 17[a,b]
18:2/8:0/18:2	244 ± 10[b]
10:0/18:2/10:0	217 ± 13[a,b]
8:0/18:2/8:0	231 ± 19[b]

[a,b]Means not sharing a common superscript letter are significantly different at $p<0.05$. Data are means ± SE of 5 to 7 rats.

[c]The 1 to 2 mixture of 8:0/8:0/8:0 and 18:2/18:2/18:2.

[d]The 2 to 1 mixture of 8:0/8:0/8:0 and 18:2/18:2/18:2.

TABLE 3

Proportion of Capric (10:0) and Linoleic (18:2) Acid Carried in Chylomicrons in the Lymph (Experiment 1)[a]

Groups	Capric acid	Linoleic acid
18:2/18:2/18:2	—	73.7 ± 5.4
10:0/10:0/10:0	78.3 ± 6.3[b]	—
18:2/10:0/18:2	60.7 ± 6.1	56.4 ± 11.1
10:0/18:2/10:0	72.3 ± 1.3	66.2 ± 4.8

[a]Data are means ± SE of 5 or 6 rats.

[b]% of total fatty acid in lymph.

TABLE 4

Proportion of Caprylic (8:0) and Linoleic (18:2) Acid Carried as Chylomicrons in the Lymph (Experiment 2)

Groups	Caprylic acid	Linoleic acid
18:2/18:2/18:2	—	80.7 ± 5.1[a]
8:0/8:0/8:0	71.2 ± 2.6[a,b]	—
18:2/8:0/18:2	72.8 ± 2.3[a]	68.0 ± 2.1[a]
8:0/18:2/8:0	62.9 ± 2.0[c]	51.8 ± 5.3[c]

[a,c]Values in the same column not sharing a common superscript letter are significantly different at $p<0.05$.

[b]% of total fatty acid in lymph.

Data are means ± SE of 5 to 7 rats.

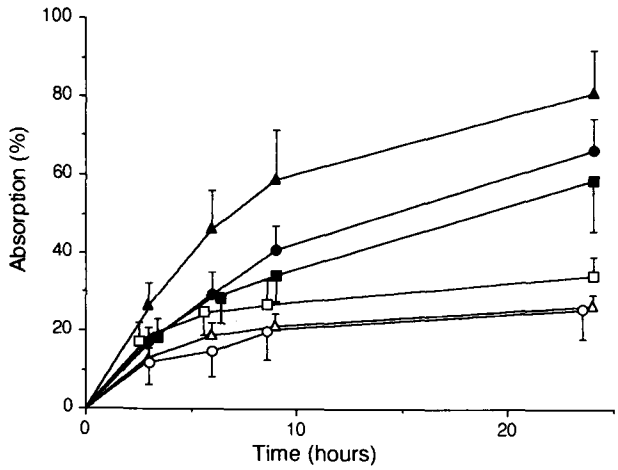


FIG. 1. Absorption of capric and linoleic acids into thoracic duct lymph of rats upon intragastric administration of fat emulsions containing sodium taurocholate and albumin. ●, ■ or ▲ shows linoleic acid absorption in rats given 18:2/18:2/18:2, 18:2/10:0/18:2 or 10:0/18:2/10:0, respectively. ○, □ or △ shows capric acid absorption in rats given 10:0/10:0/10:0, 18:2/10:0/18:2 or 10:0/18:2/10:0, respectively. Data are means ± SE of 5 or 6 rats. Significant differences were not observed in this experiment.

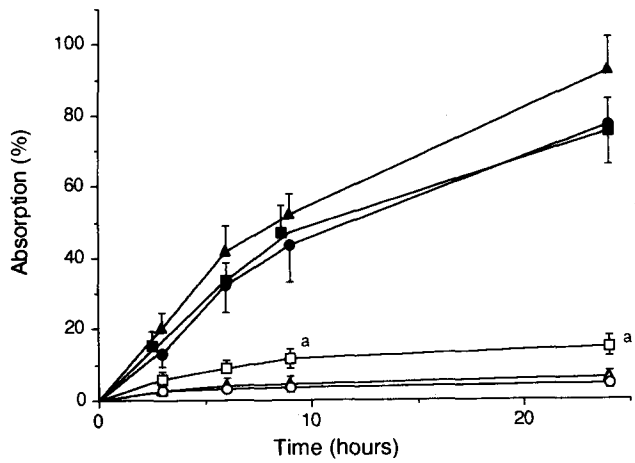


FIG. 2. Absorption of caprylic and linoleic acids into thoracic duct lymph of rats. ●, ■ or ▲ shows linoleic acid absorption in rats given 18:2/18:2/18:2, 18:2/8:0/18:2 or 8:0/18:2/8:0, respectively. ○, □ or △ shows caprylic acid absorption in rats given 8:0/8:0/8:0, 18:2/8:0/18:2 or 8:0/18:2/8:0, respectively; a, caprylic acid absorption was significantly higher in 18:2/8:0/18:2 than in 8:0/8:0/8:0 and 8:0/18:2/8:0 groups at $p<0.05$. Data are means ± SE of 5 to 7 rats.

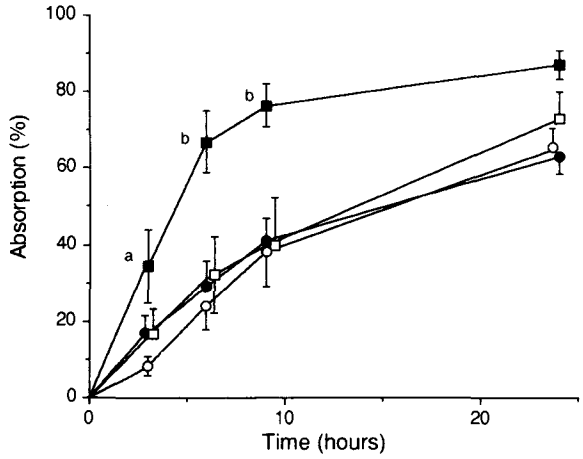


FIG. 3. Absorption of linoleic acid into thoracic duct lymph of rats. ●, ■, ○ or □ shows linoleic acid absorption in rats given 18:2/8:0/18:2, 8:0/18:2/8:0, a 1 to 2 or a 2 to 1 mixture of 8:0/8:0/8:0 and 18:2/18:2/18:2, respectively; a, significantly higher than the other three groups at $p<0.05$; b, significantly higher than the 1 to 2 mixture of 8:0/8:0/8:0 and 18:2/18:2/18:2 at $p<0.05$. Data are means ± SE of 5 or 6 rats.

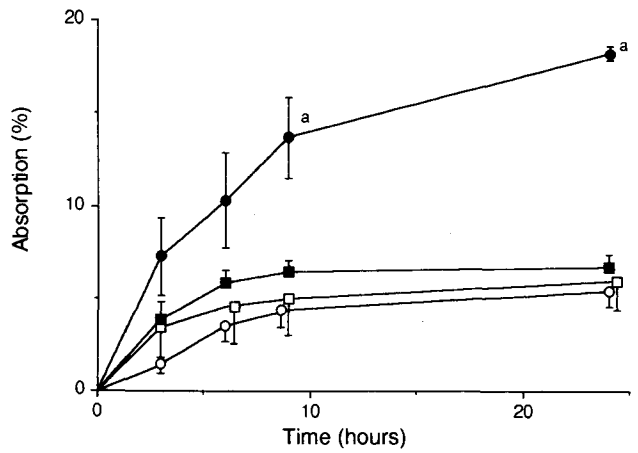


FIG. 4. Absorption of caprylic acid into thoracic duct lymph of rats. ●, ■, ○ or □ shows caprylic acid absorption in rats given 18:2/8:0/18:2, 8:0/18:2/8:0, a 1 to 2 or a 2 to 1 mixture of 8:0/8:0/8:0 and 18:2/18:2/18:2, respectively; a, significantly higher than the other three groups at $p<0.05$. Data are means ± SE of 5 or 6 rats.

8:0/8:0/8:0 is shown in Figures 3 and 4, respectively. The rate of absorption of linoleic and caprylic acid located at the 2-position of the structured lipids was more efficient than that of the fatty acids from a mixture of single-acid triglycerides and of the fatty acids located at the 1- and 3-positions of the structured lipids. Linoleic acid absorption from 8:0/18:2/8:0 for 24 hr was 87.2 ± 3.8% as compared to 73.2 ± 6.8% for the 1 to 2 mixture of 18:2/18:2/18:2 and 8:0/8:0/8:0, 63.1 ± 4.6% for 18:2/8:0/18:2, and 65.5 ± 5.0% for the 2 to 1 mixture of 18:2/18:2/18:2 and 8:0/8:0/8:0. Caprylic acid absorption from 18:2/8:0/18:2 (18.3 ± 0.4%) was significantly higher ($p<0.05$) than that from the 2 to 1 mixture of 18:2/18:2/18:2 and 8:0/8:0/8:0 (5.4 ± 0.9%), from 8:0/18:2/8:0 (6.7 ± 0.6%) and from the 1 to 2 mixture of 18:2/18:2/18:2 and 8:0/8:0/8:0 (5.9 ± 1.6%).

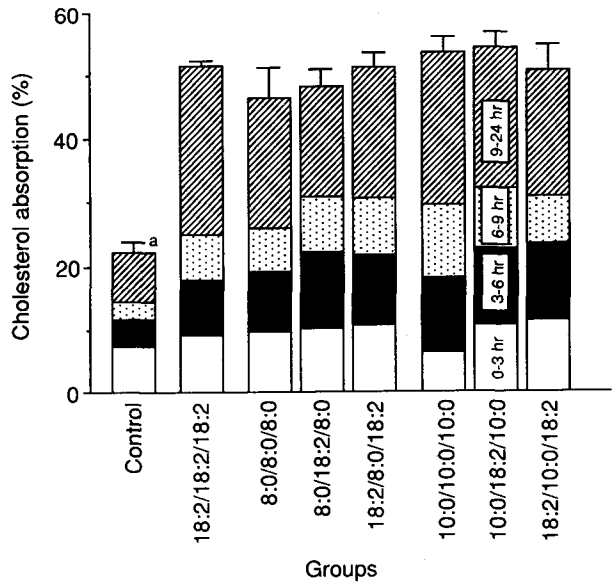


FIG. 5. Absorption of cholesterol into thoracic duct lymph of rats after intragastric administration of fat emulsions containing sodium taurocholate, labeled cholesterol and albumin; a, significantly lower than all other groups at $p<0.05$. Data are means ± SE of 5 to 7 rats.

Experiment 4. The effect of the structured lipids, 18:2/18:2/18:2, 8:0/8:0/8:0 and 10:0/10:0/10:0 on cholesterol absorption is shown in Figure 5. Lipid administration stimulated cholesterol absorption to a considerable extent as compared to the control group given a fat-free emulsion. However, the type of dietary fat did not significantly influence cholesterol absorption.

DISCUSSION

It is widely believed that medium-chain fatty acids are mainly absorbed and transported through the portal vein and that lymphatic absorption is minimal (19). However, as far as we know, the extent of lymphatic absorption of medium-chain fatty acids has not been investigated. We have shown in the present study that a portion of the medium-chain fatty acids is absorbed through lymph, but to a much smaller extent than is linoleic acid. Lymphatic absorption of capric acid is greater than that of caprylic acid. The differential absorption of these two medium-chain fatty acids may be due to their different hydrophobicity.

Based on the observed absorption characteristics of MCT and LCT, it would seem reasonable that structured lipids which contain medium-chain fatty acids in the 1- and 3-position and linoleic acid in the 2-position may have some beneficial effects both as an energy source and as a source of essential fatty acid. This could be of particular importance for patients with malabsorption syndromes receiving enteral alimentation. As shown in the present study, even in rats with normal pancreatic function, lymphatic absorption of linoleic acid from the 2-position was consistently higher than from the 1- and 3-positions, or from trilinolein (Figs. 1 and 2).

The mole amounts of linoleic acid and medium-chain fatty acids administered to the rats were different in these

experiments. Therefore, in experiment 3, the absorption of fatty acids from structured lipids was compared with that from mixtures of LCT and MCT with the same fatty acid composition. The results of the preceding experiments were confirmed and we could show that linoleic acid located in the 2-position of 8:0/18:2/8:0 was absorbed preferentially as compared to linoleic acid from the 18:2/18:2/18:2 and 8:0/8:0/8:0 mixture.

Jandacek *et al.* (20) reported that hydrolysis of triglycerides with medium-chain fatty acids in the 1- and 3-position and a long-chain fatty acid in the 2-position was more rapid *in vitro* than of LCT. The incorporation of radioactivity from 2-[^{14}C]linoleoyl-1,3-dioctanoyl-glycerol (8:0/18:2/8:0) into an isolated, irrigated intestinal loop was 2.5 times higher than that from 2-[^{14}C]linoleoyl-1,3-dioleoyl-glycerol. Therefore, the rapid and complete hydrolysis of the medium-chain fatty acids located in the 1- and 3-positions of the structured lipids appears to be responsible for the higher lymphatic absorption of linoleic acid located in the 2-position. In patients with an insufficient supply of pancreatic lipase, structured lipids with medium-chain fatty acids in the 1- and 3-position and polyunsaturated fatty acid in the 2-position should also be useful.

It has been reported that some long-chain fatty acids are transported through the portal vein (19). However, since portal absorption of linoleic acid is extremely low, the contribution of this pathway to the lower lymphatic absorption of linoleic acid in the 1- and 3-position of structured lipids can be disregarded.

Our results also show that the lymphatic absorption of medium-chain fatty acids located in the 2-position of the structured lipids was more efficient than of those located in the 1- and 3-position. The results suggest that prior to complete hydrolysis of medium-chain 2-monoglycerides to free fatty acid and glycerol, a portion of the 2-monoglycerides would be absorbed intact and utilized in triglyceride resynthesis in intestinal mucosa for lymphatic transport. It is plausible that the medium-chain fatty acids released from the 1- and 3-position are predominantly transported through the portal vein without resynthesis to triglyceride.

Several studies have shown that the concentration of serum and liver cholesterol is lower in rats fed MCT than in rats fed LCT. It would be possible that MCT decrease intestinal absorption of cholesterol (6). However, our results do not support this conclusion, as in our experiments MCT and structured lipids did not influence lymphatic cholesterol absorption. However, this would not preclude that long-term feeding of MCT or structured lipids may affect the various functions of intestinal cells including cholesterol absorption. Redgrave *et al.* (21) suggested that the 2-monoglyceride transiently formed during hydrolysis of chylomicron triglyceride by lipoprotein lipase in the capillaries may be an important factor in the metabolic control of chylomicrons and hence, serum cholesterol concentration.

Structured lipids clearly influence the incorporation of fatty acids into chylomicrons, and several dietary components affect the size and composition of chylomicrons. Phospholipids also modify the particle size of chylomicrons in the lymph (22). Vahouny *et al.* (23) furthermore showed that dietary fiber alters the rate of incorporation of cholesterol and oleic acid into lymphatic chylomicrons. However, the reasons and the metabolic significance of these differential distributions of fatty acids are not clear at present.

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Effect of 4,7,10,13,16,19-Docosahexaenoic Acid on Triglyceride Accumulation and Secretion in Rat Hepatocytes in Culture

L.J. Martin, G.B. Reaidi, G.R. Gavino and V.C. Gavino*
Department of Nutrition, University of Montréal, P.O. Box 6128 Station "A", Montréal, Québec, Canada H3C 3J7

We have investigated the effect of oleic (18:1) and 4,7,10,13,16,19-docosahexaenoic (22:6ω3) acids on triglyceride (TG) accumulation, secretion and reuptake in rat hepatocytes in culture. We also calculated the percentage of total TG, TG-esterified 18:1 and TG-esterified 22:6ω3 that were secreted relative to the total accumulation (intra + extracellular TG). Both fatty acids were incorporated mainly in the intracellular TG fraction. Treatment with 18:1 but not with 22:6ω3 increased the quantity of TG secreted into the culture medium relative to controls. Treatment with 22:6ω3 caused a greater accumulation of intracellular TG than 18:1. This arises in part from the preferential retention of 22:6ω3-enriched TG by the hepatocytes. At 24 hr, there was no longer any difference in the net secretion of TG by 18:1 and 22:6ω3-treated cells, which may be a consequence of the reuptake of TG-esterified 18:1. There was no reuptake of TG-esterified 22:6ω3. We conclude that inhibition of hepatocyte TG synthesis is not obligatory for 22:6ω3-induced diminution of TG secretion.

Lipids 26, 374-380 (1991).

It is generally stated that the hypotriglyceridemic effect of dietary fish oil arises from an inhibitory effect of long-chain ω3 polyunsaturated fatty acids (PUFA) on triglyceride (TG) synthesis in the liver (1). Several investigators have reported that the inhibition of TG synthesis may be due to changes in the activity of diacylglycerol acyltransferase (2,3), of phosphatidate phosphohydrolase (4), or of a general reduction in the activity of lipogenic enzymes involved in fatty acid synthesis (5-7).

There are, however, other reports indicating that a decrease in TG secretion induced by ω3 PUFA is not necessarily linked to inhibition of TG synthesis. These contradictory results are probably due to dissimilarity in the experimental models used, and to possible metabolic differences between 20:5ω3 and 22:6ω3, the two major PUFA in fish oil. For example, Haug and Hostmark (8) reported that dietary fish oil increases hepatic TG content despite a significant hypotriglyceridemic effect. Wong *et al.* (9) showed that 22:6ω3 but not 20:5ω3 was just as effective as 18:1 in stimulating TG accumulation in HepG2 cells although both ω3 PUFA inhibited TG secretion. Davis and Boogaerts (10) found that the degree of unsaturation of 18-carbon fatty acid (FA) was inversely related to TG secretion in cultured rat hepatocytes, but not at all correlated with intracellular TG accumulation. In an abstract, Fox and Hay (11) reported that 20:5ω3 reduced TG secretion but not TG synthesis in rat hepatoma cells.

It is in this context that we have decided to investigate further the effects of 22:6ω3 on TG accumulation, secretion and reuptake in primary cultures of rat hepatocytes. Our data show that 22:6ω3-induced decrease in TG secretion is not necessarily due to a decrease in TG synthesis, and that the TG-esterified 22:6ω3 is preferentially retained by the hepatocytes.

MATERIALS AND METHODS

Preparation and culture of hepatocytes. Hepatocytes from *ad libitum* chow-fed male 300-g Sprague-Dawley rats (Charles River, Montréal, Québec Canada) were isolated under sterile conditions by collagenase perfusion according to the method of Seglen (12). Cells were preincubated for 30 min in culture medium consisting of Dulbecco's Modified Eagle medium (Gibco, Burlington, Ontario Canada) containing 24 mM glucose, 1 mM pyruvate, 70 mM 4-hydroxyethyl-1-piperazineethanesulfonic acid (Sigma, St. Louis, MO) and 50 μg/mL of gentamycin (Gibco, Burlington, Ontario Canada). Hepatocytes were plated at a concentration of 3.0×10^6 viable cells per 25-cm^2 flask in culture medium supplemented with 20% newborn calf serum (Gibco) and insulin (10 μg/mL) as described by Nossen *et al.* (13). After 4 hr, the culture medium was changed to remove non-adherent cells. The remaining attached cells were incubated overnight at 37°C in an atmosphere of 5% CO_2 in air.

Experiments with cultured hepatocytes. The fatty acids (FA) 18:1 and 22:6ω3 were obtained from NuChek Prep (Elysian, MN). These were 97-98% pure as evaluated by gas-liquid chromatography (GLC). At the beginning of each experiment, cells were incubated with culture medium containing 4% essentially fatty acid-free bovine serum albumin (BSA) (Sigma), 1 mM FA and 1 μM butylated hydroxytoluene (BHT) as antioxidant. The cells were incubated for 2, 8 and 24 hr at 37°C. At the end of the incubation period, the media were removed and centrifuged at 5000 rpm (4°C) for 10 min prior to analysis. The attached cells were rinsed twice with phosphate buffered saline before lipid extraction.

Cells were detached from flasks with trypsin and viable cells, distinguished by Trypan blue exclusion, were counted with a hemocytometer. Protein was measured by the method of Lowry *et al.* (14). The quantity of lactate dehydrogenase released into the medium was measured as described previously (15) at the end of the 24-hr incubation period as an indicator of the extent of cell death.

TG reuptake. Parallel hepatocyte cultures were incubated with [1-^{14}C]18:1 (100 dpm/nmol), [1-^{14}C]22:6ω3 (100 dpm/nmol), unlabeled 18:1, or unlabeled 22:6ω3 under

*To whom correspondence should be addressed at Department of Nutrition, University of Montréal, P.O. Box 6128 Station "A", Montréal, Québec, Canada H3C 3J7.

Abbreviations: BHT, butylated hydroxytoluene; BSA, bovine serum albumin; CE, cholesteryl ester; FA, fatty acid; GLC, gas-liquid chromatography; NEFA, non-esterified fatty acid; PL, phospholipid; PUFA, polyunsaturated fatty acids; TG, triglyceride; TLC, thin-layer chromatography; VLDL, very low-density lipoprotein.

the conditions described above. After 8 hr, the culture media from cells treated with the labeled FA were recovered, centrifuged (5000 rpm, 4°C) for 10 min, and supplemented with 200 μM chloroquine. The recovered media contained [1-^{14}C]18:1-enriched TG or [1-^{14}C] 22:6ω3-enriched TG and were used for the next step of the experiment. After 8 hr of incubation, 200 μM chloroquine was added to the parallel hepatocyte cultures treated with non-radioactive FA. After a 30-min incubation with chloroquine, the non-radioactive culture media of these cells were discarded and replaced with radioactive media recovered from the hepatocytes treated with labeled FA. Cells originally treated for 8 hr with unlabeled 18:1 were incubated for a further 6 hr with media containing either [1-^{14}C]18:1-enriched TG or [1-^{14}C]22:6ω3-enriched TG. Cells originally treated with unlabeled 22:6ω3 were also incubated for a further 6 hr with media containing either [1-^{14}C]18:1-enriched TG or [1-^{14}C]22:6ω3-enriched TG. At the end of the 6-hr incubation period, ^{14}C-labeled TG in the culture media was isolated by thin-layer chromatography (TLC) using ^{3}H-labled triolein (DuPont, Mississauga, Ontario Canada) as recovery standard. Incubation of radioactive culture media in culture flasks without cells served as controls.

Extraction and separation of lipids. BHT was added at 4 ppt to all redistilled solvents used in the extraction, separation and storage of lipids. Lipids in the culture medium were extracted by the method of Bligh and Dyer (as described in ref. 16). Cellular lipids were extracted as previously described (17). Heptadecanoic acid, triheptadecanoin, cholesteryl heptadecanoate and 1,2-diheptadecanoyl phosphatidylcholine (Sigma) were added to cells and media as internal standards for quantitation of lipids. Non-esterified fatty acids (NEFA), TG, phospholipids (PL) and cholesteryl esters (CE) were separated by thin-layer chromatography (TLC) using pre-coated Whatman type-LK6D silica gel plates (Supelco, Oakville, Ontario Canada) with the solvent system hexane/diethyl ether/acetic acid (80:20:1, v/v/v). Radioactivity in lipid fractions was determined by liquid scintillation counting of scrapings from the TLC plates. Lipid fractions were recovered from TLC for gas-liquid chromatography (GLC) by eluting the TLC plates with diethyl ether for CE and TG, chloroform for NEFA, and methanol/water (96:4, v/v) for PL.

Determination of fatty acid composition. Lipid fractions isolated by TLC were transesterified to FA methyl esters by treatment with sodium methoxide (18). NEFA were derivatized with diazomethane. FA methyl esters were separated on a 30-m bonded Carbowax-30N fused silica capillary column installed in a Hewlett-Packard HP5890 gas chromatograph equipped with a flame ionization detector. Quantitation was achieved with a HP3392A integrator using the internal standard method.

RESULTS

Table 1 shows that FA treatments did not affect viable cell counts nor total cell protein. This indicates that under our incubation conditions, the FA treatment did not exert a toxic effect on the hepatocytes. This was confirmed by measuring lactate dehydrogenase in the culture medium as an indicator of cell death. The enzyme level was similar across all groups and was in agreement with a previous report concerning cultures of viable cells (19).

The disappearance of 22:6ω3 and 18:1 from the culture medium during the incubation period with hepatocytes is shown in Figure 1. NEFA concentration

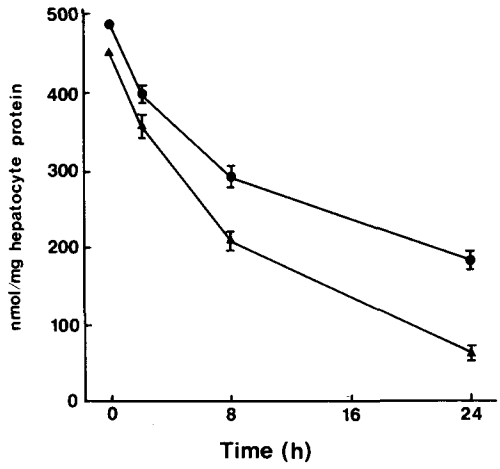


FIG. 1. Uptake of fatty acid by hepatocytes in culture. Cells were incubated with 18:1 (●) or with 22:6ω3 (▲). The decline in NEFA concentration during the incubation period, measured by quantitative GLC, was assumed to represent cellular uptake. Each point is the mean ± s.e.m. of three separate experiments.

TABLE 1

Effect of Fatty Acid Treatment on Hepatocyte Viability[a]

	Treatment group		
	Control	18:1	22:6ω3
	mean ± s.e.m. (n = 3)		
Viable cells (× 10^{-4}/cm^2)	1.67 ± 0.26	1.57 ± 0.09	1.53 ± 0.09
Cell protein (mg/25 cm^2 flask)	5.5 ± 0.4	5.4 ± 0.3	4.7 ± 0.2
LDH activity, culture medium (nmol/s × mg protein)	5.5 ± 0.2	4.7 ± 0.4	3.4 ± 0.5

[a]Rat hepatocytes were isolated by collagenase perfusion, placed in cell culture, and treated for 24 hr with 1 mM total NEFA bound to BSA. Dye-exclusion was used as the criterion for cell viability after NEFA treatment. Cell protein represents attached cells. There was no statistical significance between treatment groups at $\alpha = 0.01$ (Scheffe).

TABLE 2

Uptake of [1-^{14}C]-labeled 22:6ω3 and 18:1 Analyzed by Two-Way ANOVA

Treatment group	Incubation time (hr)	
	0[a]	8
	Dpm (mean ± s.e.m., n = 3)[b]	
[1-^{14}C] 18:1	39200 ± 4000	23200 ± 2600
[1-^{14}C] 22:6ω3	38500 ± 2600	18900 ± 1400

[a]Hepatocytes were treated with BSA-complexed 1 mM radiolabeled 18:1 or 22:6ω3 and radioactivity in the culture medium was assayed at time 0 and after 8 hr.

[b]Dpm per culture flask. The two classifications used in the ANOVA analysis were treatment group *vs* incubation time. The between group interaction term was significant at $p<0.001$.

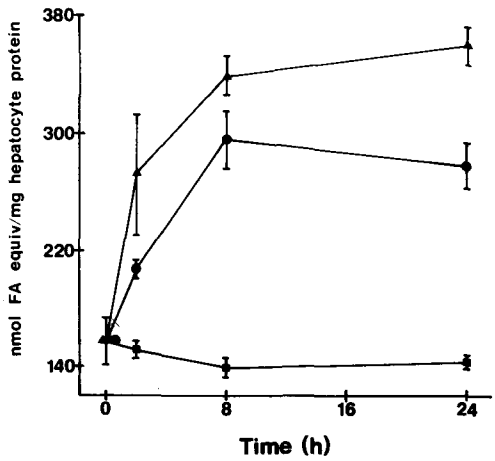


FIG. 2. Accumulation of intracellular triglyceride in hepatocytes in culture. The TG of cells, control (■) or incubated with 18:1 (●) or 22:6ω3 (▲), were extracted and separated by TLC using triheptadecanoin as a recovery standard. Each point represents the mean ± s.e.m. of three experiments. The data were subjected to ANOVA using Newman-Keuls multiple range testing to detect differences between means. Significance was declared at $p<0.05$.

did not change in culture medium incubated under the same conditions but without hepatocytes (data not shown). The rate of disappearance of 22:6ω3 was faster than that of 18:1 even when the data are normalized to initial NEFA concentrations. At 8-hr incubation time, the cells took up 40% and 54% of the initial 18:1 and 22:6ω3, respectively. These results were confirmed using [1-^{14}C]-labeled NEFA (Table 2). Two-way ANOVA analysis of the data showed that the interaction term "incubation time × FA treatment" was significant ($p<0.001$, F-test). This means that the extent of decrease in radioactivity differed significantly between the two treatment groups. At 8-hr incubation, 41% and 51% of the initial radioactivity in 18:1 and 22:6ω3, respectively, was taken up by the hepatocytes.

Figure 2 shows that 22:6ω3 stimulated more TG accumulation in hepatocytes than did 18:1 during the incubation period. However, statistically significant differences between means were found only at the 24-hr incubation time. TG accumulation in cells treated with 18:1 appeared

TABLE 3

Uptake of [1-^{14}C]NEFA in Different Lipid Classes

	Treatment group[a]	
	18:1	22:6ω3
	mean ± s.e.m. (n = 4)	
Cholesteryl ester[b]	1.50 ± 0.08[c]	0.50 ± 0.02[d]
TG	87.2 ± 0.4	90.9 ± 0.2[d]
NEFA	3.1 ± 0.2	2.8 ± 0.1
PL	8.2 ± 0.2	5.8 ± 0.2[d]

[a]Cultured hepatocytes were treated with 1 mM of the indicated [1-^{14}C]NEFA for 8 hr.

[b]Intracellular lipids were extracted and separated into lipid classes by TLC. The radioactivity in each fraction was determined by liquid scintillation counting.

[c]Data are presented as the relative percentage of radioactivity in each lipid class. Arcsin transformation of data was performed prior to statistical analysis. Significant differences between treatment group means within each lipid class is denoted by "d" ($p<0.001$, Student's t-test).

to be slower and to reach a plateau earlier than 22:6ω3-treated cells. The relative distribution of [1-^{14}C]-labeled FA in the major lipid classes after an 8-hr incubation is shown in Table 3. Both 18:1 and 22:6ω3 were largely incorporated in the TG fraction with a slightly but statistically significant higher TG-incorporation for 22:6ω3. In contrast, more labeled 18:1 than 22:6ω3 accumulated in the CE and PL fractions.

The amount of intracellular CE was unaffected by the FA treatments (Table 4). However, the enrichment of the CE fraction with 18:1 was greater than with 22:6ω3. This is consistent with the known specificity of acyl-CoA:cholesterol acyltransferase for 18:1 (20,21). No retroconversion or chain elongation derivatives of the treatment FA were detected in the CE fraction.

The treatment FA did not significantly affect the amount of cellular total PL (Table 5). Treatment with 22:6ω3 resulted in the accumulation of 20:5ω3 in the PL (Table 5) and of 20:5ω3 and 22:5ω3 in the TG (Table 6) fractions. The distribution of 22:6ω3 and 20:5ω3 was, however, dissimilar in these two lipid fractions. The molar ratio of 22:6ω3 to 20:5ω3 was 1.7 in PL but was 7.6 in TG, indicating that the metabolic control for these two ω3 PUFA is distinct from each other.

Table 7 shows TG secretion by hepatocytes treated with NEFA over a period of 24 hr. The medium of cells treated with 22:6ω3 for 2 and 8 hr contained less TG relative to cells treated with 18:1. However, at 24 hr, the three treatment groups no longer differed in the amounts of TG in the culture medium. We were unable to detect CE in any of the culture media.

We next investigated TG reuptake as a factor that could have affected TG accumulation in the culture medium (Table 8). This experiment was designed to mimic a continuous incubation with the treatment FA but without the confounding effect of radiolabel recycling. It therefore permitted the testing of the effect of 18:1-*versus* 22:6ω3-enrichment of hepatocytes on TG reuptake. First, the incubation conditions in the parallel hepatocyte cultures were identical in all respects except for the use of radiolabeled-NEFA in one and not the other. Therefore, the transfer of culture medium from the radioactive treatment group to replace the medium of the corre-

TABLE 4

Nanoequivalents of 18:1 and 22:6ω3 in Intracellular Cholesteryl Ester

Fatty acid	Treatment groups[a] (A) Control	(B) 18:1	(C) 22:6ω3	Newman-Keuls test[b]
	mean ± s.e.m. (n = 3)[c]			
Total[d]	7.34 ± 0.41	7.67 ± 0.50	7.5 ± 1.0	A C B
18:1	2.04 ± 0.13	3.60 ± 0.15	1.85 ± 0.27	C A B
22:6ω3	N.D.[e]	N.D.	0.28 ± 0.01	

[a]Hepatocytes were incubated with the indicated NEFA for 8 hr. Cholesteryl esters were isolated by TLC with the addition of cholesteryl heptadecanoate as internal standard.

[b]Data were analyzed by ANOVA using multiple range testing to detect differences between means (Newman-Keuls). Significance is declared at $p<0.05$. Homogeneous groups are connected by an underscore.

[c]Results are nanoequivalents of FA per mg hepatocyte protein.

[d]Includes all other FA.

[e]Not detected.

TABLE 5

Nanoequivalents of Selected FA in Intracellular Phospholipid

Fatty acid	Treatment groups[a] (A) Control	(B) 18:1	(C) 22:6ω3	Newman-Keuls test[b]
	mean ± s.e.m. (n = 3)[c]			
Total[d]	200 ± 10	230 ± 16	220 ± 4.0	A C B
18:1	11.0 ± 0.8	28.0 ± 1.8	7.9 ± 0.3	C A B
20:5ω3	N.D.[e]	N.D.	12.5 ± 0.4	
22:6ω3	9.0 ± 0.5	9.5 ± 0.4	21.1 ± 0.6	A B C

[a]Hepatocytes were incubated with the indicated NEFA for 8 hr. Total PL were isolated by TLC with the addition of diheptadecanoyl phosphatidylcholine as internal standard.

[b]Data were analyzed as in Table 4. Significance is declared at $p<0.05$. Homogeneous groups are connected by an underscore.

[c]Results are nanoequivalents of FA per mg hepatocyte protein.

[d]Includes all other FA.

[e]Not detected.

TABLE 6

Nanoequivalents of Selected FA in Intracellular TG

Fatty acid	Treatment groups[a] (A) Control	(B) 18:1	(C) 22:6ω3	Newman-Keuls test[b]
	mean ± s.e.m. (n = 3)[c]			
18:1	32.6 ± 1.9	169 ± 12	33.6 ± 1.1	A C B
20:5ω3	1.11 ± 0.02	1.42 ± 0.09	19.7 ± 0.68	A B C
22:5ω3	2.68 ± 0.05	3.12 ± 0.17	7.52 ± 0.30	A B C
22:6ω3	4.74 ± 0.04	5.83 ± 0.33	150 ± 5	A B C

[a]Hepatocytes were incubated with the indicated NEFA for 8 hr.

[b]Data were analyzed as in Table 4. Significance is declared at $p<0.05$. Homogeneous groups are connected by an underscore.

[c]Nanoequivalents of FA per mg hepatocyte protein.

sponding non-radioactive group at the same stage of incubation should perturb the system minimally. Second, the target hepatocytes were treated with chloroquine, a known inhibitor of very low density lipoprotein (VLDL) secretion (22). In our system, chloroquine reduced TG secretion to less than 5% of that in NEFA-treated hepatocytes (data not shown). Consequently, the decrease in TG radioactivity subsequent to the culture medium exchange would be directly due to reuptake, *i.e.*, it is not a net result of simultaneous TG secretion and reuptake. Results shown in Table 8 indicate that TG reuptake may affect TG accretion in the culture medium of hepatocyte culture systems treated with 18:1 but not with 22:6ω3.

The results for the homologous experiments (Table 8,

TABLE 7

The Effect of FA Treatments on the Secretion of TG by Cultured Hepatocytes

Incubation time (hr)	Treatment group[a] (A) Control	(B) 18:1	(C) 22:6ω3	Newman-Keuls test[b]
	mean ± s.e.m. (n = 3)			
2	6.4 ± 0.5[c]	6.3 ± 0.5	4.4 ± 0.1	C B A
8	12.2 ± 0.7	16.1 ± 0.9	10.8 ± 0.7	C A B
24	19.2 ± 0.9	20.3 ± 2.2	20.4 ± 1.0	A B C

[a]Hepatocytes were incubated with 1 mM of the indicated NEFA in the presence of 4% BSA.

[b]Data were analyzed as in Table 4. Significance is declared at $p<0.5$. Homogeneous groups are connected by an underscore.

[c]At the indicated times, the culture medium was extracted and the TG fraction isolated by TLC with the addition of a TG-internal standard. The results, analyzed by GLC, are expressed as FA nanoequivalents in TG in culture medium per mg hepatocyte protein.

TABLE 8

Hepatocyte Reuptake of Secreted TG

Incubation experiments[a]	TG source[b] 18:1-cells	22:6ω3-cells
	dpm in TG/culture well[c], mean ± s.e.m. (n = 3)	
(A) Without cells (control)	6620 ± 680	2860 ± 130
(B) With cells preincubated with same FA (homologous)	4830 ± 320	2730 ± 160
(C) With cells preincubated with other FA (crossover)	5410 ± 310	2740 ± 150
Range test (LSD)[d]	B C A	B C A

[a]Parallel hepatocyte cultures were incubated with either [1-^{14}C]-labeled or cold 18:1 or 22:6ω3. At 8 hr, the culture medium of cells incubated with radio-labeled NEFA was transferred to plates without cells (A), or used to replace the culture media of cells incubated in parallel with the same but unlabeled NEFA (B), as well as of cells incubated with the opposite and unlabeled NEFA (C). The hepatocytes were treated with 200 μM chloroquine in the 30-min interim period between the media exchange, and through the subsequent 6-hr incubation period.

[b]The TG source refers to culture media taken from cultures of hepatocytes incubated for 8 hr with [1-^{14}C]-labeled 18:1 or [1-^{14}C]-labeled 22:6ω3 (see footnote "a" above). As a result of the preliminary 8-hr incubation, the culture media contained the corresponding radio-labeled TG.

[c]TG in culture medium were separated by TLC before counting. The disappearance of TG-radioactivity was considered to represent reuptake by the hepatocytes.

[d]Statistical analysis was similar to Table 4 except that the criterion for significance ($p<0.05$) was calculated by the least significant difference (LSD) method. Homogeneous groups are connected by an underscore.

row B) demonstrate that during the course of a 14-hr incubation with 18:1 and 22:6ω3, there is significant reuptake by hepatocytes of TG-esterified 18:1, but not of TG-esterified 22:6ω3. The lower initial radioactivity in the culture medium from [1-^{14}C]22:6ω3-treated cells (Table 8, row A) reflects the lower secretion of TG-esterified 22:6ω3 relative to TG-esterified 18:1 (see Table 7). The crossover experiments (Table 8, row C) involved replacing the incubation medium of the FA-treated hepatocytes with non-homologous culture medium obtained from parallel cultures incubated with the other FA. There was no reuptake of TG-esterified 22:6ω3 by cells pre-treated with 18:1 (row C, last column). In contrast, there was a reduction in radioactivity when 22:6ω3-treated cells were treated with TG-esterified [1-^{14}C]18:1 (row C, middle column). The reduction in radioactivity, however, was not statistically significant, and was lower than what was observed when TG-esterified (1-^{14}C)18:1 was incubated with 18:1-pretreated cells (row B, middle column). Taken together, the results indicate that 22:6ω3-enrichment of either the hepatocyte or the secreted TG interferes with TG reuptake. Reuptake of 18:1-TG but not of 22:6ω3-TG may explain why at 24 hr, there was no longer any difference between the FA-treatment groups with respect to TG accumulation in the culture media (Table 7). No further attempt was made to distinguish which cellular lipid enriched with 22:6ω3 was linked to the inhibition of TG-reuptake.

DISCUSSION

The calculations presented in Table 9 indicate that 22:6ω3 esterified in the TG fraction is not efficiently secreted by the hepatocyte relative to 18:1. The comparisons were made based on a calculated "index of TG secretion" which is the percentage of TG that has accumulated in the culture medium (TG_o) relative to the net total TG (TG_{tot}) synthesized by the cell. TG_{tot} is the sum of intracellular TG and TG_o. Specific indices of TG secretion were also calculated for 18:1-containing TG ($18:1TG_o$) relative to 18:1-

containing total TG ($18{:}1TG_{tot}$), and for 22:6ω3-containing TG ($22{:}6\omega3TG_o$) relative to 22:6ω3-containing total TG ($22{:}6\omega3TG_{tot}$). The first set of indices for total TG at 2- and 8-hr incubation (Table 9, row 1) reflects the results shown in Figure 2 and Table 7, in that 22:6ω3 treatment diminished total TG secretion in spite of the fact that it also caused a greater accumulation of intracellular TG. At 24 hr, the total TG secretion indices were no longer statistically different although the lower index for the 22:6ω3-treated cells reflected the higher intracellular TG content for these cells (Fig. 2). The second set of indices which are specific for 18:1 (Table 9, row 2) shows that the secretion of TG-esterified 18:1 is not sensitive to either FA treatment. The third set of indices, which are specific for the secretion of TG-esterified 22:6ω3 (Table 9, row 3), was significantly lower than the corresponding 18:1-indices for either 18:1- or 22:6ω3-treated hepatocytes. The 22:6ω3-specific indices for the 18:1-treated cells were zero because there was no detectable 22:6ω3 in these cell cultures. These calculations demonstrate that TG-esterified 22:6ω3 is not as efficiently secreted as TG-esterified 18:1. This conclusion is consistent with the results in Table 7 and 8 (row A, middle and right columns) in that at 8-hr incubation: i) extracellular TG-esterified [1-^{14}C]18:1 was 2.3-times higher than the corresponding extracellular TG-esterified [1-^{14}C]22:6ω3; whereas ii) total extracellular TG in the 18:1-treated hepatocytes was actually only 1.5-times higher than that in 22:6ω3-treated cells.

Yao and Vance (23) reported that phosphatidylcholine synthesis is obligatory for the secretion of VLDL particles. We did not evaluate the effect of 18:1 and 22:6ω3 on the rate of synthesis of this phospholipid in our system. However, inhibition of phosphatidylcholine synthesis by 22:6ω3 would not account for the differential secretion of TG-esterified 18:1 and TG-esterified 22:6ω3. One would otherwise have expected identical effects of 22:6ω3 on the secretion efficiency on all types of TG (Table 9). Nossen *et al.* (22) reported that 95% of TG secreted by cultured hepatocytes is recovered with the VLDL fraction. It is therefore unlikely that secretion of TG in a form other than VLDL will account for the differences in the secretion efficiency of TG-esterified 18:1 and 22:6ω3.

Rustan *et al.* (3) reported that for 20:5ω3 and 18:1, the FA to BSA ratio affected the rate of TG synthesis and secretion by cultured hepatocytes. In contrast, Wong *et al.* (9) reported that changing the FA to BSA ratio from 5:1 to 2:1 did not change the relative effects of 18:1, 20:5ω3 and 22:ω3 on TG production by HepG2 hepatocytes. In our experiments, we used a constant and physiological FA to BSA ratio of 1.67 (24,25) at which to compare 18:1 to 22:6ω3. While it is true that the absolute values of the results of our experiments may vary depending on the FA to BSA ratio (3), the relative metabolic effects of 18:1 and 22:6ω3 in our system (Tables 2, 3, 6 and 7) will likely remain the same. Rustan *et al.* (3) also reported that 22:6ω3 as well as 18:1 stimulated TG secretion by cultured hepatocytes and to a much higher degree than what we observed relative to controls (Table 7). We cannot account for this discrepancy, except for differences in the source of hepatocytes, in the culture conditions and in methods for the assay of TG secretion. During the review of this manuscript, Lang and Davis (26) published a similar study on the effects of 18:1, 20:5ω3 and 22:6ω3 on TG secretion by cultured hepatocytes. It is interesting to note that their results, when expressed as absolute TG mass, are consistent with our observations on TG synthesis and secretion. However, when they used [^{3}H]glycerol incorporation to measure TG synthesis and secretion, the effects of 18:1 and 22:6ω3 relative to controls were similar to those reported by Rustan *et al.* (3) who had used (^{3}H)glycerol incorporation as the basis for their TG measurements.

TABLE 9

Efficiency of Secretion of 18:1- and 22:6ω3-Containing TG

		Incubation time (hr)		
		2	8	24
Treatment groups[a]	Index of TG secretion[b]	mean ± s.e.m. (n = 3)[c]		
18:1	$\frac{TG_o}{TG_{tot}} \cdot 100$	2.96 ± 0.18	5.18 ± 0.14	6.88 ± 0.73
22:6ω3		1.66 ± 0.19[d]	3.09 ± 0.16[d]	5.37 ± 0.24
18:1	$\frac{18{:}1TG_o}{18{:}1TG_{tot}} \cdot 100$	3.05 ± 0.32	4.79 ± 0.31	6.72 ± 0.73
22:6ω3		2.29 ± 0.41	5.25 ± 0.55	6.88 ± 0.75
18:1	$\frac{22{:}6\omega3TG_o}{22{:}6\omega3TG_{tot}} \cdot 100$	0	0	0
22:6ω3		0.98 ± 0.13[e]	2.00 ± 0.28[e]	5.31 ± 0.11[e]

[a]Hepatocytes were incubated with 1 mM 18:1 or 22:6ω3 for the indicated time periods.

[b]Index of TG secretion is the percentage of secreted TG (TG_o) relative to the total accumulated TG (TG_{tot}) in the culture system. It represents the efficiency by which TG is secreted by the hepatocytes. Specific indices of TG secretion were also calculated to reflect the efficiency by which TG-esterified 18:1 ($18{:}1TG_o$) or TG-esterified 22:6ω3 ($22{:}6\omega3TG_o$) were secreted by the cells.

[c]Intracellular and extracellular (culture medium) TG were extracted and isolated by TLC in the presence of added internal standards. Quantitation was by GLC. Arcsin transformation was used prior to statistical analysis. Significant differences between treatment group means are denoted by "d" ($p<0.005$) for the indices of total-TG secretion and by "e" ($p<0.005$) for the indices of secretion of TG-esterified 18:1 in the 18:1-treatment group *versus* the indices of secretion of TG-esterified 22:6ω3 in the 22:6ω3 treatment group. There were no significant differences between the treatment group means for the indices of secretion of TG-esterified 18:1.

While the *in vitro* system used in our experiments is necessarily a short-term model, our data has certain similarities to *in vivo* data. For example, the differences in the uptake of 18:1 *versus* 22:6ω3 (Fig. 1, Table 2) are similar to those reported by Parks *et al.* (27) for 18:1 *versus* 20:5ω3 uptake in perfused livers from African green monkeys fed a long-term fish oil diet. Furthermore, their results on the diversion of 20:5ω3 to PL are consistent with our observations (Tables 5 and 6). Secondly, hepatic TG accumulation might be expected when TG secretion, but not synthesis, is inhibited. This was the case in fish oil-feeding experiments reported by Haug and Hostmark (8).

Our results provide further evidence that TG secretion can occur without reduced TG synthesis. Figure 3 summarizes the hepatic flux of FA through successive metabolic branchpoints that ultimately determine the rate of TG secretion. Multiple and varying effects of the ω3PUFA (described in the literature and alluded to in the introduction) may arise from changes in the relative importance of each one of the steps in Figure 3, depending on experimental conditions used. For example, Beynen and Katan (28) proposed and cited evidence that inhibition of TG secretion due to dietary PUFA may arise from diversion of these PUFA to β-oxidation away from esterification. Our results indicate that the partition of nascent TG between VLDL assembly and accumulation of cystosolic TG is directed toward the retention of 22:6ω3. In support of this hypothesis, others have shown that the three cellular TG pools, namely cytosolic, nascent microsomal and VLDL, have distinct turnover kinetics (29-31). This indicates that the interchange between these TG pools is regulated by specific mechanisms. It is conceivable that these mechanisms are able to distinguish between different molecular species of TG.

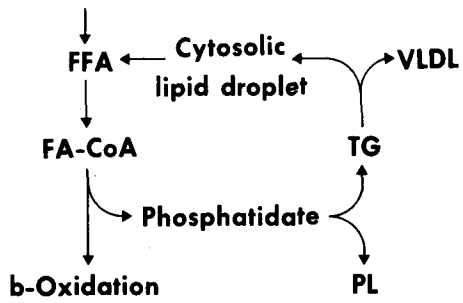


FIG. 3. Flux of fatty acids in the hepatocyte.

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Oxysterol Incorporation into Rat Aorta Resulting in Elastin Compositional Changes

J.W. Blankenship[a,b,*], C.M. Van Gent[a], L.B. Sandberg[a,c], P.J. Roos[a,d], and J.A. Scharffenberg[e]

[a]Jerry L. Pettis Memorial Veterans Hospital, Loma Linda, California 92357, Departments of [b]Nutrition, [c]Pathology, and [d]Medicine, Loma Linda University, Loma Linda, California 92350, and [e]Pacific Health Education Center, Bakersfield, California 93301

The incorporation of dietary cholestan-3β, 5α, 6β-triol (triol) into rat thoracic aortic tissue and changes in amino acid composition of the elastin were investigated to identify the cytotoxic properties of the triol. Weanling male Sprague-Dawley rats were fed the following diets for three months: (i) normal chow, (ii) normal chow with 1% (w/w) cholesterol added, or (iii) normal chow with 0.9% (w/w) cholesterol and 0.1% (w/w) triol added. Triol levels in the blood and in the thoracic aortic tissue were measured. Compositional changes of elastin were also determined. After three months on the triol-containing diet, triol was found in the thoracic aorta but was not detected in the blood. Amino acid analyses of the aortic tissue elastin revealed that the proline levels in the triol-fed animals were significantly greater than in the other two diet groups, while the elastin levels of leucine, aspartate, arginine, and phenylalanine decreased significantly. The mechanism for these observed changes induced by triol may reflect alternate splicing of elastin messenger ribonucleic acid (mRNA) resulting in structual changes in the elastin molecule. Dietary triol does contribute to tissue triol content and is associated with aortic elastin compositional changes. How these changes may contribute to the development of cardiovascular disease is not known.

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When the cytotoxicity of oxysterols in connective tissue, *e.g.* fibroblasts and vascular smooth muscle cells, was investigated in tissue culture, a particularly marked inhibition of cellular growth was observed due to cholestan-3β, 5α, 6β-triol (triol) (1). The toxicity of triol has also been observed in whole animals. Intravenous injections of triol into rabbits produced within 24 hr lesions in the luminal surface of the aorta (2). Also by long-term feeding of triol at 0.1% of the ration, Cook and MacDougall (3) observed the development of atherosclerotic lesions in rabbits resembling those found in the humans. Connective tissue involvement in triol toxicity has been noted by Matthias *et al.* (4), who found that oral administration of triol to rats produced distinct loosening of the elastin integrity at the endothelial edge of the internal elastic lamina of the aorta as evidenced by electron microscopy. The possibility of dietary cholesterol oxides being involved in human atherosclerosis has also been proposed by Jacobson (5). The observation was made on two Indian populations that used ghee (a butter with high cholesterol oxide content) and which experienced a high incidence of mortality and morbidity from atherosclerosis even though serum cholesterol levels were not elevated.

In this context, the effects of cholesterol oxidation products, which occur in normal human foods containing cholesterol, are of interest. A typical mixed diet as normally consumed by the Dutch has been analyzed for oxysterols by van de Bovenkamp *et al.* (6). They found that the normal intake of total oxysterols would amount to only a few mg per day with the known cytotoxic triol being present in only trace amounts. However, cholesterol-5α,6α-epoxide (α-epoxide) occurred at a concentration of about 0.5 mg per 500 g (dry wt) food eaten per day. Although the food contained only traces of the cytotoxic triol, Maerker *et al.* (7) have shown that the normal acidity of the stomach largely hydrolyzes the α-epoxide to the triol. Thus, the normal human food that contains cholesterol may be the source of a small but significant quantity of triol. The potential for triol absorption, incorporation into tissue, and storage or accumulation is unknown.

The present study is concerned with the contribution of dietary triol to the presence of triol in blood and thoracic aortic tissue of rats. In addition, because of reported loss of aortic elastin integrity of the internal elastic lamina (4), the amino acid compositional characteristics of aortic elastin as related to tissue incorporation of triol have been investigated.

MATERIALS AND METHODS

Animals and diets. Weanling male (21-29-day-old) Sprague-Dawley rats (37) were placed on three different diets for three months (Table 1). The triol and cholesterol for diet preparation were purchased from Sigma Chemical Company (St. Louis, MO). The cholesterol used in this feeding study did not contain measurable amounts of triol as contaminant as determined by gas-liquid chromatography (GLC). At the end of the experimental period, the thoracic aorta along with blood samples were collected from each animal. Total serum cholesterol concentra-

TABLE 1

Diet Composition (in wt%)

Group[a]	Lab chow	Corn oil	Cholesterol	Cholestan-3β, 5α, 6β-triol
1 (10)	95.0	5.0	0.0	0.0
2 (14)	94.0	5.0	1.0	0.0
3 (13)	94.0	5.0	0.9	0.1

[a]Number of animals per group in parentheses.

*To whom correspondence should be addressed at Jerry L. Pettis Memorial Veterans Hospital, Research 151, Loma Linda, California 92357.

Abbreviations: ANOVA, Analysis of variance; α-epoxide, cholesterol-5α, 6α-epoxide; GLC, gas-liquid chromatography; HPLC, high-performance liquid chromatography; TMS, trimethylsilyl ether; triol, cholestan-3β, 5α, 6β-triol.

tions were determined on 37 animals. The quantities of triol in thoracic aortic tissue and serum were determined by GLC. Samples of aortic tissue elastin were also hydrolyzed, and amino acid contents were measured. Aortic tissue from some of the animals was used in another study not reported here.

Serum cholesterol determination. The colorimetric cholesterol oxidase method for total serum cholesterol determination utilized Kit #352 from Sigma.

Isolation of the triol. Total lipid extractions were done by the Bligh and Dyer procedure (8) on plasma (1 mL) or thoracic aorta tissue (0.5-1.0 g) with 40 μg of 5α-cholestane added as an internal standard (9). Baker analyzed reagent grade $CHCl_3$ and methanol were used without further purification. The $CHCl_3$/methanol mixture contained 0.1% butylated hydroxytoluene as antioxidant. The chloroform extract was evaporated to dryness on a rotary evaporator and the lipid residue was saponified in 2 mL 1N KOH in 95% ethanol at room temperature for 12-15 hr (10), which was sufficient to complete hydrolysis of steryl esters as ascertained by thin-layer chromatography. Low temperature hydrolysis was done to avoid decomposition of triol, which has been reported to be sensitive to saponification conditions (6). Treatment of a cholesterol containing sample under these conditions did not produce a measurable amount of triol.

Park and Addis (9) isolated the nonsaponifiables from the saponification mixture by multiple diethyl ether extraction. However, we noted that the triol was much more soluble in chloroform than in diethyl ether. The extraction procedure we used was compared to that by Park and Addis (9) and was considered preferable.

The ethanolic KOH (2 mL) was diluted to 6 mL with water in a Teflon-lined screw-capped tube (12 × 125 mm). Six mL of chloroform was added, and upon mixing, the mixture was centrifuged to facilitate phase separation. The upper aqueous phase was removed by pipette and discarded. To remove residual free fatty acids, the $CHCl_3$ solution was washed two times with 0.1N KOH in 40% ethanol. Finally, the $CHCl_3$ phase was washed with 5 mL water to remove residual KOH and then evaporated to dryness on a rotary evaporator. Residual water was removed by repeated evaporation in the presence of absolue ethanol. The dried residue was redissolved in $CHCl_3$ for storage at -20°C until analyzed.

Determination of triol by GLC. The sterol containing preparation can be dissolved in $CHCl_3$ and analyzed as such or as trimethylsilyl ether (TMS). TMS derivatives are formed by dissolving the dry nonsaponifiable residue in 0.1 mL pyridine followed by the addition of 0.05 mL of Sylon BTZ (Supelco, St. Louis, MO) (9). The derivative is formed within 10-15 min and is directly injected into a capillary GLC column. Parallel analysis of derivatized and non-derivatized sterols corroborates the assignments made. A Varian model 3700 gas chromatograph equipped with a DB1 column (megabore 0.53 mm × 30 m; J&W Scientific, Inc., Rancho Cordova, CA) was used. The injector temperature was 260°C and the detector temperature was held at 280°C. Helium was used as carrier gas at a flow rate of 6 mL per min. The oven temperature was held at 100°C for 2 min, then raised at a rate of 20°C/min to 270°C, and held for 25 min. Under the above conditions, the following relative retention times were observed: 5α-cholestane (internal standard), 1.00; TMS-cholesterol, 1.40; cholesterol, 1.28; TMS-triol, 2.00; triol, 2.22. Quantifications were made by comparing peak areas with those of internal standards.

Tissue was extracted with chloroform/methanol by Bligh and Dyer extraction (8). The delipidated tissue was air-dried and weighed. The data reported in Table 3 were measured on non-derivatized sterols.

Elastin preparation, high-performance liquid chromatography (HPLC) peptide analysis and amino acid analysis. Thoracic aortic elastin was isolated by subjecting the tissue to hot alkali treatment following the Lansing procedure (11). Elastin was quantified by thermolytic peptide analysis. The thoracic aorta elastin was digested with thermolysin for 6 hr using conditions described by Sandberg *et al.* (12), and the resulting peptides were quantitated by reverse-phase HPLC.

Aliquots of each of the thermolysin digests containing the elastin peptides were hydrolyzed with 3 N mercaptoethanesulfonic acid at 110°C for 24 hr (13). The amino acid content of the elastin peptide hydrolysates was determined by ion exchange chromatography using a ninhydrin system (Biotronik model 500l, Maintal, Germany).

RESULTS AND DISCUSSION

The animals that received 1% cholesterol (group 2) in the diet showed a modest increase in serum cholesterol (Table 2), while the serum cholesterol levels of animals that received 0.9% cholesterol plus 0.1% triol (group 3) were not significantly changed by the dietary treatment. It has been reported that the triol inhibits the activity of 3-hydroxy-3-methylglutaryl coenzyme A reductase in cell culture (15). This may account for the lack of a significant difference between groups 1 and 3.

TABLE 2

Serum Cholesterol Levels of Male Rats on Three Month Diets[a]

Group			Serum cholesterol (mg/dL ± S.E.)
1 Control		(10)	76.6 ± 5.3
2 Cholesterol		(14)	96.6 ± 2.5
3 Cholesterol-triol		(13)	89.4 ± 6.4
Group 1	*vs*	Group 2	$p < .05$
Group 1	*vs*	Group 3	n.s.
Group 2	*vs*	Group 3	n.s.

[a]Number of animals per group in parentheses. Analysis of variance followed Bonferroni's procedure for the statistical comparison of mean values for groups (14).

Both the serum and thoracic aortic tissues were subjected to triol analyses. The amounts of triol in the serum were below our detection level (0.003 mg/mL) which raised questions concerning the absorption of the triol. However, the triol does accumulate in the tissues (see Table 3). The animals that received 0.1% triol in the diet over a three-month period showed significant levels of triol in the thoracic aorta. The presence of the triol appears to influence cholesterol incorporation into tissue, as cholesterol contents in arteries of triol-fed animals were higher than in controls (Table 3).

Elastin very well lends itself to compositional studies because of its unusual amino acid composition. It is high

TABLE 3

Cholesterol and Cholestan-3β, 5α, 6β-Triol Concentration in Rat Thoracic Aorta Fat Free Dry Tissue[a]

Group		Cholesterol (mg/g)	Triol (mg/g)
Control	1	3.7	<0.003
	2	4.0	<0.003
	3	3.5	<0.003
	4	3.8	<0.003
		mean = 3.75 ± 0.10[b]	<0.003
Chol.	1	4.0	<0.003
	2	3.3	<0.003
	3	4.4	<0.003
	4	4.3	<0.003
		mean = 4.0 ± 0.25	<0.003
Chol.-Triol	1	4.6	0.019
	2	4.6	0.045
	3	4.1	0.030
	4	4.1	0.029
	5	4.8	0.053
	6	4.6	0.037
		mean = 4.46 ± 0.12[b]	0.036 ± 0.005
ANOVA[c]		P < 0.05	

[a]Results expressed as mean ± SEM.

[b]Values are significant at 99% confidence level, Fisher Protected Least Significant Difference. Statistical analyses were carried out by Statview Computer Program (16). Determinations were performed on non-derivatized sterol samples.

[c]Analysis of variance.

in hydrophobic amino acids (glycine, alanine, valine, proline), and very low in hydrophilic ones (aspartate, glutamate, lysine, arginine). It is also characterized by the presence of the unique desmosine crosslinks and the absence of histidine and methionine (17). In addition, the nucleotide composition of the rat elastin message has recently been determined, allowing us to tie transcriptional and translational events to protein expression for smooth muscle cells of the aorta (18). To evaluate qualitative changes in the elastin, aliquots of all the thermolysin digests were acid hydrolyzed and the amino acid compositions determined (Table 4). In our hands, the mercaptoethanesulfonic acid hydrolysis procedure (13) gave better reproducibility than the usual 6N HCL method when applied to elastins (Blankenship, J.W., and Sandberg, L.B., unpublished observations). Furthermore the isodesmosine/desmosine contents were not different for any of the groups which indicates that the triol did not influence the degree of crosslinking during elastogenesis. Also, in the control and cholesterol-fed animals, no differences in amino acid compositions were detected. However, the elastin amino acid composition of group 3 (triol-fed rats) was significantly different from either the control or cholesterol-fed groups with respect to five amino acids (Table 4). The proline was increased in the triol group compared to both control and cholesterol groups, while leucine, aspartate, arginine, and phenylalanine were decreased in the same group (see Table 4 for statistical analysis).

In Table 5 we list the amino acid compositions resulting from translation of exons 30 through 36 in the rat (18). These exons are located at the 3′ end of the message representing the carboxyl terminal portion of the protein. The C-terminus of the tropoelastin molecule is proline-poor and arginine-rich. In elastin, the even-numbered exons code for hydrophobic regions of the protein while odd-numbered exons code for more hydrophilic regions (crosslinking). Notably, crosslink content was not affected by triol but the hydrophobic residues were. Deletion of several of the even-numbered exons shown in Table 5 would account for most of the amino acid compositional changes observed within the triol group. Alternate splicing in the rat has been noted with exon 33 (18).

TABLE 4

Amino Acid Composition of Elastin Thermolytic Digests from Thoracic Aortae of Rats Fed Cholesterol and Cholestan-3β, 5α, 6β-Triol (expressed as residues/1000)[a]

Amino acid	1-Control	2-Cholesterol	3-Cholestan-3β, 5α, 6β-triol	ANOVA[b]
Hydroxypro	17.23 ± 0.34	13.98 ± 1.13	16.46 ± 1.35	n.s.
Asp	6.35 ± 0.27[c]	6.11 ± 0.14[d]	5.42 ± 0.08[d,c]	p<0.05
Thr	12.64 ± 0.43	11.59 ± 0.61	10.59 ± 0.44	n.s.
Ser	11.08 ± 0.63	8.96 ± 1.41	8.38 ± 1.13	n.s.
Glu	15.40 ± 0.17	15.41 ± 0.17	14.84 ± 0.16	n.s.
Pro	108.40 ± 1.42[c]	109.54 ± 1.18[c]	122.14 ± 1.27[c]	p<0.001
Gly	349.93 ± 3.92	348.93 ± 2.15	357.05 ± 2.34	n.s.
Ala	213.29 ± 3.74	217.03 ± 1.61	213.46 ± 3.41	n.s.
Val	91.03 ± 1.49	93.17 ± 1.29	89.31 ± 3.93	n.s.
Ile	23.41 ± 0.07	23.02 ± 0.97	21.15 ± 0.50	n.s.
Leu	59.41 ± 1.42[c]	59.09 ± 1.04[d]	55.61 ± 0.36[c,d]	p<0.05
Tyr	31.66 ± 0.69	31.00 ± 1.01	28.89 ± 1.31	n.s.
Phe	15.63 ± 0.10[d]	15.98 ± 0.39[c]	14.50 ± 0.17[c,d]	p<0.01
Isodes-des	1.38 ± 0.04	1.36 ± 0.06	1.22 ± 0.07	n.s.
Lys	2.72 ± 0.06	2.79 ± 0.12	2.61 ± 0.09	n.s.
Arg	12.28 ± 0.41[d]	13.37 ± 0.64[c]	8.88 ± 1.46[d,c]	p<0.05

[a]Results expressed as ± SEM for four animals per group.

[b]Significance calculated by one-way analysis of variance. Group comparisons made by Fisher Protected Least Significant Difference. Statistical analyses employed the Statview Computer Program (16).

[c]Values differ at 99% confidence level, group 3 compared to groups 1 or 2.

[d]Values differ at 95% confidence level, group 3 compared to groups 1 or 2.

TABLE 5

Amino Acid Sequences (in single letter code) Resulting from Translation of Exons Located at the 3′ Translated End of Rat Tropoelastin Messenger RNA[a]

29.	G G A P A A A A A A K A A A K A A Q Y
30.	G L G G A G G L G A G G L G A G G L G A G G L G A G G L G A G G L G A G G V I P G A V G L G
31.	G V S P A A A A K A A K Y
32.	G A A G L G G V L G A R P F P G G
33.	G V A A R P G F G L S P I Y P
34.	G G G A G G L G V G
35.	G K P P K P Y G G A L G G L G Y Q
36.	G G G C F G K S C G R K R K

[a]From data by Pierce *et al.* (18). Rat elastin exons 29 to 36 represent 152 contiguous amino acid residues of the C-terminal portion of the secreted protein (one-fifth of the total protein). Proline (P) and arginine (R) are underlined. Of 9 orginine residues, 4 are contained herein, but of 110 proline residues only 11 are present. Thus the area is arginine-rich and proline-poor with respect to the remainder of the protein. These two amino acids manifested the most dramatic changes from controls in aortic elastin from rats treated with triol (proline increasing and arginine decreasing).

Because the C-terminus of tropoelastin contains the probable deletions, other mechanisms that might be invoked are premature termination of the translation process on the ribosome, or clipping of the C-terminal portion of the protein prior to its incorporation into the elastin fiber. Activation of a tissue elastase by triol could bring about the latter process.

The physiological consequences of the above alterations are presently not appreciated. However, administration of oxysterols, with triol being the most toxic, has been shown to produce arterial endothelial injury in one study (2). *In vitro* studies have demonstrated oxysterol toxicity to smooth muscle cells (1,15,19) and fibroblasts (1), and oxysterols have been shown to induce alterations in membrane structure and function (20).

Thus, modification of connective tissue elastin upon triol feeding merits further investigation inasmuch as elastin modification also affects tissue morphology (4). Formation of atherosclerotic lesions upon triol feeding (3) would suggest that triol may contribute to the pathologic changes noted in atherosclerotic disease involving connective tissue modifications.

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Effect of n-3 Fatty Acids on the Key Enzymes Involved in Cholesterol and Triglyceride Turnover in Rat Liver

Ayman AL-Shurbaji[a]*, Carin Larsson-Backström[b], Lars Berglund[a], Gösta Eggertsen[a] and Ingemar Björkhem[a]

[a]Department of Clinical Chemistry, Karolinska Institute at Huddinge University Hospital, Huddinge and [b]Kabi Nutrition, Research and Development, Stockholm, Sweden

The effect of long-chain n-3 fatty acids on hepatic key enzymes of cholesterol metabolism and triglyceride biosynthesis was investigated in two rat models. In the first model, rats were intravenously infused for two weeks with a fat emulsion containing 20% of triglycerides in which either n-6 or n-3 fatty acids predominated. The treatment with n-3 fatty acids led to a reduction primarily of serum cholesterol (45%), but also of serum triglycerides (18%). HMG-CoA reductase activity and cholesterol 7α-hydroxylase activity were reduced by 45% and 36%, respectively. There were no significant effects on diacylglycerol acyltransferase (DGAT) or phosphatidate phosphohydrolase (PAP) activities. In the second model, rats were fed a diet enriched with sucrose, coconut oil and either sunflower oil (n-6 fatty acids) or fish oil (long-chain n-3 fatty acid ethyl esters). The treatment with n-3 fatty acids decreased serum triglycerides (41%) and, to a lesser extent, serum cholesterol (17%). Neither glycerol 3-phosphate acyltransferase (GPAT) or DGAT were affected by n-3 fatty acids. In contrast, PAP activity was reduced by 26%. HMG-CoA reductase was not significantly affected, whereas cholesterol 7α-hydroxylase activity was reduced by 36%. The results indicate that part of the TG-lowering effect of long-chain n-3 fatty acids may be mediated by inhibition of the soluble phosphatidate phosphohydrolase. The effect on serum cholesterol may be partly due to inhibition of HMG-CoA reductase.

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The triglyceride (TG)-lowering effect of marine fish oils rich in n-3 polyunsaturated fatty acids is well documented (1-4). However, the underlying mechanism has not yet been elucidated in detail. Results of studies using perfused rat liver as model seem to indicate that several mechanisms might be operative. Reduced hepatic lipogenesis, retarded very low density lipoprotein (VLDL) secretion, and enhanced fatty acid oxidation have all been suggested (5).

Suppression of synthesis and secretion of triglycerides by n-3 fatty acids has been demonstrated in cultured rat hepatocytes (6), as well as in human hepatoma cells (7). Metabolic studies in humans have also indicated that n-3 fatty acids inhibit the production of VLDL TG in the liver (2), and that this inhibition is not due to a decrease in the availability of circulating fatty acid substrate. The possibility that reduced synthesis of TG induced by n-3 fatty acids might be due to an inhibition of one or more of the enzymes involved in TG biosynthesis has therefore been investigated. Infusion of eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA) in perfused rat liver has been reported to decrease total phosphatidate phosphohydrolase (PAP) activity (8). In another study, a significant reduction in the Mg^{++}-dependent PAP activity in rat liver was observed as a result of feeding a fish oil-enriched diet (9). This result is somewhat hampered, however, by the lack of suitable controls.

Different results on the effect of n-3 fatty acids on diacylglycerol acyltransferase (DGAT) activity have been reported. In some studies treatment with n-3 fatty acids reduced hepatic synthesis and secretion of TG by inhibiting DGAT activity (10). In other studies, such an inhibition could not be demonstrated (9).

n-3 Fatty acids reduce serum cholesterol levels in humans, as well as in experimental animals (1,11,12). In general, the effect on serum cholesterol is less pronounced than that on serum TG, and in some studies no effect on serum cholesterol was observed (13). Again, the underlying mechanisms are still not clear. In a recent study, HMG-CoA reductase activity was found to be decreased in rats fed sardine oil (n-3 fatty acids) compared to rats fed safflower oil (n-6 fatty acids). However, cholesterol 7α-hydroxylase was not affected (14).

In the present work we have investigated in two rat models the effect of n-3 fatty acids on the rate-limiting enzymes in the synthesis and degradation of cholesterol, and on certain key enzymes of TG biosynthesis. We found that in the infusion model, n-3 fatty acids markedly reduced serum cholesterol levels, and in the dietary model they markedly reduced serum TG levels.

MATERIALS AND METHODS

Animals and animal treatment. Male Sprague-Dawley rats were used. The animals were housed in metal cages maintained at a 12-hr light-dark cycle.

In the first animal model used, Sprague-Dawley rats weighing about 200 g were divided in two groups, one of which was intravenously infused with Intralipid® (Kabi Nutrition, Stockholm, Sweden) containing 20% soybean oil, in a dose of 25 mL/kg body weight/24 hr for two weeks. The other group was infused in the same way with a corresponding fat emulsion supplemented with 20% marine oil. The composition of both oils is shown in Table 1. The infusion was carried out as described previously (15). The animals had free access to water and pellet diet (R_3; Ewos AB, Södertälje, Sweden). Each day the infusion was stopped for a period of 4 hr (9 a.m.-1 p.m.). With the equipment used, the animals were able to move freely in the cages. There was no significant difference in weight

*To whom correspondence should be addressed at Department of Clinical Chemistry I, Huddinge University Hospital, S-141 86, Huddinge, Sweden.

Abbreviations: DGAT, diacylglycerol acyltransferase; DHA, docosahexaenoic acid; DTT, dithiotheitol; EPA, eicosapentaenoic acid; GPAT, glycerol 3-phosphate acyltransferase; PAP, phosphatidate phosphohydrolase; TG, triglycerides, triacylglycerols; VLDL, very low density lipoprotein.

gain between the two groups during the experimental period. On the day of sacrifice, the infusion was terminated (9 a.m.), 2 hr later the animals were anaesthetized using Mebumal (60 mg/kg). Blood was collected by heart puncture, and livers were removed.

TABLE 1

Fatty Acid Composition of Marine Oil and Soybean Oil Used in the Infusion Experiments[a]

Fatty acid	Marine oil %	Soybean oil %
Eicosapentaenoic acid	18	<1
Docosahexaenoic acid	12	<1
α-Linolenic acid	1	6-12
Total n-3 fatty acids	37	6-12
18:2 Fatty acid	1	47-57
Total n-6 fatty acids	4	48-58
Saturated fatty acids	25	10-20
Monoenoic fatty acids	25	19-29

[a]The soybean oil was commercial Intralipid. The fatty acid composition in the different batches used varied as shown. Only one batch of the marine oil was used, with the fatty acid composition as given.

In the second animal model, 3-week-old rats weighing about 100 g each were fed a diet containing 45% sucrose, 15% coconut oil and 5% fish oil, which contained 85% n-3 fatty acid ethyl esters (55% eicosapentaenoic acid and 30% docosahexaenoic acid) for 3 weeks. The control rats were fed a corresponding diet with the fish oil substituted by sunflower oil (containing 66% linoleic acid and less than 1% n-3 fatty acids). There were no differences in weight gain between the two experimental groups. The animals were decapitated in the morning (8 a.m.), blood was collected, and the livers were removed. No anaesthesia were used.

The animal experiments were approved by the ethical committee for animal experiments.

Materials. [1-^{14}C]Palmitic acid with a specific activity of 54 mCi/mmol, [1-^{14}C]palmitoyl-CoA with a specific activity of 56 mCi/mmol and *L*-[U-^{14}C]glycerol 3-phosphate with a specific activity of 171 mCi/mmol were purchased from the Radiochemical Centre (Amersham, England). [4-^{14}C]Cholesterol and [3-^{14}C]hydroxymethylglutaryl-CoA with specific activities of 55-60 mCi/mmol were obtained from New England Nuclear Corp. (Boston, MA). $^{2}H_3$-Labeled 7α-hydroxycholesterol was synthesized as described previously (16).

The marine oil emulsion was kindly supplied by Kabi Nutrition, Research and Development (Stockholm, Sweden). Fish oil was a generous gift from Dr. Knut Dahl, Norsk Hydro (Porsgrunn, Norway). All other chemicals used were products of Sigma Chemical Company (St. Louis, MO).

Enzyme assays. For assay of the enzymes involved in triglyceride synthesis, rat livers were rinsed, minced, and homogenized with 4 volumes of 250 mM sucrose, 20 mM Tris/HCl, pH 7.4, 1 mM EDTA, and 1 mM dithiothreitol (DDT). The homogenates were centrifuged at 20,000 × *g* for 15 min at 4°C, and the resulting supernatant was spun at 100,000 × *g* for 60 min at 4°C. The microsomes obtained were suspended in the homogenization buffer and used for GPAT and DGAT assays. The 100,000 × *g* supernatant was precipitated with ammonium sulfate as described by Lamb and Fallon (17), and used as a source of cytosolic PAP activity. PAP activity was assayed for 15 min. at 37°C as described earlier (18). The extraction procedure, thin-layer chromatography, and radio-scanning of chromatography plates were performed as described above (18).

Microsomal GPAT was assayed for 3 min at 30°C, essentially as described by Bates and Saggerson (19). The incubation mixture contained 120 mM KCl, 50 mM Tris/HCl pH 7.4, 65 μM palmitoyl-CoA, 0.7 mM DTT, 1 mM *L*-glycerol 3-phosphate, and 1.75 mg fatty acid-poor bovine serum albumin. The reaction was started by the addition of microsomes (200 μg protein) and terminated by the addition of 2.0 mL of 1-butanol. The incorporation of ^{14}C into butanol-soluble products was determined as described (19,20).

DGAT was assayed for 5 min. at 37°C. The incubation mixture contained 100 mM Tris/HCl pH 8.0, 4 mM $MgCl_2$, 250 μM 1,2-dioleoyl-*sn*-glycerol, 30 μM [1-^{14}C]palmitoyl-CoA (200,000 cpm/incubation), and 1 mg/mL fatty acid-poor bovine serum albumin in a final volume of 1 mL. The reaction was initiated by the addition of microsomes (30-40 μg protein) and terminated by the addition of 7.5 mL of 2-propanol/hexane/water (80:20:1, v/v/v). The extraction of TG into hexane was done as described by Coleman and Bell (21).

For assay of HMG-CoA reductase and cholesterol 7α-hydroxylase, a 10% (w/v) homogenate of rat liver was prepared in 50 mM Tris/HCl buffer (pH 7.4) containing 0.3 M sucrose, 50 mM NaCl, 10 mM EDTA, and 10 mM DTT. The microsomal fraction was prepared by centrifugation of the homogenate at 20,000 × *g* for 15 min and recentrifugation of the supernatant at 100,000 × *g* for 60 min. Half the microsomal fraction was resuspended in the homogenizing buffer and recentrifuged at 100,000 × *g* for 60 min. The resulting fraction was suspended in 20 mM imidazol/HCl buffer (pH 7.4) containing 10 mM DTT, and used for assay of HMG-CoA reductase. The other half of the original microsomal fraction was suspended in a homogenizing buffer lacking DTT and recentrifuged at 100,000 × *g* for 60 min. This fraction was resuspended in 0.1 M potassium phosphate buffer, pH 7.4, and used for assay of cholesterol 7α-hydroxylase.

HMG-CoA reductase activity was assayed essentially as described by Brown *et al.* (22). The incubation was run for 15 min and terminated by addition of 25 μL 6N HCl. [^{3}H]Mevalonic acid was added as internal standard. The incubation mixture was further lactonized, subjected to thin-layer chromatography (TLC), and analyzed for radioactivity (22).

For assay of cholesterol 7α-hydroxylase, the microsomal fraction was incubated with [4-^{14}C]cholesterol as described previously (16). In this procedure, the conversion of [4-^{14}C]labeled cholesterol into 7α-hydroxycholesterol is measured by radio-scanning after separation of the product from the substrate by TLC. The conversion of the endogenous microsomal cholesterol into 7α-hydroxycholesterol is measured by isotope dilution/mass spectrometry (16).

Analytical methods. Serum triglycerides and cholesterol were determined by an enzymatic procedure (Boehringer Mannheim) using a Hitachi 737 automatic enzyme analyzer (Hitachi Co., Tokyo, Japan). Triglycerides and phospholipids were extracted from liver homogenates using bonded phase (Bond Elut) columns as described by

Kalazny *et al.* (23). The fatty acid composition of the separated lipids was determined by gas chromatography (24). Protein was measured by the Lowry procedure (25). Results were evaluated for statistical significance by Student's *t*-test.

RESULTS

Several rat models were tried to obtain reproducible effects of n-3 fatty acids on the serum cholesterol and triglyceride levels. Rats on a conventional pelleted chow have low levels of serum triglycerides, and in our study, significant and reproducible effects of n-3 fatty acids were obtained only with a diet which increases serum triglyceride levels in itself. Similar results also have been obtained by another group (Hoestmark *et al.*, personal communication). In the present study, significant effects on serum cholesterol levels were only observed after prolonged and excessive exposure to n-3 fatty acids by intravenous infusion.

As shown in Table 2, treatment with dietary n-3 fatty acids resulted in a slight reduction in serum total cholesterol (17%) and a marked reduction in serum triglycerides (41%). This was accompanied by a 26% decrease in soluble PAP activity. In contrast, GPAT and DGAT were not significantly affected. No effect on HMG-CoA reductase activity was observed. However, cholesterol 7α-hydroxylase activity was depressed when assayed with exogenous substrate (Table 2).

TABLE 2

Effect of Dietary n-3 and n-6 Fatty Acids on Serum Lipids and Hepatic Enzymes[a]

Enzyme activity or lipid level	Fish oil (n-3 fatty acid-enriched)	Sunflower oil (n-6 fatty acid-enriched)
Serum cholesterol mmol/L	2.88 ± 0.28	3.45 ± 0.28
Serum triglycerides mmol/L	0.72 ± 0.15[b]	1.22 ± 0.41
Soluble phosphatidate phosphohydrolase nmol/min/mg protein	1.45 ± 0.12[b]	1.97 ± 0.11
Diacylglycerol acyltransferase nmol/min/mg protein	4.6 ± 0.4	4.3 ± 0.5
Glycerol-3-P-acyl-transferase nmol/min/mg protein	1.1 ± 0.1	1.2 ± 0.1
HMG-CoA reductase pmol/min/mg protein	471 ± 218	563 ± 299
Cholesterol 7α-hydroxylase activity (exogenous substrate) pmol/min/mg protein	3.1 ± 1.2[b]	6.2 ± 2.5
Cholesterol 7α-hydroxylase activity (endogenous substrate) pmol/min/mg protein	18.3 ± 6.3	28.4 ± 8.8

[a]The values represent the means ± SEM of two experiments, n = 12.
[b]$p<0.05$.

More intensive exposure to n-3 fatty acids by intravenous infusion resulted in a marked reduction of serum cholesterol (45%) and a slight reduction of serum triglycerides (18%) (Table 3). Under these conditions HMG-CoA reductase activity was substantially reduced (45%). In parallel, cholesterol 7α-hydroxylase activity was also reduced (64% and 36% when assayed with exogenous and endogenous substrate, respectively). However, PAP activity was not affected. Similarly, no effect on DGAT activity could be demonstrated (Table 3).

TABLE 3

Effect of Infusion of n-3 and n-6 Fatty Acids on Serum Lipids and Hepatic Enzymes[a]

Enzyme activity or lipid level	n-3 Fatty acid-enriched emulsion	n-6 Fatty acid-enriched emulsion
Serum cholesterol mmol/L	1.78 ± 0.33[b]	2.71 ± 0.28
Serum triglycerides mmol/L	0.73 ± 0.09[c]	0.89 ± 0.16
Soluble phosphatidate phosphohydrolase nmol/min/mg protein	2.11 ± 0.52	2.43 ± 1.0
Diacylglycerol acyltransferase nmol/min/mg protein	4.02 ± 0.48	4.56 ± 0.71
HMG-CoA reductase pmol/min/mg protein	265 ± 108[b]	484 ± 121
Cholesterol 7α-hydroxylase activity (exogenous substrate) pmol/min/mg protein	1.40 ± 0.51[b]	3.83 ± 1.57
Cholesterol 7α-hydroxylase activity (endogenous substrate) pmol/min/mg protein	25.9 ± 8.1[b]	40.3 ± 7.61

[a]The values represent the means ± SEM, n = 6.
[b]$p<0.01$.
[c]$p<0.05$.

Treatment with n-3 fatty acids resulted in a marked enrichment of hepatic phospholipids and neutral lipids with n-3 fatty acids in both animal models (Table 4), indicating a significant uptake and assimilation of these fatty acids. The incorporation of n-3 fatty acids into hepatic lipids was, however, not identical in the two models. As shown in Table 4, the n-6/n-3 ratio of liver phospholipids was considerably lower in the dietary model.

DISCUSSION

The TG-lowering effect of n-3 fatty acids observed may result from a reduced influx of TG from the liver or an increase in plasma TG clearance, or both. However, evidence is mounting which suggests that this effect is probably due to a reduction in the influx of TG from the liver. In humans, dietary n-3 fatty acids were found to decrease the biosynthesis of VLDL TG (26). Moreover, n-3 fatty acids have been shown to reduce the secretion of VLDL TG from cultured rat hepatocytes without inhibiting the secretory mechanism *per se* (27). Therefore, it seems probable that the TG-lowering effect was due to a reduction in TG biosynthesis. Such a reduction could result from a decrease in the amount of fatty acids available for TG biosynthesis, or an inhibition of one or more of the enzymes involved in the synthetic pathway. However, previous results argue against the former possi-

TABLE 4

Distribution of n-3 and n-6 Fatty Acids in Liver Lipids

A: Infusion model[a]

	Marine oil	Soybean oil
Phospholipids		
n-6 FA	27.7 ± 0.3	41.5 ± 0.1
n-3 FA	21.8 ± 0.4	9.8 ± 0.2
n-6/n-3 ratio	1.27 ± 0.04	4.23 ± 0.08
Neutral lipids		
n-6 FA	20.4 ± 0.7	43.6 ± 0.5
n-3 FA	27.9 ± 1.0	9.9 ± 0.5
n-6/n-3 ratio	0.73 ± 0.03	4.42 ± 0.20

B: Dietary model[b]

	Fish oil	Sunflower oil
Phospholipids		
n-6 FA	14.7	37.1
n-3 FA	35.7	11.0
n-6/n-3 ratio	0.41	3.36
Neutral lipids		
n-6 FA	11.9	36.4
n-3 FA	12.8	4.9
n-6/n-3 ratio	0.93	7.44

[a]The values represent percentage of the total fatty acid content measured in five rats, with the variation given as SEM.

[b]The values represent percentage of the total fatty acid content from a pool of six rats.

bility (2). Therefore, the latter possibility was investigated in the present study.

Glycerol 3-phosphate acyltransferase catalyzes the first esterification step in TG biosynthesis, and is a potential site for regulation. However, consistent with the results obtained by Wong *et al.* (5), this enzyme was not found to be affected by n-3 fatty acids (Table 2).

Phosphatidate phosphohydrolase is a key enzyme in the regulation of triglyceride biosynthesis. The enzyme activity has been shown to vary in response to hormonal and dietary stimuli affecting TG biosynthesis (28). In a recent study, reduction of plasma TG has been suggested to be partially due to inhibition of PAP activity (29). In the present study, inhibition of the soluble enzyme activity could be demonstrated when serum TG levels were substantially reduced (the dietary model). This strongly suggests that the TG-lowering effect could be mediated by inhibition of the soluble PAP activity. PAP occurs, however, in the microsomes as well, and it has been suggested that stimulation of the enzyme activity might be mediated by a translocation of the cytosolic enzyme onto the microsomes. Thus, an inhibition of such a mechanism could result in suppression of the enzyme activity (30,31). With the assay employed, the microsomal activity was too low to be measured under accurate enzymological conditions (18). However, in a short-term study using the perfused liver model, no effect of n-3 fatty acids on the distribution of the enzyme activity could be demonstrated (8).

The effect of n-3 fatty acids on DGAT activity has been controversial. In the present study, no significant effect on the enzyme activity (Tables 2 and 3) was observed. This is in contrast to the findings of Rustan *et al.* (10) that eicosapentaenoic acid reduced hepatic synthesis and secretion of TG by decreasing the activity of DGAT. These conclusions were, however, mainly based on the finding that eicosapentaenoyl-CoA constituted a "poor" substrate for DGAT activity. A genuine effect of n-3 fatty acids on the enzyme *per se* was not demonstrated. In accordance with our results, Marsh *et al.* (9) did not find any significant effect of n-3 fatty acids on DGAT activity. It cannot be ruled out, however, that differences in the experimental design might at least in part explain the discrepancies noted.

The finding that among the key enzymes involved in TG biosynthesis only PAP was affected by dietary n-3 fatty acids indicates that the TG-lowering effect of these fatty acids could partially be explained by inhibition of PAP activity. Moreover, the results further underscore the significance of PAP as a rate-limiting enzyme in triglyceride biosynthesis.

The effect of n-3 fatty acids on serum cholesterol levels could be due to a number of factors. Of prime significance is the possibility that this effect could be mediated through retarded cholesterol synthesis and/or increased degradation. It is well documented that HMG-CoA reductase is rate-limiting in the synthesis of cholesterol under almost all experimental conditions. Moreover, inhibition of this enzyme has been shown to reduce serum cholesterol levels (32). The infusion of n-3 fatty acids led to a depression of the enzyme activity of a magnitude which was very similar to that of the reduction of serum cholesterol. Thus, it is tempting to suggest that the effect on serum cholesterol was mediated by an inhibition of the reductase. However, the rate-limiting enzyme in the degradation of cholesterol into bile acids, *i.e.*, cholesterol 7α-hydroxylase, was also depressed to a similar degree. The effect of n-3 fatty acids on cholesterol 7α-hydroxylase was about the same in the two animal models, whereas a significant effect on HMG-CoA reductase was obtained only with the infusion model. Since a marked effect on circulating levels of cholesterol was obtained only with the latter model, it seems likely that part of this effect was mediated by suppression of HMG-CoA reductase. Other possible mechanisms include retarded VLDL secretion resulting in decreased LDL levels, increased biliary secretion of cholesterol, and enhanced LDL clearance in plasma.

Although the rats used in the two experimental models were not identical in regards to weight and sexual maturity, the observation that the physiological response varied between the two models used in the present study indicate that the mode of administration of n-3 fatty acids is important. In the dietary model, the intestinal fat load results in production of intestinally derived lipoprotein particles enriched in n-3 fatty acids. However, by infusion of synthetic triglyceride-rich particles the gastrointestinal absorption system is circumvented. It therefore seems likely that the circulating lipoprotein particles in the two models differ with respect to their properties and that the fate of these particles may be dissimilar. This is strengthened by the observation that the incorporation of n-3 into hepatic phospholipids was clearly higher in the dietary model. The fact that different results on triglyceride and cholesterol metabolism were obtained even when using two *in vivo* models underscores the complex action of long-chain n-3 fatty acids on lipoprotein metab-

olism, and emphasizes the value of using whole animal models when studying the physiological impact and mechanisms of action of these fatty acids.

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Synthesis of 6-Phosphatidyl-L-Ascorbic Acid by Phospholipase D

Akihiko Nagao*, **Nobuaki Ishida** and **Junji Terao**
National Food Research Institute, Ministry of Agriculture, Forestry and Fisheries, Tsukuba, Ibaraki, 305 Japan

Phospholipase D (EC 3.1.4.4) of *Streptomyces* species was found to catalyze transphosphatidylation to L-ascorbic acid from phosphatidylcholine (PC) in a biphasic reaction system. The product was identified as 1,2-diacyl-*sn*-glycero-3-phospho-6′-L-ascorbic acid (PA-AsA) by mass spectrometry and nuclear magnetic resonance spectroscopy. The optimal pH of transphosphatidylation was 4.5 and the rate of PA-AsA formation increased as concentrations of L-ascorbic acid increased. The conversion of PC to PA-AsA was greater than 80%. PA-AsA was found to be more resistant to hydrolysis by phospholipase D than was PC.

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Phospholipase D (PLD) catalyzes the hydrolysis of the terminal phosphoester bond of phospholipids and can bring about transphosphatidylation (1,2). By using the transphosphatidylation reaction with PLD, the head group of phospholipids can be modified easily. Thus, phosphatidylglycerol can be produced from phosphatidylcholine and glycerol by catalysis with PLD (3,4). The transphosphatidylation technique also has been shown to be useful for preparing phosphatidylserine and plasmenylethanolamine (5–10). In addition, novel types of phospholipids with various physiological or therapeutic activities can be synthesized by catalysis with PLD. Thus, Shuto *et al.* (11) synthesized phosphatidylnucleosides with antileukemic activity, and Brachwitz *et al.* (12) prepared *O*-alkylglycerophospho-L-serine with cytostatic activity.

We have been working on the cellular defense system against oxygen toxicity, and especially against membrane lipid peroxidation (13–15). The defense against active oxygen radicals at the interface between membrane lipids and the aqueous phase is of considerable importance because the attack of active oxygen radicals originating in the aqueous phase is believed to be one of the initiation steps of membrane lipid peroxidation (16,17). To prevent active oxygen radicals from attacking membrane lipids, it seems effective to arrange an appropriate antioxidant on the surface of a membrane. One possible approach would be to substitute the polar head group of a membrane phospholipid with a functional group having antioxidant activity. L-Ascorbic acid, one of the important antioxidants in the cellular defense against active oxygen radicals (18), is an excellent candidate for a polar head group substitution that would produce phospholipid with antioxidant activity. Moreover, L-ascorbic acid has a primary hydroxy group available for transphosphatidylation by PLD. In this paper, the synthesis of phosphatidyl-L-ascorbic acid by phospholipase D is described.

*To whom correspondence should be addressed.

Abbreviations: COSY, correlation spectroscopy; FAB, fast atom bombardment; HPLC, high performance liquid chromatography; MS, mass spectrometry; NMR, nuclear magnetic resonance spectroscopy; PA, phosphatidic acid; PA-AsA, 6-phosphatidyl L-ascorbic acid; PC, phosphatidylcholine; PLD, phospholipase D; TLC, thin-layer chromatography.

MATERIALS AND METHODS

Phospholipase D from *Streptomyces lydicus* (78.3 U/mg protein) was obtained from Honen Co. (Yokohama, Japan). The two PLD preparations from *Streptomyces* species (1130 and 391 U/mg protein) and PLD from cabbage (0.67 U/mg protein) were purchased from Toyo Jozo Co. (Tokyo, Japan), Sigma Chemical Co. (St. Louis, MO) and Boehringer Mannheim GmbH (Mannheim, Germany), respectively. Phosphatidylcholine (PC) from egg yolk (99% purity) and dimyristoyl PC were purchased from Sigma. Thin-layer chromatography (TLC) plates (Art. 5721) were purchased from Merck (Darmstadt, Germany). Other chemicals were reagent grade.

Enzyme reaction. The enzyme reaction was carried out in a biphasic system consisting of a diethyl ether and an aqueous phase using an 8-mL vial. The standard reaction mixture contained 1 mL of 10 mM egg yolk PC in diethyl ether, 0.8 mL of 1 M L-ascorbic acid, 0.05 mL of the PLD solution which contained 90 U of PLD from *S. lydicus* in 1 mL of 10 mM sodium acetate buffer, pH 5.1, and 0.15 mL of deionized water. The pH of 1 M L-ascorbic acid was adjusted to 4.5 with KOH. The enzyme reaction was initiated by the addition of the enzyme and the mixture was shaken (120 rpm) at 37°C. The reaction was stopped by adding 0.2 mL of 6 N HCl. The product was extracted three times from the reaction mixture with 4 mL of chloroform/methanol (2:1, v/v). The combined chloroform layer was washed with 5 mL of deionized water and evaporated in a rotary evaporator. The extract was dissolved in 5 mL of *n*-hexane/isopropanol (4:1, v/v) and then centrifuged. The supernatant was analyzed by TLC and high performance liquid chromatography (HPLC). PLD units were determined by the method of Kato *et al.* (19).

The hydrolytic activity of the PLD with PC or 6-phosphatidyl-L-ascorbic acid (PA-AsA) as substrate was determined by measuring the amount of phosphatidic acid (PA) formed. The reaction mixture contained 5 μmol of egg yolk PC or PA-AsA prepared from egg yolk PC in 1 mL of diethyl ether and 4.5 U of the PLD of *S. lydicus* in 1 mL of 0.1 M potassium citrate buffer, pH 6.0. The reaction was stopped by adding 0.2 mL of 6 N HCl, and PA was extracted as described above and analyzed by HPLC.

TLC analysis. The lipid extract of the reaction mixture was subjected to thin-layer chromatography using the developing solvent chloroform/methanol/water (65:35:5, by vol). Phospholipids were visualized with Dittmer's reagent or iodine vapor. Substances with reducing activity were detected using ferric ion reagent (20).

HPLC analysis. HPLC was performed on a Shimadzu (Shimadzu Co., Kyoto, Japan) LC-5A equipped with a Rheodyne 7125 manual loop injector (20 μL), a Shimadzu SPD-2A variable wavelength detector (AUFS 0.01) and a Shimadzu C-R3A data processor. PA-AsA was analyzed on a silica gel column (YMC-Pack SIL-06, 4.6 × 150 mm, YMC Co., Kyoto, Japan) with *n*-hexane/isopropanol/10% phosphoric acid (85:15:0.4, by vol) serving as eluent at a flow rate of 2 mL/min. The column temperature was maintained at 40°C for the analysis of PA-AsA. The retention time of PA-AsA was 3.3 min. PC was analyzed on

the same column with *n*-hexane/isopropanol/10% phosphoric acid (20:30:5, by vol) serving as eluent at a flow rate of 1.2 mL/min. The retention time of PC was 3.7 min. PA was analyzed on SHIM-PACK FLC-NH_2 column (4.6 × 50 mm, Shimadzu) with *n*-hexane/isopropanol/10% phosphoric acid (80:20:0.5, by vol) serving as eluent at a flow rate of 2 mL/min. The retention time of PA was 2.0 min. PA-AsA, PC and PA were monitored at 245, 206 and 206 nm, respectively. Sample was injected using a sample loop of 20 μL.

Spectral analyses. Infrared spectra were measured on potassium bromide pellets using a Shimadzu FTIR-4200 infrared spectrophotometer. Mass spectra were measured in the negative fast atom bombardment (FAB) mode with a JEOL (Japan Electronic Optics Laboratory Co., Tokyo, Japan) JMS-HX 100. The FAB gun was operated at 6 KV; xenon atoms were used to ionize the sample in the desorption mode from a triethanolamine matrix. ^1H and ^{13}C nuclear magnetic resonance (NMR) spectra were measured with a JEOL GSX-270 FT NMR spectrometer at 270 and 67.9 MHz, respectively. Samples were dissolved in $CDCl_3/CD_3OD$ (2:1, v/v); tetramethylsilane was used as internal standard.

RESULTS

A new product derived from egg yolk PC and L-ascorbic acid was proposed by catalysis with PLD of *S. lydicus*. The product (R_f 0.25) was separated from PC (R_f 0.35) and PA (R_f 0.41) by TLC. The function gave a blue spot with Dittmer's reagent and a red spot with ferric ion reagent indicating the presence of a phospholipid with reducing activity. The phospholipid was tentatively identified as phosphatidyl-L-ascorbic acid (PA-AsA) having an ester linkage between either the 5- or 6-hydroxy group of L-ascorbic acid and the phosphatidyl moiety.

The structure of PA-AsA was confirmed by spectral analysis of dimyristoyl PA-AsA. PA-AsA was synthesized from 1,2-dimyristoyl-*sn*-glycero-3-phosphocholine and L-ascorbic acid using the same procedure as described in Materials and Methods, except that the concentration of L-ascorbic acid in the aqueous phase of reaction mixture was 2 M. A conversion of 93.6% was attained after 24 hr. PA-AsA was purified on a silicic acid column using the same solvent mixture as for TLC; the isolated yield of PA-AsA was 54.2% relative to PC used. The infrared spectrum of the purified PA-AsA indicated the presence of a phosphate ester and of the double bond of L-ascorbic acid (KBr, cm^{-1}: 1738 $\nu_{C=O}$, 1600 $\nu_{C=C}$, 1231 $\nu_{P=O}$, 1109 $\nu_{P\text{-}O\text{-}C}$). PA-AsA showed the expected UV spectrum for the L-ascorbic acid moiety. The FAB mass spectrum of PA-AsA showed a $[M+Na-2H]^-$ ion at *m/z* 771, a $[M-H]^-$ ion at *m/z* 749, a carboxylate anion at *m/z* 227, and a phosphatidate anion at *m/z* 591 (Fig. 1).

^{13}C NMR chemical shift assignments for PA-AsA in $CDCl_3/CD_3OD$ (2:1, v/v) were made based on the chemi-

TABLE 1

^{13}C NMR Chemical Shifts of PA-AsA, PC and L-Ascorbic Acid

	PA-AsA	Dimyristoyl PC	L-Ascorbic acid
Dimyristoyl glycerol moiety			
CH_2O	62.31	63.01	
CHO	70.04[a]	70.74[a]	
CH_2OP	65.28[b]	63.93[b]	
CO	174.21	174.30	
	173.80	173.93	
CH_2	34.43-22.93	34.55-22.96	
CH_3	14.19	14.21	
Choline moiety			
CH_2OP		59.34[b]	
CH_2N		66.79	
$(CH_3)_3N$		54.39	
L-Ascorbic acid moiety			
C-1	172.03		172.43
C-2	119.15		119.05
C-3	152.62		153.45
C-4	75.67		76.40
C-5	68.12[a]		70.15
C-6	67.34[b]		62.96

[a] $^3J_{CP}$ = 8.25 Hz.

[b] $^2J_{CP}$ = 5.50 Hz.

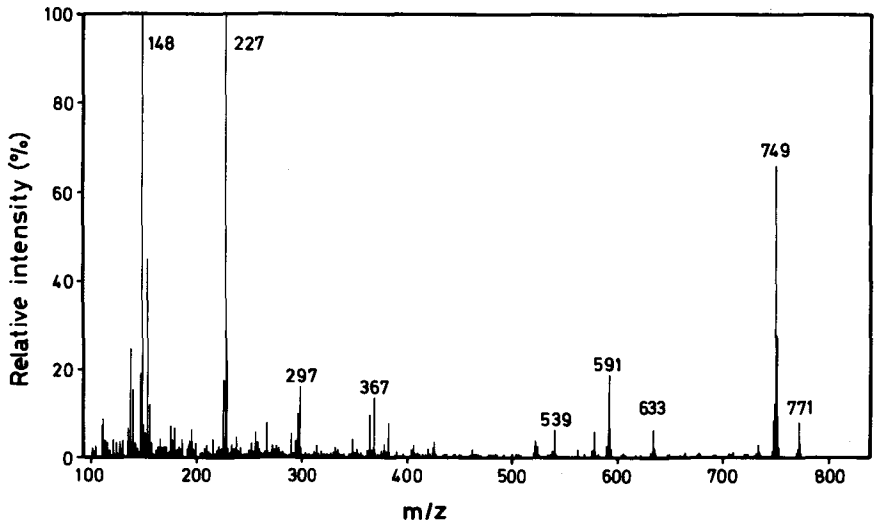


FIG. 1. Negative ion FAB mass spectrum of dimyristoyl PA-AsA.

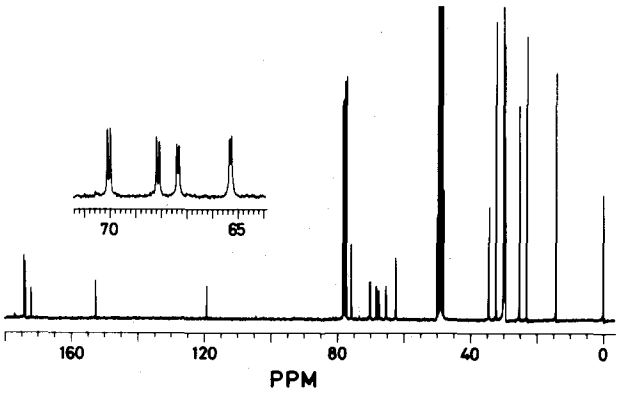


FIG. 2. ^{13}C NMR spectrum of dimyristoyl PA-AsA.

cal shifts of dimyristoyl PC and L-ascorbic acid in the same solvent (Table 1). The ^{13}C chemical shift assignments for dimyristoyl PC were consistent with those reported for dioctanoyl PC (e.g., ref. 21). The assignments for L-ascorbic acid were also based on literature values (22). The ^{13}C chemical shift assignments for PA-AsA were confirmed by DQF correlation spectroscopy (COSY) and ^{1}H-^{13}C COSY experiments. The ^{13}C chemical shifts of the dimyristoyl glycerol moiety of PA-AsA essentially resembled those of dimyristoyl PC. The ^{13}C chemical shifts of the L-ascorbic acid moiety of PA-AsA were essentially those of L-ascorbic acid, except for the C-5 and C-6 shifts. The C-6 signal of PA-AsA showed a significant downfield shift relative to that of L-ascorbic acid, while the C-5 signal was slightly shifted upfield. This ^{13}C chemical shift pattern for carbons proximal to phosphate were previously observed for other phosphoesters such as 5′-(3-*sn*-phosphatidyl) nucleosides (11) and L-ascorbic acid 6-phosphate (23). Moreover, the ^{13}C-^{31}p couplings observed (Fig. 2) for C-6(J=5.5Hz) were smaller than those for

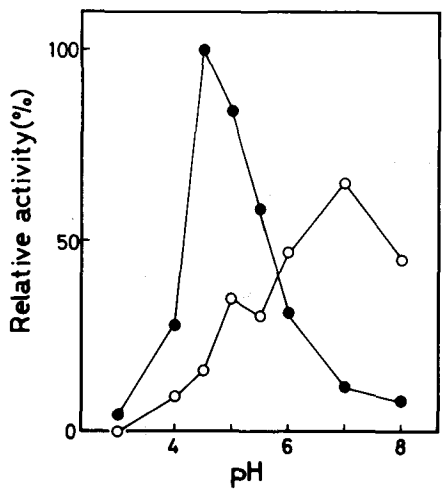


FIG. 3. Effect of pH on the synthesis of PA-AsA and on the hydrolysis to PA. The reaction was done as described in Materials and Methods, except that the pH was varied. The pH of the aqueous medium was adjusted to 3.0, 4.0, 4.5, 5.0 and 5.5 with KOH; to 6.0 with 0.1 M succinic acid-KOH buffer; and to 7.0 and 8.0 with 0.1 M Tris-HCl buffer. The reaction was terminated after 30 min. Relative activities were calculated based on the amount of PA-AsA formed at pH 4.5 (100%). ●, PA-AsA; and ○, PA.

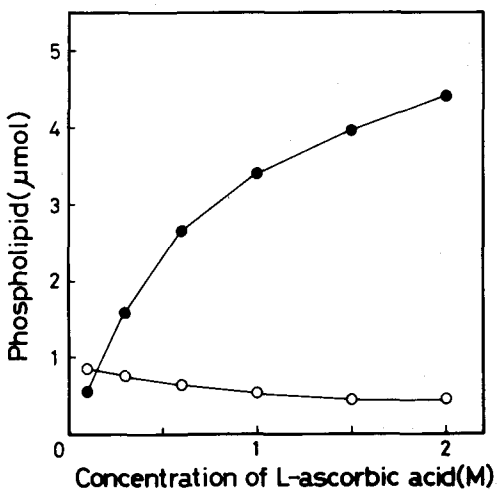


FIG. 4. Effect of L-ascorbic acid concentration on the synthesis of PA-AsA. The reaction was terminated after 30 min. ●, PA-AsA; and ○, PA.

C-5(J=8.25Hz). Based on the $^{2}J_{CP}$ and $^{3}J_{CP}$ values reported for various phospholipids (24), our data strongly suggest that the phosphatidyl moiety in PA-AsA is linked *via* the hydroxy group at C-6 of L-ascorbic acid rather than *via* C-5. Thus, PLD of *S. lydicus* catalyzes transphosphatidylation from PC to L-ascorbic acid to form 1,2-diacyl-*sn*-glycero-3-phospho-6′-L-ascorbic acid.

Figure 3 shows the effect of pH of the reaction mixture on the synthesis of PA-AsA and on the hydrolysis of PC to PA by PLD of *S. lydicus*. The optimum pH for PA-AsA synthesis was in the range of 4.5 to 5.0. The optimum pH for the hydrolysis of PC to PA in the presence of L-ascorbic acid was 7.0. Therefore, the pH of the reaction mixture of subsequent experiments was kept at 4.5.

The effect of L-ascorbic acid concentration on the synthesis of PA-AsA is shown in Figure 4. PA-AsA formation markedly increased as the concentration of L-ascorbic acid increased. The amount of PA formed decreased only slightly as the concentration of L-ascorbic acid increased. Hence, higher concentrations of L-ascorbic acid clearly favored conversion of PC to PA-AsA.

Among the organic solvents tested for use in the transphosphatidylation, ethyl acetate gave the same level of PA-AsA as did diethyl ether. However, twice as much PA was produced when ethyl acetate was used. Other solvents such as *n*-hexane, cyclohexane, chloroform, acetonitrile and *tert*-butanol gave lower conversions to PA-AsA and produced a higher ratio of PA to PA-AsA. Thus, diethyl ether appeared most suitable as organic phase for the synthesis of PA-AsA.

The synthesis of PA-AsA proceeded in a linear fashion during the initial 30 min and then gradually reached a plateau. After 6 hr, conversion of PC to PA-AsA reached about 80%. PA was also formed, but at a much lower level. PC decreased rapidly and entirely disappeared after prolonged reaction. After 24 hr, PA-AsA started to decrease gradually while PA increased (Fig. 5).

The susceptibility of PA-AsA to hydrolysis by PLD was compared with that of PC in the biphasic reaction system (Fig. 6). PC was rapidly hydrolyzed to release PA, where as PA-AsA was more resistant to hydrolysis. The initial rate of hydrolysis of PA-AsA was about 6% of that of PC in this condition.

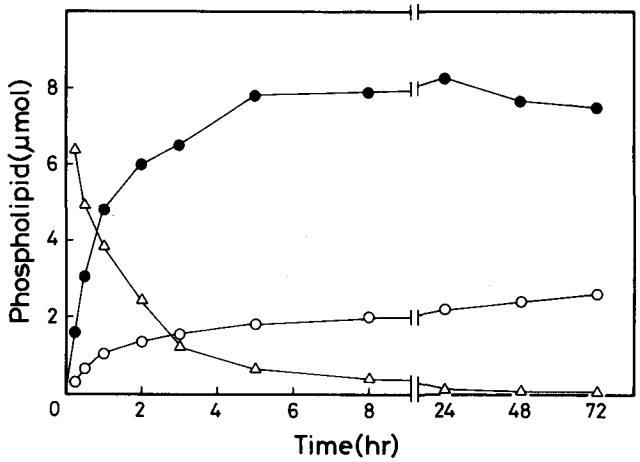


FIG. 5. Time course for the synthesis of PA-AsA. ●, PA-AsA; △, PC; and ○, PA.

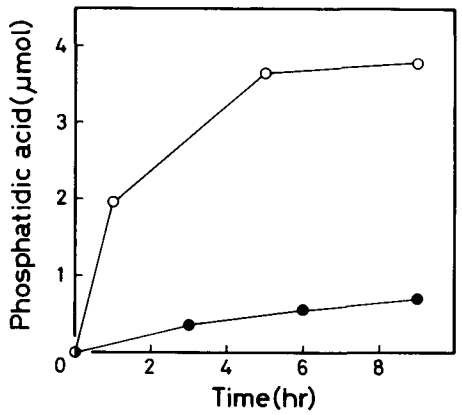


FIG. 6. Hydrolysis of PA-AsA and PC to PA. ●, PA-AsA; and ○, PC.

The synthetic activities of four different phospholipase D were compared using the same reaction conditions (see Materials and Methods) except that in the case of cabbage PLD the reaction mixture contained 40 mM $CaCl_2$ (Table 2). PLD-A showed the highest conversion of PC to PA-AsA. On the other hand, PA-AsA could not be synthesized and PC could not be significantly hydrolyzed by cabbage PLD under the conditions tested. However, PC was completely hydrolyzed by cabbage PLD within 30 min, when the 0.8 M L-ascorbic acid was replaced by 80 mM sodium acetate buffer, pH 4.5. This may suggest that the activity of cabbage PLD is suppressed at the higher L-ascorbic acid concentration.

DISCUSSION

The present study demonstrates that 6-phosphatidyl-L-ascorbic acid can be produced in high yield by utilizing the transphosphatidylation activity of phospholipase D. Maximum conversion of PC to PA-AsA was 93.6%. This value is similar to the conversion reached for phosphatidylethanolamine to phosphatidylglycerol (97.3%) and for PC to phosphatidylglycerol (83%) by this enzyme (25,26). PLD of *Actinomycetes* has been shown to have a broad substrate specificity for acceptor alcohols including geraniol, glucose and nucleosides (27-29). L-ascorbic acid was found to also be a good acceptor alcohol for the phospholipase D of three different *Streptomyces* species. Transphosphatidylation *vs.* hydrolysis varied among the phospholipases. The transphosphatidylation activity of cabbage PLD in respect to L-ascorbic acid remains unknown because activity is suppressed in the presence of ascorbic acid at high concentrations. However, it is likely that cabbage PLD does not exhibit transphosphatidylation activity in respect to L-ascorbic acid, because cabbage PLD was shown to catalyze transphosphatidylation to several primary alcohols, but not to sucrose, glucose, galactose (2) and nucleosides (29).

TABLE 2

Synthesis of PA-AsA by Different Phospholipases D

	PLD-A[a]	PLD-B[b]	PLD-C[c]	PLD-D[d]
	μmol			
30 min reaction				
PA-AsA	2.86	3.25	4.33	0
PA	0.50	1.36	1.36	0.44
5 hr reaction				
PA-AsA	7.79	6.33	—	0
PA	1.82	2.86	—	0.47
24 hr reaction				
PA-AsA	8.85	6.22	7.16	0
PA	2.07	3.24	3.46	0.48

[a]PLD of *Streptomyces lydicus* (Honen Co.).
[b]PLD of *Streptomyces* sp. (Toyo Jozo Co.).
[c]PLD of *Streptomyces* sp. (Sigma Co.).
[d]PLD of cabbage (Boehringer Mannheim GmbH).

It has been reported that the optimal pH was 6.0 for the hydrolysis of egg yolk PC (solubilized with Triton X-100) by PLD from *S. lydicus*. However, in the present study, optimal formation of PA occurred at pH 7.0 in the presence of L-ascorbic acid. This difference may be due to the different physical states of PC which would affect the enzyme reaction at the ether/water interface. Shuto *et al.* (29) reported that the optimum pH for the synthesis of 5'-phosphatidyl-nucleosides by PLD was equal to the pK of nucleoside bases. Similarly, the optimum pH for the synthesis of PA-AsA (pH 4.5) was about equal to the pK (4.17) of L-ascorbic acid. It appears that PLD has a higher affinity for the non-ionized form of L-ascorbic acid.

Juneja *et al.* (30) reported that the initial rate of transphosphatidylation from PC to ethanolamine by several phospholipases D, including *S. lydicus*, reached a maximum at about 0.3 M ethanolamine. At higher concentrations of ethanolamine, the initial reaction rate decreased. The rate of hydrolysis of PC was also low at higher concentrations of ethanolamine. Both transphosphatidylation and hydrolysis activities of cabbage PLD decreased at higher concentrations of acceptor alcohol, such as glycerol, choline, ethanolamine, methanol and ethanol (6,8). By contrast, the rate of formation of PA-AsA was not suppressed at higher concentrations of L-ascorbic acid and the Km value for ascorbic acid was *ca.* 1 M in the present study. A similar dependence of transphosphatidylation on the concentration of an acceptor alcohol was observed in the case of L-serine (6,31). The activity of PLD was not depressed at higher concentrations of L-serine and maximum conversion to PS was attained at the saturation level of L-serine.

Several solvents, such as diethyl ether, chloroform and ethyl acetate, have been used to facilitate dispersions of phospholipids in the transphosphatidylation mixture. Surfactants such as Triton X-100 and SDS have alternately been used. It also has been reported that chloroform can be a suitable solvent to disperse dimyristoyl PC for the synthesis of phosphatidyl-L-homoserine (9) and phosphatidylnucleosides (29). In the synthesis of PA-AsA, however, chloroform resulted in low yields and high PA-to-PA-AsA ratios. Diethyl ether was found to be the best solvent for the transphosphatidylation reaction of L-ascorbic acid. Although dimyristoyl PC does not dissolve completely in diethyl ether, 93.6% of the PC was converted to PA-AsA.

Conversion to PA-AsA reached a maximum (about 80%) after 24 hr. Thereafter, PA-AsA decreased gradually while PA increased slowly. It is likely that the decrease of PA-AsA is due to its hydrolysis to PA after consumption of PC. This is consistent with the fact that PA-AsA can be hydrolyzed to PA by PLD, although the rate of hydrolysis is much slower than that of PC. The resistance of PA-AsA to hydrolysis by the PLD favors the synthesis of PA-AsA. It also seems unnecessary to stop the reaction at a specific time point as prolonged reaction does not significantly diminish final PA-AsA levels.

The phospholipase D of *Streptomyces* catalyze the transphosphatidylation to L-ascorbic acid and the PA-AsA formed retains the reducing activity of L-ascorbic acid. We have confirmed the antioxidant activity of PA-AsA in the peroxidation of methyl linoleate in apolar solvent and of egg yolk phosphatidylcholine in liposomal suspension (32).

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Effect of Chitosan Feeding on Intestinal Bile Acid Metabolism in Rats

Yasuhiko Fukada, Koji Kimura and **Yoshikazu Ayaki***
Department of Biochemistry, Tottori University School of Medicine, Yonago, Tottori 683, Japan

The effect of chitosan feeding (for 21 days) on intestinal bile acids was studied in male rats. Serum cholesterol levels in rats fed a commercial diet low in cholesterol were decreased by chitosan supplementation. Chitosan inhibited the transformation of cholesterol to coprostanol without causing a qualitative change in fecal excretion of these neutral sterols. Increased fiber consumption did not increase fecal excretion of bile acids, but caused a marked change in fecal bile acid composition. Lithocholic acid increased significantly, deoxycholic acid increased to a lesser extent, whereas hyodeoxycholic acid and the 6β-isomer and 5-epimeric 3α-hydroxy-6-keto-cholanoic acid(s) decreased. The pH in the cecum and colon became elevated by chitosan feeding which affected the conversion of primary bile acids to secondary bile acids in the large intestine. In the cecum, chitosan feeding increased the concentration of α-,β-, and ω-muricholic acids, and lithocholic acid. However, the levels of hyodeoxycholic acid and its 6β-isomer, of monohydroxy-monoketo-cholanoic acids, and of 3α, 6ξ, 7ξ-trihydroxy-cholanoic acid decreased. The data suggest that chitosan feeding affects the metabolism of intestinal bile acids in rats.

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Chitosan (glucosamine polymer) is known to have a marked hypocholesterolemic effect in cholesterol-fed rats (1-4). Sugano *et al.* (5) recently reported that the hypocholesterolemic effect of chitosan did not differ within the viscosity range tested (17-1620 cps), while glucosamine oligomer proved ineffective. Vahouny *et al.* (6) found that chitosan was effective in lowering cholesterol absorption in thoracic duct lymph.

Sugano *et al.* (1,4) also showed that chitosan lowered the transformation of cholesterol to coprostanol and increased the excretion of these neutral sterols. In contrast, the fecal excretion of bile acids was essentially unchanged (4). It is well known that primary bile acids are converted into secondary bile acids by intestinal bacteria (7-9). The secondary bile acids, lithocholic acid and deoxycholic acid, have been correlated with the risk of bowel cancer and gallstones (10-12). Because the ingestion of some types of dietary fibers has been shown to influence the intestinal flora (13-15), it appeared possible that chitosan may also influence bile acid metabolism. In the present study we investigated the effect of chitosan supplementation of a normal diet (0.1% cholesterol) on the metabolism of intestinal bile acids in rats.

MATERIALS AND METHODS

Materials. The chitosan used in this study was donated by Professor S. Hirano, Faculty of Agriculture, Tottori University, Tottori, Japan. It was a high viscosity grade preparation with a mean molecular weight of 250,000. The degree of deacetylation was approximately 94%.

3α-Hydroxy-6-keto-5β-cholanoic acid and 3α-hydroxy-6-keto-5α-cholanoic acid were synthesized according to Thomas *et al.* (16) and Bergström *et al.* (17), respectively.

Reagents and organic solvents were of analytical grade. Solvents were distilled, if necessary, before use.

Animals and diet. Male Wistar strain rats were purchased from the Shizuoka Agricultural Cooperative Association for Laboratory Animals (Shizuoka, Japan), and were housed individually in an air-conditioned room (23 ± 1°C) with lighting from 6 a.m. to 6 p.m. After acclimation for two weeks, the animals were divided into three groups. One group was fed a control diet consisting of a commercial powder diet (Clea CA-1, Nihon Clea Co., Tokyo, Japan), supplemented with 0.5% cellulose and 4.5% sucrose; the second group was fed a diet supplemented with 0.5% cellulose, 2.5% sucrose and 2% chitosan; the third group was fed a 5% chitosan-supplemented diet. The stock powder diet contained 25.6% protein, 4.6% fat, 4.0% fiber and all vitamins at recommended values. The diet contained 1.05 ± 0.09 mg/g of cholesterol (mean ± S.D., n=4) and 98.0 ± 5.1 μg/g (mean ± S.D., n=4) of cholic acid as analyzed in our laboratory. The animals were maintained on these diets *ad libitum* for 21 days. Food intake was measured daily, and body weight was determined every second day. Feces were collected for the two days prior to killing. Animals were sacrificed by cardiac puncture under diethyl ether anesthesia. The serum was separated from blood, and the intestine was excised. The entire small intestine was divided into three segments: duodenum, jejunum and ileum, as described by Dietschy and Siperstein (18). The large intestine was divided into two parts: the cecum and colon. The intestinal contents were removed from each segment for measurement of pH and bile acid analysis. The pH of the intestinal contents was estimated directly from the samples using a Horiba Electrometer pH meter. All sampling procedures were performed between 9 and 11 a.m., and all samples were stored at -20°C prior to analysis.

Analysis. Frozen fecal samples were lyophilized, pulverized, and weighed before analysis; 500 mg specimens of blended feces were used for crude extraction of bile acids by refluxing in 90% ethanol for 2 hr. Refluxing was repeated twice, and the pooled extracts were evaporated. The extracts were hydrolyzed by refluxing in 5% NaOH/50% ethanol for 2 hr, and after cooling, the neutral sterols were extracted with n-hexane. The neutral sterols, analyzed by gas-liquid chromatography, were cholesterol and coprostanol. The alkaline solution was adjusted to neutrality by adding dilute HCl dropwise and then evaporated to dryness. The extracts were chromatographed on Amberlite XAD-2 (Rohm and Haas, Philadelphia, PA) to obtain the bile acid fraction. The steps used in the analysis of bile acids were absorption, elution, solvolysis, hydrolysis, extraction, and preparation of methyl ester acetate

*To whom correspondence should be addressed.

Abbreviations: GC/MS, gas-liquid chromatography/mass spectrometry; MC, muricholic acid; HD, hyodeoxydiolic acid.

derivatives. All these steps have been previously described in detail, including the analysis of samples by GLC at 230°C using a Shimadzu Model GC-3BF unit with a coiled glass column (3 mm × 1.75 m), filled with 1.5% OV-1 on Gas Chrom Q 100-120 mesh (Shimadzu Co., Kyoto, Japan) (19). Quantitative results were obtained by comparing peak areas with those of known amounts of reference compounds.

In order to identify the fecal bile acids, aliquots of the bile acid fraction were fractionated on a column of silicic acid (AR, 100 mesh; Mallinckrodt, St. Louis, MO) (20), and the bile acid samples were analyzed by gas-liquid chromatography/mass spectrometry (GC/MS) using a JMS-DX 303 mass spectrometer (JEOL, Tokyo, Japan) connected to a Hewlett-Packard HP 5890A gas chromatograph. A glass column (2 mm × 1.2 m) packed with 1% OV-1 on Gas Chrom Q (100-120 mesh) was used. The conditions for analysis were: injection temperature, 270°C; column temperature, 250°C; ion source temperature, 250°C; ionization voltage, 70 eV; ionization current, 300 μA; carrier gas, He (27.5 mL/min).

The significance of differences between the means of the control and test groups was determined by one-way analysis of variance followed by Student's t-test.

RESULTS

As shown in Table 1, there were no significant differences in growth, food intake, liver weight and dried fecal weight between the control and either of the chitosan-fed groups after 21 days. The serum cholesterol level was depressed by the 5% chitosan-supplemented diet group compared with the control group. There was no significant difference in fecal excretion of neutral sterols and bile acids between the control group and either of the chitosan-treated groups.

The same bile acids that were found in the intestine and/or feces of control rats were also present in the respective samples from both chitosan-treated groups (Table 2). Three of the compounds found in the feces—X, Y, Z—remain unidentified. The fecal bile acids were fractionated into monohydroxy (I and II), dihydroxy (III and IV), dihydroxy plus trihydroxy (V and VI), and trihydroxy (VII and VIII) fractions by chromatography on a silicic acid column. Both compounds X and Y were eluted in fraction II. The mass spectra of these compounds showed peaks at *m/z* 446 (M^+), 386 (M^+-60), 371, 313, 271 and 253 [M^+-(18+60+115)], indicating that they were monohydroxy-monoketo-cholanoic acid deriva-

TABLE 1

Growth, Food Intake, Liver Weight, Fecal Weight, and Fecal Neutral Sterols and Bile Acids in Rats[a]

Observations	Control (n=5)	2% Chitosan (n=4)	5% Chitosan (n=6)
Growth[b] (g)	70.0 ± 6.5	65.0 ± 11.0	78.0 ± 9.4
Food intake (g/day)	23.5 ± 0.9	24.2 ± 1.1	24.8 ± 0.6
Liver weight (g/100g body weight)	5.1 ± 0.3	4.6 ± 0.3	4.6 ± 0.2
Dried fecal weight (g/day)	3.0 ± 0.3	3.2 ± 0.3	2.9 ± 0.1
Serum cholesterol (g/dL)	45.2 ± 2.9	41.8 ± 3.3	36.1 ± 2.3[c]
Fecal neutral sterol excretion (mg/day)	15.87 ± 1.97	14.87 ± 2.21	17.68 ± 0.97
Fecal bile acid excretion (mg/day)	12.16 ± 0.90	9.94 ± 1.14	12.64 ± 0.80

[a]Data are expressed as mean ± SEM.

[b]Weight gain.

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

TABLE 2

Profiles of Bile Acids by GLC and GC/MS Analysis

Bile acid	Retention time relative to deoxycholic acid (18.2 min) OV-1	Characteristic ions *(m/z)* in GC/MS analysis[a]
Lithocholic	0.77	
Deoxycholic	1.00	
Chenodeoxycholic	1.18	
Compound X	1.23	446 (61.8), 428 (3.7), 386 (82.3), 371 (10.3), 313 (17.6), 271 (25.0), 253 (20.6), 213 (100), 121 (69.9)
Cholic	1.31	
Compound Y	1.33	446, 386, 371, 313, 271, 253, 244, 229
Hyodeoxycholic and its 6β-isomer	1.50	
α-Muricholic	1.99	
Compound Z	2.12	428 (17.0), 386 (100), 368 (18.8), 353 (8.9), 313 (10.7), 253 (17.0), 211 (8.9), 159 (42.9)
ω-Muricholic	2.35	
β-Muricholic	2.48	

[a]Number in parentheses shows the relative intensity.

TABLE 3

Composition of Fecal Bile Acids and Neutral Sterols in Rat Feces[a]

Composition	Dietary group		
	Control (n=5)	2% Chitosan (n=4)	5% Chitosan (n=6)
Bile acid (%)			
Lithocholic	3.5 ± 0.8	5.9 ± 1.6	15.3 ± 2.7[b]
Deoxycholic	12.0 ± 2.6	13.4 ± 4.1	20.0 ± 4.1
Hyodeoxycholic and its 6β-isomer (HD)	22.4 ± 6.8	20.9 ± 6.4	1.3 ± 0.2[b]
α-Muricholic	0.3 ± 0.1	2.2 ± 0.1	1.8 ± 0.1
β-Muricholic	5.5 ± 1.2	5.2 ± 0.2	5.6 ± 0.1
ω-Muricholic (ω-MC)	21.0 ± 5.6	18.7 ± 4.6	29.2 ± 3.6
3α-Hydroxy-12-keto-5β-cholanoic	16.7 ± 3.5	16.5 ± 4.1	20.0 ± 3.1
5-Epimeric 3α-hydroxy-6-keto-cholanoic	14.3 ± 2.7	13.4 ± 4.4	1.1 ± 0.1[b]
3α, 6ξ, 7ξ-Trihydroxycholanoic	4.3 ± 0.7	3.8 ± 1.2	5.7 ± 0.4
ω-MC/HD ratio	0.94	0.89	22.5
Neutral sterol (%)			
Coprostanol	57.5 ± 13.0	54.2 ± 13.1	21.6 ± 3.6[b]
Cholesterol	42.5 ± 8.1	45.8 ± 12.7	78.4 ± 6.1[b]

[a]Data are expressed as mean ± SEM.

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

TABLE 4

pH of Intestinal Contents[a]

Intestine	Control (n=5)	2% Chitosan (n=4)	5% Chitosan (n=6)
Duodenum	6.32 ± 0.10	6.28 ± 0.11	6.45 ± 0.10
Jejunum	6.25 ± 0.13	6.32 ± 0.13	6.45 ± 0.05
Ileum	7.13 ± 0.26	7.05 ± 0.13	7.01 ± 0.06
Cecum	5.88 ± 0.06	6.18 ± 0.20	6.52 ± 0.09[b]
Colon	5.79 ± 0.04	6.06 ± 0.20	6.52 ± 0.09[b]

[a]Data are expressed as mean ± SEM.

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

TABLE 5

Composition of Bile Acids in the Duodenum[a]

Bile acid	Composition (%)	
	Control (n=5)	5% Chitosan (n=6)
Deoxycholic	7.0 ± 1.4	4.4 ± 0.7
Chenodeoxycholic	4.0 ± 0.0	2.7 ± 0.4[b]
Hyodeoxycholic	11.1 ± 1.5	4.8 ± 0.8[b]
Cholic	53.1 ± 3.4	59.7 ± 6.2
α-Muricholic	3.5 ± 0.5	3.3 ± 0.5
β-Muricholic	21.5 ± 2.5	25.1 ± 4.6

[a]Data are expressed as mean ± SEM.

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

tives. Fragments *m/z* 231 and 121 from compound X are indicative of a keto group at C-12. Compound Y showed an ABC ring fragmentation ion at *m/z* 229, indicating a keto group at position 3 or 6. Fragment *m/z* 244 is indicative of a keto group at C-6. 6-Keto-5β-cholanoic acids are transformed to their 5α-forms in the presence of excess OH^- or H^+ (21), but this allomerization is incomplete (data from capillary gas chromatographic analysis, not shown). The mass spectra of compound Z showed peaks at *m/z* 428, 368, 353, 313, and 253 [M^+-(3×60+115)], indicative of a triacetoxy derivative. The intense peak at *m/z* 386 [M^+-(60 plus an epoxide between C-6 and C7)] is characteristic for the 3,6,7-triacetoxy compound but there were no specific fragments at *m/z* 446 and 271 as found for α- and β-muricholic acid. From these results, compounds X and Y appear to be 3α-hydroxy-12-keto-5β-cholanoic acid and 5-epimeric 3α-hydroxy-6 ketocholanoic acid(s), respectively. Compound Z appears to be 3α, 6ξ, 7ξ-trihydroxycholanoic acid.

The composition of fecal bile acids and neutral sterol is shown in Table 3. In control rats, 3α-hydroxy-12-keto-5β-cholanoic acid, ω-muricholic acid, and hyodeoxycholic acid and its 6β-isomer were the predominant bile acids (>15%); small amounts (<5%) of α-muricholic acid, lithocholic acid, and 3α,6ξ,7ξ-trihydroxycholanoic acid were also present. Feeding 5% chitosan to rats resulted in an increase in lithocholic acid, a smaller increase in deoxycholic acid ($p<0.2$), and a decrease in hyodeoxycholic acid and its 6β-isomer, and in 5-epimeric 3α-hydroxy-6-ketocholanoic acid(s). There was a decrease in coprostanol and an increase in cholesterol in the 5% chitosan-treated group compared with controls.

Table 4 shows the pH of the contents of various intestinal segments. The pH of the contents of the cecum and of the colon in the 5% chitosan-treated group was higher than for the controls.

Table 5 shows the bile acid composition in the duodenum. In the chitosan-treated group, hyodeoxycholic acid and its 6β-isomer, as well as chenodeoxycholic acid were decreased as compared to controls.

As shown in Table 6, in the cecum of the chitosan-fed group, lithocholic acid, and α-, β- and ω-muricholic acids increased. By contrast, hyodeoxycholic acid and its 6β-isomer, monohydroxy-monoketo-cholanoic acids, and 3α,6ξ,7ξ-trihydroxy-cholanoic acid decreased. Chitosan feeding expanded the neutral sterol pool and concomitantly caused a decrease in coprostanol and an increase in cholesterol.

TABLE 6

Composition of Bile Acids and Neutral Sterols in the Cecum[a]

Composition	Control (n=5)	5% Chitosan (n=6)
Bile acid[b] (%)		
Lithocholic	3.1 ± 0.7	5.0 ± 0.5[c]
Deoxycholic	25.2 ± 4.1	30.3 ± 2.5
Hyodeoxycholic and its 6β-isomer (HD)	25.9 ± 2.2	4.6 ± 0.6[c]
α-Muricholic	0.9 ± 0.0	4.1 ± 1.1[c]
β-Muricholic	8.2 ± 1.1	17.5 ± 2.2[c]
ω-Muricholic (ω-MC)	21.0 ± 2.4	38.5 ± 3.6[c]
3α-Hydroxy-12-keto-5β-cholanoic	2.5 ± 0.2	Tr[c,d]
3α-Hydroxy-6-keto-cholanoic	10.3 ± 1.4	Tr[c,d]
3α, 6ξ, 7ξ-Trihydroxycholanoic	2.9 ± 0.5	Tr[c,d]
ω-MC/HD ratio	0.81	8.37
Neutral sterol[e] (%)		
Coprostanol	49.7 ± 2.0	24.2 ± 0.8[c]
Cholesterol	50.3 ± 1.2	75.8 ± 3.0[c]

[a]Data are expressed as mean ± SEM.

[b]Total bile acid content of cecum (mg): control, 7.14 ± 0.21; 5% chitosan, 8.62 ± 0.16.

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

[d]Trace (<0.3%).

[e]Total neutral sterol content of cecum (mg): control, 3.16 ± 0.12; 5% chitosan, 5.46 ± 0.14[c].

DISCUSSION

Chitosan feeding induced a significant reduction of plasma cholesterol in rats fed a hypercholesterolemic diet consistent with previous reports (1-5). The present study also shows that chitosan feeding (5%) reduces serum cholesterol levels in rats fed a low cholesterol diet (0.1%). Dietary fiber may alter the excretion or metabolism of cholesterol and bile acids in the gut and thus influence plasma cholesterol levels. Cholesterol is converted to coprostanol by intestinal microflora (22). In our experiments, chitosan feeding suppressed the formation of coprostanol, as was observed earlier by Sugano *et al.* (1,4). While chitosan increased the excretion of neutral sterols in rats fed a high cholesterol diet, there was no increase in fecal neutral sterols in our experiments with rats fed a low cholesterol diet. The cholesterol pool in the cecum was expanded by chitosan feeding; however, the amount of cholesterol that accumulated in the cecum may have been insufficient to increase the excretion of cholesterol. It is, therefore, likely that cholesterol that was not absorbed by the proximal region of the small intestine, the site of cholesterol absorption (23), accumulated in the cecum which may explain decreased plasma cholesterol levels.

Dietary fiber affected the composition of fecal bile acids but did not alter their excretion. Primary bile acids are converted to secondary bile acids by intestinal bacteria in the cecum and colon (24). Bacterial 7α-dehydroxylation converts cholic acid to deoxycholic acid (7), and chenodeoxycholic acid to lithocholic acid (8). Lithocholic acid and deoxycholic acid are carcinogenic and toxigenic (10, 11). Although lithocholic acid and deoxycholic acid are detoxified by hydroxylation in liver in the course of enterohepatic circulation (25-28), chitosan feeding caused an increase in lithocholic acid and a smaller increase in deoxycholic acid in cecum and feces. 7α-Dehydroxylation of primary bile acids *in vitro* is stimulated at a pH greater than 6.5 (29). Chitosan may cause an increase in pH in the cecum by degradation to glucosamine or ammonia by colonic bacteria.

Hyodeoxycholic acid and ω-muricholic acid are the most abundant bile acids in the feces of normal male rats (30-33). Chitosan feeding caused a marked increase in the ω-muricholic acid (ω-MC)/hyodeoxycholic acid (HD) ratio in the cecum and feces. If one would assume that β-muricholic acid is converted to hyodeoxycholic acid and ω-muricholic acid by colonic bacteria, the increased ω-MC/HD ratio would suggest that one of these metabolic pathways is blocked causing the accumulation of β-muricholic acid (34,35). The bile acid composition in cecum and feces is specifically changed by chitosan feeding. In rats, chitosan feeding caused a marked effect on the metabolism of bile acids as well as cholesterol in the large intestine, with no quantitative changes being observed in the excretion of neutral sterols and bile acids. However, the changes in bile acid composition may not always be significant, particularly when high cholesterol is included in the diet, because cholesterol feeding can mask the effects of dietary fiber on intestinal bile acids (36).

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Digestion and Absorption of Free and Esterified Fish Oil Fatty Acids in Rats

Remi De Schrijver*, **Daniel Vermeulen** and **Suzanne Backx**
Laboratory of Nutrition, Catholic University of Leuven, B-3001 Leuven, Belgium

This study was designed to test the hypotheses that digestibility and post-absorption metabolism of fish oil are influenced by impaired lipolysis and by the stereospecific composition of its triacylglycerols. Male Wistar rats were fed nonpurified diets containing one of the following fat sources: 9% native fish oil (NFO), 9% autorandomized fish oil (RFO), 8.1% fish oil-derived free fatty acids (FO-FFA) plus 0.9% glycerol, or 9% soybean oil (SO) as a reference fat. In a 24-day balance study, apparent digestibility of total dietary fat averaged 93.1% in the SO, NFO and RFO groups, and 90.9% in the FO-FFA group. Randomization of fish oil had no effect on apparent digestibility of individual fatty acids. In rats fed FO-FFA, apparent absorption of saturated and monounsaturated fatty acids was lower when compared to the NFO and RFO groups. Feeding the FO-FFA diet tended to increase plasma triglyceride content. The hypocholesterolemic effect of polyunsaturated n-3 fatty acids was not influenced by the dietary source. Similar effects on fatty acid profiles of plasma and liver phospholipids were caused by the NFO, RFO and the FO-FFA diets. We conclude that once polyunsaturated n-3 fatty acids are absorbed, their effect on lipid metabolism is not determined by the dietary source.

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Fish oil consumption has been shown to have beneficial effects on cardiovascular disease. The anti-atherogenic effect of fish oils has been attributed to long-chain n-3 polyunsaturated fatty acids, in particular eicosapentaenoic (EPA) and docosahexaenoic (DHA) acids, which appear to reduce the plasma concentrations of total triglyceride and VLDL-triglyceride in animals and humans (1–4). The effect of dietary fish oil on plasma cholesterol is equivocal (5). Fish oil is ingested either as a component of fish or as encapsulated dietary supplement consisting of the stabilized and purified oil. Controversy exists regarding the digestibility of fish oil and the absorbability of the digested products. Fatty acid recovery in the lymph of rats was considerably less when fish oil instead of corn oil was fed (6). An explanation for this could be that the EPA-glycerol ester bond may be more resistant to hydrolysis by pancreatic lipase. On the other hand, fish oil was readily digested in neonatal piglets as determined by analysis of stomach and intestinal contents collected 12 hr after fish oil intubation (7). Digestion of fish oil may be different when ingested as part of a diet rather than as a single dose. Also, the stereospecific composition of the triacylglycerols of fish oil may determine digestibility and, possibly, the metabolic fate of the fatty acids absorbed (8). It has been shown for peanut oil (9) and oleoyl stearoyl triacylglycerols (10) that the triacylglycerol structure can influence lipid metabolism.

In the present study carried out with rats, we compared the digestibility of fat in nonpurified diets supplemented with native (NFO) or randomized fish oil (RFO). In order to determine whether lipolysis of fish oil is a factor contributing to impaired fatty acid absorption (6), the digestive hydrolysis process in one of the experimental groups was circumvented by feeding fish oil-derived free fatty acids (FO-FFA) as dietary supplement. Because of its high digestibility, soybean oil (SO) was also included as a control in this study. Plasma lipid contents and fatty acid profiles of plasma and liver phospholipids were determined in order to establish whether the chemical form in which fish oil fatty acids are ingested affects post-absorption lipid metabolism.

MATERIALS AND METHODS

Experimental design. Male Wistar rats (University Animal Center, Leuven, Belgium), weighing approximately 150 g each, were individually housed in metabolism cages. The animal room was maintained at 23°C at a 12-hr light-dark cycle. Rats were fed a nonpurified, cereal-based diet containing 9% beef tallow for a 25-day adaption period. The diet contained 205 g crude protein, 130 g crude fat, 35 g crude fiber, 65 g crude ash, and 1 g butylhydroxytoluene per kg. A beef tallow diet was chosen in order to induce a hyperlipidemic effect before feeding the experimental fat sources. At the end of the pre-experimental period, after a 24-hr fast, six rats were killed under diethyl ether anesthesia and plasma lipids were measured. Twenty-four rats were assigned to four dietary treatment groups; the average starting weight in each of the groups was 230 $\pm$ 3 g (mean $\pm$ SEM). The experimental diets were similar to the diet fed during the adaption period, except that beef tallow was replaced by NFO, RFO, SO or FO-FFA, respectively. However, the latter diet contained 8.1% FO-FFA and 0.9% added glycerol to equalize fatty acid and glycerol contents of the diets. Beef tallow and soybean oil were purchased at a local store. Native but purified fish oil was obtained from Seven Seas Health Care Ltd. (Kingston-upon-Hull, U.K.); it contained 1 g of α-tocopherol and 0.1 g of dodecylgallate per kg. Diets were prepared every six days and kept at 0°C under N_2 to minimize lipid autoxidation. Diets were fed as meal and provided fresh daily. Animals had free access to tap water and food. The experimental period lasted 24 days. Food intake was measured daily, with corrections made for spilled food. Feces were collected daily and frozen at -18°C. At the time of analysis, feces samples from each rat were pooled per 6-day subperiod. At the end of the assay period, rats were fasted for 24 hr and weighed. Trunk blood from each rat was obtained after decapitation under diethyl ether anesthesia. The liver was removed,

*To whom correspondence should be addressed at Laboratory of Nutrition, Catholic University of Leuven, 92 Kard. Mercierlaan, B-3001 Leuven, Belgium

Abbreviations: DHA, docosahexaenoic acid; EPA, eicosapentaenoic acid; FO-FFA, fish oil-free fatty acids; NFO, native fish oil; RFO, randomized fish oil; SO, soybean oil; VLDL, very low density lipoproteins.

washed with cold saline/phosphate solution, blotted dry, and frozen at -70°C until lipid analysis.

Analytical procedures. Crude fat contents of diets and feces were analyzed by diethyl ether extraction, following hydrolysis with 3N HCl (11). Fatty acid profiles of diets, dietary supplemented fats, feces, and plasma and liver phospholipids were determined by gas-liquid chromatography of the methyl esters prepared by transmethylation with BF_3/methanol. A Shimadzu gas chromatograph (Model 15A, Shimadzu Corp., Kyoto, Japan) was used, equipped with an on-column injection system and a flame ionization detector. Samples were injected onto a fused silica capillary column (RSL 500, 25 m × 0.25 mm, 0.2 μm film, Bio-Rad RSL, Eke, Belgium). Retention times were verified by comparison with standards (Nu-Chek Prep, Elysian, MN). To isolate phospholipids from plasma and homogenized liver, samples were extracted with chloroform/methanol (2:1, v/v) containing 0.1% butylhydroxytoluene, and the extracts were washed with 0.9% KCl in water. The solvent was evaporated by a stream of N_2, and total lipids were redissolved and concentrated in chloroform. The neutral and polar lipid fractions were separated on a silica cartridge (Extract Clean column, Alltech Associates Inc., Laarne, Belgium) by elution with chloroform and methanol, respectively. Plasma triglyceride and total cholesterol were determined by enzymatic methods using Boehringer kits (Boehringer Pharma, Mannheim, Germany).

Preparation of randomized fish oil. Autorandomized fish oil was prepared by adding 0.2% sodium methoxide, heating at 75°C for 3 hr under continuous stirring, and flushing with N_2. The reaction was stopped by cooling to room temperature and adding 1% citric acid. The randomized fish oil was freed from free fatty acids by adding 0.5 N NaOH and washing with water containing 3% NaCl. The oil was dried over anhydrous Na_2SO_4. The efficiency of the washing procedure was checked by thin-layer chromatography using silica gel LK plates (Whatman, Maidstone, U.K.) and hexane/diethyl ether/formic acid (80:2:2, v/v/v) as solvent. The interesterified fish oil used in the nutritional experiments consisted almost exclusively of triglycerides. The acid values (11) of the native and randomized fish oils were 0.06 and 0.13%, respectively. Redistribution of fatty acids by randomization was tested by hydrolysis with pancreatic lipase (12) and by subsequent separation of the monoacylglycerol fraction by thin-layer chromatography on borate impregnated silica gel G plates using chloroform/acetone (8:12, v/v) as solvent (13). Gas-liquid chromatography of the fatty acids in the 2-acylglycerol fraction showed that 31-34% of each fatty acid was present in position 2 relative to its abundance in the oil. The fatty acid patterns of NFO and RFO were very similar (Table 1).

Preparation of free fatty acids from fish oil. Saponification of fish oil was brought about by adding 2 L of 10% NaOH in 70% ethanol to 1 kg of oil. The reaction was carried out at 50°C for 12 hr while flushing with N_2. Water was added, and the mixture was acidified (pH 1) while cooling. The free fatty acid layer was separated, washed with 3% NaCl in water until neutral, and dried over anhydrous Na_2SO_4. Thin-layer chromatography on silica gel LK plates (Whatman, Maidstone, U.K.) with hexane/diethyl ether/formic acid (80:2:2, v/v/v) as solvent as well as determination of the acid value (11) showed that the prepared fraction consisted entirely of free fatty acids.

TABLE 1

Fatty Acid Composition (wt %) of Dietary Fats[a]

Fatty acid[b]	BT	SO	NFO	RFO	FO-FFA
14:0	3.0	—	7.2	7.7	6.7
14:1	0.9	—	0.2	—	0.3
16:0	23.9	10.9	19.5	18.4	15.8
16:1n-7	3.2	—	11.1	11.1	11.2
17:0	1.1	—	0.8	0.7	0.7
18:0	18.5	2.0	2.6	2.4	2.2
18:1	40.3	23.8	15.6	15.2	15.6
18:2n-6	4.1	57.0	1.2	1.2	1.3
18:3n-3	0.4	6.0	0.5	0.7	0.7
18:4	—	—	3.2	3.1	3.2
20:0	0.8	—	—	—	—
20:1n-9	0.2	—	5.6	6.0	6.3
20:4n-6	—	—	1.0	1.0	1.2
22:1n-9	—	—	2.0	1.7	1.7
22:5n-3	—	—	1.4	1.6	1.6
20:5n-3	—	—	17.6	17.9	18.4
22:6n-3	—	—	9.1	9.3	10.4

[a]BT, beef tallow; SO, soybean oil; NFO, native fish oil; RFO, randomized fish oil; FO-FFA, free fatty acids from fish oil.

[b]The difference between the sum of the percentages and 100 represents trace amounts (<0.5%) and unidentified components.

Data analysis. Results are presented as means ± SEM. Data were analyzed by one-way analysis of variance, except for the digestibility data. The latter results were subjected to two-way analysis of variance with repeated measures in order to evaluate possible differences that occurred over time. The least significant difference test with equal replication was used to compare individual means when significant F ratios were calculated (14). Differences were considered significant when $p<0.05$.

RESULTS AND DISCUSSION

Body weight and food consumption. Feeding the experimental diets for 24 days resulted in an average body weight gain of 169 g. Average daily food intake was 18.8 g. There were no significant effects of the dietary treatment on final body weight and food consumption (data not shown).

Effects of dietary fat on lipid digestibility. Apparent digestibility of total dietary fat as well as of individual fatty acids was not affected by age. Therefore, only average data regarding the entire 24-day experimental period are presented in Table 2. Digestibility of total fat was not significantly different among experimental groups, although the group fed the FO-FFA diet showed a tendency toward lower utilization. This was even more apparent from the digestibility data of individual fatty acids. Whereas apparent fatty acid digestibility was not different in rats fed NFO or RFO, feeding FO-FFA resulted in significantly lower digestibility of the saturated (14:0, 16:0, 18:0) and the monounsaturated (18:1, 20:1n-9, and 22:1n-9) fatty acids. By contrast, digestibility data for polyunsaturated fatty acids as well as for 16:1 in the FO-FFA group were not significantly different from the corresponding data in the NFO and the RFO groups. Also, within each group, lowest digestibility was obtained for 18:0, while 20:5n-3 and 22:6n-3 showed the highest digestibilities. These results indicate i) that lipolysis of fish oil and absorption of digestive products is not

TABLE 2

Effects of Experimental Diets[a] on Apparent Digestibility of Fat and Fatty Acids[b]

	SO (%)	NFO (%)	RFO (%)	FO-FFA (%)
Fat	93.1 ± 2.5	92.7 ± 3.1	93.5 ± 1.3	90.9 ± 2.2
Fatty acids				
14:0	88.7 ± 0.4[c]	96.1 ± 0.4[d]	96.5 ± 1.3[d]	92.1 ± 1.1[e]
16:0	89.3 ± 0.4[c]	92.1 ± 0.2[d]	91.7 ± 2.3[d]	84.4 ± 2.4[e]
16:1n-7	80.2 ± 2.0[c]	97.4 ± 0.3[d]	97.7 ± 0.4[d]	95.5 ± 1.3[d]
18:0	67.6 ± 0.3[c]	80.0 ± 5.3[d]	76.3 ± 4.2[d]	63.6 ± 6.2[c]
18:1	92.6 ± 0.4[c]	94.0 ± 3.2[c]	93.3 ± 2.3[c]	89.6 ± 1.0[d]
18:2n-6	97.0 ± 0.1	96.2 ± 1.5	96.6 ± 1.1	96.3 ± 1.4
18:3n-3	97.8 ± 0.2	97.1 ± 2.7	94.8 ± 1.8	94.6 ± 1.4
20:1n-9	—	95.8 ± 3.5[c]	94.3 ± 2.1[c]	90.1 ± 1.3[d]
20:4n-6	—	95.0 ± 2.1[c]	95.2 ± 1.4[c]	93.4 ± 1.4[c]
22:1n-9	68.7 ± 1.3[c]	88.6 ± 4.7[d]	86.3 ± 4.4[d]	79.6 ± 2.0[e]
20:5n-3	91.1 ± 4.3	99.4 ± 0.2	99.5 ± 0.2	99.3 ± 0.3
22:6n-3	82.3 ± 16.6	99.5 ± 0.3	99.6 ± 0.2	99.5 ± 0.2

[a]Diets containing SO, soybean oil; NFO, native fish oil; RFO, randomized fish oil; FO-FFO, free fatty acids from fish oil.

[b]Means ± SEM of six rats; apparent digestibility of fat or fatty acid was determined in a 24-day experiment and was calculated according to the following formula: 100 × difference between intake and fecal excretion of fat or fatty acid/fat or fatty acid intake.

[c–e]Values having different superscript letters in one row are significantly different ($p<0.05$).

influenced by positional redistribution of fatty acids in the triglycerides; ii) that micellar solubilization and absorption of fatty acids are favored by a higher degree of unsaturation and by a shorter chain length (Table 2) thus confirming earlier findings (15); and iii) that the monoglycerides formed in digestion appear to serve as emulsifiers of fatty acids and thus contribute to micellar solubilization; the latter was concluded from the greater apparent digestion of total fat and of saturated and monounsaturated fatty acids in the NFO and RFO groups as compared to the FO-FFA group. Also, the 16:0, 18:0 and 18:1 fatty acids, which were less digestible when fed as free acids, showed higher digestibility when fed as SO, probably due to the micellization effect of 2-monoglycerides. This suggested role of monoglycerides in lipid digestion is consistent with the finding that digestibility of beef tallow in piglets decreased when fed in hydrolyzed form (16). Similarly, rats showed higher fat absorbability when fed corn oil instead of free fatty acids isolated from corn oil (17). In our experiments, the digestibility of the total fat in the FO-FFA group was, on average, only 2.2% lower than in the other groups. This could be explained by i) the types of fatty acids derived from fish oil, which are characterized by low levels of fatty acids that are less readily solubilized; and ii) the 4% crude fat inherently present in the dietary ingredients, which may have contributed to absorption of FO-FFA by virtue of the digestive products liberated. It is not known whether the presence of a high amount of free fatty acids in the intestinal lumen changed the pH in such a way that formation of micelles was impaired (18) leading to somewhat lower fat absorption in the FO-FFA group. The effect of glycerol addition to the FO-FFA diet on absorption of free fatty acids was probably small. This follows from experiments with rats (17) which showed only a 4% difference ($p<0.05$) in apparent absorbability when purified diets were fed containing either 35% corn oil or 35% corn oil-derived fatty acids without having equalized the glycerol contents of the diets.

The present finding that apparent absorption of EPA and DHA was not influenced by the dietary source is in contradiction with the reported substantially lower absorption in humans when these fatty acids were ingested as fish oil (19) or as 1,3-dioctanoyl-2-eicosapentaenoyl glycerol (20). This was explained by assumed reduced activity of pancreatic lipase toward triacylglycerols containing EPA and DHA and by the suggestion that once triglycerides are hydrolyzed, the n-3 fatty acids are totally absorbed. In addition, discrepancy with the present results may also be explained by the way lipid absorption was measured. In the studies mentioned (19,20), a single dose of test fat was administered and the transient rise of EPA and DHA *vs.* either 18:3n-3 or 20:4n-6 in plasma triglycerides was measured. In the present experiments, a possibly slower rate of lipolysis of FO-triglycerides, and thus slower absorption of fatty acids, did not affect digestibility because of the extended experimental time periods. Slower EPA and DHA absorption in lymph, when rats were administered menhaden oil *vs.* both fatty acids in the free form, has recently been reported (21). Moreover, our finding regarding the high digestibility and absorbability of FO and FO-FFA was substantiated by the fact that the rats fed these supplemented fat sources showed similar EPA and DHA contents in the plasma (Table 3). Also, FO-FFA and FO-fed rats showed the same efficiency of food utilization as the rats fed the SO diet. Thus the availability of FO and FO-FFA was not substantially different from SO, which is usually regarded as highly digestible. This conclusion is supported by the finding that rats digested fish oil at least as efficiently as peanut oil in experiments based on measurement of fecal digestibility (De Schrijver, R., Vermeulen, D., and Viaene, E., unpublished data).

Digestibility of 14:0, 16:0, 18:0 and 16:1 was significantly higher in groups fed NFO or RFO, as compared to rats fed SO. As a consequence, apparent digestibility of fatty acids, which are less readily absorbed, appeared to depend on the nature of the other fatty acids present in the dietary

TABLE 3

Effects of Experimental Diets[a] on Fatty Acid Composition (wt %) of Plasma Phospholipids

Fatty acids[b]	SO	NFO	RFO	FO-FFA
14:0	0.5 ± 0.2[c]	1.0 ± 0.3[d]	1.0 ± 0.2[d]	1.1 ± 0.2[d]
16:0	17.3 ± 1.7[c]	21.5 ± 0.9[d]	21.5 ± 0.2[d]	22.3 ± 0.7[d]
16:1	0.8 ± 0.2[c]	2.1 ± 0.5[d]	2.0 ± 0.3[d]	2.5 ± 0.3[d]
17:0	0.4 ± 0.1	0.6 ± 0.1	0.6 ± 0.1	0.6 ± 0.1
18:0	16.1 ± 1.6[c]	13.4 ± 0.6[d]	14.2 ± 0.4[d]	13.1 ± 1.4[d]
18:1	10.0 ± 1.1[c]	13.4 ± 1.1[d]	13.1 ± 0.7[d]	13.8 ± 1.3[d]
18:2n-6	19.2 ± 1.6[c]	17.7 ± 0.6[c,d]	16.8 ± 1.0[d]	17.4 ± 1.0[d]
18:3n-3	0.7 ± 0.2	0.4 ± 0.3	0.4 ± 0.3	0.6 ± 0.3
20:4n-6	32.0 ± 4.1[c]	17.3 ± 2.5[d]	19.0 ± 1.7[d]	16.6 ± 2.5[d]
20:5n-3	1.0 ± 0.2[c]	5.7 ± 0.6[d]	5.2± 0.6[d]	6.2 ± 0.2[d]
22:6n-3	1.9 ± 0.4[c]	5.4 ± 0.5[d]	5.1 ± 0.6[d]	5.5 ± 0.4[d]

[a]Diets containing SO, soybean oil; NFO, native fish oil; RFO, randomized fish oil; FO-FFO, free fatty acids from fish oil.

[b]Means ± SEM of six rats, only the major fatty acids are listed.

[c,d]Values having different superscript letters in one row are significantly different ($p<0.05$).

triacylglycerols. The higher apparent absorption of 18:0, when fed as NFO or RFO, was remarkable and may be related to the better emulsifying properties of the digestive products formed from fish oils. This could also explain the controversy regarding the degree of absorption of 18:0 (22-25).

Effects of dietary fat on plasma and liver lipids. Plasma triglyceride contents (Table 4) were similar in the groups fed SO, NFO or RFO. Moreover, the transfer from the beef tallow diet to the diets containing one of the unsaturated oils did not affect plasma triglyceride content. By contrast, elevated plasma triglyceride levels were found in rats which received the FO-FFA diet. A similar tendency toward hypertriglyceridemia, although not statistically significant, was observed in rats given a purified diet containing 35% free fatty acids derived from corn oil (17), as well as in experiments with chicks fed nonpurified diets containing 6% free fatty acids isolated from either soybean oil, palm oil or beef tallow (26). The high availability of dietary free fatty acids indicates that the glycerol phosphate pathway must have become activated in the course of mucosal triglyceride synthesis. The increased amount of plasma triglyceride in animals fed free fatty acids may result from over-stimulation of the glycerol phosphate pathway in the enterocytes, as well as from increased endogenous triglyceride contributions during fat absorption (27).

TABLE 4

Effects of Experimental Diets on Plasma Lipid Concentrations[a]

Dietary groups	Total cholesterol (mg/dL)	Triglyceride (mg/dL)
Beef tallow[b]	166 ± 14[c]	72 ± 8[c,d]
Soybean oil[e]	178 ± 18[c]	65 ± 10[c]
Native fish oil[e]	99 ± 15[d]	72 ± 16[c,d]
Randomized fish oil[e]	104 ± 15[d]	63 ± 13[c]
Free fatty acids from fish oil[e]	92 ± 10[d]	92 ± 16[d]

[a]Means ± SEM of six rats.

[b]Values at start of experimental period.

[c,d]Values having different superscript letters in one column are significantly different ($p<0.05$).

[e]Values at termination of experimental period.

Contrary to SO, feeding NFO or RFO resulted in decreased plasma cholesterol contents (Table 4) by approximately 40%. Moreover, rats fed the FO-FFA diet showed a similarly large decrease in cholesterol consistent with the finding that FO-FFA and fish oils were equally digested and absorbed. Given the difference in the positional distribution of fatty acids in native and randomized fish oil and admitting that the 2-position of the dietary triglycerides survived intestinal lipolysis, it may be assumed that the fatty acids in the 2-position of chylomicron triglycerides differed in both experimental groups. Nevertheless, differences in the positional distribution of fatty acids in triglycerides released from the enterocytes influenced neither the contents of plasma triglyceride and cholesterol, nor the fatty acid profiles of plasma (Table 3) and liver phospholipids (Table 5). Thus, post-absorptive effects from ingested fish oil were only related

TABLE 5

Effects of Experimental Diets[a] on Fatty Acid Composition (wt %) of Liver Phospholipids

Fatty acids[b]	SO	NFO	RFO	FO-FFA
14:0	0.4 ± 0.1	0.6 ± 0.2	0.8 ± 0.5	0.3 ± 0.1
16:0	15.9 ± 1.1[c]	19.4 ± 1.4[d]	19.8 ± 1.0[d]	18.6 ± 0.7[d]
16:1	0.3 ± 0.2[c]	1.1 ± 0.5[d]	1.1 ± 0.4[d]	1.0 ± 0.1[d]
17:0	0.4 ± 0.1	0.6 ± 0.1	0.6 ± 0.1	0.6 ± 0.1
18:0	22.6 ± 1.6	20.3 ± 2.1	20.8 ± 2.3	20.6 ± 2.7
18:1	7.3 ± 1.0[c]	9.9 ± 2.2[c,d]	10.3 ± 2.0[d]	8.7 ± 0.6[c,d]
18:2n-6	19.1 ± 1.5[c]	16.1 ± 0.8[d]	15.8 ± 1.2[d]	15.3 ± 1.5[d]
18:3n-3	0.6 ± 0.1[c]	0.4 ± 0.1[cd]	0.2 ± 0.2[d]	0.3 ± 0.2[d]
20:4n-6	25.9 ± 2.7[c]	15.3 ± 1.8[d]	14.2 ± 2.1[d]	15.8 ± 0.8[d]
20:5n-3	0.4 ± 0.1[c]	4.2 ± 0.7[d,e]	3.8± 0.7[d]	4.9 ± 0.8[e]
22:6n-3	3.9 ± 0.3[c]	9.7 ± 1.9[d]	9.1 ± 2.2[d]	10.4 ± 1.3[d]

[a]Diets containing SO, soybean oil; NFO, native fish oil; RFO, randomized fish oil; FO-FFO, free fatty acids from fish oil.

[b]Means ± SEM of six rats, only the major fatty acids are listed.

[c-e]Values having different superscript letters in one row are significantly different ($p<0.05$).

to the nature of its constituting fatty acids. This conclusion was supported by the observation that the animals fed FO-FFA also showed the same plasma cholesterol content and the same fatty acid profiles in plasma and liver as did rats fed NFO or RFO; the only exception was the plasma triglyceride content. However, as mentioned above, slightly elevated triglyceride levels in plasma upon feeding free fatty acids may be a normal phenomenon (17,26) which may not necessarily be related to the nature of these fatty acids.

In conclusion, our experiments demonstrated i) that fish oil is readily digested in rats; ii) that autorandomization of fish oil has no effect on its metabolism; and iii) that despite possibly slow digestion rates, fish oil is somewhat better utilized than are fish oil-derived fatty acids.

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METHODS

Separation of Cholesterol, and Fatty Acylglycerols, Acids and Amides by Thin-Layer Chromatography[1]

Alexander Bilyk, George J. Piazza, Raymond G. Bistline, Jr. and Michael J. Haas*
U.S. Department of Agriculture, ARS, Eastern Regional Research Center, Philadelphia, Pennsylvania 19118

A rapid unidimensional thin-layer chromatographic (TLC) method for the separation of neutral lipids is described, using two sequential solvent systems of different polarity. Excellent separations of mono-, di- and triglycerides, fatty acids, fatty amides, and cholesterol are thereby achieved. Separation is accomplished at room temperature and requires 25 min.
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In the course of our investigations on the modification and hydrolysis of natural lipid mixtures, we found it necessary to rapidly separate and detect fatty acids, fatty amides, mono-, di, and triacylglycerols, as well as cholesterol. Numerous reports (1–9) describe the separation of several of these compounds from each other using thin-layer chromatography (TLC). However, none of the methods was capable of separating all of these compounds from one another. We describe here a TLC method that can achieve this goal. The procedure is efficient, rapid, and reproducible and achieves excellent resolution for both analytical and preparative applications.

MATERIALS AND METHODS

Solvents. Toluene was purchased from J. T. Baker Chemical Co. (Phillipsburg, NJ). Methanol and n-hexane were from Burdick and Jackson (Muskegon, MI). Ethyl acetate and formic acid were from Eastman Kodak Co. (Rochester, NY). Acetone, diethyl ether, chloroform and sulfuric acid were from Mallinckrodt (Paris, KY). Acetic acid was from Aldrich Chemical Co. (Milwaukee, WI). Lipid standards were from Sigma Chemical Co. (St. Louis, MO). *N*-Butylpalmitamide was prepared according to the Krafft-Stauffer procedure (10). Silica Gel G TLC plates (20 × 20 cm, 250 μm) were obtained from Analtech (Newark, DE).

Thin-layer chromatography. Stock solutions were prepared by dissolving 25 mg of each of the reference compounds in 2.5 mL of chloroform. Part of the stock solutions were combined to prepare a standard mixture containing triolein, 1,3-diolein, 1,2-diolein, 1-monoolein, oleic acid, *N*-butylpalmitamide and, as required, cholesterol, each at a concentration of 1.67 mg/mL. TLC plates were washed in a tank of methanol for 5 min. After removal, plates were air-dried for at least 30 min. Samples were applied with a Hamilton syringe 2 cm from the lower edge of the plates. Plates were developed sequentially with solvent A (toluene:diethyl ether:ethyl acetate:acetic acid, 75:10:13:1.2, by vol) to 8 cm above the level of application and dried under nitrogen. The TLC plates were then developed with solvent B (hexane:diethyl ether:formic acid, 80:20:2, v/v/v) to 14 cm above the level of application. After air-drying of the plates, the samples were made visible by spraying the TLC plates with 60% aqueous sulfuric acid and charring on a hot plate.

RESULTS AND DISCUSSION

With the dual development system described in Materials and Methods, the compounds under examination are separated on the basis of polarity, with triacylglycerols exhibiting the greatest mobility, followed by fatty acids (which are protonated in the acidic solvent systems), diacylglycerols, fatty amides, and, finally, monoacylglycerols. Figure 1 illustrates the need for dual solvent development to separate these components. As shown in lane 1 of Figure 1, the attempted separation of individual components with solvent system B alone produced very poor results. Two of the components remained unresolved and another (1-monoacylglycerol) remained at the origin. With solvent system A (Fig. 1, lane 2), all acylglycerols had greater mobility. However, oleic acid and 1,3-diolein

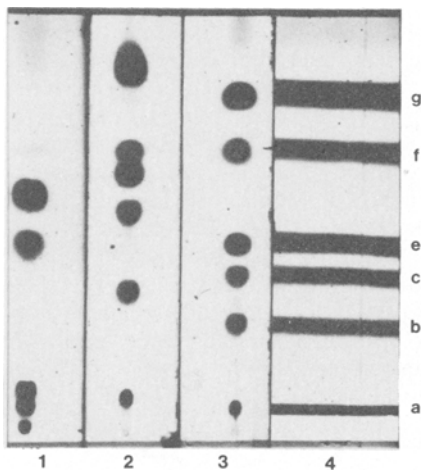


FIG. 1. Thin-layer chromatography of a standard mixture. Lane 1—standard mixture, 25 μg of each component, developed in solvent system B; lane 2—standard mixture, 25 μg of each component, developed in solvent system A; lane 3—standard mixture, 25 μg of each component, developed sequentially in solvent systems A and B; lane 4—preparative TLC of standard mixture, 1.67 mg of each component per plate, developed sequentially in solvent systems A and B; for lanes 3 and 4: a, 1(3)-monoolein; b, *N*-n-butyl palmitamide; c, 1,2-diolein; e, 1,3-diolein; f, oleic acid; g, triolein.

[1]Reference to brand or firm name does not constitute endorsement by the United States Department of Agriculture over others of a similar nature not mentioned.

*To whom correspondence should be addressed at U.S. Department of Agriculture, ARS, 600 East Mermaid Lane, Philadelphia, PA 19118.

Abbreviation: TLC, thin-layer chromatography.

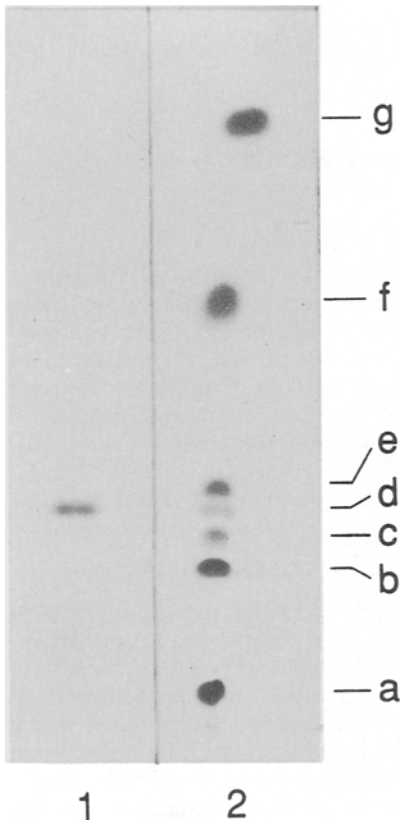


FIG. 2. Separation and detection of cholesterol by sequential development with solvent systems A and B. Lane 1—cholesterol, 25 μg; lane 2—standard mixture, 25 μg each component. a, monoolein; b, *N*-n-butylpalmitamide; c, 1,2-diolein; d, cholesterol; e, 1,3 diolein; f, oleic acid; g, triolein.

were not separated (lane 2). By sequentially using the two solvent mixtures, A and B, excellent separation of the acylglycerols, fatty acid and fatty amide was achieved (Fig. 1, lane 3). Individual components were well resolved from one another and migrated as tight, circular entities. The relative mobilities and the extent of separation of these compounds are unaffected by chain length (at least between C_8 and C_{22}) and degree of unsaturation (up to at least five double bonds; data not shown). However, hydroxylated fatty acids, and acylglycerols containing them, do not exhibit the characteristic mobilities reported here. For example, ricinoleic acid (12-hydroxy-(9Z)-octadecenoic acid) migrates to a level just below that of 1,2-diacylglycerols. Castor oil, which is rich in triacylglycerols containing ricinoleic acid, exhibits two prominent species, one just below ricinoleic acid, and the other just above the 1,2-diacylglycerol fraction. In this system, fatty acid methyl esters and triacylglycerols migrate together.

Successful separations were achieved when the TLC technique described here was used for preparative-scale separations of the components of the standard mixture: lane 4 of Figure 1 illustrates the separation of more than 1.5 mg of each of the components of the standard mixture. We have also found this method useful for the quantitation of individual lipid components, which can be achieved by means of a TLC plate scanner after the plates have been sprayed with sulfuric acid and charred. Alternatively, spraying the preparative plates with an ethanolic solution of 2,7-dichlorofluorescein, visualizing the bands under ultraviolet light, scraping the fractions from the plate and extracting and weighing each fatty component gives quantitative results (data not shown).

The separation of sterols in crude lipid mixtures from the glyceride components is often difficult. Figure 2 illustrates the effectiveness of the method described here in resolving cholesterol from acylglycerols and free fatty acids. As shown in lanes 1 and 2, the mobility of cholesterol is intermediate to that of 1,3- and 1,2-diacylglycerol. The detection and identification of this compound is facilitated by the fact that when the plate is sprayed with aqueous sulfuric acid and then heated for 2–5 min a very bright pink color arises from cholesterol. With further heating, the spot turns black. The separation of cholesterol from diacylglycerols is critically dependent upon the height to which solvent mixture A is allowed to rise on the TLC plate. The optimal development height (usually between 6 and 10 cm) is dependent upon the materials used, and must be determined by trial and error using standard compounds.

The two-solvent, unidimensional thin-layer chromatographic procedure described here is a rapid means for identifying components of fatty mixtures containing mono-, di- and triacylglycerols, fatty acids, fatty amides and cholesterol. The procedure uses inexpensive, readily available solvents and simple laboratory equipment, and results are obtained in a short period of time. We have found this technique to give satisfactory and highly reproducible separations of the components of a wide range of natural lipid mixtures, and to be useful in studies of the hydrolysis and other modifications of these materials.

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Regio- and Stereochemical Analysis of Trihydroxyoctadecenoic Acids Derived from Linoleic Acid 9- and 13-Hydroperoxides

Mats Hamberg*
Department of Physiological Chemistry, Karolinska Institutet, S-104 01 Stockholm, Sweden

The methyl esters of 9*S*,10*S*,13*R*-trihydroxy-11*E*-octadecenoic acid, 9*S*,10*S*,13*S*-trihydroxy-11*E*-octadecenoic acid, 9*S*,10*R*,13*S*-trihydroxy-11*E*-octadecenoic acid, and 9*S*,10*R*,13*R*-trihydroxy-11*E*-octadecenoic acid were prepared from 9*S*-hydroperoxy-10*E*,12*Z*-octadecadienoic acid *via* the epoxy alcohols methyl 10*R*,11*R*-epoxy-9*S*-hydroxy-12*Z*-octadecenoate and methyl 10*S*,11*S*-epoxy-9*S*-hydroxy-12*Z*-octadecenoate. The trihydroxyesters, as well as four stereoisomeric methyl 9,12,13-trihydroxy-10*E*-octadecenoates earlier prepared [Hamberg, M., *Chem. Phys. Lipids 43,* 55–67 (1987)], were characterized by thin-layer chromatography, gas-liquid chromatography, mass spectrometry, and by chemical degradation. Availability of these chemically defined trihydroxyoctadecenoates made it possible to design a method for regio- and stereochemical analysis of 9,10,13- and 9,12,13-trihydroxyoctadecenoic acids obtained from various sources. Application of the method revealed that the mixture of 9,10,13- and 9,12,13-trihydroxyoctadecenoic acids formed during autoxidation of linoleic acid in aqueous medium contained comparable amounts of the sixteen possible regio- and stereoisomers. Furthermore, hydrolysis of the allylic epoxy alcohol, methyl 9*S*,10*R*-epoxy-13*S*-hydroxy-11*E*-octadecenoate, yielded a major trihydroxyoctadecenoate, *i.e.,* methyl 9*S*,10*S*,13*S*-trihydroxy-11*E*-octadecenoate, together with smaller amounts of methyl 9*S*,10*R*,13*S*-trihydroxy-11*E*-octadecenoate, methyl 9*S*,12*R*,13*S*-trihydroxy-10*E*-octadecenoate, and methyl 9*S*,12*S*,13*S*-trihydroxy-10*E*-octadecenoate.

Lipids 26, 407–415 (1991).

Trihydroxyoctadecenoic acids containing the 1,2,5-trihydroxy-3*E*-pentene moiety were first described by Graveland (1) in a study of oxygenation of linoleic acid in suspensions of wheat flour. Two trihydroxy acid isomers were isolated and characterized, *i.e.,* 9,10,13-trihydroxy-11*E*-octadecenoic acid and 9,12,13-trihydroxy-10*E*-octadecenoic acid. Since then, a large number of studies have been concerned with the formation of trihydroxyoctadecenoic acids from linoleic acid *via* linoleic acid 9- and 13-hydroperoxides (2–10). In addition, trihydroxy acids derived from linoleic and/or α-linolenic acids have been isolated from the marine blue-green alga *Lyngbya majuscula* (11), from higher plants (12–16), from the culture medium of the primitive fungus *Saprolegnia parasitica* (17), and from beer (18). Furthermore, trihydroxyoctadecenoic acids constitute a considerable portion of the stable end-products formed on autoxidation of linoleic acid in aqueous systems (19). Linoleic acid-derived trihydroxyoctadecenoic acids have been reported to exert prostaglandin E-like activity on a number of smooth muscle preparations (14) and to be bitter tasting (20).

The present paper is concerned with preparation and characterization of eight stereoisomeric 9,10,13- and 9,12,13-trihydroxyoctadecenoic acids and with a method for regio- and stereochemical analysis of these compounds and their enantiomers.

MATERIALS AND METHODS

Linoleic acid was purchased from Nu-Chek Prep (Elysian, MN). [1-^{14}C]Linoleic acid and $H_2{}^{18}O$ (98 atom% ^{18}O) were obtained from Amersham Laboratories (Amersham, UK).

9*S*-[1-^{14}C]HPOD (specific radioactivity, 3.0 kBq/μmol; enantiomeric composition: 9*S*/9*R*, 98:2) was prepared by incubation of [1-^{14}C]linoleic acid with whole homogenate of tomato essentially as earlier described (21). 13*S*-[1-^{14}C]HPOD (specific radioactivity, 7.2 kBq/μmol; enantiomeric composition: 13*S*/13*R*, 99:1) was obtained by incubation of [1-^{14}C]linoleic acid with soybean lipoxygenase (22). Methyl 9*R*,12*S*,13*S*-trihydroxy-10*E*-octadecenoate (**9a**), methyl 9*S*,12*S*,13*S*-trihydroxy-10*E*-octadecenoate (**10a**), methyl 9*S*,12*R*,13*S*-trihydroxy-10*E*-octadecenoate (**11a**), and methyl 9*R*,12*R*,13*S*-trihydroxy-10*E*-octadecenoate (**12a**) were prepared by hydrolysis of the epoxy alcohols methyl 11*R*,12*R*-epoxy-13*S*-hydroxy-9*Z*-octadecenoate (**3**) and methyl 11*S*,12*S*-epoxy-13*S*-hydroxy-9*Z*-octadecenoate (**4**) as described (ref. 9; see Fig. 1). Methyl *erythro*- and *threo*-2,3-dihydroxyoctanoates were prepared by OsO_4 hydroxylation of methyl 2*Z*- and 2*E*-octenoates, respectively (23).

Methyl 9S,10R-epoxy-13S-hydroxy-11E-octadecenoate. A mixture of methyl 9*S*,10*R*-epoxy-13*S*-hydroxy-11*E*-octadecenoate and methyl 9*R*,10*S*-epoxy-13*S*-hydroxy-11*E*-octadecenoate (91:9, w/w) was obtained following incubation of 13*S*-HPOD with *Vicia faba* epoxygenase as recently described (24). The minor epoxy alcohol was removed by thin-layer chromatography (TLC) using ethyl acetate/toluene (3:7, v/v) as solvent (9*S*,10*R*-epoxy alcohol, R_f, 0.44; 9*R*,10*S*-epoxy alcohol, R_f, 0.48).

Methyl 10R,11R-epoxy-9S-hydroxy-12Z-octadecenoate (1) and methyl 10S,11S-epoxy-9S-hydroxy-12Z-octadecenoate (2). Treatment of the methyl ester of 9*S*-HPOD (10 mg) with 10 mL of hexane saturated with vanadium oxyacetylacetonate at 22°C for 1 hr (9) yielded two diastereomeric α,β-epoxy alcohols in a 1:1 ratio. The compounds were obtained in pure form following thin-layer chromatography (TLC) using ethyl acetate/hexane (3:7, v/v) as solvent. As recently described, the more polar epoxy alcohol (R_f, 0.49) was identical with methyl 10*R*,11*R*-epoxy-9*S*-hydroxy-12*Z*-octadecenoate (10).

The less polar epoxy alcohol (R_f, 0.59) was found to be the diastereomeric methyl 10*S*,11*S*-epoxy-9*S*-hydroxy-12*Z*-octadecenoate on basis of the following results. Infrared spectrometry showed bands at 3620–3350 cm^{-1}

*To whom correspondence should be addressed at Department of Physiological Chemistry, Karolinska Institutet, Box 60400, S-104 01 Stockholm, Sweden.

Abbreviations: CC, cyclic carbonate; GC-MS, gas chromatography-mass spectrometry; GLC, gas-liquid chromatography; 9*S*-HOD, 9*S*-hydroxy-10*E*,12*Z*-octadecadienoic acid; 9*S*-HPOD, 9*S*-hydroperoxy-10*E*,12*Z*-octadecadienoic acid; 13*S*-HOD, 13*S*-hydroxy-9*Z*,11*E*-octadecadienoic acid; 13*S*-HPOD, 13*S*-hydroperoxy-9*Z*,11*E*-octadecadienoic acid; IR, infrared; MC, (−)-menthoxycarbonyl; Me_3Si, trimethylsilyl; TLC, thin-layer chromatography; UV, ultraviolet.

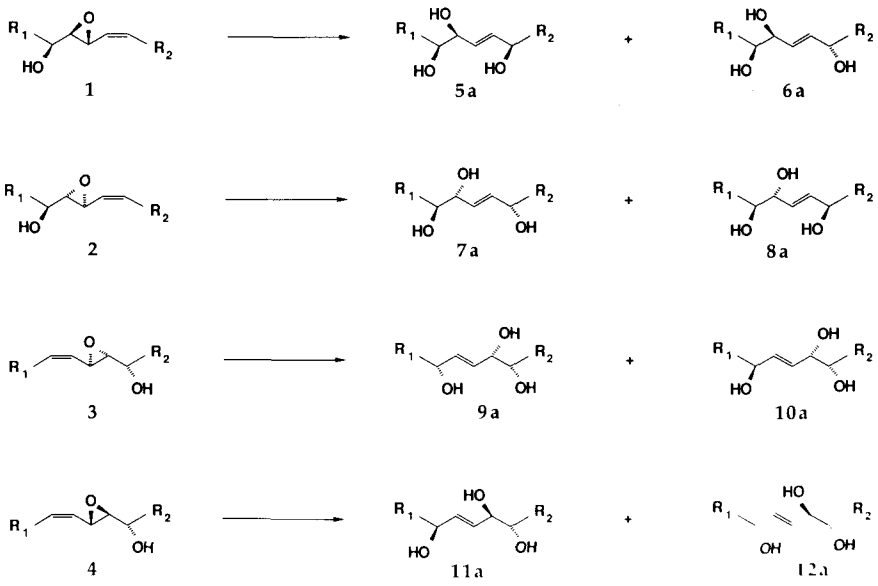


FIG. 1. Structures of trihydroxyesters prepared by hydrolysis of epoxy alcohols. R_1 = $(CH_2)_7$-$COOCH_3$; R_2 = $(CH_2)_4$-CH_3.

(hydroxyl), 1735 cm^{-1} (ester carbonyl) and 890 cm^{-1} (*trans* epoxide). No absorption band appeared in the range 950–1000 cm^{-1}, demonstrating the absence of *E* double bond(s). The mass spectrum of the trimethylsilyl (Me_3Si) ether derivative was virtually identical to that of the corresponding derivative of the 10*R*,11*R*-epoxy alcohol (10). Furthermore, $KMnO_4$ oxidation (23) performed on the (−)-menthoxycarbonyl (MC) derivative afforded the MC derivative of 2*S*-hydroxysebacate showing the presence of a hydroxyl group at C-9 (*S* configuration). Catalytic hydrogenation yielded methyl *erythro*-9,10-dihydroxyoctadecanoate as demonstrated by gas-liquid chromatography (GLC) using references of authentic methyl *erythro*- and *threo*-9,10-dihydroxyoctadecanoates (C-values of Me_3Si derivatives, 21.41 and 21.25, respectively; see ref. 24). Assignment of the absolute configuration of the epoxide carbons C-10 and C-11 as *S,S* followed from the established configuration at C-9 (*S*) coupled with the facts that the relative configuration of C-9/C-10 was *erythro*, and that the epoxide group had the *trans* configuration.

Dimethyl 2E-*undecene-1,11-dioate.* Malonic acid (6.4 g; 62 mmol) was slowly added to pyridine (14 mL). The solution was stirred at 22°C for 30 min and subsequently added to methyl 9-oxononanoate (8.8 g, 47 mmol) in pyridine (10 mL) containing piperidine (0.4 mL). The mixture was kept at 55°C for 1 hr and at 85°C for 4 hr. Extraction with diethyl ether afforded an oily residue that was treated with 10% (w/v) sodium hydroxide in 80% aqueous methanol at 22°C for 15 hr. The crude 2*E*-undecene-1,11-dioic acid (12 g) thus obtained was purified by crystallization from ethyl acetate/hexane. The identity of the compound was based on i) ultraviolet (UV) spectrometry (λ_{max} = 208 nm in ethanol; α,β-unsaturated carboxyl), ii) infrared spectrometry [dimethyl ester; 1730 cm^{-1} (ester carbonyls), and 985 cm^{-1} (*E* double bond)], iii) mass spectrometry [dimethyl ester; prominent ions at *m/e* 211 (M-31, 8%), 182 (M-60; loss of $COOCH_3$ + H, 20), 178 (M-2*32, 27), 150 (182-32, 60), and 55 (100)], and iv) oxidative ozonolysis (performed on the dimethyl ester; affording methyl hydrogen azelate).

Dimethyl erythro-*2,3-dihydroxyundecane-1,11-dioate.* Dimethyl 2*E*-undecene-1,11-dioate (484 mg; 2 mmol) was refluxed for 72 hr with *m*-chloroperbenzoic acid (500 mg) in methylene chloride (20 mL). Material obtained by extraction with diethyl ether was subjected to preparative TLC [solvent system, ethyl acetate/hexane (2:8, v/v)] to afford pure dimethyl *trans*-2,3-epoxyundecane-1,11-dioate [mass spectrum, *m/e* 226 (M-32; 1%), 209 (M-[18+31]; 4), 201 (23), 167 (M-[59+32], 22), 137 (33), and 55 (100)].

Attempts to hydrolyze the epoxide by treatment with perchloric acid in aqueous dimethoxyethane were unsuccessful. Instead, the diol was obtained by treatment of the epoxide (200 mg) with formic acid (10 mL) at 22°C for 15 hr followed by hydrolysis of the formate ester by treatment with 5% (w/v) sodium hydroxide in 80% aqueous methanol at 50°C for 1 hr. Material obtained by extraction with ethyl acetate was esterified by treatment with diazomethane and subjected to preparative TLC [solvent, ethyl acetate/hexane (1:1, v/v; R_f, 0.30)]. Analysis of the Me_3Si derivative of the dimethyl *erythro*-2,3-dihydroxyundecane-1,11-dioate gave a C-value of 17.70 and a mass spectrum which showed prominent ions at *m/e* 405 (M-15, 1%), 389 (M-31, 2), 373 [M-(15+32), 2], 361 (M-59; loss of $\cdot COOCH_3$, 2), 345 (5), 273 (14), 271 [M-(59+90), 9], 259 (Me_3SiO^+=CH-$(CH_2)_7$-$COOCH_3$, 42), 234 (M-186; migration of Me_3Si from C-3 to C-1 followed by elimination of OHC-$(CH_2)_7$-$COOCH_3$, 50), 155 (OHC-$(CH_2)_7$-C≡O^+; 43), and 73 (100).

Dimethyl threo-*2,3-dihydroxyundecane-1,11-dioate.* Dimethyl 2*E*-undecene-1,11-dioate (50 mg) was *cis*-hydroxylated by treatment with OsO_4 as previously described (23). The resulting dimethyl *threo*-2,3-dihydroxyundecane-1,11-dioate was purified by preparative TLC [solvent system, ethyl acetate/hexane (1:1, v/v; R_f, 0.34)]. An aliquot was analyzed by gas chromatography-mass spectrometry (GC-MS) as the Me_3Si derivative. The C-value

was 17.81 and the mass spectrum was virtually identical to that of the corresponding *erythro* isomer given above.

Cyclic carbonate derivatives of dimethyl erythro- *and* threo-*2,3-dihydroxyundecane-1,11-dioates.* Dimethyl *erythro-* and *threo*-2,3-dihydroxyundecane-1,11-dioates (200 μg) were converted into their cyclic carbonate (CC) derivatives by treatment with phosgene in chloroform/toluene/pyridine (23; see also below). Material obtained by extraction with diethyl ether was purified by TLC using ethyl acetate/hexane (25:75, v/v) as the solvent system. The CC derivatives were analyzed by GLC as shown in Figure 2B. The CC derivatives of methyl *erythro-* and *threo*-2,3-dihydroxyoctanoates (23) were analyzed in a similar way (Fig. 2A).

Autoxidation of linoleic acid. Linoleic acid (250 mg) was dissolved in ethanol (50 mL). The mixture was heated and water (75 mL) was slowly added. The solution was refluxed under a stream of oxygen gas for 42 hr. Material obtained by extraction with diethyl ether was treated with diazomethane. Methyl 10*Z*-nonadecenoate (1.47 mg) was added to an aliquot (5%) of the esterified autoxidation product. The mixture was subjected to silicic acid column chromatography and the fatty acid ester fraction was isolated and analyzed by GLC. From the ratio of the areas of the peaks of methyl linoleate and of the added internal standard it was calculated that the amount of remaining linoleate was 4–5 mg, *i.e.,* the extent of autoxidation of linoleic acid was *ca.* 98%. The yield of 9,10,13- and 9,12,13-trihydroxyoctadecenoates present in the autoxidation product was not precisely determined in the present study. However, from the weights of trihydroxyesters recovered following TLC it was estimated that the yield of trihydroxy acids under the conditions used for autoxidation was 5–10% based on the starting amount of linoleic acid.

Steric analysis. Determination of the absolute configuration of allylic alcohol groups was carried out by gas-liquid chromatographic analysis of MC derivatives of short-chain esters formed upon oxidative ozonolysis (25). Epoxy alcohols (100–500 μg) were derivatized by treatment with toluene (50 μL), pyridine (10 μL) and a toluene solution (50 μL) of (−)-menthoxycarbonyl chloride (Aldrich-Chemie, Steinheim, Germany; 1 μmol/μL) at 22°C for 30 min. The MC derivatives were purified by TLC [solvent system, ethyl acetate/hexane (1:9, v/v)] and subjected to oxidative ozonolysis. The esterified product containing the MC derivatives of methyl 2-hydroxyheptanoate or dimethyl 2-hydroxysebacate was analyzed by GLC using authentic reference compounds (25). Methyl trihydroxyoctadecenoates were derivatized by treatment with toluene (50 μL), pyridine (25 μL) and menthoxycarbonyl chloride solution (100 μL) at 60°C for 3 hr. The tris-MC derivatives were purified and further analyzed as described above. Formation of MC derivatives of dienols, *i.e.,* the methyl esters of 9- and 13-HOD, was found to be accompanied by partial racemization with the batch of reagent used in the present study. In order to avoid this problem, these compounds were directly ozonized and subsequently treated with toluene (50 μL), pyridine (10 μL), and menthoxycarbonyl chloride solution (50 μL) at 22°C for 30 min. Hexane (3 mL) was added and the organic phase was washed twice with 0.5 mL of water. The residue obtained after evaporation of the solvent was oxidized with peracetic acid at 50°C for 15 hr (25). The product was esterified and subjected to TLC [solvent system, ethyl acetate/hexane (1:9, v/v)] using references of the MC derivatives of methyl 2*R,S*-hydroxyheptanoate and dimethyl 2*R,S*-hydroxysebacate. The zones corresponding to the spots of the reference compounds were scraped off and the recovered materials were analyzed by GLC.

The relative configuration of the 1,2-diol structure of trihydroxyoctadecenoates was determined by gas-liquid chromatographic analysis of CC derivatives of 2,3-dihydroxyesters obtained following oxidative ozonolysis. The trihydroxyesters (100–500 μg) were dissolved in chloroform (0.2 mL) and pyridine (0.1 mL) and treated with 0.05 mL of a solution of phosgene in toluene (20%, w/w) at 0°C for 5 min. After an additional 30 min at 22°C, excess reagent was destroyed by treatment with methanol (2 mL) for 15 min. Material obtained by extraction with diethyl ether was subjected to oxidative ozonolysis (25), and the esterified product was analyzed by GLC.

Chromatographic and instrumental methods. For most analyses TLC was carried out with precoated plates (Kieselgel 60, 0.25 mm) from E. Merck (Darmstadt, Germany). *Erythro-* and *threo*-isomers of methyl trihydroxyoctadecenoates were separated using plates coated with sodium arsenite/silica gel Gel G (1:9, w/w) and a solvent system consisting of methanol/chloroform (3:97, v/v). Material was located by spraying with 2′,7′-dichlorofluorescein and viewing under UV light. Radioactivity on TLC plates was determined by a Berthold Dünnschichtscanner II (Wildbad, Germany) interfaced with a Macintosh SE/30 PC. GLC was performed with a Hewlett-Packard (Avondale, PA) model 5890 gas chromatograph equipped with a methyl silicone capillary column (length, 25 m; film thickness, 0.33 μm). Helium at a flow rate of 25 cm/s was used as the carrier gas. GC-MS was carried out with a Hewlett-Packard model 5970B mass selective detector connected to a Hewlett-Packard model 5890 gas chromatograph. Ultraviolet spectrometry was carried out with a Hewlett-Packard model 8450A UV/VIS

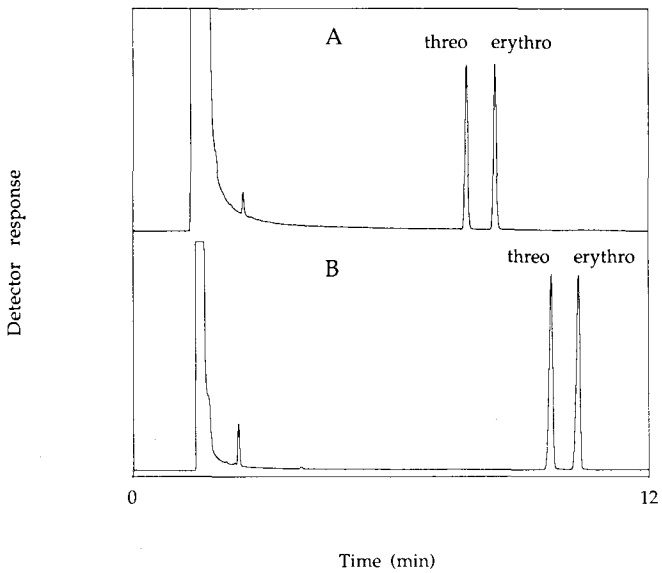


FIG. 2. Gas chromatograms of CC derivatives of methyl *erythro-* and *threo*-2,3-dihydroxyoctanoates (A), and of dimethyl *erythro-* and *threo*-2,3-dihydroxyundecane-1,11-dioates (B). Column temperatures, 160°C (A) and 220°C (B); carrier gas, helium at a flow rate of 25 cm/s.

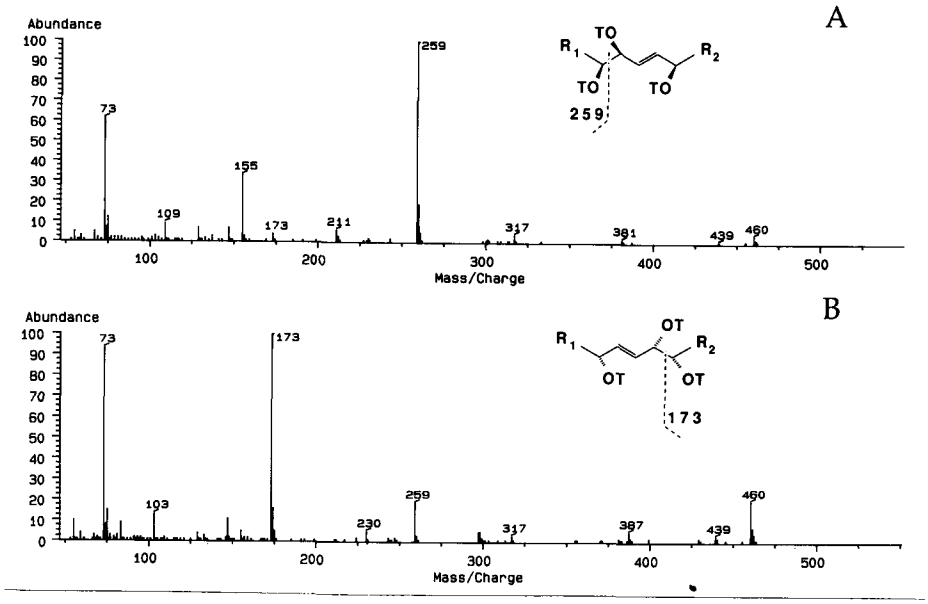


FIG. 3. Mass spectra of Me_3Si derivatives of compounds 5a (A) and 9a (B). "T" = Me_3Si; R_1 =$(CH_2)_7$-$COOCH_3$; R_2 = $(CH_2)_4$-CH_3.

spectrophotometer and infrared spectra were recorded with a Perkin-Elmer (Überlingen, Germany) model 257 infrared (IR) spectrophotometer. Radioactivity was determined with a Packard (Downers Grove, IL) Tri-Carb model 4450 liquid scintillation counter.

RESULTS

Methyl 9S,10S,13R-trihydroxy-11E-octadecenoate (5a) and methyl 9S,10S,13S-trihydroxy-11E-octadecenoate (6a). Methyl [1-^{14}C]10*R*,11*R*-epoxy-9*S*-hydroxy-12*Z*-octadecenoate (2 mg; compound 1 in Fig. 1) was dissolved in methanol (0.5 mL) and glass-distilled water (50 mL) was added. The solution was acidified to pH 3 by addition of dilute hydrochloric acid. After 5 min at 22°C the solution was extracted with diethyl ether, and the material obtained was subjected to radio-TLC using ethyl acetate as solvent. Two major peaks of radioactivity appeared (ratio, *ca.* 1:1), *i.e.,* compounds **5a** and **6a**. In addition, smaller peaks of less polar material were observed. This material was not fully characterized although the mass spectra of the Me_3Si derivatives indicated that it was due to two stereoisomeric methyl 9,10,11-trihydroxyoctadecenoates (cf. ref. 9).

The UV spectra of **5a** and **6a** did not show any specific absorption in the range 205–300 nm. The IR spectra of the compounds were similar and showed absorption bands at 3620-3200 cm^{-1} (hydroxyls), 1730 cm^{-1} (ester carbonyl), and 980 cm^{-1} (*E* double bond).

The mass spectra of the Me_3Si derivatives of **5a** and **6a** were virtually identical and showed prominent ions at *m/e* 460 (M-100; rearrangement with elimination of OHC-$(CH_2)_4$-CH_3, 4%), 439 [M-(31+90), 1], 387 [(CH=CH-CH($OSiMe_3$)-CH($OSiMe_3$)-$(CH_2)_7$-$COOCH_3$)$^+$, 1], 317 (4), 259 (Me_3SiO^+=CH-$(CH_2)_7$-$COOCH_3$, 100), 211 (6), 173 (Me_3SiO^+=CH-$(CH_2)_4$-CH_3, 4), 155 (OHC-$(CH_2)_7$-C≡O^+, 33), 109 (9), and 73 (Me_3Si^+, 61) (Fig. 3A). For comparison, Figure 3B shows the mass spectrum of the Me_3Si derivative of a methyl 9,12,13-trihydroxyoctadecenoate, *i.e.,* compound **9a**. This spectrum showed prominent ions at *m/e* 460 (M-100; rearrangement with elimination of OHC-$(CH_2)_4$-CH_3, 20%), 439 [M-(31+90), 3], 387 (Me_3SiO^+=CH-CH=CH-CH($OSiMe_3$)-$(CH_2)_7$-$COOCH_3$, 5), 317 (3), 259 (Me_3SiO^+=CH-$(CH_2)_7$-$COOCH_3$, 19), 230 (5), 173 (Me_3SiO^+=CH-$(CH_2)_4$-CH_3, 100), 103 (Me_3SiO^+=CH_2, 13), and 73 (Me_3Si^+, 94).

Oxidative ozonolysis performed on the MC derivatives of **5a** and **6a** yielded the MC derivatives of 2*R*- and 2*S*-hydroxyheptanoic acids, respectively. This result showed that both compounds possessed a double bond in the Δ^{11} position as well as a hydroxyl group at C-13 (*R* in **5a**, *S* in **6a**). Oxidative ozonolysis of the CC derivatives of **5a** and **6a** followed by esterification afforded the CC derivative of dimethyl *threo*-2,3-dihydroxyundecane-1,11-dioate. On the basis of these results, **5a** was identified as methyl 9*S*,10*S*,13*R*-trihydroxy-11*E*-octadecenoate and **6a** as methyl 9*S*,10*S*,13*S*-trihydroxy-11*E*-octadecenoate (Fig. 1).

Methyl 9S,10R,13S-trihydroxy-11E-octadecenoate (7a) and methyl 9S,10R,13R-trihydroxy-11E-octadecenoate (8a). Methyl [1-^{14}C]10*S*,11*S*-epoxy-9*S*-hydroxy-12*Z*-octadecenoate (2 mg; compound 2 in Fig. 1) was hydrolyzed at pH 3 as described above and the product was subjected to TLC. Two major labeled compounds appeared, *i.e.,* compounds **7a** and **8a**. In addition, less polar compounds tentatively identified as two stereoisomeric methyl 9,10,11-trihydroxyoctadecenoates were observed in the chromatogram.

The UV, IR, and mass spectral data obtained for compounds **7a** and **8a** were similar to those obtained for **5a** and **6a** as described above, indicating that **7a** and **8a** were stereoisomeric methyl 9,10,13-trihydroxy-11*E*-octadecenoates. Oxidative ozonolysis performed on the MC derivatives of **7a** and **8a** produced the MC derivatives of 2*S*- and 2*R*-hydroxyheptanoic acids, respectively, whereas ozonolysis of the CC derivatives followed by esterification yielded the CC derivative of dimethyl *erythro*-2,3-di-

hydroxyundecane-1,11-dioate. On the basis of these results, compound **7a** was identified as methyl 9*S*,10*R*,13*S*-trihydroxy-11*E*-octadecenoate and compound **8a** as methyl 9*S*,10*R*,13*R*-trihydroxy-11*E*-octadecenoate (Fig. 1).

Characterization of trihydroxyesters by TLC and GLC. Figure 4 and Table 1 show results of thin-layer chromatographic analysis of trihydroxyesters **5a–12a**. As seen, a group separation was observed between trihydroxyesters **5a, 7a, 9a, 11a**, and trihydroxyesters **6a, 8a, 10a, 12a**. The factor underlying this separation was the steric relationship between the two allylic hydroxyl groups, *i.e.*, those trihydroxyesters in which the two hydroxyl groups were located on the same side with respect to the plane of the double bond were more polar than those in which the hydroxyl groups were located on opposite sides. The terms "*cis*-1,4-diol" and "*trans*-1,4-diol" were introduced to designate trihydroxyesters in which the two allylic, non-vicinal hydroxyls were located on the same and opposite sides, respectively, with respect to the plane of the double bond. A small superimposed effect on the TLC separation was exerted by the 1,2-diol structure, *i.e.*, the presence of a *threo* diol in a trihydroxyester slightly reduced the polarity compared to that of the corresponding *erythro* compound. On the other hand, 9,10,13- and 9,12,13-trihydroxyoctadecenoates having the same steric relationship did not separate.

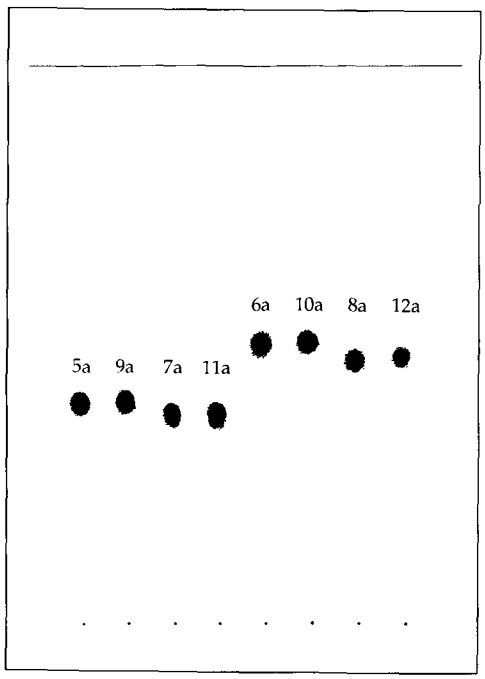


FIG. 4. TLC separation of trihydroxyesters 5a–12a. Solvent system, ethyl acetate.

Erythro- and *threo*-trihydroxyesters were separated by sodium arsenite-TLC (Fig. 5, Table 1). As expected (26), a *threo* trihydroxyester was less polar than the corresponding *erythro* compound. In addition, a superimposed effect of the relative configuration of the 1,4-diol structure was observed. As with regular TLC, pairs of 9,10,13- and 9,12,13-trihydroxyoctadecenoates having the same stereochemical features did not separate.

GLC was useful for separation of *erythro/threo* isomers and of positional isomers (Table 1). However, because of overlapping of the retention times of the Me_3Si derivatives of certain trihydroxyesters, analysis of mixtures of trihydroxyoctadecenoates by GLC was performed following separation of trihydroxyesters into *cis*- and *trans*-1,4-diols by regular TLC. This is illustrated by Figure 6 which shows analysis by GLC of Me_3Si derivatives of trihydroxyesters formed upon autoxidation of linoleic acid.

Method for determination of individual isomers of 9,10,13- and 9,12,13-trihydroxyoctadecenoic acids. The following method was developed for complete analysis of the 16 possible isomers of 9,10,13- and 9,12,13-trihydroxyoctadecenoates. In the first step, the mixture of methyl trihydroxyoctadecenoates (0.2–1 mg) was subjected to TLC using ethyl acetate as the solvent. Two bands of

TABLE 1

Thin-Layer Chromatographic and Gas-Liquid Chromatographic Data of Compounds 5a–12a

Compound	R_f value		C-value[c]
	Plain silica gel[a]	$NaAsO_2$-silica gel[b]	
5a	0.39	0.31	22.29
6a	0.50	0.39	22.36
7a	0.37	0.22	22.57
8a	0.47	0.30	22.58
9a	0.39	0.30	22.37
10a	0.50	0.40	22.45
11a	0.37	0.23	22.62
12a	0.47	0.31	22.65

[a] Solvent system, ethyl acetate.

[b] $NaAsO_2$/silica gel (1:9, w/w); solvent system, methanol/chloroform (3:97, v/v).

[c] Analyses were performed on the Me_3Si derivatives. Column temperature, 230°C; carrier gas, helium at a flow rate of 25 cm/s.

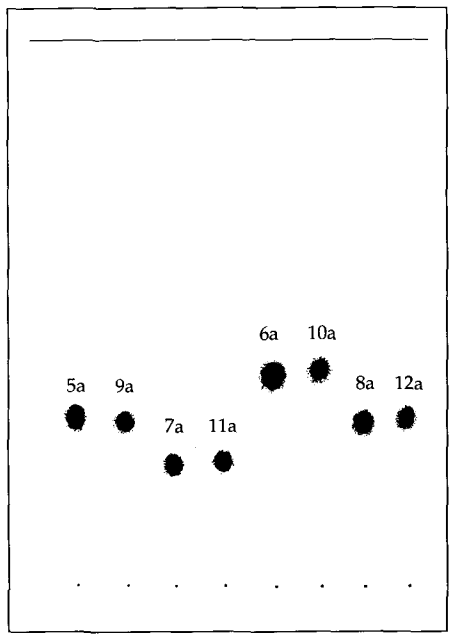


FIG. 5. TLC separation of trihydroxyesters 5a–12a using silica gel impregnated with $NaAsO_2$. Solvent system, methanol/$CHCl_3$ (3:7, v/v).

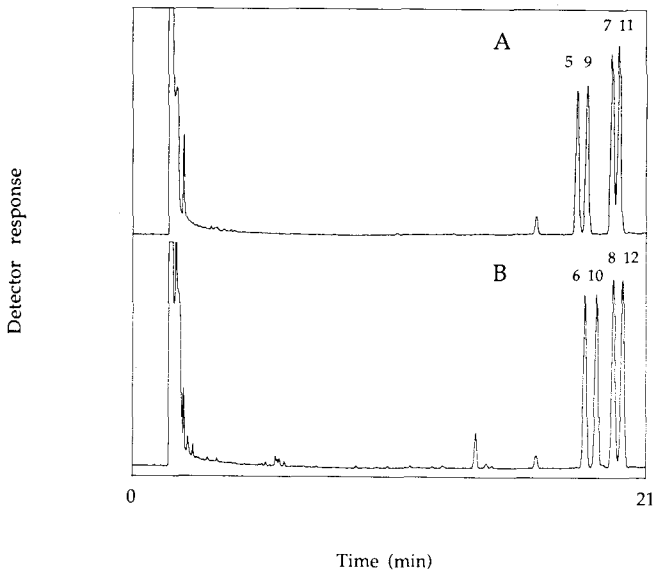


FIG. 6. Gas chromatograms of Me_3Si derivatives of trihydroxyesters obtained by autoxidation of linoleic acid. Separate analyses were performed of trihydroxyesters containing the *cis*-1,4-diol structure (compounds 5, 7, 9, and 11; panel A) and trihydroxyesters containing the *trans*-1,4-diol structure (compounds 6, 8, 10, and 12; panel B). Column temperature, 230°C; carrier gas, helium at a flow rate of 25 cm/s.

material appeared, *i.e.*, trihydroxyesters having the *cis*- and *trans*-1,4-diol structures (R_f = 0.37–0.39 and 0.47–0.50, respectively; see Fig. 4 and Table 1). Methyl ricinoleate (221 μg) was added to the fractions of *cis*- and *trans*-1,4-diols to serve as an internal standard. An aliquot of each fraction was converted into the Me_3Si derivatives and analyzed by GLC. The percentage composition of the mixture of compounds 5–12 was calculated from the peak areas of the trihydroxyesters and the peak area of the internal standard. The remaining parts of the fractions of *cis*- and *trans*-1,4-diols were used to determine the enantiomeric composition of the compounds detected by GLC. In situations where GLC analysis had shown that the fractions of *cis*- and *trans*-1,4-diols were due to a single diastereomer of methyl 9,10,13-trihydroxy-11*E*-octadecenoate and/or of 9,12,13-trihydroxy-10*E*-octadecenoate steric analysis was performed directly on the material present in the two fractions. With more complex mixtures of trihydroxyesters, each fraction contained two 9,10,13- and/or two 9,12,13-trihydroxyoctadecenoates (*erythro* and *threo* isomers). In this case, materials present in the *cis*- and *trans*-1,4-diol fractions were separately subjected to sodium arsenite-TLC in order to obtain fractions of *threo*/*cis*-1,4-, *threo*/*trans*-1,4-, *erythro*/*cis*-1,4-, and *erythro*/*trans*-1,4-diols. These fractions, which contained only one methyl 9,10,13-trihydroxyoctadecenoate and/or one methyl 9,12,13-trihydroxyoctadecenoate (Table 2), were subjected to steric analysis.

TABLE 2

Characteristics and Structures of Trihydroxyoctadecenoates 5–12

Compound	Regiochemical feature	Stereochemical feature	Enantiomer	Structure
5	9,10,13-trihydroxy	*threo*/*cis*-1,4-diol	5a	Methyl 9*S*,10*S*,13*R*-trihydroxy-11*E*-octadecenoate
			5b	Methyl 9*R*,10*R*,13*S*-trihydroxy-11*E*-octadecenoate
6	9,10,13-trihydroxy	*threo*/*trans*-1,4-diol	6a	Methyl 9*S*,10*S*,13*S*-trihydroxy-11*E*-octadecenoate
			6b	Methyl 9*R*,10*R*,13*R*-trihydroxy-11*E*-octadecenoate
7	9,10,13-trihydroxy	*erythro*/*cis*-1,4-diol	7a	Methyl 9*S*,10*R*,13*S*-trihydroxy-11*E*-octadecenoate
			7b	Methyl 9*R*,10*S*,13*R*-trihydroxy-11*E*-octadecenoate
8	9,10,13-trihydroxy	*erythro*/*trans*-1,4-diol	8a	Methyl 9*S*,10*R*,13*R*-trihydroxy-11*E*-octadecenoate
			8b	Methyl 9*R*,10*S*,13*S*-trihydroxy-11*E*-octadecenoate
9	9,12,13-trihydroxy	*threo*/*cis*-1,4-diol	9a	Methyl 9*R*,12*S*,13*S*-trihydroxy-10*E*-octadecenoate
			9b	Methyl 9*S*,12*R*,13*R*-trihydroxy-10*E*-octadecenoate
10	9,12,13-trihydroxy	*threo*/*trans*-1,4-diol	10a	Methyl 9*S*,12*S*,13*S*-trihydroxy-10*E*-octadecenoate
			10b	Methyl 9*R*,12*R*,13*R*-trihydroxy-10*E*-octadecenoate
11	9,12,13-trihydroxy	*erythro*/*cis*-1,4-diol	11a	Methyl 9*S*,12*R*,13*S*-trihydroxy-10*E*-octadecenoate
			11b	Methyl 9*R*,12*S*,13*R*-trihydroxy-10*E*-octadecenoate
12	9,12,13-trihydroxy	*erythro*/*trans*-1,4-diol	12a	Methyl 9*R*,12*R*,13*S*-trihydroxy-10*E*-octadecenoate
			12b	Methyl 9*S*,12*S*,13*R*-trihydroxy-10*E*-octadecenoate

Regio- and stereochemical analysis of trihydroxyoctadecenoates formed by autoxidation. The method described was used to determine the percentage amounts of individual isomers present in the mixture of 9,10,13- and 9,12,13-trihydroxyoctadecenoic acids formed upon autoxidation of linoleic acid in aqueous medium. An aliquot of the esterified autoxidation product (21 mg; containing 1–2 mg of 9,10,13- and 9,12,13-trihydroxyoctadecenoic acid isomers) was subjected to TLC (solvent, ethyl acetate). The bands due to the *cis*- and *trans*-1,4-diol isomers of methyl trihydroxyoctadecenoates were scraped off and added to methyl ricinoleate (221 μg). One-tenth of each fraction was converted into the Me_3Si derivatives and analyzed by GLC (cf. Fig. 6) and the percentages of compounds **5–12** were determined. The remaining part of each fraction was subjected to sodium arsenite-TLC. The materials present in the fractions of *threo/cis*-1,4-, *threo/trans*-1,4-, *erythro/cis*-1,4-, and *erythro/trans*-1,4-diols were recovered and subjected to steric analysis. As expected, the methyl 2-hydroxyheptanoates and the dimethyl 2-hydroxysebacates formed upon oxidative ozonolysis were racemic. This fact coupled with the results of determination of **5–12** by GLC showed that the trihydroxyoctadecenoate fraction formed upon autoxidation of linoleic acid was a mixture of isomers in the following percentage amounts: **5a**, 5%; **5b**, 5%; **6a**, 6%; **6b**, 6%; **7a**, 7%; **7b**, 7%; **8a**, 7%; **8b**, 7%; **9a**, 5%; **9b**, 5%; **10a**, 6%; **10b**, 6%; **11a**, 7%; **11b**, 7%; **12a**, 7%; and **12b**, 7% (Fig. 7).

Regio- and stereochemical analysis of trihydroxyoctadecenoates formed by hydrolysis of methyl 9S,10R-epoxy-13S-hydroxy-11E-octadecenoate. Methyl 9*S*,10*R*-epoxy-13*S*-hydroxy-11*E*-octadecenoate (0.4 mg) was dissolved in methanol (0.5 mL) and water (50 mL) was added. The solution was acidified to pH 3 and kept at 22°C for 5 min during which time the initially cloudy solution became clear. The product isolated by extraction with diethyl ether was subjected to TLC. Part of the materials forming the bands of *cis*- and *trans*-1,4-diols was added to the methyl ricinoleate internal standard and analyzed by GLC. Four isomers were detected, *i.e.*, compounds **6** and **10** (*threo/trans*-1,4-diol isomers of methyl 9,10,13- and 9,12,13-trihydroxyoctadecenoates, respectively; see Table 2), as well as compounds **7** and **11** (*erythro/cis*-1,4-diol isomers of methyl 9,10,13- and 9,12,13-trihydroxyoctadecenoates, respectively). Steric analysis of the tris-MC derivatives of these materials showed that the 9,10,13-trihydroxyoctadecenoates present in the two fractions had the *S* configuration at C-13 (1% or less of the *R* configuration) and that the 9,12,13-trihydroxy isomers had the *S* configuration at C-9 (1% or less of the *R* configuration). The composition of trihydroxyoctadecenoate isomers formed by hydrolysis of methyl 9*S*,10*R*-epoxy-13*S*-hydroxy-11*E*-octadecenoate was thus found to be: compounds **6a**, 61%; **7a**, 16%; **11a**, 15%; and **10a**, 8% (Fig. 7).

Hydrolysis of methyl 9S,10R-epoxy-13S-hydroxy-11E-octadecenoate in $H_2{}^{18}O$. Methyl 9*S*,10*R*-epoxy-13*S*-hydroxy-11*E*-octadecenoate (0.4 mg) was dissolved in methanol (40 μL) and treated with $H_2{}^{18}O$ (250 μL) and 2 M hydrochloric acid (2 μL) at 22°C for 5 min. Material extracted with diethyl ether was esterified and subjected to TLC (solvent, ethyl acetate). The *cis*-1,4-diol fraction (containing **7** and **11**) and the *trans*-1,4-diol fraction (containing **6** and **10**) were recovered, and the Me_3Si derivatives were analyzed by selected monitoring of the ions *m/e* 173 and 175 (Me_3SiO^+=CH-$(CH_2)_4$-(CH_3)), 259 and 261 (Me_3SiO^+=CH-$(CH_2)_7$-$COOCH_3$), and 387, 389, and 391 (due to Me_3SiO^+=CH-CH=CH-CH$(OSiMe_3)$-$(CH_2)_7$-$COOCH_3$ in the mass spectra of **10** and **11**, and to $[CH{=}CH\text{-}CH(OSiMe_3)\text{-}CH(OSiMe_3)\text{-}(CH_2)_7\text{-}COOCH_3]^+$ in the mass spectra of **6** and **7**). The fragment containing the C-9 hydroxyl group (*m/e* 259) and that containing the C-13 hydroxyl group (*m/e* 173) did not contain ^{18}O (less than 0.5% ^{18}O species), whereas the fragment containing the C-9 hydroxyl group and either of the C-10 or C-12 hydroxyl groups (*m/e* 387) was due to 95% $^{18}O_1$ species and 5% $^{18}O_0$ species. These findings demonstrated that solvent attack in epoxy alcohol hydrolysis occurred exclusively at C-10 and C-12.

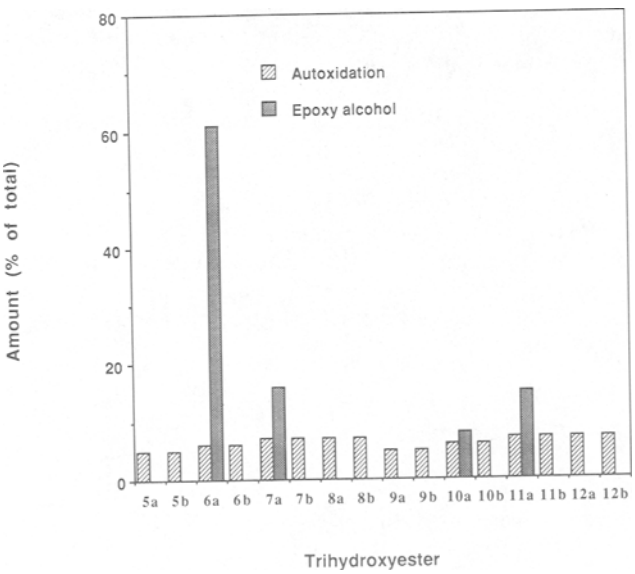


FIG. 7. Percentage compositions of isomeric trihydroxyoctadecenoates formed upon autoxidation of linoleic acid, and upon hydrolysis of methyl 9*S*,10*R*-epoxy-13*S*-hydroxy-11*E*-octadecenoate.

DISCUSSION

Several studies have been concerned with the formation of 9,10,13- and 9,12,13-trihydroxyoctadecenoic acids following incubation of linoleic acid with preparations of plant and animal tissue (1,2,4,5,7,8,10) and with the isolation of linoleic- and α-linolenic acid-derived trihydroxy acids as natural products from various sources (11–18). Arachidonic acid serves as the precursor of related series of compounds, *e.g.* trioxilin A_3 (27). The mechanism of formation of trihydroxyoctadecenoic acids containing the 1,2,5-trihydroxy-3*E*-pentene structure has been studied *inter alia* in chemical model systems (3,6,9) and has been found to involve initial lipoxygenase-catalyzed oxygenation of linoleic acid into its 9- or 13-hydroperoxide derivatives. The hydroperoxides can be further converted, enzymatically as well as non-enzymatically, into epoxy alcohols. Non-allylic epoxy alcohols are fairly stable compounds, whereas the allylic ones (containing either the 2,3-epoxy-1-hydroxy-4-pentene structure or the 4,5-epoxy-1-hydroxy-2-pentene structure) are rapidly hydrolyzed into isomeric trihydroxyoctadecenoic acids. As would be expected, the rate of hydrolysis is greatly accelerated at

acidic pH. For example, the half-lives of the arachidonic acid-derived allylic epoxy alcohols methyl 11*S*,12*R*-epoxy-15*S*-hydroxy-5*Z*,8*Z*,13*E*-eicosatrienoate and methyl 13*R*,14*R*-epoxy-15*S*-hydroxy-5*Z*,8*Z*,11*Z*-eicosatrienoate in aqueous medium at pH 3.0 and 22°C were found to be about 50 s and 100 s, respectively (28).

Linoleic acid-derived trihydroxyoctadecenoates possessing the 1,2,5-trihydroxy-3*E*-pentene structure can exist as 2 positional isomers, each having 3 asymmetrical carbon atoms. Thus, 16 isomers, *i.e.*, 8 separable compounds (5–12) each consisting of 2 enantiomers (**5a**, **5b**, etc.) are possible. In the present study, one of the two enantiomers of each of the 8 compounds was characterized by chemical and chromatographic methods. Preparation of **5a–8a** (Fig. 1) was accomplished by intermolecular epoxidation of the methyl ester of 9*S*-HPOD in the presence of vanadium oxyacetylacetonate into two diastereomeric *trans*-10,11-epoxy-9*S*-hydroxy-12*Z*-octadecenoates (1 and 2) followed by hydrolysis. Trihydroxyesters **9a–12a** were obtained in an analogous way starting with the methyl ester of 13*S*-HPOD (9). The structures of **5a–12a** were established by IR spectrometry and mass spectrometry, as well as by chemical degradation of the cyclic carbonate and (−)-menthoxycarbonyl derivatives followed by steric analysis of the short-chain esters by GLC.

The main factor determining the mobility of **5a–12a** on regular TLC (Fig. 4; Table 1) was the relative configuration of the two hydroxyl groups which are allylic to the *E* double bond. In agreement with previous work (9,10,23), trihydroxyesters having a *cis*-1,4-diol structure were more polar than trihydroxyesters having a *trans*-1,4-diol structure. A slight effect of the relative configuration of the 1,2-diol structure was also noticed. No separation between positional isomers having the same stereochemical features was observed. Separation of trihydroxyesters on sodium arsenite-TLC was influenced by the relative configurations of both the 1,2- and the 1,4-diol structures but not significantly by positional isomerism (Fig. 5; Table 1). As seen from Figure 6 and Table 1, separation of the Me_3Si derivatives of trihydroxyesters by GLC was affected mainly by the relative configuration of the 1,2-diol structure *(erythro/threo)*. In addition, GLC was capable of separating positional isomers (9,10,13-trihydroxy *vs* 9,12,13-trihydroxy-) having the same stereochemical features. By contrast, the retention times found on GLC were only marginally influenced by the relative configuration of the 1,4-diol structure.

The method developed for regio- and stereochemical analysis of individual isomers of trihydroxyoctadecenoates involved preliminary separation of the trihydroxyesters into *cis*- and *trans*-1,4-diols by regular TLC. Part of each fraction was analyzed by GLC in order to determine the percentages of compounds **5–12**, *i.e.*, isomers having known regiochemistry and known relative configurations of the 1,2- and 1,4-diol structures (Table 2). The absolute stereochemistry of the compounds detected by GLC was established by oxidative ozonolysis of the tris-MC derivatives followed by gas-liquid chromatographic analysis of the MC derivatives of methyl 2-hydroxyheptanoate and dimethyl 2-hydroxysebacate. The configuration of the first and last mentioned compound corresponded to the configuration of C-13 of the 9,10,13-trihydroxyesters and to the configuration of C-9 of the 9,12,13-trihydroxyesters, respectively. Application of the method to trihydroxyesters formed upon autoxidation of linoleic acid showed the presence of comparable amounts of compounds **5–12** (Fig. 7). As expected, each diastereomer was racemic. In another application, trihydroxyesters formed by hydrolysis of the epoxy alcohol methyl 9*S*,10*R*-epoxy-13*S*-hydroxy-11*E*-octadecenoate were analyzed. Four compounds appeared. Steric analysis showed that each of these compounds were pure enantiomers, *i.e.* **6a**, **7a**, **10a**, and **11a** (Fig. 7).

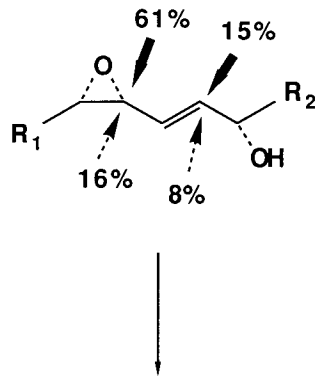


FIG. 8. Hydrolysis of methyl 9*S*,10*R*-epoxy-13*S*-hydroxy-11*E*-octadecenoate. Percentages indicate the extent of attack by solvent at the two faces of C-10 and C-12. R_1 = $(CH_2)_7$-$COOCH_3$; R_2 = $(CH_2)_4$-CH_3.

The composition of trihydroxyoctadecenoates formed upon hydrolysis of methyl 9*S*,10*R*-epoxy-13*S*-hydroxy-11*E*-octadecenoate, coupled with the $H_2{}^{18}O$ experiments which showed that solvent attack occurred exclusively at C-10 and C-12, demonstrated that hydrolysis of the epoxy alcohol occurred mainly (61%) by solvent attack at C-10 with inversion of the configuration of that carbon atom (Fig. 8). In addition, hydrolysis occurred by solvent attack at C-10 with retention of the configuration (16%), as well as by attack at C-12 with isomerization of the double bond into the Δ^{10} position and opening of the epoxide function (23%). Interestingly, solvent attack at C-12 did not occur to the same extent from the two sides of the plane of the Δ^{11} double bond. It may be speculated that water molecules behind the plane of the double bond (Fig. 8) were less reactive in attacking C-12 because of hydrogen bonding to the C-13 hydroxyl group.

The regio- and stereochemical profiles of trihydroxyoctadecenoic acids obtained from a number of natural sources and from epoxy alcohols in the presence of a plant epoxide hydrolase are presently under investigation in our laboratory.

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Comparative Specificity of Plasma Lecithin:Cholesterol Acyltransferase from Ten Animal Species

Douglas Grove and **Henry J. Pownall***
Division of Atherosclerosis and Lipoprotein Research, Department of Medicine, Baylor College of Medicine and The Methodist Hospital, Houston, Texas 77030

The molecular specificities of plasma lecithin:cholesterol acyltransferase (LCAT) from ten animal species have been compared. Using a reassembled high density lipoprotein containing a mixture of phosphatidylcholines, the relative rates of liberation of different species of cholesteryl ester were measured. All but two species of LCAT clustered according to one of three patterns of substrate specificity. The LCAT from six species, including human, did not transfer highly polyunsaturated fatty acyl chains. In addition, human LCAT transesterified saturated fatty acyl chains more effectively than unsaturated fatty acyl chains. We conclude that the structures of the active sites of the enzymes differ, and that this may be related to size constraints that prevent efficient binding of large bulky phosphatidylcholines.
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Lecithin:cholesterol acyltransferase (LCAT) catalyzes the production of nearly all plasma cholesteryl esters and is one of the key enzymes involved in reverse cholesterol transport (1–4). The enzyme is composed of a single polypeptide chain containing six cysteines, two of which are essential for activity; the other four form disulfide bridges that are believed to stabilize the enzyme structure (5,6). LCAT has both acyltransferase and phospholipase A_2 activity; the major protein of high density lipoprotein (HDL), apoA-I, stimualtes both activities (7). The major cholesteryl esters found in human plasma are cholesteryl linoleate, oleate and palmitate (2). In model systems in which reassembled high density lipoproteins (R-HDL) are used as substrates, the most reactive acyl donors are phosphoglycerides containing myristic and palmitic acid (8,9). The differences between the *in vitro* and *in vivo* specificities originate from two sources. First, the fatty acyl moieties of native phosphatidylcholines (PC) in HDL, which typically consist of palmitoyl or stearoyl groups in the *sn*-1 position and linoleoyl or oleoyl in the *sn*-2 position, are important determinants of the composition of the resulting cholesteryl esters. Thus the high yield of cholesteryl linoleate and oleate is due to the high abundance of these acids at the *sn*-2 position, which is the preferred, but not the exclusive acyl donor site (7). Second, the model systems were composed of symmetrical diacyl phosphatidylcholines, which are in low abundance in native HDL. There is also some evidence that the identity of the chain at the *sn*-1 position will determine, in part, the reactivity of the acyl group at *sn*-2 (10). The major advantage of model systems is that substrate composition can easily be controlled. Therefore, such a system was used to show that the specificities of human and rat LCAT differ, with the latter having a greater specificity for arachidonate (11,12). This finding suggests that the two enzymes differ, with the rat LCAT having a greater specificity for arachidonate (11,12). This, in turn, may indicate that the two enzymes differ, with the rat LCAT active site being larger and thus more readily able to accommodate bulky groups, such as arachidonate. To understand the differences in specificity, we compared the activity of LCAT derived from 10 species. The data show different patterns of specificity for plasma LCAT activity.

*To whom correspondence should be addressed at Baylor College of Medicine, The Methodist Hospital, 6565 Fannin Street, M.S. A-601, Houston, TX 77030.

Abbreviations: HDL, high density lipoproteins; HPLC, high-performance liquid chromatography; LCAT, lecithin:cholesterol acyltransferase; PC, phosphatidylcholines; POPC ether, 1-*O*-hexadecyl-2-*O*-*cis*-9′-octadecenyl-*sn*-glycero-3-phosphocholine; R-HDL, reassembled HDL.

EXPERIMENTAL PROCEDURES

Materials. LCAT was isolated from the pooled sera of human and nine animal species. The animals were chicken *(Gallus gallus),* rabbit *(Oryctolagus cuniculus),* pig *(Sus scrofa),* horse *(Equus caballus),* dog *(Canis familiaris),* rat *(Rattus species),* sheep *(Ovis aries),* guinea pig *(Cavia cobaya)* and cow *(Bos species).* All animal sera were obtained from Pel-Freeze Biologicals (Rogers, AR).

Preliminary tests showed that the molecular specificities of partially purified and of purified human and rat LCAT were identical. Therefore, the enzymes used in our tests were partially purified. Partial purification was accomplished by flotation of the plasma lipoproteins at d = 1.21 g/mL, after which the colorless zone in the middle one-third of the centrifuge tube was passed through phenyl sepharose in high salt. The activity was then eluted with distilled water, dialyzed against a standard buffer composed of 0.1 M NaCl and 10mM Tris (pH 7.4), and used in this form for assays (8,13). Sodium dodecylsulfatepolyacrylamide gel electrophoresis of these preparations demonstrated the absence of apoA-I. Human apoA-I was obtained from human plasma as previously described (14,15).

Synthetic PC were either purchased from Avanti Polar Lipids (Birmingham, AL) or synthesized (16,17). Gas chromatographic analyses of the fatty acid methyl esters liberated by transesterification of PC gave compositions consistent with their respective structures. 1-*O*-hexadecyl-2-*O*-*cis*-9′-octadecenyl-*sn*-glycero-3-phosphocholine (POPC ether) was prepared from 3-benzylglycerol, as described previously (8). All phospholipids migrated as a single fraction in thin-layer chromatography on silica gel using chloroform/methanol/water (65:25:4, v/v/v) as the developing solvent.

LCAT assays. R-HDL were prepared by a detergent removal technique using sodium cholate (18,19). POPC ether, cholesterol and the other phospholipids were combined with human apoA-I, and sodium cholate was added to the mixture until optical clarity was achieved. The mixture was then exhaustively dialyzed against standard buffer, giving R-HDL with a defined chemical composition. Four different mixtures of PC in equimolar amounts were combined with POPC ether, which composed 67% of the

phospholipid content of the R-HDL. All R-HDL were prepared with a 100:1 molar ratio of phospholipid (PC mixture plus POPC ether) to apoA-I and contained 2 mol% cholesterol and a trace of [^{3}H]cholesterol that was used to quantify cholesteryl ester formation. The POPC ether, which provides a fluid environment similar to that of HDL, was used as an inert matrix that would be similar in all test mixtures (8). The four groups of substrates that were prepared contained equimolar amounts of the following phosphatidylcholines, all of which were symmetrically substituted and in which the chain length, n, and number of double bonds, m, were represented by n:m. The four groups are as follows: I. 9:0, 12:0, 14:0, 16:0, 18:0. II. 16:0, 18:1, 18:2, 18:3, 22:1. III. 14:0, 20:1, 20:3, 20:4ω6, 22:6ω3. IV. 16:0, 18:2, 20:0.

Typically, LCAT assays were performed at 37°C in a final volume of 0.2 mL containing 2% human serum albumin, 0.5 mM dithiothreitol, 100 mM NaCl and 10 mM Tris, pH 7.4. All assays were performed within a time range where the velocity was linear with respect to time. The substrate concentration was high enough to give a rate that was greater than 90% V_{max}. Each assay used an amount of activity approximately equal to 1 μg of the pure enzyme.

The relative production rates for various molecular species of cholesteryl esters were determined by high-performance liquid chromatography (HPLC) (20,21). Briefly, the reaction mixtures were quenched by the addition of 0.1 mL methanol and 0.5 mL hexane. After vortexing, the upper phase was transferred to vials, and the solvent was removed under a stream of nitrogen. The individual species of cholesteryl esters were separated and quantified on a Hewlett Packard (Andover, MA) 1090 HPLC equipped with an LKB Model 1208 Betacord radioisotope monitor. Chromatography was performed on a 4.6 × 250 mm C_{18} Adsorbosphere column (All-Tech Applied Science, State College, PA). The cholesteryl esters were applied to the column in 50–100 μL of acetonitrile/tetrahydrofuran (68:32, v/v) and then eluted with the same solvent at a flow rate of 0.75 mL/min.

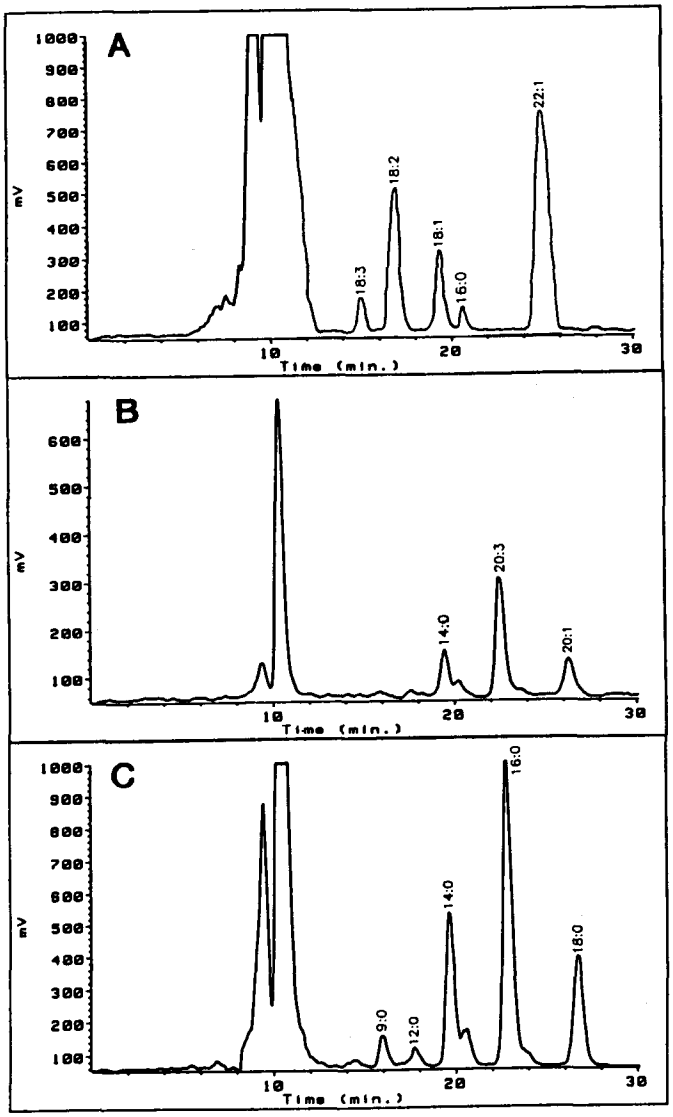


FIG. 1. High-pressure liquid chromatography analysis of human LCAT activity. Substrates for the reactions were R-HDL containing 2.5 μmoles of five symmetrical diacyl phosphatidylcholines with various chain lengths and numbers of double bonds, 25 μmoles POPC ether, 375 nmoles apoA-I, 2 mol% free cholesterol and a trace of [^{3}H]cholesterol to permit quantification of ester formation. Incubations were performed at 37°C for 6 min in final volumes of 220 μL. All experiments were conducted at substrate concentrations of > 90% of V_{max}. Molecular species of cholesteryl esters were liberated by human LCAT using R-HDL containing diacyl PC with the following chain lengths: (A) 18:3, 18:2, 18:1, 16:0, and 22:1 (B) 14:0, 20:3, 20:1, 20:4, and 22:6. (C) 9:0, 12:0, 14:0, 16:0, and 18:0.

RESULTS AND DISCUSSION

The molecular specificity of LCAT from ten different species was analyzed by measuring the relative rates of production of cholesteryl esters liberated from a series of mixtures of R-HDL having equimolar amounts of various PC. Figure 1 (A–C) is representative chromatograms obtained from the cholesteryl esters formed by the incubation of human LCAT with three different kinds of R-HDL. Even though the acyl donors were in the R-HDL in equimolar concentrations, the relative amount of each molecular species of cholesteryl ester that was formed differed. In the mixture that contained PC with the acyl chains 18:0, 18:1, 18:2, 18:3 and 22:1, the last was the best acyl donor (Fig. 1A). The PC that contained 20:3 was the most reactive of those analyzed in Figure 1B. Of the cholesteryl esters that were liberated from the series of saturated PC shown in Figure 1C, 14:0 and 16:0 were the most reactive.

The reactivities of the various PC in these and other HPLC runs were compiled using either 16:0 or 14:0, one of which was present in all of the mixtures, to normalize pairs of runs. These data are shown in Figure 2 for all ten mammalian species studied. The data on the ordinate are plotted on a logarithmic scale so the differences in reactivity are much larger than they appear. In the case of human LCAT (Fig. 2A), the data show that the enzyme from this species prefers saturated chains, with those for 20:4 and 22:6 being almost totally unreactive. Under our experimental conditions, the radioactivity corresponding to the formation of the cholesteryl esters of 20:4 and 22:6 was close to background. In contrast, some of the other species readily utilized these two fatty acids—these were cow, dog, rat and sheep. A log-log plot compared the normalized data from each non-human species with that of

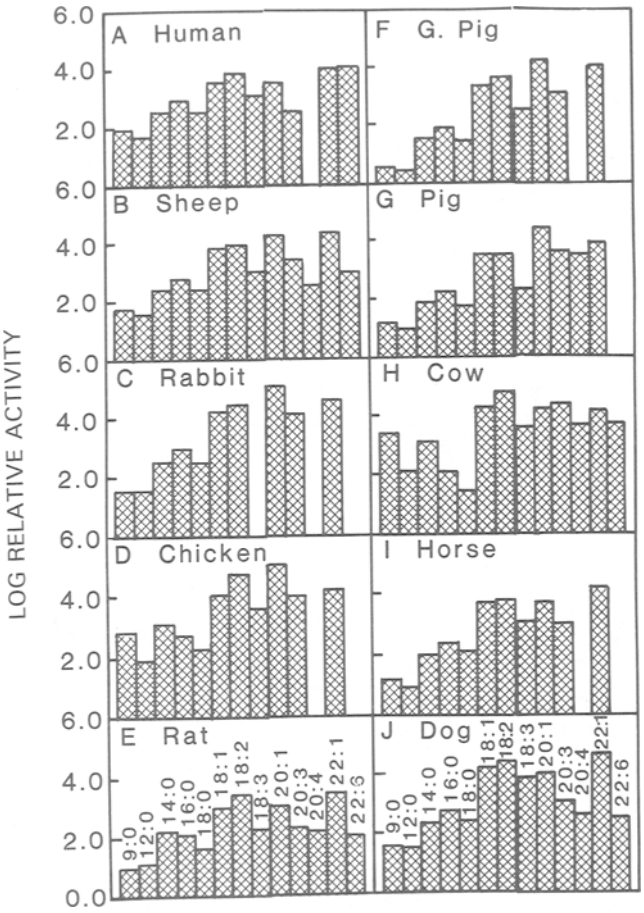


FIG. 2. Relative LCAT specificity for various species using R-HDL containing diacyl PC of differing lengths and numbers of double bonds. (A) human; (B) sheep; (C) rabbit; (D) chicken; (E) rat; (F) guinea pig; (G) pig; (H) cow; (I) horse; (J) dog. Activity on ordinate is relative to the total amount of cholesteryl ester formed.

the human (Fig. 3). The data show that human LCAT prefers saturated diacyl chains (points below the lines in Fig. 3) relative to the nine other species shown; the diacyl chains 20:3 and 20:1 are utilized to a larger extent by all species except human. More striking is the preference of all species, relative to human, for the polyunsaturated diacyl PC chains.

Visual inspection of Figure 3 leads to some qualitative statements. LCAT specificities of rat and dog were very similar to that of the human enzyme, with both having a correlation coefficient of 0.97 (Fig. 3C and 2E). LCAT from chicken and cow were the least similar to human LCAT, with correlation coefficients of 0.75 and 0.64, respectively (Fig. 3, A and D). (These correlations exclude 20:4 and 22:6, for which human LCAT had no measurable activity). Both chicken and cow LCAT were similar to human LCAT in their preference for saturated diacyl PC. The other species utilized unsaturated PC to a greater extent than humans except that chicken and cow LCAT utilized diacyl PC 9:0 far more than human LCAT did.

Those species that used di20:4 and di22:6 as a substrate were clearly distinct from those, such as human, that did not. Cow LCAT transesterified di20:4 and di22:6 from PC to cholesterol relatively efficiently. Sheep, dog and rat followed cow in their utilization of di20:4 and di22:6 PC as substrates. The LCAT from all other species, like human LCAT, did not use these two diacyl chains efficiently (Fig. 2).

To determine whether the molecular species specificity of the LCAT from different sources formed clusters of reactivity, a more quantitative approach was employed. If all species are compared in a normalized correlation matrix using a transposed data matrix according to the equation:

$$[Z]_n = [D]^T [D] \quad [1]$$

and analyzed using principal component analysis (PCA), a scatter plot can be generated (22). In producing this plot (Fig. 4), the data matrix was decomposed into a product of an abstract row matrix and an abstract column matrix as defined previously using the equation:

$$[D] = [R]_c \text{ PCA } [C]_c \text{ PCA} \quad [2]$$

Factors were ranked according to their accounting for variation in the data. To avoid reproducing experimental error, two factors were used. These factors were associated with the most significant eigenvalues and, therefore, the greatest variation in the data.

The scatter plot of the principal component analysis reveals clusters of data points that represent different species of LCAT of similar enzyme specificity (these data include diacyl di20:4 and di22:6, which were not LCAT substrates for six species). Five distinct clusters were identified. Human LCAT was closely associated with chicken (Cluster I), while rabbit, pig and horse formed their own group (Cluster II). Dog, rat and sheep formed another distinct cluster of similar enzyme specificity (Cluster III). Guinea pig (Cluster IV) and cow (Cluster V) had a unique specificity relative to the other LCAT from other mammalian species.

The original data obtained with human LCAT were compared with those corresponding to each of the other four clusters to determine the structural features that distinguish the human enzyme from the others. Table 1 shows that Cluster I, which represents human and chicken LCAT, is much more reactive than Clusters II, III and IV when the substrate contains diacyl chains 9:0 and 12:0. Cluster I is distinct from Clusters II, IV and V with

TABLE 1

Comparison of Clusters of LCAT Activity[a]

Cluster	Cholesteryl esters formed (% change)			
I *vs* II	9:0 (46)	12:0 (36)	14:0 (25)	20:3 (−36)
I *vs* III	9:0 (38)	12:0 (20)	20:4 (−100)	22:6 (−100)
I *vs* IV	9:0 (75)	12:0 (73)	14:0 (44)	16:0 (32)
	18:0 (38)	18:3 (26)	20:3 (−26)	
I *vs* V	19:0 (40)	9:0 (−30)	20:3 (−48)	20:4 (−100)
	22:6 (−100)			

[a]This Table identifies the major differences in the reactivities of di n:m PC in cluster I, which includes human LCAT, with the other clusters, cluster II, rabbit, pig, horse; cluster III, dog, rat, sheep; cluster IV, guinea pig; and cluster V, cow. The value in parentheses represents the percent change in reactivity relative to human LCAT. Only the acyl donors that exhibit more than 25% difference are shown.

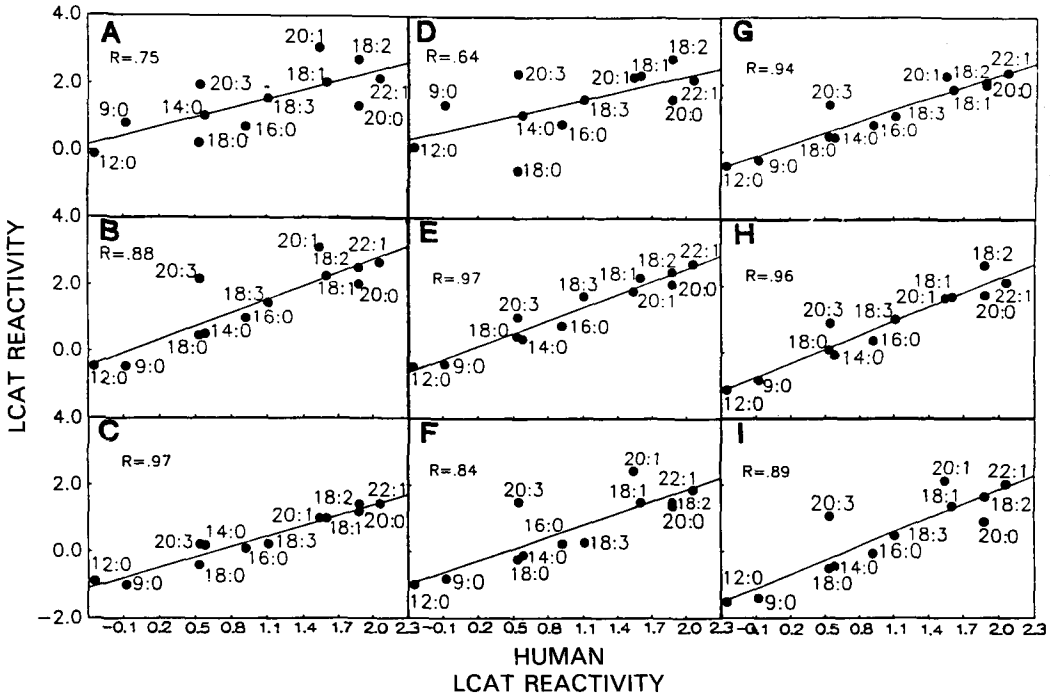


FIG. 3. Correlation of specificity between human LCAT (abscissa) and various other species (ordinate) for diacyl PC of differing chain lengths and double bonds in R-HDL. The PC to apolipoprotein ratio (by weight) of the R-HDL was 100:1, with a free cholesterol content of 2 mol% and a trace of [^{3}H]cholesterol for quantification. Incubations were performed at 37°C in final volumes of 220 μL using 143 μg LCAT in each experiment. Substrate concentrations were > 90% of V_{max}. Measurements were made using high-pressure liquid chromatography; the results are presented in log% area/min. (A) chicken, r = 0.75; (B) rabbit, r = 0.88; (C) rat, r = 0.97; (D) cow, r = 0.64; (E) dog, r = 0.97; (F) pig, r = 0.84; (G) sheep, r = 0.94; (H) horse, r = 0.96; (I) guinea pig, r = 0.89.

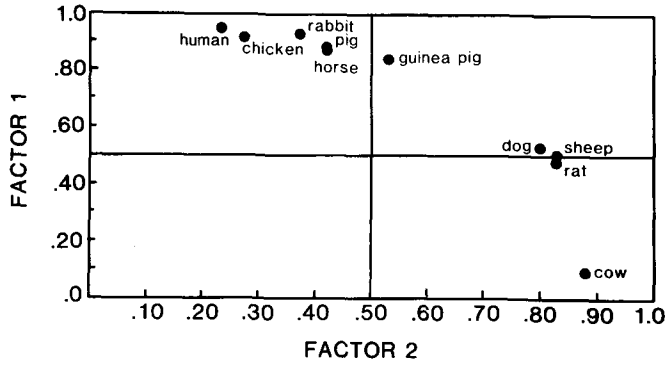


FIG. 4. The scatter plot generated when all species of LCAT were analyzed using principal component analysis. Two factors were associated with the most significant eigenvalues and, therefore, the greatest variation in data. The scatter plot reveals clusters of data points that represent LCAT of similar specificity (this data includes all diacyl chains). Three distinct clusters can be identified. These are human and chicken LCAT; rabbit, pig and horse; dog, rat, and sheep. The LCAT specificities of guinea pig and cow did not cluster with those of any other species.

respect to its relatively low reactivity towards diacyl chain 20:3. Compared to Cluster I, Cluster IV, which consists solely of guinea pig, was relatively inactive in six of the thirteen diacyl chains tested. The other cluster (Cluster V, cow) consisted of only one species and was more reactive with the diacyl chains 9:0, 12:0, 20:4 and 22:6 than was Cluster I.

In general, substrate specificity is caused by molecular species selectivity and not a peculiar microenvironment or surface afforded by a particular phosphatidylcholine (8,9,12,23,24). The unique specificity for substrates of different diacyl chain lengths and double bonds exhibited by various species of LCAT is consistent with different active site structures. The tendency for LCAT from all species except human to transesterify highly unsaturated phosphatidylcholines to cholesterol suggests that the active sites of LCAT from these species are suited to handle the larger molecular volumes that are characteristic of polyunsaturated substrates. Diacyl chains 20:4 and 20:6, which were the bulkiest substrates used, were utilized by only four of the ten species (cow, sheep, dog and rat). The high content of plasma cholesteryl esters of arachidonate in rat HDL correlates well with the unusually high amounts of arachidonate at the *sn*-2 position of phosphatidylcholine and the high reactivity of the LCAT with respect to arachidonate (12).

LCAT from cow was also unique in its fairly indiscriminate specificity for substrates. Only the diacyl chains 18:0 and 16:0 had a relatively low reactivity. Guinea pig and chicken LCAT also transesterified diacyl PC chains 18:0 and 16:0 to cholesterol to a lesser extent than human LCAT, which revealed an active site compatible with bulky polyunsaturated substrates, but it was not as compatible with longer, fully saturated PC.

The source of the specificity differences of LCAT from different species is not clear. As the primary structures

of the various LCAT species become available, it may be possible to assign differences in specificity to specific parts of the polypeptide chain. These, in turn, may permit a correlation of the specificity and structure of different species with their evolutionary relatedness.

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Fatty Acid Composition of Brain Phospholipids in Aging and in Alzheimer's Disease

M. Söderberg*, C. Edlund, K. Kristensson and G. Dallner
Division of Medical Cell and Neurobiology, Clinical Research Center, Huddinge University Hospital, Karolinska Institutet, S-141 86 Huddinge, Sweden

The two major phospholipid classes, namely, phosphatidylethanolamines (PE) and phosphatidylcholines (PC), were studied in four different regions of human brain, *i.e.*, in frontal gray matter, frontal white matter, hippocampus and in pons. The fatty acid (FA) compositions of these phospholipids were found to be specific for the different regions. PC contains mostly saturated and 18:1 FA, while PE is rich in polyunsaturated FA. Aging has no influence on the FA compositions, while in Alzheimer's disease (AD) PE is modified in all four regions, particularly in frontal gray matter and in hippocampus. The abundance of the major monounsaturated FA of PE, 18:1, is not significantly altered in Alzheimer's disease, but there is a substantial increase in the relative amounts of the saturated components 14:0, 16:0 and 18:0. This is paralleled by a decrease in the polyunsaturated FA 20:4, 22:4 and 22:6. It is not clear whether the changes observed are specific for AD. Changes in saturated/polyunsaturated FA ratio are likely to influence cellular function, which in turn may cause certain neural deficiencies. The findings do not support the hypothesis that AD reflects an accelerated aging process.
Lipids 26, 421–425 (1991).

Phospholipids (PL) are structurally and functionally important constituents of membranes. They regulate membrane structure and permeability, as well as the activities of membrane-bound enzymes. The lipid composition of the brain—especially in regard to the high concentrations of cholesterol, of the polyisoprenoid compounds dolichol and ubiquinone, and of glycolipids—differs significantly from those of other tissues (1,2). It is generally assumed that cholesterol in model membranes preferentially associates with phosphatidylcholine (PC) (3) and that isoprenoids located in the hydrophobic portion of phospholipid bilayers prefer phosphatidylethanolamine (PE) (4). Changes in the amounts and types of PL could thus, in turn, also affect the neutral lipid components of membranes.

Changes in the composition and metabolism of brain lipids have been proposed to contribute to the deterioration of central nervous system function with increasing age (2,5). It has also been proposed that phosphoinositide levels in brain, and of some of the metabolites of PE and PC, are involved in the processes that cause various types of senile dementia (6,7). In one study, the content of polyunsaturated fatty acids (FA) (PUFA) in total PL from frontal brain cortex was found to decrease with increasing age, while the PUFA content in Alzheimer's disease (AD) varied (8). In contrast, another investigation found no differences in FA composition in aging (9). In this latter study a decreased n-6 PUFA content was observed in PC from the frontal cortex of AD patients, while no significant changes in the FA composition of PE were found.

In light of these facts, it appeared timely and important to initiate a study of the lipid compositions of different parts of the brain in AD. The study is concerned with changes in the FA composition of PE and PC in four different regions of the brain.

*To whom correspondence should be addressed.

Abbreviations: AD, Alzheimer's disease; FA, fatty acids; GLC, gas-liquid chromatography; NADH, nicotinamide adenine dinucleotide; PC, phosphatidylcholine; PE, phosphatidylethanolamine; PL, phospholipids; PUFA, polyunsaturated fatty acids; TLC, thin-layer chromatography.

MATERIALS AND METHODS

Brain samples were obtained during autopsy approximately 24 hr after death. The contralateral brain hemisphere was fixed for histopathological examination. In histological preparations various silver staining techniques were employed. Patients with clinical dementia were diagnosed according to the criteria established for AD by the National Health Institute/American Association of Retired People (NHI/AARP) working group (10). All patients included in this study showed substantial quantities of neuritic plaques, well above the minimum number required to verify the diagnosis by the above-mentioned criteria. In addition, neurofibrillary tangles were present in the neocortex in all patients with AD.

Patients from which control samples were taken died from causes unrelated to the nervous system and showed no clinical manifestations of dementia or other mental disorders. Microscopic investigation of the control brains revealed normal cytoarchitecture in the neocortex. In some of the older patients small numbers of neuritic plaques were found as is usual at advanced age.

Brain regions chosen for lipid analysis were removed and homogenized in 0.25 M sucrose with an Ultra-Turrax blender (Janke & Kunkel, Staufen, Germany) for 2 min (24000 rpm). The resulting 10% homogenate was stored at −20°C until assayed, which was usually within 1–2 wk. White matter was taken from the frontal lobe.

To extract lipids, 6 mL methanol and 6 mL chloroform were added to 2 mL of the homogenate to obtain a final chloroform/methanol/water ratio of 1:1:0.3 (v/v/v). Extraction was performed at 40°C for 60 min with magnetic stirring. Phase separation was obtained by addition of chloroform and water to give a final chloroform/methanol/water ratio of 3:2:1 (v/v/v). The tubes were then centrifuged for 5 min at 250 × *g*. The lower phase was washed 4 times with theoretical upper phase (chloroform/methanol/water, 3:48:47, v/v/v) and evaporated under nitrogen. The lipid residue was dissolved in 5 mL chloroform and placed onto a Silica-SepPak cartridge (Millipore, Waters Associates, Milford, MA), which was then washed with 25 mL chloroform to remove neutral lipids. PL were eluted from the Silica-SepPak with 25 mL methanol. The solvent was evaporated under N_2 and lipids were dissolved in 200 μL chloroform/methanol 2:1 (v/v). Twenty μL of the extract was applied to a Silica gel 60 plate (Merck, Darmstadt, Germany) for two-dimensional thin-layer chromatography (TLC). The plates were developed with

TABLE 1

Phospholipid Compositions of Different Regions of the Human Brain[a]

Region	Age (yrs)	Total phospholipid amount (mg/g)	Phospholipid[b] (mg/g)		PC/PE ratio
			PE	PC	
Frontal gray	33–36	36.4 ± 3.0	13.3 ± 1.5	16.1 ± 1.5	1.21 ± 0.08
	54–57	35.4 ± 2.6	12.6 ± 1.6	15.5 ± 1.8	1.23 ± 0.07
	69–72	34.8 ± 3.8	12.3 ± 1.4	14.7 ± 1.7	1.20 ± 0.09
	89–92	32.8 ± 3.2[c]	12.0 ± 1.0	13.9 ± 1.2[c]	1.16 ± 0.08
White matter	33–36	70.0 ± 4.6	29.5 ± 3.9	19.4 ± 2.4	0.66 ± 0.05
	54–57	69.0 ± 8.6	29.9 ± 4.3	20.1 ± 2.0	0.67 ± 0.06
	69–72	59.8 ± 8.4[c]	25.1 ± 2.5[c]	17.2 ± 2.4[c]	0.69 ± 0.04
	89–92	57.6 ± 7.6[c]	24.7 ± 3.5[c]	16.5 ± 2.6[c]	0.67 ± 0.06
Hippocampus	33–36	46.0 ± 4.0	18.1 ± 2.4	19.3 ± 1.8	1.07 ± 0.09
	54–57	42.8 ± 4.4	17.0 ± 2.0	17.4 ± 1.5	1.02 ± 0.05
	69–72	37.0 ± 4.2[c]	14.4 ± 1.5[c]	15.0 ± 1.6[c]	1.04 ± 0.06
	89–92	35.0 ± 4.4[c]	13.4 ± 1.8[c]	14.0 ± 1.0[c]	1.05 ± 0.07
Pons	33–36	64.8 ± 4.6	31.4 ± 3.4	20.0 ± 1.7	0.64 ± 0.05
	54–57	62.2 ± 5.0	30.4 ± 2.2	19.5 ± 2.5	0.64 ± 0.04
	69–72	56.4 ± 6.2[c]	27.1 ± 2.7[c]	17.5 ± 1.8[c]	0.65 ± 0.05
	89–92	53.2 ± 7.2[c]	25.4 ± 2.7[c]	15.8 ± 1.6[c]	0.66 ± 0.04

[a]The values are the means ± SD of 8–10 measurements.

[b]PE, phosphatidylethanolamine; PC, phosphatidylcholine.

[c]Significantly decreased compared with the youngest age group ($p<0.05$). Statistics were performed using Kruskal-Wallis one-way analysis of variance followed, if group differences existed, by Wilcoxon-Mann-Whitney test.

chloroform/methanol/acetic acid/water (106:50:12:6, by vol) in the first direction and with chloroform/acetone/methanol/acetic acid/water (50:20:10:10:5, by vol) in the second direction. The plates were dried and sprayed with an aqueous solution of Rhodamin 6G to visualize fractions. Fractions were scraped off and PL were extracted with 5 mL chloroform/methanol, 2:1 (v/v). The recoveries of PE and PC were between 79 and 92%. Half of the solution was used to measure phosphate content, as described previously (2). The other half was used to determine the FA composition of the PL after transesterification with boron trifluoride/methanol (11). Antioxidants were not added. PUFA recoveries in the presence and absence of antioxidants were identical, when methanolysis was performed in a N_2 atmosphere. FA methyl esters were analyzed by gas chromatography on a Shimadzu GC-9A (Kyoto, Japan) with a flame ionization detector and with hydrogen as the carrier gas. The column was a fused silica capillary with cyanosiloxane 60 as the stationary phase (30 m × 0.25 mm I.D., df = 0.18 μm). From an initial temperature of 140°C, which was maintained for 2 min, the temperature was raised 2.5°C/min for 18 min, then 0.4°C/min for 5 min and, finally, 2.0°C/min until a final temperature of 195°C was attained, which was then maintained for 4 min. The total time per gas-liquid chromatography (GLC) run was 33 min. Peaks were integrated on a Shimadzu Chromatopac C-R3A. FA methyl esters were identified by comparison with commercial FA methyl ester standards (Sigma Chemical Co., St. Louis, MO). Solvents were from Merck.

RESULTS

Phospholipid composition. The amount of PL in human brain varies between 32 and 70 mg per gram wet weight, depending on the anatomical location and age (Table 1). In white matter and pons, the amount is about double that found in frontal gray matter and hippocampus. At ages above 70, a moderate decrease in the total amount of PL is observed at all locations, which agrees with previous findings (2). About 80% of total PL consists of the two major components, PE and PC. PE plasmalogen content was determined in some samples. The amounts of plasmalogen found in AD patients and controls were identical (not shown in tables). For practical reasons, plasmalogen content was therefore not routinely determined.

Fatty acids of PE in aging. The FA composition of PE shows extensive variation depending on the source of the lipid (Table 2). While some FA, such as 16:0 and 20:4, occur in similar amounts in all regions, others vary within wide limits. For example 18:0 and 18:1, respectively, constitute 8–35% and 10–46% of the total FA in PE in various regions of the brain. Saturated FA in white matter and in pons contributes 16% and 18% of the total, respectively, while for gray matter and hippocampus these values are 45% and 35% (see also Fig. 1). The specific role of PUFA makes the distribution of these species particularly interesting. While arachidonic acid levels fluctuate only to a limited extent, the relative amounts of 22:4 and 22:6 vary substantially, with 22:6 being the most variable with levels ranging from 4% to 24% for different regions

TABLE 2

Fatty Acid Composition of PE in Different Regions of the Human Brain[a]

Region	Age (yrs)	Fatty acid[b] (% of total)						
		16:0	18:0	18:1	20:1	20:4	22:4	22:6
Frontal gray	33-36	10.3	36.2	10.7	1.2	9.4	8.2	21.5
	54-57	9.4	34.0	9.7	0.8	10.6	9.1	24.0
	69-72	9.9	34.7	10.2	1.0	9.6	8.5	21.7
	89-92	9.3	35.2	9.5	0.9	10.7	9.3	23.2
White matter	33-36	6.4	10.1	44.4	7.5	7.3	18.3	3.7
	54-57	6.6	9.3	45.5	8.8	7.3	17.1	4.0
	69-72	7.1	9.7	44.0	7.4	8.8	16.3	4.6
	89-92	6.8	10.7	43.8	8.2	8.0	17.0	4.1
Hippocampus	33-36	9.1	27.7	20.0	2.0	11.4	12.9	15.3
	54-57	7.9	27.8	19.7	2.3	12.4	12.5	15.9
	69-72	9.0	25.5	22.1	2.1	11.6	12.2	16.0
	89-92	8.7	25.9	19.9	1.8	12.2	13.8	16.4
Pons	33-36	10.7	7.7	41.5	11.5	7.8	10.8	5.0
	54-57	10.5	8.5	42.1	12.0	8.2	11.8	6.0
	69-72	9.6	8.8	43.4	11.2	7.5	10.2	6.5
	89-92	9.2	9.2	41.1	10.6	8.7	12.3	5.8

[a]The values are the means of measurements from 8-10 different individuals. SDs were 2-12% of the means.

[b]Small amounts (<1%) of other FA, *e.g.* 16:1 and 18:2, also occurred.

of the brain. FA species comprising less than 1% of the total amount, *e.g.*, 20:3 and 22:5, are not included in the tables.

Fatty acids of PE in Alzheimer's disease. The FA composition of PE is characteristically altered in both the gray matter and the hippocampus in the brains of patients afflicted by AD (Table 3). Most typically, a sizeable increase in the relative amounts of saturated FA (14:0, 16:0 and 18:0) is apparent. By contrast, the levels of the three PUFA species (20:4, 22:4 and 22:6) are decreased considerably in gray matter and in hippocampus. To some extent, similar changes can be observed in white matter and pons.

The calculated values for total saturated, monounsaturated and polyunsaturated FA in PE in various regions of the brain in control patients and Alzheimer victims are presented in Figure 1. Taking into consideration the great variations in the extent of histological changes in the brains affected by AD, it is not surprising that there are also considerable differences in the degrees to which the FA compositions of these brains are modified. The principal changes are decreases in PUFA content in gray matter and hippocampus, compensated for by an increase in their saturated counterparts. The white matter demonstrates similar, but less accentuated changes. In pons the difference between control and diseased brains is even smaller, but still significant.

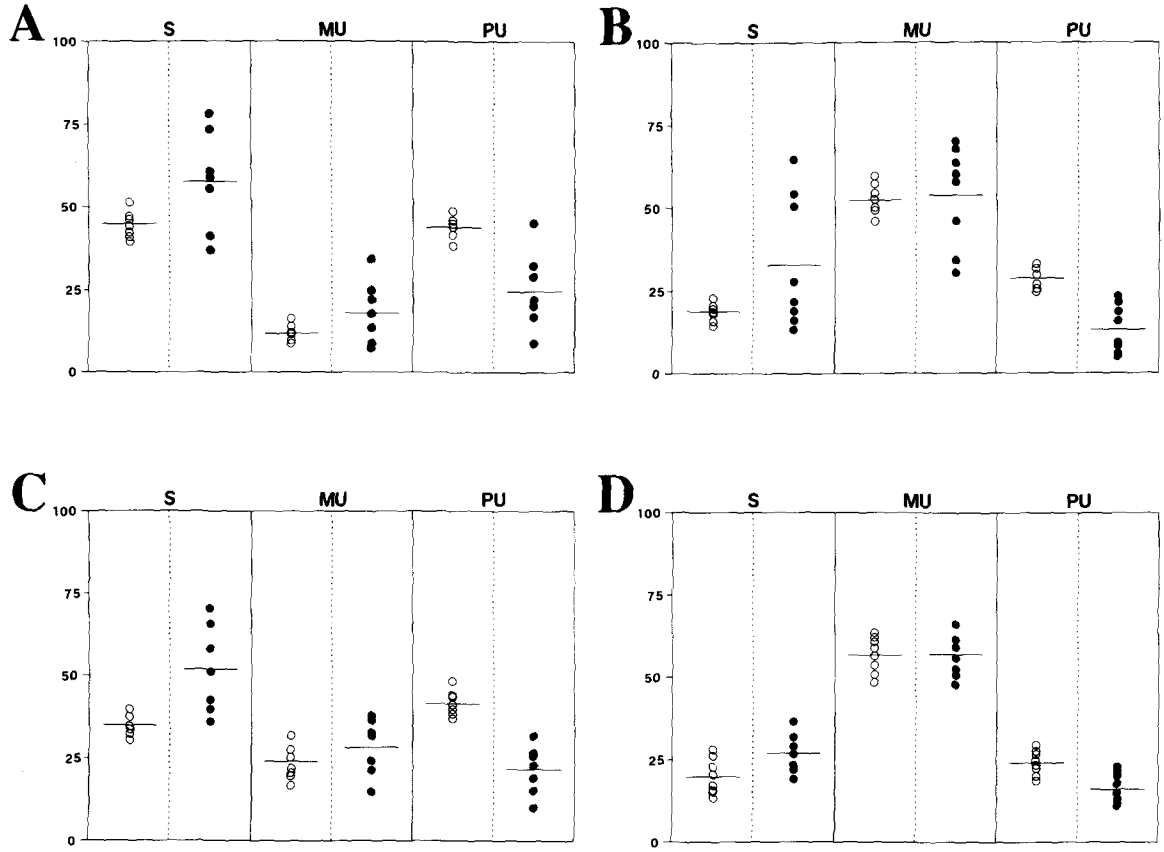


FIG. 1. Distributions of saturated and unsaturated fatty acids in PE of various regions of the brain. A: frontal gray matter; B: white matter; C: hippocampus; D: pons. The individual values were plotted separately. Open circle, control; filled circle, Alzheimer's disease; S, saturated fatty acids; MU, monounsaturated fatty acids; PU, polyunsaturated fatty acids. The horizontal bars represent the means. The numbers on the vertical axis represent percentage of total FA.

TABLE 3

Fatty Acid Composition of PE in Alzheimer's Disease[a,b]

Region	Fatty acid (% of total)							
	14:0	16:0	18:0	18:1	20:1	20:4	22:4	22:6
Frontal gray								
Control	0.7	9.4	35.0	10.6	1.0	10.7	9.7	23.5
AD[c]	2.5[d]	12.6[d]	42.7[d]	12.5	1.1	6.9[d]	5.6[d]	12.6[d]
White matter								
Control	0.4	7.7	10.5	44.6	6.9	8.0	15.8	4.6
AD	2.4[d]	11.3[d]	17.4[d]	44.9	6.2	3.7[d]	8.8[d]	2.0[d]
Hippocampus								
Control	0.6	8.6	25.6	21.3	1.8	12.4	11.9	16.9
AD	2.5[d]	14.1[d]	33.0[d]	23.2	1.8	8.1[d]	7.0[d]	7.9[d]
Pons								
Control	0.9	8.7	9.5	44.0	10.8	8.6	10.6	5.7
AD	1.0	9.1	16.7[d]	46.4	9.5	4.9[d]	6.2[d]	4.1[d]

[a]The values are the means of measurements from 8–10 different individuals. SDs were 3–30% of the means.

[b]The mean age ± SD was 80.0 ± 7.9 for AD and 79.2 ± 11.6 for controls.

[c]Alzheimer's disease.

[d]These values are significantly different from the controls ($p<0.05$), using Wilcoxon-Mann-Whitney test.

TABLE 4

Fatty Acid Composition of PC in Different Regions of the Human Brain[a]

Region		Fatty acid (% of total)							
		14:0	16:0	18:0	18:1	20:1	20:4	22:4	22:6
Frontal gray	33–36	2.9	52.4	12.8	23.8	0.5	2.9	0.4	1.5
	54–57	3.0	52.1	13.5	24.0	0.4	3.0	0.6	1.7
	69–72	3.6	48.2	12.3	26.9	0.4	2.8	0.5	1.9
	89–92	3.3	51.0	13.3	25.6	0.5	2.5	0.4	1.3
White matter	33–36	2.3	33.8	17.0	40.0	1.0	2.0	0.8	tr
	54–57	2.2	32.7	15.5	42.9	0.9	1.8	0.7	0.4
	69–72	2.4	31.6	14.9	42.8	1.3	2.5	0.9	0.6
	89–92	2.5	31.7	17.1	41.5	1.1	2.2	0.5	0.4
Hippocampus	33–36	2.2	44.2	17.2	30.5	tr	3.2	tr	0.8
	54–57	2.8	44.1	16.2	30.0	tr	2.8	0.5	1.0
	69–72	3.3	42.1	15.3	31.7	tr	3.3	0.6	1.0
	89–92	2.5	44.3	16.9	30.8	tr	2.7	tr	0.7
Pons	33–36	0.7	33.4	23.9	37.6	2.1	tr	tr	tr
	54–57	1.2	34.2	22.4	35.4	2.8	0.8	tr	tr
	69–72	1.0	33.6	21.5	37.1	2.0	0.5	tr	0.5
	89–92	0.7	32.9	24.8	35.9	2.4	0.7	tr	tr

[a]The values are the means of measurements from 8–10 different individuals. SDs were 5–16% of the means. Traces are identified as tr.

Fatty acids of PC in aging. The FA composition of PC is very different from that of PE. Saturated components constitute one-half to two-thirds of the total, and 18:1 is the only unsaturated component present in appreciable amounts (Table 4). The only significant polyunsaturated FA present is arachidonic acid (2–3% of the total). Thus, the heterogeneity of FA patterns of PC is less pronounced than for PE, but an individual pattern characteristic for the histological location is preserved. Two- to three-fold differences in the relative amounts of certain FA are observed between regions. As is the case for PE, no significant changes in the FA pattern of PC occur during aging.

TABLE 5

Fatty Acid Composition of PC in Alzheimer's Disease[a,b]

Region	Fatty acid (% of total)							
	14:0	16:0	18:0	18:1	20:1	20:4	22:4	22:6
Frontal gray								
Control	3.5	51.2	12.3	27.0	0.4	2.6	0.4	1.4
AD[c]	2.8	53.8	14.0	25.1	tr	2.0[d]	tr	0.8[d]
White matter								
Control	2.3	31.7	16.7	42.6	1.4	2.2	0.6	tr
AD	1.8	33.8	19.8	40.8	1.0	1.3[d]	0.4	tr
Hippocampus								
Control	2.7	43.7	16.1	31.2	tr	2.9	tr	0.9
AD	2.1	46.9	17.6	29.0	tr	2.1[d]	tr	0.6
Pons								
Control	0.7	33.5	22.5	37.0	2.2	0.7	tr	0.5
AD	0.9	35.4	19.0	38.7	1.9	0.8	tr	0.6

[a]The values are the means of measurements from 8–10 different individuals. SDs were 2–15% of the means.

[b]The mean age ± SD was 80.0 ± 7.9 for AD and 79.2 ± 11.6 for controls.

[c]Alzheimer's disease.

[d]The values are significantly different from the controls ($p<0.05$).

Fatty acids of PC in Alzheimer's disease. Because of the more limited regional variations in the FA compositions of PC, the changes observed in brains affected by AD were much less pronounced than those found in PE (Table 5). The small decreases seen in 18:1 and 20:4 are paralleled by minor increases in certain saturated components.

DISCUSSION

Compared with other organs, the brain has an unusually high content of lipids, some two-thirds of which consists of PL. Even limited alterations in PL structure are therefore thought to be of considerable functional importance. Many physical properties of membranes, such as permeability and fluidity, are greatly influenced by phospholipid FA composition (12), and several integral membrane proteins are dependent on certain types of PL (13). Membrane phospholipids also participate in compartmentalization (14–16). Variations in the FA compositions of certain PL in various regions of the brain are extensive. At all locations PC differs from PE, in that the former contains mostly saturated and 18:1 FA, while the latter possesses 30–40% PUFA. Variations in different regions create specific patterns, particularly in the case of PE. In most cases, the ratio of saturated-to-monounsaturated FA varies widely, while the percentage of PUFA is more stable. The lipid compositions of specific brain regions are highly dependent on the abundance of myelin and synapses, since these structures have markedly different FA profiles (17).

During aging some of the brain lipids are considerably modified (2). However, the FA compositions of PL are not changed. In AD the FA composition of PC exhibits minimal changes. In PE, the amounts of the saturated FA 14:0, 16:0 and 18:0 are elevated, while the polyunsaturated FA 20:4, 22:4 and 22:6 are significantly decreased. The

relatively modest changes in PC may be explained by the absence of substantial amounts of PUFA in PC even in normal brain tissue, thereby reducing the margin for change.

PE in the brain is made up of two subclasses, namely diacylglycerophospholipids and plasmalogens. The ratio between these two subclasses does not appear to be changed in AD. We have not examined individual FA compositions, nor the composition of the ether-linked chains in the 1-position of the plasmalogens. Still, the decrease in PUFA is likely to affect both PE subclasses. It is established that PUFA occur almost exclusively in ester-linked form in PE (18).

It is not known whether the changes in FA patterns found in the brains of Alzheimer victims are specific for this disease, or whether patterns similar to those found in AD appear in other neurodegenerative diseases. The changes observed do, however, not appear to be typical of normal aging, because no change in the relative amounts of PUFA is seen with advancing age. This would also speak against the possibility that AD just represents an accelerated aging process (19).

The moderate FA changes that were observed in a previous study on AD material (9) may be explained by different sampling procedures. FA changes varied greatly between affected brains (see Fig. 1), and in less advanced cases the PUFA levels were close to the control values.

There could be several reasons for the disappearance of PUFA in AD. One, for example, could be the modification of the microsomal desaturation system (20). Since no intermediary PUFA appeared in our material, a possible mechanism could be a decrease in nicotinamide adenine dinucleotide (NADH)/cytochrome b_5 reductase activity, the initial enzyme common to various desaturation processes (21). An alternative explanation for the absence of PUFA in AD may be a deficiency in uptake thus decreasing the supply of appropriate substrates. Linoleic and linolenic acids are essential FA, and their depletion in the brain would explain the loss of the longer PUFA, most of which are synthesized in the brain from linoleic and linolenic acids (22). A third possible explanation for the altered FA composition may be a change in the cellular makeup of the brain in AD, possibly involving gliosis or a reduction in the number of dendrites. Such changes also occur, however, in normal aging (23), which is not accompanied by a decrease in PUFA. A specific decrease in the number of synaptic structures, where PE rich in PUFA is abundant (24), has been proposed to occur in AD (25). This would also result in some reduction of total PUFA content. Isolated and purified subcellular fractions from AD brains are presently being investigated in our laboratory.

The substantial decrease in polyunsaturated FA may have serious consequences for cellular function. The large decrease in arachidonic acid content, for example, could hamper the production of important active metabolites, such as prostaglandins and leukotrienes, which, in turn, could cause the functional changes observed in Alzheimer's disease.

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Activation and Solubilization by Triton X-100 of Membrane-Bound Phospholipase D of Rat Brain

Hiroyuki Kanoh*, Yasunori Kanaho and Yoshinori Nozawa
Department of Biochemistry, Gifu University School of Medicine, Gifu 500, Japan

In the present study, we examined the ability of detergents to stimulate and solubilize phospholipase D (PLD) of a particulate fraction of rat brain. PLD activity was assayed by measuring the [^{3}H]choline produced from the exogenous substrate dipalmitoyl phosphatidyl[^{3}H]choline (dipalmitoyl [^{3}H]PC). In the absence of detergents, PLD activity was not detectable. Of the detergents examined, Triton X-100 was found to markedly enhance PLD activity, whereas other detergents including sodium deoxycholate, sodium cholate, CHAPS and Lubrol-PX caused only a small, if any increase in activity. Enhancement by Triton X-100 was maximal at 0.1–0.2% (w/v) and decreased at higher concentrations. The optimal pH was 7.1–7.3. Both Ca^{2+} and Mg^{2+} inhibited enzyme activity stimulated by Triton X-100 in a concentration-dependent manner. Triton X-100 effectively solubilized PLD from the particulate fraction of rat brain; more than 70% of the activity of the particulate fraction was extracted by 0.5–1.0% (w/v) Triton X-100. Furthermore, when the PLD activities in brains of three different species (rat, rabbit and bovine) were measured under optimal conditions, the activities were found to differ greatly. PLD activity was highest in rabbit brain, followed by rat and bovine brains; the activity in bovine brain was extremely low compared to the activities in rat and rabbit brains. We conclude that Triton X-100 is potentially useful for the purification of PLD and that rabbit and rat brains are the preferred sources.
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Phospholipase D (PLD, EC 3.1.4.4) is a unique enzyme which catalyzes the hydrolysis of phospholipids as well as transphosphatidylation reaction. Hydrolysis of phospholipids by PLD leads to the generation of phosphatidic acid and alcohols, *e.g.*, choline, ethanolamine and serine. Based on the phosphatidyltransferase activity of PLD, the phosphatidyl moiety of substrate phospholipids is transferred to nucleophilic primary alcohols to produce their corresponding phosphatidylalcohols; *e.g.*, in the presence of ethanol, PLD produces phosphatidylethanol (PEt) (1–3). In intact cells, whose endogenous phosphatidylcholine (PC) is radiolabeled, PLD activity can be easily detected by measuring the radiolabeled PEt formed in the presence of ethanol.

More recent studies have demonstrated that PLD exists in mammalian cells (4–10), although it had earlier been believed that PLD is present only in plant tissue. Recent studies have furthermore shown that PLD is activated in response to a variety of specific receptor agonists in several types of cells (5,8,9,11–15), which suggests that PLD activation of cells by agonists is part of a signal transduction pathway.

*To whom correspondence should be addressed at Department of Biochemistry, Gifu University School of Medicine, Tsukasamachi 40, Gifu 500, Japan.

Abbreviations: PC, phosphatidylcholine; PEt, phosphatidylethanol; PLD, phospholipase D; PNPP, *p*-nitrophenylphosphate.

Kanfer and co-workers (16–18) have reported that the particulate fraction of rat brain contains PLD, but the activity was barely detectable in the absence of appropriate activators, such as unsaturated fatty acids. The fatty acids, however, did not appear to have a detergent effect in the sense that they failed to solubilize membrane-bound PLD (17). Although Taki and Kanfer (19) have solubilized and partially purified PLD from rat brain using Miranol, this detergent is not commercially available.

In the present study, we have therefore tested the ability of various commercially available detergents to stimulate and solubilize PLD using a particulate fraction from rat brain, and have attempted to establish optimum conditions for the assay of PLD activity. Furthermore, we have compared PLD activities in the brain of three different species (rat, rabbit and bovine) to find an optimal source for the purification of PLD.

MATERIALS AND METHODS

Materials. 1,2-Dipalmitoyl-*sn*-glycero-3-phospho[*methyl*-^{3}H]choline (dipalmitoyl [^{3}H]PC, 73 Ci/mmol) was purchased from Amersham (Buckinghamshire, U.K.). 1,2-Dipalmitoyl-*sn*-glycero-3-phosphocholine (dipalmitoyl PC) and [*methyl*-^{3}H]choline chloride (85 Ci/mmol) were obtained from Avanti Polar Lipids Inc. (Pelham, AL) and American Radiolabeled Chemicals Inc. (St. Louis, MO), respectively. AG 50W-X8 resin (100–200 mesh, hydrogen form) was from Bio-Rad (Richmond, CA).

Preparation of particulate, crude membrane and microsomal fractions. Rats and rabbits were sacrificed by decapitation or by injection of air into a vein, respectively. Immediately after sacrifice, brains were removed and frozen in liquid nitrogen. Bovine brain was removed immediately after sacrifice, cut in about 0.5-cm thick slices, and then frozen in liquid nitrogen. Brains were stored at −80°C until use.

To study the effect of detergents, pH and divalent cations on PLD activity, the particulate fraction of rat brain was prepared as follows. Rat brains (6 g, wet wt) were minced and homogenized in 30 mL of buffer A consisting of 20 mM Tris-HCl (pH 7.4), 1 mM EDTA, 1 mM dithiothreitol and 0.2 mM phenylmethylsulfonyl fluoride, and then an additional 30 mL of buffer A was added. After sedimentation of nuclei and unbroken cells at 400 × *g* for 10 min, the resultant supernatant was centrifuged at 105000 × *g* for 60 min to obtain the particulate fraction.

To study species difference in PLD activity, crude membrane and microsomal fractions of brain of three different species (rat, rabbit and bovine) were prepared according to Natarajan *et al.* (20). Rat, rabbit and bovine brains were minced and homogenized (20% w/v) in 0.32 M sucrose containing 1 mM EGTA. The homogenate was centrifuged at 1000 × *g* for 10 min and the postnuclear supernatant was centrifuged at 22000 × *g* for 30 min to obtain the crude membrane fraction that contained myelin, synaptosomes and mitochondria. The supernatant was centrifuged at 105000 × *g* for 60 min to obtain the microsomal

fraction. The protein concentration was determined with a BCA protein assay kit (Pierce Chemical Company, Rockford, IL) using bovine serum albumin as standard.

Assay of PLD activity. Dipalmitoyl [^{3}H]PC was used as a substrate for PLD. Unilamellar vesicles were prepared in buffer warmed above the phase transition temperature of dipalmitoyl PC (41.5°C) (21). To dipalmitoyl [^{3}H]PC dried under nitrogen was added 100 mM Tris-maleate buffer (pH 7.0) prewarmed to 55°C. The dipalmitoyl [^{3}H]PC suspension was vigorously vortexed and sonicated for 5–10 min in a bath sonicator (Funakoshi A-10F, Tokyo, Japan) which was kept with water at 55°C.

PLD activity was assayed by measuring [^{3}H]choline generated from dipalmitoyl [^{3}H]PC. For the standard assay, the reaction mixture contained 40 mM Tris-maleate (pH 7.0), 30 μM dipalmitoyl [^{3}H]PC (0.2 μCi), 0.1% (w/v) Triton X-100, 0.1 mM EDTA and 100 μg protein in a total volume of 100 μL. Incubations were carried out at 37°C for 30 min. PLD activity was also assessed in the presence of sodium oleate, which is known to activate PLD activity (17,18), according to the method by Chalifa *et al.* (22) except that [^{3}H]choline produced in the absence of ethanol was determined. Briefly, the reaction mixture (100 μL) contained 50 mM Na-Hepes buffer (pH 7.2), 2.7 mM dipalmitoyl [^{3}H]PC (0.4 μCi), 4 mM sodium oleate, 1 mM EGTA, 1 mM $MgCl_2$, 5 mM *p*-nitrophenylphosphate (PNPP) and 30 μg protein of crude membrane or microsomal fraction. The phosphatase inhibitor PNPP was included to prevent the conversion of phospho[^{3}H]choline formed by the PC-specific phospholipase C to [^{3}H]choline by phosphocholine phosphatase (23,24). Incubations were carried out at 37°C for 15 min. The reaction was stopped by adding 400 μL of chloroform/methanol (1:2, v/v), and the phases were separated by adding 100 μL of chloroform and 100 μL water. [^{3}H]Choline partitioned into the resultant aqueous phase was separated on an AG 50W-X8 column and radioactivity was counted.

Isolation of [^{3}H]choline by AG 50W-X8 column chromatography. [^{3}H]Choline was separated from other radiolabeled [^{3}H]choline derivatives by AG 50W-X8 cation-exchange column chromatography using a slight modification of the method reported by Cook and Wakelam (25). The AG 50W-X8 column (0.5 mL) was washed with 5 mL of 1 M HCl and then with 10 mL of water before use. Samples diluted to 5 mL with water were applied to the column, and the column was washed three times with 5 mL of water to elute glycerophosphocholine and phosphocholine. [^{3}H]Choline was eluted by five successive additions of 1 mL of 1 M KCl, with the recovery being over 90%, and radioactivity was counted. When 1 M HCl was employed to elute [^{3}H]choline as reported by Cook and Wakelam (25), 12 mL of 1 M HCl was required to recover more than 90% of [^{3}H]choline in a relatively broad fraction (data not shown). Use of 1 M KCl considerably shortened [^{3}H]choline elution time.

Solubilization of PLD from the particulate fraction of rat brain by Triton X-100. One milliliter of the particulate fraction of rat brain in buffer A (5.5 mg protein/mL) was mixed with an equal volume of buffer A containing various concentrations of Triton X-100. The mixture was incubated at 4°C for 2 hr with stirring and then centrifuged at 105000 × *g* for 1 hr to obtain the Triton X-100 extract. Twenty microliters of the extract was used for the assay of PLD activity and 100 μL was used for the protein determination. The concentration of Triton X-100 was adjusted to 0.2% (w/v) in the PLD assay.

RESULTS AND DISCUSSION

Effects of detergents on PLD activity in the particulate fraction of rat brain. Of the detergents employed in this study, including Triton X-100, sodium deoxycholate, sodium cholate, CHAPS and Lubrol-PX, only Triton X-100 was found to significantly activate PLD in the particulate fraction of rat brain; maximum stimulation occurred at 0.1–0.2% (w/v) (Fig. 1). Other detergents caused only little activation. In the absence of detergents, PLD activity was not detectable. In the presence of 0.1% (w/v) Triton X-100, [^{3}H]choline formation was linear with time for 30 min and with protein concentration up to 50 μg/assay (data not shown). This would indicate that [^{3}H]choline was produced enzymatically. [^{3}H]Choline can also be produced from [^{3}H]PC by the sequential action of PC specific-phospholipase C and phosphocholine phosphatase. When the PLD assay was carried out in the presence of 5 mM PNPP, a phosphatase inhibitor which inhibits phosphocholine hydrolysis (23,24), [^{3}H]choline formation was the same as in the absence of PNPP (data not shown). This confirms that the [^{3}H]choline formed under our experimental conditions reflects PLD activity only.

Solubilization of PLD from the particulate fraction of rat brain by Triton X-100. We found that Triton X-100 significantly stimulates PLD activity, which confirms what was reported by Qian *et al.* (24). If Triton X-100 can solubilize PLD of the particulate fraction, this should prove useful for the purification of the enzyme. As shown in Table 1, Triton X-100 solubilized PLD from the

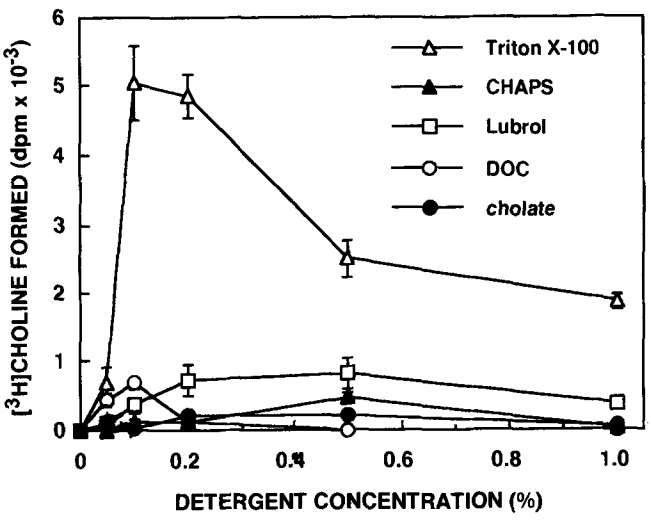


FIG. 1. Effects of detergents on phospholipase D activity in the particulate fraction of rat brain. The reaction mixture (100 μL) contained 40 mM Tris-maleate (pH 7.0), 30 μM dipalmitoyl [^{3}H]PC (0.2 μCi), 0.1 mM EDTA, 100 μg protein of the particulate fraction of rat brain and the indicated concentration of sodium deoxycholate (DOC), (○), sodium cholate (●), Triton X-100 (△), CHAPS (▲) or Lubrol-PX (□). After incubation at 37°C for 30 min, [^{3}H]choline formed was determined as described in Materials and Methods. The results shown are the mean ± S.D. of three independent experiments.

TABLE 1

Solubilization of Phospholipase D from the Particulate Fraction of Rat Brain by Triton X-100[a]

	Triton X-100 (%)			
	0.1	0.2	0.5	1.0
Protein (mg/mL)	0.95 ± 0.07 (31.9 ± 2.5)[b]	1.02 ± 0.16 (35.1 ± 6.6)[b]	1.20 ± 0.20 (41.0 ± 4.0)[b]	1.21 ± 0.28 (41.5 ± 7.3)[b]
Specific activity (nmol/hr/mg protein)	0.53 ± 0.18	1.58 ± 0.71	1.76 ± 0.80	1.94 ± 0.87
Extractability[c] (%)	21.2 ± 6.9	53.6 ± 14.9	73.6 ± 27.9	79.0 ± 31.0

[a] PLD was extracted from the particulate fraction of rat brain as described in Materials and Methods. The concentration of Triton X-100 in the reaction mixture of the PLD assay was adjusted to 0.2% (w/v). PLD activity of the particulate fraction before solubilization, that was also assayed in the presence of 0.2% (w/v) Triton X-100, was 0.99 ± 0.24 nmol/hr/mg protein. The results shown are the mean ± S.D. of four independent experiments.

[b] Values in parentheses represent the percentage of protein solubilized when protein of the particulate fraction before solubilization was taken as 100%.

[c] Extractability represents the percentage of PLD activity in the extracts when the activity of the particulate fraction before solubilization was taken as 100%.

particulate fraction of rat brain in a concentration-dependent manner. Maximal solubilization was observed at 0.5–1.0% (w/v) Triton X-100, with more than 70% of the initial activity present in the particulate fraction being extracted. The efficiency varied substantially from one experiment to another. Although PLD activity decreased at Triton X-100 concentrations higher than 0.2% (w/v) (Fig. 1), the activity recovered when the concentration of Triton X-100 was lowered again to 0.2% (w/v). The results may suggest that higher concentrations of Triton X-100 could interfere with interaction of PLD with the substrate. The specific activity of PLD in the 1.0% (w/v) Triton X-100 extract was about 4 times higher than that in the 0.1% (w/v) Triton X-100 extract (Table 1). Thus, rat brain PLD appears to be tightly associated with the particulate fraction. The sum of the PLD activities in the extract and in the residual pellet exceeded the initial activity in the particulate fraction present before extraction (data not shown). This would indicate that PLD activity is enhanced by solubilization by Triton X-100. We thus conclude that Triton X-100 is highly effective in activating and solubilizing membrane-bound PLD and that this detergent could be utilized for the purification of PLD.

Effects of pH and divalent cations on PLD activity in the particulate fraction of rat brain. To optimize PLD assay conditions in the presence of Triton X-100 (0.1%, w/v), we have examined the pH dependency and the effects of divalent cations Ca^{2+} and Mg^{2+}. The PLD activity in the particulate fraction of rat brain was maximal at pH 7.1–7.3 (Fig. 2). Either above pH 7.5 or below pH 6.5, the activity was significantly decreased.

Figure 3 shows the effects of Ca^{2+} and Mg^{2+} on PLD activity. In order to suppress the effect of endogenous Mg^{2+} contained in water and other reagents, the reaction mixtures for studying the effects of Mg^{2+} contained 0.1 mM EDTA. It has been reported that phosphatidylinositol 4,5-bisphosphate-specific phospholipase C is activated by free Ca^{2+} at micromolar concentrations (26), whereas phospholipase A_2 requires higher concentrations

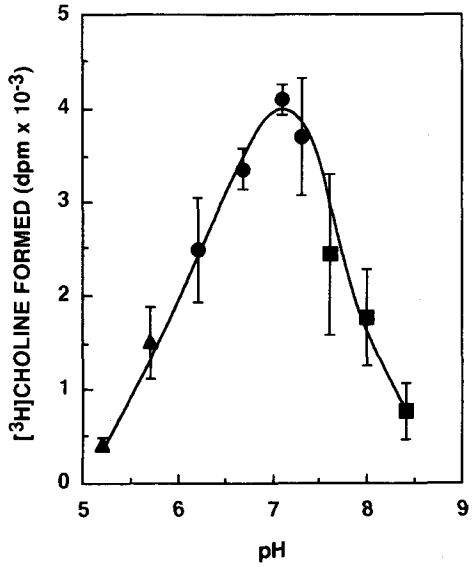


FIG. 2. Effects of pH on phospholipase D activity of the particulate fraction of rat brain. The reaction mixture (100 μL) contained 30 μM dipalmitoyl [^{3}H]PC (0.2 μCi), 0.1% (w/v) Triton X-100, 0.1 mM EDTA, 100 μg protein of the particulate fraction of rat brain and the following buffers: 40 mM citrate, pH 5.2 and 5.7 (▲); 40 mM Tris-maleate, pH 6.2, 6.7, 7.1 and 7.3 (●); and 40 mM Tris-HCl, pH 7.6, 8.0 and 8.4 (■). Incubations were carried out at 37°C for 30 min. After termination of the reaction, [^{3}H]choline formed was determined as described in Materials and Methods. The results shown are the mean ± S.D. of three independent experiments.

of free Ca^{2+} (27). This would indicate that it is advisable to examine the effects of Ca^{2+} over a wide range. To provide such a wide range of free Ca^{2+} concentrations, Ca^{2+}-EGTA buffer was used by supplementing with 2 mM EGTA. Under these conditions, both Ca^{2+} and Mg^{2+} inhibited PLD activity in the particulate fraction of rat brain in a concentration-dependent manner. By contrast, it has been reported that Ca^{2+} is required for PLD

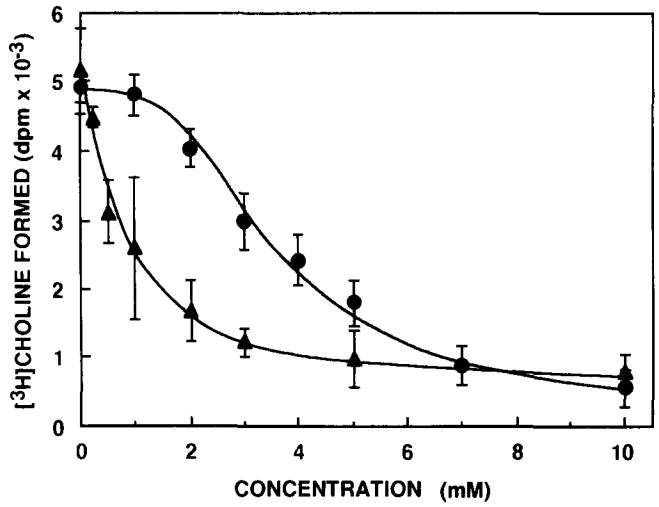


FIG. 3. Effects of Ca^{2+} and Mg^{2+} on phospholipase D activity of the particulate fraction of rat brain. The reaction mixture (100 μL) contained 40 mM Tris-maleate (pH 7.0), 30 μM dipalmitoyl [^{3}H]PC (0.2 μCi), 0.1% (w/v) Triton X-100, 100 μg protein of the particulate fraction of rat brain and the indicated concentration of Ca^{2+} (●) in the presence of 2 mM EGTA or Mg^{2+} (▲) in the presence of 0.1 mM EDTA. After incubation at 37°C for 30 min, [^{3}H]choline formed was determined as described in Materials and Methods. The results shown are the mean ± S.D. of three independent experiments.

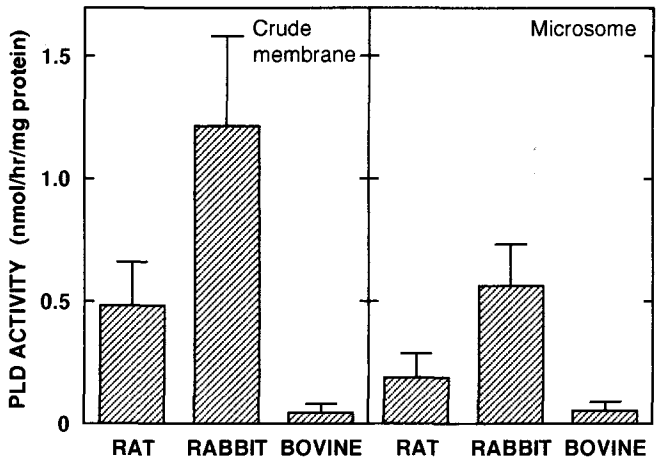


FIG. 4. Species differences in phospholipase D activity in brain stimulated by Triton X-100. Crude membrane and microsomal fractions from brain of rat, rabbit and bovine were prepared as described in Materials and Methods. The reaction mixture (100 μL) contained 40 mM Tris-maleate (pH 7.0), 30 μM dipalmitoyl [^{3}H]PC (0.2 μCi), 0.1% (w/v) Triton X-100, 0.1 mM EDTA and 50 μg protein of crude membrane or microsomal fraction. After incubation at 37°C for 30 min, [^{3}H]choline formed was determined as described in Materials and Methods. The results shown are the mean ± S.D. of three independent experiments.

activation in intact cells and homogenates. For example, in human neutrophils, agonist-stimulated PLD activity requires extracellular Ca^{2+} (28). Calcium ionophore A23187 in the presence but not in the absence of extracellular Ca^{2+} activates PLD in human neutrophils (29), erythroleukaemia cells (14) and HL-60 granulocytes (30). Furthermore, PLD activity in HL-60 homogenates is activated by Ca^{2+} (31). These findings, taken together with our result that Ca^{2+} is not required for PLD activity in the particulate fraction of rat brain, suggest that some Ca^{2+}-dependent factor(s) such as calmodulin may be involved in the Ca^{2+}-induced activation of PLD.

Species difference of PLD activity in brains. We have studied PLD activity in rabbit and bovine brain as well as in rat brain under optimum conditions (Fig. 4). Crude membrane and microsomal fractions were prepared to localize PLD activity. PLD activity in the crude membrane fraction from rat and rabbit brain was higher than in the microsomal fraction, whereas no significant difference was observed between the respective fractions of bovine brain. Furthermore, we found that rabbit brain had the highest PLD activity, followed in order of decreasing activity by rat and bovine brain. The activity of bovine brain was extremely low compared to the activity of rat or rabbit brain. Thus, it appears that PLD activity in brain varies between animal species. To determine whether species differences in PLD activity are assay-specific, we also measured PLD activity upon stimulation with sodium oleate, a well-established activator of brain PLD (17,18) (Fig. 5). Again, PLD activity in bovine brain was extremely low compared to the activity in the two other species. However, PLD activity in rat brain was higher than in rabbit brain and the activity in crude membrane of rat brain was lower than in the microsomal fraction, differing from the result obtained with Triton X-100 (Figs. 4 and 5). Although, these discrepancies have not

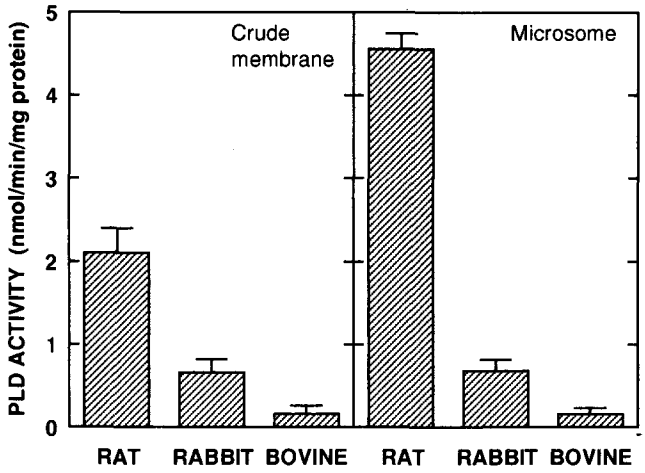


FIG. 5. Species differences in phospholipase D activity in brain stimulated by sodium oleate. Crude membrane and microsomal fractions from brain of rat, rabbit and bovine were prepared as described in Materials and Methods. The reaction mixture (100 μL) contained 50 mM Na-Hepes buffer (pH 7.2), 2.7 mM dipalmitoyl [^{3}H]PC (0.4 μCi), 4 mM sodium oleate, 1 mM EGTA, 1 mM $MgCl_2$, 5 mM *p*-nitrophenylphosphate and 30 μg protein of crude membrane or microsomal fraction. After incubation at 37°C for 15 min, [^{3}H]-choline formed was determined as described in Materials and Methods. The results shown are the mean ± S.D. of three independent experiments.

further been explored, it is conceivable that different isozymes of PLD, which have different sensitivity to Triton X-100 and sodium oleate, are expressed in rabbit and rat brains, and in the crude membrane and microsomal fractions.

Stimulation of PLD activity with sodium oleate was much greater than with Triton X-100 (Figs. 4 and 5). The

level of stimulation attained with sodium oleate in the present study was similar to that previously reported by others (16-18,22). Although, both Triton X-100 and sodium oleate activate PLD, the enzyme can be solubilized only with Triton X-100 (Table 1), not with sodium oleate (17). Furthermore, the optimal concentration of sodium oleate to stimulate PLD activity depends on the concentration of substrate PC (17,18). This may suggest that Triton X-100 directly activates the enzyme, whereas sodium oleate interacts with the substrate thereby giving rise to an apparent stimulation of enzyme activity.

In summary, we conclude that Triton X-100 should prove useful for the purification of PLD because of its ability to stimulate and to solubilize PLD. Rabbit and rat brain are the preferred sources for the isolation and purification of the enzyme.

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Lipid Composition and Peroxide Levels of Mucosal Cells in the Rat Large Intestine in Relation to Dietary Fat

M.E. Turini, A.B.R. Thomson and **M.T. Clandinin***
Nutrition and Metabolism Research Group, Departments of Foods & Nutrition and Medicine, University of Alberta, Edmonton, Alberta, Canada T6G 2C2

To examine whether dietary fat alters membrane lipid composition and peroxidation of polyunsaturated fatty acids in "non-proliferative" and "proliferative" cells in the large intestine, Sprague-Dawley rats were fed diets providing a polyunsaturated-to-saturated fatty acid ratio of 1.2 or 0.3 at a high or low level of fat intake for a 25-day period. Cell populations were isolated and the effect of dietary fat on membrane polyunsaturated fatty acid content and peroxide levels was determined. Neither fat level nor fatty acid composition of diet influenced total cholesterol, total phospholipids, and percentage of phospholipid classes in membrane phospholipids. Feeding the high fat and/or high polyunsaturated-to-saturated fatty acid ratio diet increased polyunsaturated fatty acid content of mucosal cell phospholipids. Increase in polyunsaturated fatty acid content was paralleled by a decrease in the monounsaturated fatty acid content of mucosal cell phospholipids. Membrane content of total saturated fatty acids was not significantly affected by diet. Variation in phospholipid fatty acid composition between "non-proliferative" and "proliferative" cells was observed. Lipid peroxide levels in mucosal cell lipid fractions were altered by dietary fat treatment. Animals fed high fat diets, compared to groups fed low fat diets, exhibited higher membrane peroxide levels when results are expressed as nmol/mg protein. Higher peroxide levels were observed in mucosal cells for rats fed high polyunsaturated-to-saturated fatty acid ratio diets when results were expressed per nmol of phospholipid. It is concluded that changes in fat level and fatty acid composition of the diet alters the mucosal cell membrane lipid composition in the rat large intestine and influences susceptibility of mucosal cell lipid to peroxidation. Further research is required to delineate which dietary factors — fat level, polyunsaturated-to-saturated fatty acid ratio, or both — have a primary influence on the degree of lipid peroxidation.

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Dietary fat intake has been associated with increased incidence of skin, breast and colon cancer (1-5). Colon cancer is the leading cause of death among all patients with malignancies in the United States. Previous studies have shown that dietary fat influences membrane structure and function (6,7) and have also demonstrated that membranes with a high unsaturation index are more sensitive to peroxidation (8,9). Susceptibility to peroxidation is influenced by free radical scavengers and antioxidants such as vitamin E, vitamin C, glutathione, superoxide dismutase, catalase, and Se^{++} and non-Se^{++}-dependent glutathione peroxidases (10-12). As vitamin E is a membrane soluble antioxidant, interactions with cystolic antioxidants occur (10,13-15). Lipid peroxides may affect regulations of DNA synthesis and cell division (16-18) and may also be involved in a number of diseases such as inflammation of the large bowel and cancer.

Studies of mucosal cell membranes from the small and large intestine have been reported (19-23). However, the effect of dietary fat on colonocyte cell membrane lipid composition and peroxidation is not well understood. The objective of this study was first to determine if dietary fat influences colonocyte cell membrane lipid and phospholipid fatty acid composition, and secondly to determine if dietary fat content of unsaturated fatty acids influences colonocyte lipid peroxide levels. Colonocytes were fractionated into "non-proliferative" and "proliferative" cell populations to determine differences in the lipid composition between these cell types.

MATERIALS AND METHODS

Animals and diets. Four weanling, male, Sprague-Dawley rats were obtained weekly for an eight-week period. Animals were fed chow (Allied Mills, Inc., Specialty Feeds Department, Chicago, IL) for 3-5 days and then fed semi-purified diets (Table 1) for 25 days. Each week, four rats were distributed into one of four treatment groups (total n=8 for each diet group). Diets and water were supplied *ad libitum* to rats housed individually in a temperature (22°C) and light controlled room with a 12/12 hr light/dark cycle. Diets were freshly prepared each week and animals were fed on alternate days. The four semi-purified diets fed were high fat or low fat with high (P/S=1.2) or low (P/S=0.3) polyunsaturated-to-saturated fatty acid (P/S) ratios (Table 2). High fat diets provided 50% of energy from fat while low fat diets provided 15% of energy from fat. Mixtures of safflower oil, hydrogenated beef tallow and linseed oil were used to provide the dietary fat source. To ensure similar essential nutrient intakes, the essential nutrient densities for non-fat components were similar for the four diets on a per calorie basis. Vitamin E levels of the four diets were equalized to the vitamin E content of the high fat high polyunsaturated-to-saturated fatty acid ratio diet by addition of *d*-α-tocopheryl acetate (a gift from Veris, La Grange, IL) to give a final vitamin E density of 17.63 IU/1000 kcal for each diet. Diets were prepared using vitamin-free constituents and a vitamin mix free of vitamin E. This vitamin mix was otherwise prepared according to the composition of the AOAC vitamin mix (Teklad Test Diets, Madison, WI). Rats were sacrificed by decapitation and

*To whom correspondence should be addressed at the Nutrition & Metabolism Research Group, 533 Newton Research Building, University of Alberta, Edmonton, Alberta, Canada T6G 2C2.

Abbreviations: AOAC, Association of Analytical Chemists; BHT, butylhydorxylated toluene; BSA, bovine serum albumin; HPLC, high performance liquid chromatography; MOPS, 3-[*N*-morpholino] propanesulfonic acid; POPOP, 1,4-*bis*[5-phenyl-2-oxazolyl]benzene; PPO, 2,5-diphenyloxazole.

TABLE 1

Composition of Experimental Diets

Fat level	High (g)		Low (g)	
P/S ratio[a]	High	Low	High	Low
Fat mixture	267.9	266.7	65.3	64.9
High protein casein (vit. free)	292.2	292.7	237.4	237.4
Cornstarch	81.5	81.6	406.9	406.9
Glucose	225.1	225.5	182.9	182.9
Non-nutritive cellulose	53.19	53.29	42.49	42.89
Vitamin mix[b] (vit. E free)	11.0	11.0	8.9	8.9
Mineral mix[c]	55.3	55.4	44.9	44.9
Choline	4.3	4.3	3.5	3.5
Inositol	6.7	6.7	5.4	5.4
L-Methionine	2.8	2.8	2.3	2.3
DL-α-tocopheryl acetate	—	45.61	49.35	60.35
Energy (kcal)	4791	4799	3893	3893

[a]P/S, polyunsaturated-to-saturated fatty acid ratio.

[b]Vitamin mix provided the following (per kilogram): Vitamin A, 2,000,000 IU; vitamin D, 200,000 IU; menadione, 0.5 g; choline, 200 g; *p*-aminobenzoic acid, 10 g; inositol, 10 g; niacin, 4 g; *d*-calcium pantothenate, 4 g; riboflavin, 0.8 g; thiamin-HCl, 0.5 g; pyridoxine-HCl, 0.5 g; folic acid, 0.2 g; biotin, 0.04 g; and vitamin B_{12}, 3.0 g.

[c]Bernhart-Tomarelli mineral mix (Teklad Test Diets, Madison, WI) provided the following (per kilogram): $CaCO_3$, 21.0 g; $CaHPO_4$, 735 g; MgO, 25.0 g; K_2HPO_4, 81.0 g; K_2SO_4, 68.0 g; NaCl, 30.6 g; Na_2PO_4, 21.4 g; cupric citrate, 0.46 g; ferric citrate (16.7% Fe), 5.58 g; manganese citrate (13.9% Mn), 8.35 g; KI, 0.0072 g; zinc citrate, 1.33 g; and citric acid, 2.2728 g.

TABLE 2

Fatty Acid Composition of Experimental Diets

	(%, w/w)			
	High fat		Low fat	
Fatty acid	High P/S[a]	Low P/S	High P/S	Low P/S
14:0	2.0	3.5	1.9	3.7
14:1	0.1	0.3	0.1	0.2
15:0	0.3	0.5	0.3	0.5
15:1	0.1	0.2	0.1	0.2
16:0	16.5	24.1	16.4	24.8
16:1	0.3	0.4	0.3	0.4
17:0	0.9	1.7	0.9	1.7
18:0	22.0	41.2	22.4	41.1
18:1ω9	8.2	4.7	8.3	4.7
18:2ω6	47.3	20.6	44.6	17.5
18:3ω6	0.1	0.3	0.1	0.2
18:3ω3	1.4	1.5	4.2	4.3
20:0	0.3	0.5	0.1	0.5
22:2ω6	0.2	0.2	0.1	0.1
22:0	0.1	0.1	ND[d]	ND
22:5ω3	0.1	0.2	0.1	0.1
22:6ω3	0.1	ND	0.1	ND
Σsaturated[a]	42.1	71.6	42.0	72.3
Σmonounsaturated[a]	8.7	5.6	8.8	5.5
Σpolyunsaturated[a]	49.2	22.8	49.2	22.2
Σω6[a]	47.6	21.1	44.8	17.8
Σω3[a,b]	1.6	1.7	4.4	4.4
P/S[a,c]	1.17 ± 0.05	0.32 ± 0.01	1.17 ± 0.03	0.31 ± 0.02

[a]Abbreviations: P/S, polyunsaturated-to-saturated fatty acid ratio; Σsaturated, total saturated fatty acids; Σmonounsaturated, total monounsaturated fatty acids; Σpolyunsaturated, total polyunsaturated fatty acids; Σω6, total ω6 fatty acids; and Σω3, total ω3 fatty acids.

[b]Densities (g/1000 kcal) of ω3 fatty acids are similar for the four experimental diets.

[c]Values are means ± S.D. of 11 freshly prepared diets.

[d]ND, not detected.

the large intestine removed for mucosal cell isolation. For serum vitamin E determination, blood was collected into non-heparinized glass test tubes and protected from light. Tubes were kept in the dark at room temperature for 30 min before being centrifuged at 500 × *g* for 15 min at 10-15°C. The supernatant (serum) was collected and stored in the dark at -20°C until analyzed.

Cell isolation. The cell isolation procedure used is a modification of methods described by Brasitus (24) and Weiser (25). Solutions used were: cold 0.9% (w/w) saline solution; solution A containing 1.5 mM HCl, 96.0 mM NaCl, 27.0 mM sodium citrate, 8.0 mM KH_2PO_4, 5.6 mM Na_2HPO_4, 0.5 mM dithiothreitol at pH 7.3; solution 8 consisting of Ca^{++}- and Mg^{++}-free Hank's buffer containing 10.0 mM triethanolamine-HCl and 0.5 mM dithiothreitol at pH 7.3; and standard buffer containing 139 mM NaCl and 10 mM Na_2HPO_4 at pH 7.3. It was crucial to eliminate contamination by intestinal bacteria. Thus, solutions A and B, as well as cold saline solution, were supplemented with Penicillin-G (K-salt, 660 U/mL) and streptomycin (1.6 mg/mL). Solution B was also supplemented with mycostatin (118.2 U/mL); 0.1 mM phenylmethanesulfonyl fluoride was added to solution A and B just before use as were antibiotics to solution A. Ca^{++}- and Mg^{++}-free Hank's buffer and antibiotics were stable for only one to two weeks. Therefore, these solutions were freshly prepared each week.

The large intestine was exised, feces carefully removed by means of cold saline solution, and the intestine was flushed three times with 40 mL of cold saline. The intestine was then tied at its proximal end, filled with solution A and closed at its other end with a hemostat. This slightly distended sac was placed in a 250-mL flask containing 125 mL of standard buffer solution (37°C) and incubated for 15 min at 37°C in a shaking waterbath (75 osc./min). The sac was then emptied, the solution discarded and replaced by solution B (pre-incubated at 37°C) and incubated. After each incubation, the solution containing each fraction of cells was collected in a 15-mL Corex glass centrifuge tube and the sac was refilled. Subsequent fractions of cells were collected after 2 × 10-min followed by 10 × 15-min incubation times. The 12 tubes were kept on ice until centrifuged at 484 × *g* at 4°C for 10 min. Supernatants were discarded and cell pellets were resuspended in 5 mL of standard buffer. Cell fractions were pooled as follows: fractions 2 and 3 (tube I), 4 and 5 (tube II), 6 and 7 (tube III), 9 and 10 (tube IV), and 11 and 12 (tube V), and the tubes were centrifuged at 484 × *g* at 4°C for 10 min. Supernatants were discarded. Cell pellets were resuspended in standard buffer, filtered on a 100-micrometer nylon mesh (Nitex) and pooled. Fractions I-III, and fractions IV and V were pooled to give two 12-mL fractions (A and B, respectively). These two tubes were centrifuged (484 × *g* at 4°C for 10 min), supernatants were discarded, and pellets were resuspended in 1 mL of standard buffer. Cell fractions were assessed for bacterial contamination by microscopy. Fractions prepared in this manner were essentially free of bacterial contamination. Protein was determined by a modified method of Lowry *et al.* (26) using bovine serum albumin (BSA) as a standard.

[^{3}H]Thymidine incorporation. Preliminary experiments were performed to determine fractions containing proliferative cells using [*methyl*-^{3}H]thymidine as a marker for DNA synthesis. Rats weighing 300-305 g were injected intraperitoneally with 100 μCi of 2-[*methyl*-^{3}H]thymidine 3 hr prior to sacrifice (24,25). Rats were killed by decapitation and cells isolated similarly to the method previously described. Fractions were not pooled and cell pellets were transferred into flint tubes (10 mm × 75 mm), dissolved in 500 μL of protosol, transferred into a scintillation cocktail (100 mg POPOP, 5 g PPO per liter of toluene) in 7-mL counting vials and counted to determine [^{3}H]thymidine incorporation. Counting was done in a Beckman LS 5801 liquid scintillation spectrometer at an efficiency of 45%. Results were expressed as dpm/mg protein.

Thymidine kinase assay. To differentiate "proliferative" from "non-proliferative" cells during the main experiment, thymidine kinase activity was measured according to the method of Salser and Balis (27). Reactions were carried out at 37°C in capped flint tubes (10 mm × 75 mm) in a waterbath. The reaction mixture containing 90-110 μg of homogenate protein (100-μL volume), adjusted if necessary by using cell resuspending buffer (pH 7.3), 12 mol Tris-HCl (pH 8.0), 1.8 mol $MgCl_2$, 1.8 mol ATP, 1.5 mol 3-phosphoglyceric acid, and 0.6 μmol NaF, was pre-incubated for 10 min at 37°C. Reactions were initiated by addition of 50 μL of 2 [2-^{14}C]deoxythymidine (9 nmoles; 500,000 dpm), bringing the total volume to 250 μL, and were incubated for 45 min. Reactions were stopped by immersing the tubes into boiling water for 3 min and then cooling the tubes in an ice bath until centrifugation (3020 × *g*) at 10°C for 15 min. Aliquots (50 μL) were applied onto DEAE-cellulose filters (Whatman No. DE-81; 2 cm diam.) and filters washed according to the method described by Bresnick and Karjala (28). The filters were quickly immersed in a beaker containing 30 mL of 0.001 M ammonium formate for 10 min. The discs were then washed in distilled H_2O and placed in a new beaker containing 30 mL of 0.001 M ammonium formate for 10 min. The discs were again washed with distilled water, placed in distilled water for 5 min and finally immersed in 95% (v/v) ethanol before being dried at 80°C and placed in counting vials containing 5 mL of a toluene-based cocktail (5 g PPO, 100 mg POPOP per liter of toluene). This procedure removes the precursor [2-^{14}C]deoxythymidine while retaining [2-^{14}C]deoxythymidinemonophosphate. Counting was done in a Beckman LS 5801 liquid scintillation system (Beckman Instruments, Fullerton, CA) with an efficiency of 97%.

Lipid analysis. Total lipids were extracted (29), total phospholipid was estimated (30), and total cholesterol was determined (31-33) using an enzymatic kit (cholesterol C-system kit, Boehringer Mannheim Canada, Montreal, Canada). Different phospholipids were estimated by densitometry (34). For densitometry, samples were spotted on high performance thin-layer chromatography plates (Whatman, HP-K high performance Silica Gel plates, 10 cm × 10 cm) and phospholipids were separated using a solvent system comprised of chloroform/methanol/propanol/triethylamine/0.25% (w/v) KCl (14:4.5:12.5: 6.5:3.5, by vol).

For fatty acid analysis, phospholipids were separated using Silica Gel H plates (20 × 20 cm) and developed in a solvent system composed of chloroform/methanol/2-propanol/0.25% (w/v) KCl/triethylamine (30:9:25:6:18, by vol) (35). Fatty acids were methylated for 1 hr at 100°C using borontrifluoride/methanol reagent (36). Separation of fatty acid methylesters was performed by auto-

mated gas-liquid chromatography (Vista 6000 GLC and Vista 654 data system; Varian) Instruments, Georgetown, Ontario, Canada) using a fused silica BP20 capillary column (25 mm × 0.25 mm i.d.; Varian) and a splitless injection mode (37). Helium was used as the carrier gas at a flow rate of 1.8 mL/min. Injector and detector temperatures were maintained at 250°C. The initial oven temperature of 90°C was increased to 172°C at 20°C/min and held for 13.2 min, then increased again to 220°C at 3.5°C/min for a total analysis time of 45 min.

Lipid peroxide determination. Lipid peroxides were determined using a "Determiner LPO" or leuco-methylene blue derivative assay kit (a generous gift from Kyowa Medics Co., Tokyo, Japan) with some modifications to increase test sensitivity (38). Tests were performed in borosilicate glass test tubes (13 mm × 100 mm). The first reagent (150 μL of 30 U/mL of ascorbic oxidase in 100 mM 3-[*N*-morpholino]propanesulfonic acid, MOPS, pH 5.8, (stabilizer) was added to 100 μL of lipid extract containing 1.0-1.5 mg protein equivalent in methanol. Tubes were vortexed and incubated 5 min at 30°C in a shaking water bath before adding 300 μL of the second reagent (52.7 mM of 10-*N*-methyl-carbamoyl-3, 7-dimethylamine-10H-phenothiazine, 100 mM MOPS pH 5.8, 68 mg/L of hemoglobin, surface active reagent, chelating agent and stabilizer). Tubes were vortexed and incubated for 10 more min. Absorbency was determined at 675 nm in a Perkin-Elmer model Lambda 3b spectrophotometer. Sample concentrations were calculated using a standard curve in the range of 0-1.0 nmole of cumen hydroperoxide. Change in blank absorbency with time was determined against water. The test was performed in a dark room with red light. Lipid peroxide determination was performed on batches of one blank and five samples starting the reaction in each tube at 1-min intervals. Results are expressed as nmole equivalents of cumen hydroperoxide.

Vitamin E determination. Oil samples for vitamin E determination were prepared using a modification of the method described earlier (39). Safflower oil supplemented with *d*-α-tocopheryl acetate was dissolved in distilled hexane and injected directly onto the high performance liquid chromatography (HPLC) column. Serum samples were prepared using the simplified clarification method described by Nierenberg and Lester (40). The organic upper layer (250 μL) was transferred by automatic pipette into a borosilicate glass tube (10 mm × 75 mm) and dried in 5 to 10 min at 75°C under N_2. No loss occurred during this process when verified with a standard mixture of *dl*-α-tocopherol and *d*-α-tocopheryl acetate. The residue was resuspended in 250 μL of distilled hexane, transferred to a 0.4-mL polypropylene microcentrifuge tube and centrifuged at 13,000 × rpm for 1 min at 4°C. The clarified distilled hexane (100 μL) was injected onto the HPLC column. Butylated hydroxytoluene (BHT) was present in the extraction system to avoid vitamin E oxidation.

Tocopherols were analyzed using the HPLC method described by Buttris and Diplock (41). Chromatography utilized a Micropack Si-5 column (4.5 mm I.D. × 15 cm; Varian). Solvents were filtered and degassed by N_2 prior to use. The system was standardized by injection of a 100-μL mixture containing 50 μg/mL of *dl*-α-tocopherol (Sigma Chemical Co., St. Louis, MO) and *d*-α-tocopheryl acetate (Veris) in hexane after being checked for linearity in the range of 5-100 μg/mL.

Statistical analysis. The effect of diet treatment and differences between "non-proliferative" and "proliferative" cell fractions were examined by least square analysis of variance (ANOVA) (42). Effect of diet treatment on total protein released, serum vitamin E concentrations and lipid peroxide levels were examined by two-way ANOVA procedures. [^{3}H]Thymidine incorporation into DNA of "non-proliferative" and "proliferative" cells was analyzed by a Student's *t*-test.

RESULTS

Effect of diet treatment. Mean body weights of animals after the diet treatments were not significantly different. Body weights (mean ± S.D.) were 77.6 ± 4.5 g on initiation of treatment and 252.7 ± 20.8 at the time of sacrifice.

Mucosal cell isolation and thymidine kinase assay. Prior to initiation of experimental procedures, rats fed chow were injected with [^{3}H]thymidine to localized "proliferative" cell vs. "non-proliferative" cell fractions. Initial experiments were performed without pooling the collected fractions (data not shown). Fractions to be pooled as "non-proliferative" and "proliferative" cell fractions were determined from these preliminary experiments based upon [^{3}H]thymidine incorporation. "Proliferative" cell fractions incorporated 2.5-fold more [^{3}H] thymidine than "non-proliferative" fractions ($p<0.002$). Diet fatty acid composition, but not diet fat level, altered the total yield of cells isolated ($p<0.005$; Table 3). During experimental procedures the relative yield of cell protein was similarly distributed between "non-proliferative" and "proliferative" cell fractions (34.26% and 65.74%, respectively) irrespective of the diet treatment. Linearity between time and rate of thymidine incorporation was observed for up to 45 min (data not shown). No effect of treatment was observed on thymidine kinase activity. Mean enzyme specific activity was 4.1 nmole of product formed per mg protein in the "proliferative" cell fraction as compared to 2.9 nmole/mg protein in the "non-proliferative" cell population ($p<0.05$; data not shown).

TABLE 3

Effect of Diet Treatment on Mucosal Cells Released from the Rat Large Intestine (Total mg of Protein[a])

	Fat level	
	High	Low
High P/S ratio[b]	4.8 ± 1.4	6.1 ± 1.2
Low P/S ratio	8.9 ± 2.7	8.1 ± 3.3
Effect of treatment[c]:		
Fat level	NS[d]	
P/S ratio	0.005	
Fat × P/S ratio	NS	

[a]Sum of protein (mg) from "non-proliferative" cells and "proliferative" cells.

[b]P/S ratio, polyunsaturated-to-saturated fatty acid ratio.

[c]Significant effects ($p<$) by two-way analysis of variance procedures are indicated.

[d]NS, not significantly different.

TABLE 4

Effect of Diet Treatment on Total Cholesterol, Total Phospholipids, and Cholesterol-to-Phospholipid Ratio of Mucosal Cells

Fat level	High				Low			
P/S ratio[a]	High		Low		High		Low	
Cell fraction	Non-prolif.[b]	Prolif.[b]	Non-prolif.	Prolif.	Non-prolif.	Prolif.	Non-prolif.	Prolif.
Cholesterol (nmol/mg protein)[d]	70.3 ± 11.1[c]	60.1 ± 7.1	64.4 ± 12.8	59.1 ± 7.3	68.9 ± 14.5	60.9 ± 14.5	66.4 ± 15.9	61.0 ± 9.4
Phospholipids (nmol/mg protein)[d]	147 ± 40[c]	122 ± 48	150 ± 47	171 ± 34	142 ± 40	106 ± 26	149 ± 46	142 ± 9
Cholesterol/phospholipids (nmol/nmole)	0.51 ± 0.14[c]	0.60 ± 0.37	0.45 ± 0.11	0.36 ± 0.08	0.50 ± 0.10	0.59 ± 0.14	0.42 ± 0.12	0.43 ± 0.05

Effect of Treatment[e]:	Fat level	P/S ratio	Cell fraction	Interactions at $p<0.05$
Cholesterol	NS	NS	<0.05	NS
Phospholipids	NS	NS	NS	P/S ratio × cell fraction
Cholesterol/phospholipid ratio	NS	<0.05	NS	NS

[a]Abbreviations: P/S Ratio, polyunsatured-to-saturated fatty acid ratio; n, number of replicates.

[b]Non-prolif. cells, "non-proliferative" cell fraction; Prolif. cells, "proliferative" cell fraction.

[c]Values are means ± S.D.; number of replicates, n = 5-7.

[d]Cholesterol, MW 386.7; Phospholipids, MW 730.

[e]Significant effects by least square analysis of variance procedures are indicated ($p<0.05$); NS. not significantly different.

Effect of diet treatment on lipid composition of mucosal cells. Experimental day, amount of fat, or polyunsaturated-to-saturated fatty acid ratio in the diet did not alter mucosal cell total cholesterol content. The "non-proliferative" cell population exhibited a 12% higher total cholesterol level as compared to the "proliferative" cell fraction ($p<0.05$; Table 4). Day of experiment, diet fat level, polyunsaturated-to-saturated fatty acid ratio in the diet, or cell fraction tested did not significantly affect the total phospholipid content of mucosal cells in the large intestine (Table 4). Cholesterol to phospholipid molar ratio was not affected by day of cell isolation or diet fat level. "Non-proliferative" and "proliferative" cell fractions exhibited similar ratios of cholesterol to phospholipid. Groups fed diets providing a high polyunsaturated-to-saturated fatty acid ratio (P/S = 1.2) showed a 32.5% increase ($p<0.05$) in cholesterol-to-phospholipid ratio compared to groups fed diets containing a low polyunsaturated-to-saturated fatty acid ratio (P/S = 0.3; Table 4). Phosphatidylethanolamine and phosphatidylcholine represented greater than 50% and 22% of total mucosal cell phospholipid, respectively (Table 5). Minor phospholipids consisted of phosphatidylinositol, phosphatidic acid plus phosphatidylserine, and sphingomyelin compared to the "non-proliferative" cell population ($p<0.05$). The phosphatidylcholine content in the "proliferative" cell fraction was 20.8% lower ($p<0.05$) as compared to the "non-proliferative" cell population.

Effect of diet treatment on fatty acid composition of mucosal phospholipid. Each phospholipid class responded similarly to the diet. Major fatty acids in phosphatidylethanolamine were 20:4ω6, 18:0, 18:1ω9, and 18:2ω6, representing 32.8%, 18.9%, 13.3%, and 9.1%, respectively (Table 6). For groups fed high fat diets, phosphatidylethanolamine 18:2ω6 and total ω6 fatty acid content increased ($p<0.005$), as did the unsaturation index ($p<0.01$). The content of 16:1, 18:1ω9, 18:1(ω5, ω7), total monounsaturated fatty acids, and total ω3 fatty acid decreased ($p<0.05$) when compared to groups fed low fat diets. Membrane phosphatidylethanolamine from rats fed the low polyunsaturated fatty acid diet exhibited a significantly higher level of 18:1ω9, 18:1(ω5, ω7) and total monounsaturated fatty acids when compared to animals fed diets high in polyunsaturated fatty acid content ($p<0.005$). Amounts of 18:2ω6, total ω6 fatty acids, and the unsaturation index were significantly lower for rats fed diets providing the low polyunsaturated-to-saturated fatty acid ratio as compared to animals fed diets providing the high polyunsaturated fatty acid-to-saturated fatty acid ratio ($p<0.01$). Levels of 20:4ω6, 16:1, 16:0, 18:0, as well as the total saturated fatty acid content of phosphatidylethanolamine, were not altered by change in the diet fatty acid composition.

"Non-proliferative" and "proliferative" cell fractions also showed differences in the fatty acid profile. The "proliferative" cells exhibited lower ($p<0.005$) content of total monounsaturated fatty acid, and of 16:0 and 18:0 in phosphatidylethanolamine compared to "non-proliferative" cells. Increased levels of total monounsaturated fatty acids, 18:1ω9, and polyunsaturated fatty acid, ω6 series and 20:4ω6 ($p<0.05$), as well as increased unsaturation index ($p<0.005$) in phosphatidylethanolamine of the "proliferative" cell population was observed when compared to the "non-proliferative" cell population. No significant difference between cell fractions for 18:1 (ω5,ω7), 18:2ω6, and the total ω3 fatty acid content was observed.

Diet altered the major fatty acyl constituents of phosphatidylcholine in both "non-proliferative" and "proliferative" cell fractions (Table 7). A significant decrease in 16:1, 18:1ω9, and 18:1(ω5,ω7) accounted for the lower total monounsaturated fatty acid content of phosphatidylcholine in animals fed high fat diets as compared to animals fed low fat diets ($p<0.005$). Higher levels of 18:2ω6 and 20:4ω6 occurred in phosphatidylcholine for animals fed high fat diets ($p<0.005$). While the total ω6 fatty acid content of phosphatidylcholine increased ($p<0.005$) in rats fed high fat diets, neither the total ω3 nor total

TABLE 5

Effect of Diet on Relative Percentage (% w/w) of Total Phospholipid in "Non-Proliferative" and "Proliferative" Mucosal Cells

Diet level	High				Low							
P/S ratio[a]	High		Low		High		Low		Effect of treatment[d]			
									Main effects		Interaction	
Cell fraction	Non-prolif.[b]	Prolif.[b]	Non-prolif.	Prolif.	Non-prolif.	Prolif.	Non-prolif.	Prolif.	P/S ratio	Cell fraction	Fat level cell fraction	P/S ratio cell fraction
Sphingomyelin	3.8 ± 0.5[c]	8.7 ± 4.8	4.5 ± 0.6	6.7 ± 3.3	5.2 ± 0.8	5.4 ± 3.0	6.1 ± 2.0	7.6 ± 3.1	NS	0.05	0.05	0.05
Phosphatidylcholine	32.7 ± 4.6	26.4 ± 11.3	31.6 ± 5.3	27.3 ± 7.8	28.7 ± 3.2	16.2 ± 7.2	28.3 ± 4.4	26.2 ± 8.5	NS	0.05	NS	0.05
Phosphatidic acid phosphatidylserine	6.8 ± 0.9	5.8 ± 4.8	5.9 ± 3.8	5.5 ± 3.9	4.9 ± 2.1	8.6 ± 3.1	5.3 ± 0.6	7.2 ± 3.9	NS	NS	NS	NS
Phosphatidylinositol	8.4 ± 1.7	4.4 ± 2.4	10.0 ± 3.4	8.9 ± 2.3	8.9 ± 1.5	8.7 ± 1.1	8.1 ± 3.2	7.2 ± 1.5	0.01	NS	NS	NS
Phosphatidylethanolamine	48.2 ± 4.2	53.4 ± 3.6	49.1 ± 5.6	51.6 ± 9.3	52.4 ± 5.0	61.0 ± 7.0	57.7 ± 4.6	51.8 ± 9.7	NS	NS	NS	NS

Abbreviations: [a]P/S Ratio, polyunsaturated-to-saturated fatty acid ratio.

[b]Non-prolif. cells, "non-proliferative" cell fraction; Prolif. cells, "proliferative" cell fraction.

[c]Values are means ± S.D.; number of replicates, n = 3-6.

[d]Significant effects by least square analysis of variance procedures are indicated ($p<0.05$); NS, not significantly different. Effect of fat level and P/S ratio was not significant.

TABLE 6

Effect of Diet Treatment on Fatty Acid Composition of Phosphatidylethanolamine in "Non-Proliferative" and "Proliferative" Mucosal Cells

	Fatty acid composition (%, w/w)														
Diet level	High				Low				Effect of treatment[d]						
P/S ratio[a]	High		Low		High		Low								
Cell fraction	Non-prolif.[b]	Prolif.[b]	Non-prolif.	Prolif.	Non-prolif.	Prolif.	Non-prolif.	Prolif.	Fat level	P/S ratio	Fat level P/S ratio	Cell fraction	Fat level cell fraction	P/S ratio cell fraction	Fat level ratio cell fraction
16:0	8.7 ± 1.7[c]	7.9 ± 1.3	8.0 ± 1.0	6.4 ± 0.3	8.3 ± 0.7	7.1 ± 0.3	11.2 ± 1.3	7.1 ± 0.2	NS	NS	0.01	0.005	NS	0.05	NS
16:1	1.7 ± 0.5	1.4 ± 0.2	1.2 ± 0.3	1.3 ± 0.2	1.7 ± 0.2	1.8 ± 0.4	2.2 ± 0.7	2.1 ± 0.2	0.005	NS	NS	NS	NS	NS	NS
18:0	21.4 ± 0.6	19.0 ± 0.7	20.5 ± 2.1	18.3 ± 1.5	17.9 ± 1.3	17.7± 1.7	20.3 ± 4.0	15.7 ± 0.5	NS	NS	NS	0.005	NS	NS	NS
18:1(9)	10.0 ± 0.9	9.5 ± 0.8	12.1 ± 1.0	13.3 ± 1.3	12.7 ± 1.4	14.0 ± 1.4	15.9 ± 2.1	18.7 ± 0.8	0.005	0.005	NS	0.005	NS	NS	NS
18:1(5+7)	1.3 ± 0.3	0.9 ± 0.4	1.3 ± 0.2	1.6 ± 0.4	2.1 ± 0.3	2.1 ± 0.2	2.5 ± 0.5	3.0 ± 0.3	0.005	0.005	NS	NS	NS	NS	NS
18:2(6)	12.5 ± 2.0	11.6 ± 1.1	10.2 ± 0.8	9.4 ± 1.6	7.6 ± 0.7	7.4 ± 0.7	7.4 ± 1.3	7.0 ± 0.4	0.005	0.01	0.05	NS	NS	NS	NS
20:4(6)	30.9 ± 3.7	33.1 ± 2.8	33.5 ± 3.3	35.5 ± 1.6	35.3 ± 1.7	35.7 ± 2.2	26.5 ± 3.3	31.6 ± 1.5	NS	NS	0.005	0.05	NS	NS	NS
Σsaturated[a]	33.5 ± 2.5	29.5 ± 2.3	32.7 ± 2.9	27.3 ± 1.3	29.8 ± 1.2	27.2 ± 1.6	35.8 ± 4.6	26.6 ± 1.1	NS	NS	0.05	0.005	NS	NS	NS
Σmonounsaturated[a]	13.7 ± 1.1	12.4 ± 0.4	15.2 ± 0.7	16.7 ± 1.9	17.4 ± 1.4	18.5 ± 1.8	21.1 ± 2.7	24.3 ± 0.9	0.005	0.005	NS	0.05	NS	0.05	NS
Σ(ω6)[a]	49.6 ± 3.7	54.3 ± 2.3	49.7 ± 2.7	51.9 ± 1.9	48.9 ± 1.8	50.0 ± 2.7	39.2 ± 3.6	44.8 ± 1.4	0.005	0.005	0.005	0.005	NS	NS	NS
Σ(ω3)[a]	2.4 ± 0.7	2.9 ± 1.0	2.9 ± 1.8	3.6 ± 0.7	3.6 ± 0.9	3.7 ± 0.5	3.1 ± 0.8	3.8 ± 0.4	0.05	NS	NS	NS	NS	NS	NS

[a]Abbreviations: P/S Ratio, polyunsaturated-to-saturated fatty acid ratio; Σsaturated, total saturated fatty acids; Σmonounsaturated, total monounsaturated fatty acids; Σ(ω6), total ω6 fatty acids; Σ(ω3), total ω3 fatty acids.

[b]Non-Prolif., "non-proliferative" cell fraction; Prolif., "Proliferative" cell fraction.

[c]Values are means ± S.D. for major fatty acids; number of replicates, n = 5-7.

[d]Significant effects by least square analysis of variance procedures are indicated.

TABLE 7

Effect of Diet Treatment on Fatty Acid Composition of Phosphatidylcholine in "Non-Proliferative" and "Proliferative" Mucosal Cells

Diet level	Fatty acid composition (%, w/w)								Effect of treatment[d]						
	High				Low										
P/S ratio[a]	High		Low		High		Low								
Cell fraction	Non-prolif.[b]	Prolif.[b]	Non-prolif.	Prolif.	Non-prolif.	Prolif.	Non-prolif.	Prolif.	Fat level	P/S ratio	Fat level P/S ratio	Cell fraction	Fat level cell fraction	P/S ratio cell fraction	Fat level ratio cell fraction
16:0	23.2 ± 2.0[c]	25.5 ± 3.8	24.0 ± 1.0	25.5 ± 1.2	25.3 ± 1.2	29.3 ± 2.6	24.1 ± 1.2	27.0 ± 0.9	0.05	NS	NS	0.005	NS	NS	NS
16:1	1.0 ± 0.2	0.8 ± 0.2	1.1 ± 0.4	1.7 ± 0.2	1.2 ± 0.6	2.2 ± 0.7	2.0 ± 0.9	3.4 ± 0.4	0.005	0.005	NS	0.005	0.005	0.05	NS
18:0	12.0 ± 0.9	12.4 ± 0.9	10.4 ± 0.5	10.4 ± 1.4	10.5 ± 1.8	9.9 ± 0.6	10.5 ± 2.9	8.1 ± 0.7	0.005	0.005	NS	NS	NS	NS	NS
18:1(9)	10.0 ± 1.7	10.3 ± 0.6	15.3 ± 2.2	15.2 ± 1.8	16.5 ± 2.6	15.7 ± 2.6	22.1 ± 1.2	20.7 ± 2.4	0.005	0.005	NS	NS	NS	NS	NS
18:1(5)+(7)	1.8 ± 0.3	1.7 ± 0.4	2.5 ± 0.2	2.5 ± 0.3	4.8 ± 0.6	5.4 ± 1.1	5.7 ± 0.5	7.6 ± 1.2	0.005	0.005	NS	0.01	0.005	NS	NS
18:2(6)	24.0 ± 1.8	23.6 ± 1.5	19.5 ± 1.2	18.3 ± 0.6	15.2 ± 1.1	13.7 ± 1.0	12.0 ± 0.9	11.2 ± 1.5	0.005	0.01	0.05	0.05	NS	NS	NS
20:4(6)	14.1 ± 1.4	13.3 ± 1.7	14.2 ± 1.7	13.1 ± 1.7	14.1 ± 1.5	12.6 ± 1.5	11.2 ± 1.2	9.7 ± 0.6	0.01	0.05	0.05	0.005	NS	NS	NS
Σsaturated[a]	41.1 ± 2.7	42.2 ± 4.7	39.7 ± 1.4	40.1 ± 1.7	40.9 ± 1.1	43.6 ± 2.1	40.0 ± 1.9	40.6 ± 0.8	NS	NS	NS	0.05	NS	NS	NS
Σmonounsaturated[a]	13.7 ± 1.1	13.6 ± 0.9	20.5 ± 2.0	21.1 ± 1.8	24.0 ± 2.3	24.8 ± 2.5	31.4 ± 2.0	33.6 ± 1.9	0.005	0.005	NS	0.01	0.05	0.05	NS
Σ(ω6)[a]	42.7 ± 2.1	42.0 ± 3.6	38.0 ± 2.7	36.6 ± 2.2	33.2 ± 2.0	29.8 ± 2.3	27.1 ± 1.2	24.5 ± 1.7	0.005	0.005	NS	0.01	NS	NS	NS
Σ(ω3)[a]	0.7 ± 0.3	0.6 ± 0.2	0.9 ± 0.2	0.8 ± 0.3	1.1 ± 0.4	0.9 ± 0.4	0.7 ± 0.4	1.0 ± 0.2	NS	NS	NS	NS	NS	NS	NS

[a]Abbreviations: P/S Ratio, polyunsaturated-to-saturated fatty acid ratio; Σsaturated, total saturated fatty acids; Σmonounsaturated, total monounsaturated fatty acids; Σ(ω6), total ω6 fatty acids; Σ(ω3), total ω3 fatty acids.

[b]Non-Prolif., "non-proliferative" cell fraction; Prolif., "proliferative" cell fraction.

[c]Values are means ± S.D. for major fatty acids; number of replicates, n = 5–7.

[d]Significant effects by least square analysis of variance procedures are indicated.

saturated fatty acid content was significantly altered. However, 16:0 increased ($p<0.05$) and 18:0 decreased ($p<0.005$) in animals fed low fat diets. The unsaturation index of phosphatidylcholine decreased ($p<0.005$) for animals fed low fat diets.

Diet fatty acid composition also influenced phosphatidylcholine fatty acid composition (Table 7). Levels of 16:1, 18:1ω9, and 18:1(ω5,ω7), as well as the sum of monounsaturated fatty acids, were significantly lower ($p<0.005$) for rats fed diets having a high polyunsaturated fatty acid content. Increased levels ($p<0.005$) of total ω6 fatty acids were observed in groups fed diets of high polyunsaturated-to-saturated fatty acid ratio. However, 16:0 was not significantly different between groups fed diets varying in fatty acid composition; phosphatidylcholine 18:0 content was lower in animals fed diets with a low polyunsaturated-to-saturated fatty acid ratio (16.0%, $p<0.005$). Variation in level of these fatty acids led to significant alteration of the unsaturation index with a 13.5% decrease observed in mucosal cell phosphatidylcholine for animals fed low fat diets compared to animals fed high fat diets.

Phosphatidylcholine fatty acid composition also varied between cell populations (Table 7). The fatty acid content of 16:0, 16:1, and 18:1(ω5,ω7) was higher by 11.1% ($p<0.005$), 64.2% ($p<0.005$), and 16.2% ($p<0.01$) in phosphatidylcholine of "proliferative" cells compared to "non-proliferative" cells, respectively. The level of 18:2ω6 and 20:4ω6 was lower by 5.5% ($p<0.05$) and 9.1% ($p<0.005$) in "proliferative" cells. 18:0 and 18:1ω9 content was not significantly different between cell fractions. Higher total saturated ($p<0.05$) and total monounsaturated ($p<0.01$) fatty acids, and lower total ω6 ($p<0.01$) fatty acids were observed for the "proliferative" cell population as compared to "non-proliferative" cells. No significant difference occurred in phosphatidylcholine total polyunsaturated ω3 fatty acid content between cell fractions. The unsaturation index was higher by 6.8% in "proliferative" cells compared to "non-proliferative" cells.

Effect of diet treatment of serum vitamin E and lipid peroxide levels in mucosal cell lipid. Serum vitamin E levels. To avoid the saponification step, which may be accompanied by loss of vitamin E, a modification of the clarification method described by Nierenberg and Lester was used (40). Recoveries for *dl*-α-tocopherol and *d*-α-tocopheryl acetate were 105% and 123%, respectively. Neither experimental day, fat level, nor fatty acid composition of the diet altered the serum vitamin E level when determined as α-tocopherol (1495 μg/dL; n=23).

Spectra comparison between cumen hydroperoxide and peroxide from the mucosal cell lipid fraction demonstrated no interference due to turbidity or other factors within the linear range of 0–1.0 nmole of cumen hydroperoxide (data not shown). The fat level did not affect lipid peroxide values when expressed as nmol equivalent of cumen hydroperoxide/mg cell membrane phospholipid. However, animals fed a polyunsaturated-to-saturated fatty acid diet ratio of 1.1 exhibited a 73% increase ($p<0.006$) in lipid peroxide content compared to groups fed a polyunsaturated-to-saturated fatty acid diet ratio of 0.3 (Table 8). Feeding high fat diets increased "proliferative" cell lipid peroxide values by 64% ($p<0.05$) compared to animals fed the low fat diets (Table 8) when results are expressed as nmol equivalent of cumen hydroperoxide/mg cell protein.

TABLE 8

Effect of Diet Treatment on Lipid Peroxidation of "Proliferative" Mucosal Cell Lipid Fraction from the Rat Large Intestine

Lipid peroxide values[a]						
P/S ratio[b]	High	Low	High	Low	Significant fat level	Effects of P/S ratio[d]
nmole/mg protein[c]	0.11 ± 0.02	0.07 ± 0.04	0.05 ± 0.01	0.06 ± 0.01	0.05	NS
nmole/μmole phospholipids	0.80 ± 0.22	0.38 ± 0.21	0.66 ± 0.24	0.44 ± 0.11	NS	0.006
n	5	5	2	6		

[a]Lipid peroxide values are expressed as nmole of cumen hydroperoxide.

[b]Abbreviations: P/S ratio, polyunsaturated-to-saturated fatty acid ratio.

[c]Values are means ± S.D.

[d]Significant effects ($p<$) by two-way analysis of variance procedures are indicated; NS, not significantly different.

DISCUSSION

Previous studies demonstrate that dietary fat affects membrane lipid content and phospholipid fatty acyl composition (7). Membrane lipid peroxide levels also increase as membrane polyunsaturated fatty acid content and membrane unsaturation index increase (8,9). Experiments examining brush-border membrane and basolateral membrane in the small and large intestine demonstrate alteration of membrane structure and function by change in diet fat treatment (7,22,43). The present experiment extends current knowledge by establishing that change in dietary fat level and fatty acid composition alters colonocyte membrane content of polyunsaturated fatty acids which, in turn, affects lipid peroxide levels in this cell type.

"Non-proliferative" and "proliferative" cell populations from the small (25,44,45) and large intestine (24,46,47) have been successfully isolated. Direct comparisons with other studies are difficult as the procedure and tissue sites used varied. For example, Brasitus (24) utilized the cecum and proximal colon while Craven and DeRubertis (46) sampled the distal colon from the colonic flexure to 1 cm above the anal orifice, pooling cells from two colons. The "non-proliferative" or differentiated cell population is complex consisting of columnar epithelial cells, goblet cells, some endocrine cells and lymphocytes (48). As observed by others (49), variability in total colonocyte material released (mg of protein) was found between animals. Unexpectedly, diet treatment exhibited an effect on the cell isolation procedure, with a 55% greater amount of cell material collected from groups fed diets with low polyunsaturated-to-saturated fatty acid ratio as compared to animals fed diets with a high polyunsaturated-to-saturated fatty acid ratio. This effect is apparently not due to the procedure as the percentage of protein distributed between "non-proliferative" and "proliferative" cell populations appeared similar for each dietary group. It is conceivable that diet induced change in cell membrane composition altered cell cohesion or mucus properties.

Cholesterol and phospholipid values and cholesterol-to-phospholipid ratios reported for brush-border membrane of rat small (7,48,50-53) and large (22,54) intestine are generally higher than values observed in the present study for mucosal cells from the large bowel.

In aggreement with results obtained for brush-border membrane of the small intestine (43,48), no significant effect of fat level or diet fatty acid composition was observed on the phospholipid class content. Colonocytes contain a high percentage of phosphatidylethanolamine (55) in the microsomal fraction of rat small intestine, thus differing from most other membranes with phosphatidylcholines being predominant.

Dietary fat altered the phospholipid fatty acid composition and the unsaturation index of "non-proliferative" and "proliferative" mucosal cells from rat large intestine. The effects observed will primarily be attributed to dietary fat manipulation, since intake of all other essential nutrients remained constant for non-fat components. Feeding either high fat diets (50% as energy) or high polyunsaturated fatty acid diets (P/S=1.17) decreased the total monounsaturated fatty acid level and increased the total polyunsaturated fatty acid content of the colonic cell phospholipds. Increasing the dietary polyunsaturated-to-saturated fatty acid ratio altered the $\omega 6$ fatty acid series in phospholipids but did not change $\omega 3$ fatty acids. This finding is consistent with previous observations (7,56) and indicates that dietary fat alters both the phospholipid fatty acid composition and the unsaturation index of mucosal cells from the rat large intestine. The effect of diet varied between cell populations for "non-proliferative" and "proliferative" cell types.

The serum α-tocopherol values observed in the present study are in the upper range of those reported earlier (57-60). No difference in serum α-tocopherol status for animals reflects a similar vitamin E intake between each group and thus it is likely that the vitamin E status in "proliferative" cells of the rat large intestine was similar among dietary groups.

Lipid peroxides and free radicals are known to induce cell membrane damage and DNA mutation. Increased lipid peroxidation occurs under various conditions, including septic conditions (61), in vitamin E deficient animals (62), as well as after irradiation with gamma-rays (63) and UV light (8), or after injection of various types of carcinogens (64). Several methods for lipid peroxide determination are available (65). To assay peroxides, the thiobarbituric acid reactive substance procedure is most widely used. However, this method does not measure lipid peroxide *per se*, but the end product, malondialdehyde, resulting from the breakdown of lipid peroxides and other non-lipid compounds. The procedure is sensitive to parameters such as tempera-

ture and pH, involves an acid-heating step which may initiate lipid peroxidation, thus potentially increasing product formation. It is also important to differentiate results obtained by non-induced lipid peroxidation systems (66-68) from those measuring susceptibility of sample to lipid peroxidation induced by ascorbate-iron or NADPH-iron (69-71). Recently, Ohishi *et al.* (38) described a new methylene blue derivative for use in a calorimetric method to specifically detect hydroperoxides, endoperoxides and peroxy radicals. This method has also been used by others to determine lipid peroxide levels in rat liver homogenates (72). Although animals fed diets that reflect human consumption of dietary fats exhibit very low levels of hydroperoxide, change in fat level and fatty acid composition of the diet significantly altered the membrane level of lipid peroxides.

Discrepancies between data published on membrane peroxide levels may partly be explained by differences in physiological states of the animals examined but also in the antioxidant status and in the activity of various enzymes (73) in the tissue or membrane studied. A radical scavenger or the collision of two free radicals is needed to stop propagation of lipid peroxidation. An environment with high polyunsaturated fatty acid levels will facilitate the propagation step compared to an environment with lower polyunsaturated fatty acid levels, assuming both contain similar free radical defense systems. The present experiment supports the hypothesis that increased membrane polyunsaturated fatty acid content in animals fed high fat or high polyunsaturated-to-saturated fatty acid ratio diets compared to groups fed low fat or low polyunsaturated-to-saturated fatty acid ratio diets, will increase membrane lipid peroxide levels in proliferative cells. However, it is not yet possible to determine which of the dietary factors—fat level, polyunsaturated-to-saturated fatty acid ratio, or both—primarily affect lipid peroxidation as both factors influence substrate availability for peroxidation and, secondly, both factors also affect membrane peroxide levels depending on the units used to express the results. These findings contradict those of Buckingham (74) who, based on a different assay method, reports an effect of diet vitamin E content, but not of diet fatty acid composition (P/S ratio) on lipid peroxidation in rats. The biological significance of the difference in the lipid peroxide levels observed between diet treatments is not yet known. Further studies are needed to determine the extent of possible cell damage induced by small amounts of peroxides and to delineate the precise relationships between dietary fat and cell membrane injury.

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Effect of Dietary Fat on Diabetes-Induced Changes in Liver Microsomal Fatty Acid Composition and Glucose-6-Phosphatase Activity in Rats

J.T. Venkatraman[a,b,1], D. Pehowich[a,b], B. Singh[c], R.V. Rajotte[d], A.B.R. Thomson[a,e], and M.T. Clandinin[a,b,e,*]

[a]Nutrition and Metabolism Research Group, Departments of [b]Foods & Nutrition, [c]Immunology, [d]Surgery and [e]Medicine, University of Alberta, Edmonton, Alberta, Canada T6G 2C2

Experimental diabetes may manifest itself in a defect in liver microsomal fatty acid desaturation and increased activity of glucose-6-phosphatase (G-6-Pase). The present study was designed to determine whether these changes could be normalized by a change in the dietary fat consumed. Control and streptozotocin-induced diabetic rats were fed nutritionally adequate diets which varied in fatty acid composition. Fatty acid analysis of liver microsomal phospholipids revealed that non-diabetic control animals fed saturated fat (beef tallow) or a diet high in ω3 fatty acids (fish oil) exhibited a significantly higher level of 18:2ω6 and a lower level of 20:4ω6 in the phosphatidylcholine and phosphatidylethanolamine fractions compared with diabetic animals. Control and diabetic animals fed the high linoleic acid diet had similar levels of 18:2ω6 in the microsomal phosphatidylcholine and phosphatidylserine fractions. Microsomal G-6-Pase activity was higher in diabetic than in control animals. Activity of G-6-Pase was lower in microsomes of control animals fed the soybean oil or the fish oil diet, but was not significantly reduced in diabetic animals fed high polyunsaturated fats. Blood glucose levels were similar in control groups fed the different diets, but the plasma hemoglobin A1c level was lower in diabetic animals fed the soybean oil diet. Cholesterol and triglyceride levels were lower in diabetic animals fed the fish oil-based diet. The results suggest that dietary fat manipulation has the potential to change at least some of the abnormalities in the microsomal membrane in experimental diabetes.

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A defect in fatty acid desaturation is one of the complications associated with experimental diabetes (1-4). As diabetes causes alterations in lipid composition and in the physical state of liver microsomal membranes (5,6), these changes may also affect microsomal membrane-bound enzymes. Microsomal glucose-6-phosphatase (G-6-Pase), which plays a key role in dephosphorylation and transport of liver glucose into the blood (7), was reported to increase in the diabetic state (8). Abnormalities in lipoprotein metabolism have also been described in patients with non-insulin-dependent diabetes mellitus (9). Dietary polyunsaturated fatty acids may alter lipoprotein metabolism and are generally associated with a cholesterol-lowering effect and altered membrane lipid composition (10,11). The present study was designed to determine whether saturated ω6 or ω3 fatty acids normalize the changes that occur in microsomal membrane phospholipid fatty acid composition, plasma lipids, glucose and glucose-6-phosphatase activity in the experimental diabetes.

MATERIALS AND METHODS

Three semipurified diets containing 20% w/w fat were fed to rats. All diets were nutritionally adequate and differed only in fatty acid composition (Table 1). The beef tallow diet was based on a blend of beef tallow (18%), safflower oil (1%) and soybean oil (1%). The high linoleic acid diet contained 20% soybean oil. The fish oil diet consisted of a blend of 10% fish oil concentrate, 8% beef tallow and 2% safflower oil.

Male Wistar-Furth strain rats, weighing 230-250 g, were placed in individual metabolic cages and monitored weekly for plasma glucose, urine volume, urine glucose, food consumption and body weight. Animals were rendered diabetic using 55 mg/kg streptozotocin (a gift from the Upjohn Company, Kalamazoo, MI) which was injected intravenously *via* the penile vein using citrate buffer (pH 4.5). Following induction of diabetes, the animals were

TABLE 1

Composition of Experimental Diets

	Beef tallow diet	Soybean oil diet	Fish oil diet
	(g/kg Diet)		
Casein	270	270	270
Starch	200	200	200
Glucose	207.7	207.7	207.7
Cellulose	50	50	50
Vitamin mix[a]	10	10	10
Mineral mix[b]	50.85	50.85	50.85
Choline chloride	2.75	2.75	2.75
Inositol	6.25	6.25	6.25
L-Methionine	2.5	2.5	2.5
Soybean oil	10	200	—
Hydrogenated beef tallow	190	—	80
Safflower oil	10	—	20
Fish oil	—	—	100

[a]AOAC vitamin mix (Teklad test diets, Madison, WI) provided the following per kg of complete diet: 20,000 IU vitamin A; 2,000 IU vitamin D; 100 mg vitamin E; 5 mg menadione; 5mg thiamin-HCl; 8 mg riboflavin; 40 mg pyridoxine-HCl; 40 mg niacin; 40 mg pantothenic acid; 2,000 mg choline; 100 mg myothiositol; 100 mg *p*-aminobenzoic acid; 0.4 mg biotin; 2 mg folic acid; and 30 mg vitamin B-12.

[b]Berrhart Tomarelli mineral mix (General Biochemicals, Chagrin Falls, OH).

[1]Present address: Division of Clinical Immunology, Department of Medicine, The University of Texas Health Science Center, 7703 Floyd Curl Drive, San Antonio, TX 78284.

*To whom all correspondence should be addressed.

Abbreviations: G-6-Pase, glucose-6-phosphatase; HgA1c, hemoglobin A1c.

fed Wayne lab Blox diet (Teklad Premier, Madison, WI) for three weeks and then the experimental diets for seven weeks. Non-diabetic control rats were similarly injected with citrate buffer, fed chow and then fed the experimental diets for seven weeks. Blood samples were taken for determination of plasma cholesterol and triglyceride (Dri-Stat, Beckman, Fullerton, CA) during the baseline period and at four and eight weeks. Clinical parameters of urine volume, urine glucose, plasma glucose, body weight, food consumption, plasma cholesterol, plasma triglyceride and HgA1c were recorded. Plasma glucose concentration was measured with glucose oxidase using a Beckman (Fullerton, CA) ERA 2001 analyzer. Plasma total cholesterol was measured using the Dri-Stat (Beckman, Fullerton, CA) cholesterol-ES Reagent kit (12); plasma triglyceride concentration was measured using the Dri-Stat triglycerides-UV endpoint reagent (13). HgA1c (hemoglobin A1c) was assayed courtesy of Dr. L. Oliver (The Upjohn Company). Microsomes were prepared from the livers, and phospholipid fatty acid composition and G-6-Pase activity were determined.

Isolation of liver microsomes. The liver was rinsed in 0.25 M sucrose, and 5 g liver tissue has homogenized in 25 mL of 0.25 M sucrose buffer. The homogenate was centrifuged twice at 16,000 × *g* for 20 min and the postmitochondrial supernatant was centrifuged at 115,000 × *g* for 90 min to sediment microsomes. The surface of the microsomal pellet was washed with 0.1 M potassium citrate buffer (pH 6.5) and resuspended in the same buffer to provide a microsomal isolate of high purity (4). Protein content of the microsomal membrane was determined (14).

Enzyme assay. G-6-Pase activity was measured in freshly prepared microsomes, as described by Freedland and Harper (15), by incubating 0.1 mL of freshly prepared microsomal membrane (100 μg protein) in 0.1 mL potassium citrate buffer (pH 6.5) in the presence of 100 mM glucose-6-phosphate at 37.5°C for 15 min. The reaction was stopped by adding 2 mL of 10% trichloroacetic acid (w/v). After centrifugation, 1-mL aliquots were taken to determine the amount of inorganic phosphate released (16).

Lipid extraction. Total lipids were extracted from liver microsomes (17) in the presence of 2-ethoxyquin as an antioxidant. Lipid extracts were dried at 40°C under nitrogen. Liver microsomal phospholipids were separated on silica gel H plates using the solvent system chloroform/methanol/2-propanol/0.25% potassium chloride/triethylamine (30:9:25:6:18, by vol) (18). Individual phospholipid fractions were visualized under UV light after spraying with 1% (w/v) 8-anilino-1-naphthalenesulfonic acid. Fractions were scraped off and methyl esters were prepared. Phospholipid fatty acids were transmethylated with boron trifluoride in methanol (19). Methyl esters were analyzed by fused silica capillary gas-liquid chromatography (20).

Statistical analysis. The effect of treatments was determined by analysis of variance procedures (21). Individual treatments were compared by student Neuman Keuls multiple range test after a significant effect of treatment was shown by analysis of variance. Significant interactions are indicated where found.

RESULTS

Body weight, plasma glucose and plasma lipid and urinary glucose levels. Weight gain was lower in diabetic animals than in control animals. Diabetic animals fed the soybean oil diet had higher body weights as compared with the other two groups (Table 2). Plasma glucose levels in diabetic animals were not altered by diet treatment. The diabetic condition resulted in a significant increase in plasma cholesterol levels, with diabetic animals fed fish oil having significantly lower cholesterol levels compared with the other dietary groups. Diabetes increased the level of plasma triglycerides but dietary treatment did not influence plasma triglyceride level. Hemoglobin A1c levels were lower in diabetic animals fed soybean oil as compared with the other diabetic groups.

Fatty acid composition. Fatty acid analysis of liver microsomal phospholipids revealed that fatty acid composition was influenced by the lipid composition of the diets as well as by the diabetic condition (Tables 3 and 4).

Phosphatidylcholine fraction. Diabetes increased 16:0 and decreased 18:0 levels in the microsomal phosphatidylcholine fraction compared with controls (Table 3). The level of ω6 fatty acids decreased in the diabetic state. A decrease in 20:5ω3 and an increase in 22:6ω3 concentration was also associated with diabetes. Feeding diets high in beef tallow or fish oil resulted in a significant increase

TABLE 2

Effect of Feeding Diets Containing Beef Tallow, Soybean Oil or Fish Oil on Body Weight, Plasma Glucose, Plasma Cholesterol, Plasma Triglyceride and Urinary Glucose Levels in Control and Diabetic Rats[a]

	Beef tallow		Soybean oil		Fish oil		Pooled main effects		
	Control	Diabetic	Control	Diabetic	Control	Diabetic	SEM	Disease	Diet
Body weight (g)	317^{cd}	171^{a}	337^{d}	198^{b}	294.1^{c}	175^{ab}	8.9	***	**
Plasma									
Glucose (mg/dL)	99	393^{b}	99^{a}	407^{b}	97^{a}	388^{b}	15.6	***	NS
Cholesterol (mg %)	137^{b}	166^{c}	132^{b}	134^{b}	88^{a}	122^{b}	7.6	***	***
Triglycerides	249^{a}	776^{b}	161^{a}	876^{b}	157^{a}	620^{b}	93.1	***	NS
Hemoglobin Alc (%)	1.5	3.3	1.7	3.0	1.5	3.3			
Urine									
Glucose (g/day)	0.007^{a}	3.04^{b}	0.004^{a}	4.31^{c}	0.006^{a}	3.15^{b}	0.148	***	***

[a]Values without a common superscript are significantly different at $p<0.05$ level.

**$p<0.01$; ***$p<0.001$; NS, not significant.

TABLE 3

Effect of Feeding Diets Containing Beef Tallow, Soybean Oil or Fish Oil on the Fatty Acid Composition of Phosphatidylcholine and Phosphatidylethanolamine Fractions in Normal (C) and Diabetic (D) Rat Liver Microsomes[a]

	Phosphatidylcholine									Phosphatidylethanolamine								
	Beef tallow		Soybean oil		Fish oil			Main effects		Beef tallow		Soybean oil		Fish oil			Main effects	
	C	D	C	D	C	D	SEM	Disease	diet	C	D	C	D	C	D	SEM	Disease	diet
16:0	24.0^{b}	27.6^{c}	20.2^{a}	21.8^{a}	26.6^{c}	30.5^{d}	0.6	***	***	20.7^{b}	18.4^{ab}	18.2^{ab}	17.0^{a}	20.3^{ab}	23.0^{c}	1.1	NS	**
18:0	23.0^{bc}	20.3^{a}	27.0^{d}	24.4^{c}	22.5^{b}	20.2^{a}	0.6	***	***	27.1^{a}	24.6^{a}	27.8^{a}	27.9^{a}	27.7^{a}	25.2^{a}	1.4	NS	NS
18:1ω9	8.5^{d}	12.4^{e}	3.0^{a}	4.2^{b}	5.8^{c}	4.7^{b}	0.4	***	***	2.7^{ab}	5.3^{c}	3.0^{b}	3.0^{b}	1.9^{ab}	1.5^{a}	0.4	*	***
18:1ω7	0.9^{a}	0.8^{a}	1.9^{b}	0.9^{a}	1.0^{a}	0.7^{a}	0.2	***	**	0.7^{b}	0.5^{ab}	1.3^{c}	0.6^{ab}	0.5^{ab}	0.3^{a}	0.1	***	***
18:2ω6	12.2^{b}	8.3^{a}	17.7^{d}	19.3^{d}	14.6^{c}	11.9^{b}	0.6	**	***	7.8^{b}	3.5^{a}	11.7^{d}	9.6^{c}	7.5^{b}	4.6^{a}	0.5	***	***
20:3ω6	0.5^{bc}	0.5^{b}	0.0^{a}	0.0^{a}	0.7^{c}	0.5^{b}	0.1	NS	***	0.3^{b}	0.3^{b}	0.0^{a}	0.0^{a}	0.4^{b}	0.2^{b}	0.05	NS	***
20:4ω6	24.6^{d}	22.0^{c}	26.0^{d}	20.8^{c}	12.8^{b}	8.9^{a}	0.6	***	***	30.2^{c}	27.1^{cd}	23.0^{b}	22.8^{b}	13.5^{a}	11.8^{a}	1.6	NS	***
20:5ω3	0.2^{a}	0.1^{a}	0.1^{a}	0.1^{a}	5.2^{c}	3.9^{b}	0.2	***	***	0.3^{ab}	0.4^{ab}	0.8^{b}	0.1^{a}	8.9^{d}	5.2^{c}	0.2	***	***
22:5ω3	0.4^{a}	0.8^{b}	0.4^{a}	0.6^{ab}	1.1^{c}	1.5^{d}	0.1	***	***	0.7^{a}	1.4^{bc}	1.6^{c}	1.2^{b}	2.8^{a}	2.5^{a}	0.1	NS	***
22:6ω3	4.5^{ab}	5.8^{bc}	3.5^{a}	7.2^{cd}	8.1^{d}	14.4^{e}	0.6	***	***	6.3^{a}	15.0^{c}	12.4^{bc}	10.3^{b}	16.0^{c}	24.4^{d}	1.3	***	***

[a]Values without a common superscript are significantly different at $p<0.05$ level. Replicates of five determinations.
*$p<0.05$; **$p<0.01$; ***$p<0.001$; NS, not significant.

TABLE 4

Effect of Feeding Diets Containing Beef Tallow, Soybean Oil or Fish Oil on the Fatty Acid Composition of Phosphatidylinositol and Phosphatidylserine Fractions in Normal (C) and Diabetic (D) Rat Liver Microsomes

	Phosphatidylinositol									Phosphatidylserine								
	Beef tallow		Soybean oil		Fish oil			Main effects		Beef tallow		Soybean oil		Fish oil			Main effects	
	C	D	C	D	C	D	SEM	Disease	diet	C	D	C	D	C	D	SEM	Disease	diet
16:0	4.8^{b}	4.8^{b}	3.8^{a}	3.2^{a}	3.9^{a}	4.8^{b}	0.3	NS	***	7.0	7.3	5.3	6.1	6.7	5.8	0.7	NS	NS
18:0	41.2^{a}	37.8^{a}	46.1^{b}	46.1^{b}	40.2^{a}	40.9^{a}	1.2	NS	***	39.3^{a}	40.8^{a}	47.7^{b}	50.7^{b}	42.6^{a}	39.4^{a}	1.2	NS	***
18:1ω9	1.6^{b}	2.6^{c}	0.7^{a}	1.8^{b}	0.5^{a}	0.9^{a}	0.2	***	***	3.4	3.6	2.8	3.2	2.8	2.3	0.5	NS	NS
18:1ω7	0.4^{bc}	0.5^{c}	0.2^{ab}	0.1^{a}	0.1^{a}	0.1^{a}	0.1	NS	***	0.4	0.2	0.4	0.3	0.4	0.2	0.1	NS	NS
18:2ω6	2.1^{abc}	1.9^{ab}	2.7^{c}	4.0^{d}	1.7^{a}	2.5^{bc}	0.2	***	***	3.3^{ab}	2.4^{a}	3.8^{ab}	4.1^{b}	3.0^{ab}	2.4^{a}	0.4	NS	*
20:3ω6	0.3^{b}	1.2^{d}	0.0^{a}	0.0^{a}	0.8^{c}	1.1^{d}	0.1	***	***	0.7^{c}	0.8^{d}	0.0^{a}	0.0^{a}	0.6^{b}	0.6^{bc}	0.04	*	***
20:4ω6	43.7^{b}	42.2^{b}	45.2^{b}	41.9^{b}	43.4^{b}	37.5^{a}	1.2	***	*	2.9	5.8	5.8	2.3	6.8	6.6	1.5	NS	*
20:5ω3	0.9^{b}	1.1^{b}	0.1^{a}	0.1^{a}	2.9^{d}	2.0^{c}	0.2	NS	***	0.4^{bc}	0.2^{bc}	0.2^{ab}	0.0^{a}	0.6^{c}	0.5^{bc}	0.1	NS	**
22:5ω3	0.04^{a}	0.8^{bc}	0.3^{a}	0.5^{ab}	1.2^{c}	2.0^{d}	0.3	***	***	0.2	0.2	0.2	0.2	0.6	0.4	0.2	NS	NS
22:6ω3	0.7^{a}	1.4^{ab}	0.5^{a}	1.6^{ab}	2.6^{b}	5.7^{c}	0.4	***	***	4.1^{a}	5.9^{ab}	7.7^{b}	3.8^{a}	5.5^{ab}	3.9^{a}	1.1	NS	NS

[a]Values without a common superscript are significantly different at $p<0.05$ level. Replicates of five independent determinations.
*$p<0.05$; **$p<0.01$; ***$p<0.001$; NS, not significant.

in 16:0 levels and decreased 18:0 and 18:2ω6 levels compared with animals fed the soybean oil-based diet. Feeding fish oil led to increased levels of ω3 fatty acids and decreased levels of ω6 fatty acids in the phosphatidylcholine fraction of liver microsomes.

Phosphatidylethanolamine fraction. Saturated fatty acid levels in the microsomal phosphatidylethanolamine fraction were not affected by the diabetic state while 18:2ω6 and 20:4ω6 levels decreased (Table 3). Levels of 22:5ω3 increased in diabetes; 18:2ω6 levels were high when the soybean oil diet was fed. Omega-3 fatty acid levels were considerably higher for the diabetic group fed fish oil.

Phosphatidylinositol fraction. Saturated fatty acid and 20:5ω3 contents of the phosphatidylinositol fraction were not altered by diabetes, while all other fatty acid levels were significantly changed (Table 4). Elevation in 18:2ω6 and 22:6ω3 occurred in the diabetic condition and diabetic animals fed fish oil exhibited markedly reduced levels of 20:4ω6. A significant increase in ω3 fatty acid levels was observed in animals fed the fish oil diet, while the change induced by diet was not very pronounced for animals fed a diet high in ω6 fatty acids.

Phosphatidylserine fraction. Unlike the other phospholipid fractions, diabetes did not alter the fatty acid composition of the phosphatidylserine fraction (Table 4). Levels of 18:0 and 18:2ω6 were higher when soybean oil was included in the diet while the level of 20:5ω3 in the microsome increased when fish oil was fed.

G-6-Pase activity. Liver microsomal G-6-Pase activity was influenced by the diabetic state as well as by feeding the polyunsaturated fats to control animals (Fig. 1). G-6-Pase activity was significantly higher in liver microsomes of diabetic animals as compared with control groups, but was not reduced by feeding the polyunsaturated fat diet.

DISCUSSION

Streptozotocin-induced diabetes is associated with impaired conversion of linoleic acid to arachidonic acid due to depressed activity of liver microsomal fatty acid desaturases (1-4). The present study demonstrates a

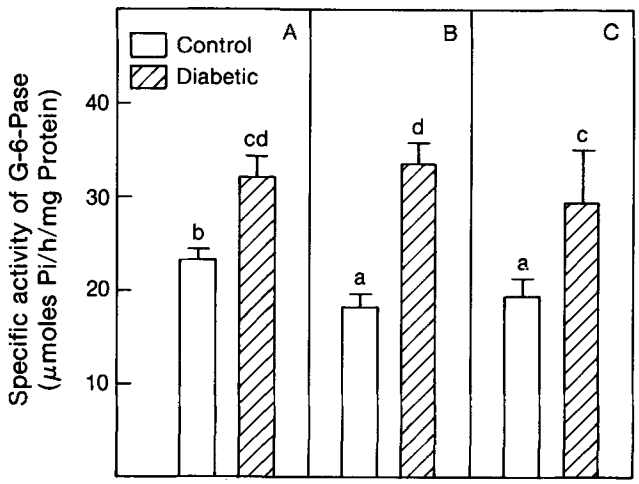


FIG. 1. Effect of diet on liver microsomal glucose-6-phosphatase activity for control and diabetic rats. Values are mean ± S.D. Values without common superscript are significantly different at 5% level. A, Beef tallow diet; B, soybean oil diet; C, fish oil diet.

decrease in 20:4ω6 and an increase in 22:6ω3 in the liver microsomal phospholipid fractions of the diabetic rats. Feeding a diet high in saturated fat (beef tallow) or fish oil decreased the levels of 18:2ω6 in diabetic animals, while the fish oil diet also markedly reduced the 20:4ω6 levels in the phosphatidylcholine and phosphatidylethanolamine fractions. Although desaturase activity was not measured in this study, it is conceivable that altered desaturase activity may be responsible for the changes that occur in the microsomal membranes in the diabetic state. Insulin treatment has been suggested to repair this defect (3), although the arachidonic acid level was shown to be further diminished by insulin treatment (3). Several other factors, such as fatty acid chain elongation, membrane lipid turnover, and fatty acid oxidation, may also contribute to the changes observed in membrane lipid composition.

Diabetes is known to change G-6-Pase activity in rat liver (8). Our results indicate that liver microsomal G-6-Pase activity significantly increases in the diabetic state as compared to controls. Feeding fish oil significantly lowered G- 6-Pase activity in liver microsomes of diabetic rats as compared with diabetic rats fed diets containing beef tallow or soybean oil. G-6-Pase is known to play a key role in the maintenance of normal blood glucose levels because of its involvement in the terminal reactions of glycogenolysis and gluconeogenesis (22). G-6-Pase is a membrane-bound enzyme and has been reported to be lipid-dependent (13,22), functioning in a system composed of a specific G-6-Pase transporter and a nonspecific phosphohydrolase phosphotransferase (8). In the diabetic liver, an increase in the phosphohydrolase portion of the enzyme has been reported, while the enzyme's transport component is limited (8). The change in microsomal G-6-Pase activity observed may be explained on the basis of changes occurring in the phospholipid fatty acid composition and associated effects on the lipid microenvironment affecting glucose-6-phosphatase function. Transitions occurring in substrate availability in transcriptional or translational events, and possibly in the phosphorylation state may also explain the changes observed in G-6-Pase activity. It is suggested that glucose-6-phosphatase activity can be modulated in the diabetic state by careful control of dietary ω6 and ω3 fatty acid intake.

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The Effect of Dietary Linoleic Acid on the Fatty Acid Composition of Individual Phospholipids and Lipoxygenase Products from Gills and Leucocytes of Atlantic Salmon (*Salmo salar*)

J. Gordon Bell[a,*], Robert S. Raynard[b] and John. R. Sargent[a]

[a]NERC Unit of Aquatic Biochemistry, University of Stirling, Scotland, U.K. and [b]D.A.F.S. Marine Laboratory, Aberdeen, Scotland

Diets containing either fish oil or sunflower oil, both of which supplied the minimum required level of n-3 fatty acids, were given to Atlantic salmon *(Salmo salar)* post-smolts for a period of 16 weeks. In fish fed sunflower oil, the phospholipids of gills showed increased 18:2n-6 (2–13-fold), 20:2n-6 (4.5–12-fold) and 20:3n-6 (2–8-fold). In addition, phosphatidylethanolamine had increased 20:4n-6 (1.5-fold). Changes of a similar magnitude were observed in the phospholipids of blood leucocytes except that, in addition, 20:4n-6 was elevated in phosphatidylserine (1.7-fold) and phosphatidylinositol (1.4-fold). Both tissues showed a general decrease in phospholipid 20:5n-3 (up to 3-fold), which caused an increase in 20:4n-6/20:5n-3 ratio (1.3–6-fold). The elongation and desaturation products of 20:4n-6, 22:4n-6 and 22:5n-6 were not increased as a result of feeding sunflower oil. When isolated gill cells were stimulated with the calcium ionophore A23187, 12-hydroxy-8,10,14,17-eicosapentaenoic acid (12-HEPE) was the major lipoxygenase product from salmon given fish oil. 12-HEPE was significantly reduced in salmon given sunflower oil. When stimulated with A23187, the lipoxygenase products derived from whole blood of fish given sunflower oil showed decreased levels of leukotriene B_5, 12-HEPE and 12-hydroxy-5,8,10,14-eicosatetraenoic acid. *Lipids 26,* 445–450 (1991).

Most mammals can metabolize linoleic acid (LA), 18:2n-6 by Δ6 desaturation to gamma-linolenic acid 18:3n-6 which, after elongation to dihomogamma-linolenic acid 20:3n-6, can subsequently undergo Δ5 desaturation to arachidonic acid (AA), 20:4n-6 (1). Generally freshwater fish are capable of utilizing the same pathway (2), whereas marine species, such as turbot *(Scophthalmus maximums),* apparently lack Δ5 desaturase and thus require their diet to supply the highly unsaturated fatty acids (HUFA), eicosapentaenoic acid (EPA), docosahexaenoic acid (DHA) and probably AA (3,4). Studies with an anadramous species, Atlantic salmon *(Salmo salar),* indicated an apparent inability to metabolize LA to AA during smolting (the transition from freshwater to seawater) (5), although post-smolts given linoleic acid as corn oil showed increased AA in the phospholipids of liver and muscle (6).

AA and EPA can be metabolized by cyclooxygenase and lipoxygenase enzymes to yield a group of biologically active products known collectively as eicosanoids. While the AA-derived eicosanoids are powerful agonists in a number of pathophysiological conditions such as psoriasis and cardiovascular disease (7,8), the EPA-derived eicosanoids are generally of decreased efficacy, and thus their generation *in vivo* may allow them to function as anti-inflammatory mediators (9). This fact underlies the use of n-3 HUFA-rich fish oils in the treatment of a number of inflammatory conditions in humans (10).

Fish are an ideal model system for studying the production and interaction of AA- and EPA-derived eicosanoids because their tissues are naturally rich in n-3 HUFA. In this study Atlantic salmon post-smolts were given diets in which the lipid was supplied either as fish oil or sunflower oil. Although the latter diet had a markedly reduced n-3/n-6 ratio, it contained sufficient n-3 HUFA derived from the dietary fish meal to meet the normally recommended requirements of salmon (11). Our aims were i) to study the incorporation of LA and its metabolic products into the individual phospholipids of gills and leucocytes. These tissues are highly active in eicosanoid production (12–14). ii) To determine lipoxygenase products from endogenous fatty acid precursors in isolated gill cells and whole blood challenged with the calcium ionophore A23187.

MATERIALS AND METHODS

Materials. Leukotriene B_5 (LTB_5), 12-hydroxyeicosatetraenoic acid (12(R,S)-HETE) and 12-hydroxyeicosapentaenoic acid (12(S)-HEPE) were purchased from Cascade Biochem Ltd. (Reading, U.K.). Leukotriene B_4 (LTB_4), A23187 and "Sigmacote" were from Sigma Chemical Co. Ltd. (Poole, U.K.). 15-Hydroxyeicosatetraenoic acid (15(S)-HETE) and 5-hydroxyeicosatetraenoic acid (5(R,S)-HETE) were purchased from Novabiochem Ltd. (Nottingham, U.K.).

Thin-layer chromatography (TLC) plates (20 × 20 cm × 0.25 mm) precoated with Silica Gel 60 were obtained from Merck (Darmstadt, Germany). All solvents were of high-performance liquid chr tography (HPLC) grade and were obtained from Rathb..n Chemicals Ltd. (Walkerburn, U.K.).

Animals and diets. Three hundred Atlantic salmon S1 smolts (Salmon undergoing transition to seawater in one year, mean wt. *ca.* 51 g) were obtained from the D.A.F.S. Fish Cultivation Unit (Aultbea, Wester Ross, Scotland) and transferred to sea water tanks at the same unit. The diets were supplied by automatic feeders which were adjusted to provide 20 g/kg biomass per day, 7 days each week. Fish were bulk weighed every 28 days and the ration adjusted accordingly. Samples for analytical measurements were collected after 16 weeks of the feeding experiment. The fish meal-based diets were formulated to meet the nutritional requirements of salmon (15) and contained 46% crude protein and 17% lipid (Table 1). The

*To whom correspondence should be addressed.

Abbreviations: AA, arachidonic acid; BHT, butylated hydroxytoluene; DHA, docosahexaenoic acid; EPA, eicosapentaenoic acid; FO, fish oil; HEPE, hydroxy-5,8,10,14,17-eicosapentaenoic acid; HEPES, *N*-(2-hydroxyethyl)piperazine-*N'*-(2-ethanesulfonic acid); HETE, hydroxy-5,8,10,14-eicosatetraenoic acid; HPLC, high-performance liquid chromatography; HUFA, highly unsaturated fatty acid; LA, linoleic acid; LTB_4, leukotriene B_4; LTB_5, leukotriene B_5; PC, phosphatidylcholine; PE, phosphatidylethanolamine; PI, phosphatidylinositol; PS, phosphatidylserine; PUFA, polyunsaturated fatty acid; SFO, sunflower oil; TLC, thin-layer chromatography.

TABLE 1

Composition of the Basal Diet

Constituent	Amount (g/kg)
Fishmeal	650
Starch[a]	150
Oil[b]	100
Vitamin mix[c]	10
Mineral mix[d]	24
α-Cellulose	65.5
Antioxidant mix[e]	0.4
Choline chloride (40% w/v)	10 mL

[a] Passelli WA4, Tunnel Avebe Starches Ltd., Gillingham, U.K.

[b] Fosol, Seven Seas Ltd. (Hull, U.K.) or Sunflower oil (Tesco Ltd. U.K.).

[c] Supplied (mg/kg): α-tocopherol acetate, 40; menadione, 10; ascorbic acid, 1000; thiamin hydrochloride, 10; riboflavin, 20; pyridoxine hydrochloride, 12; calcium pantothenate, 44; nicotinic acid, 150; biotin, 1; folic acid, 5; cyanocobalamin, 0.02; myo-inositol, 400; retinyl acetate, 2500 I.U.; and cholicalciferol, 2400I.U.

[d] Supplied per kg diet: KH_2PO_4, 22 g; $FeSO_4.7H_2O$, 1.0 g; $ZnSO_4.7H_2O$, 0.13 g; $MnSO_4.4H_2O$, 52.8 mg; $CuSO_4.5H_2O$, 12 mg; $CoSO_4.7H_2O$, 2 mg; and KI, 2 mg.

[e] Contained (g/L): Butylated hydroxy anisole, 60; propyl gallate, 60; citric acid, 40; dissolved in proylene glycol.

TABLE 2

Fatty Acid Composition of Diets

Fatty acids	Fish oil diet (weight %)	Sunflower oil diet (weight %)
14:0	5.7	2.0
16:0	13.8	9.7
18:0	2.5	3.4
16:1n-7	5.2	2.2
18:1n-9	12.0	14.7
20:1n-9	8.9	3.2
22:1n-11	13.8	4.0
24:1	1.4	0.4
18:2n-6	1.4	42.1
18:3n-6	0.2	1.1
20:2n-6	0.2	<0.1
20:3n-6	0.2	0.1
20:4n-6	0.6	0.4
18:3n-3	1.3	0.4
18:4n-3	2.9	0.7
20:5n-3	7.2	3.4
22:5n-3	1.2	0.5
22:6n-3	10.4	4.6
Total n-6	2.5	43.6
Total n-3	23.8	9.9
n-3/n-6	9.4	0.2

dietary lipid was supplied either as fish oil (FO) or sunflower oil (SFO), and both diets fulfilled the requirement for n-3 fatty acids to be *ca.* 1% of the dry diet. The fatty acid compositions of the two diets are shown in Table 2.

Leucocyte preparation and lipid extraction. Five mL of blood were collected in heparinized syringes from the caudal vein of six fish from each dietary treatment. The blood was diluted with two volumes of phosphate buffered saline (PBS). Four mL of the diluted blood were layered onto 6 mL of lymphocyte separation medium (Gibco Ltd., Paisley, Scotland) and centrifuged at 400 × *g* for 30 min at 4°C. The cells around the interface of the erythrocytes were harvested and washed in PBS. If erythrocyte contamination was greater than 1%, the gradient centrifugation was repeated. The leucocytes obtained from six samples per dietary treatment were pooled and the lipids extracted by the method of Folch *et al.* (16). The extracts were stored at −20°C prior to further analysis. All solvents contained 0.05% butylated hydroxytoluene (BHT) as an antioxidant.

Extraction of gill lipids. Intact gill arches were dissected from three fish per treatment, blotted on paper tissue to remove excess blood and immediately frozen in liquid nitrogen. Samples were stored at −20°C prior to lipid extraction. After thawing, the gill filaments were dissected from the gill arches, weighed and homogenized in chloroform/methanol (2:1, v/v) using a "Polytron" tissue macerator. The remaining procedures were as described for leucocyte lipid extraction. Lipids were extracted from 1.0 g samples of the diets by a similar procedure.

Lipid class separation and fatty acid analysis. Lipid extracts were separated into phosphatidylcholine (PC), phosphatidylethanolamine (PE), phosphatidylserine (PS) and phosphatidylinositol (PI) fractions by TLC as described by Vitiello and Zanetta (17). The plates were sprayed with 0.1% 2′,7′-dichlorofluorescein in 97% methanol containing 0.05% BHT, and the lipid bands were visualized under UV light. Acid-catalyzed transmethylation was performed overnight at 50°C by the method of Christie (18). The fatty acid methyl esters were separated and quantified by gas-liquid chromatography (Carlo Erba Fractovap 4160, Fisons Ltd., Crawley, U.K.) using a capillary column (50 m × 0.22 mm i.d.) coated with free fatty acid phase (S.G.E. Ltd., Milton Keynes, U.K.). Individual methyl esters were identified by comparison with known standards and by reference to published data (19,20).

Preparation of isolated gill cells. Fish were killed by a blow to the head. The pericardium was opened and the bulbus arteriosus was perfused with 20 mL of ice-cold 0.9% NaCl (w/v) to flush blood from the gills. The gill arches were then removed and placed in ice-cold Hank's medium. This is a calcium-free medium which is specially modified for use with salmonid fish as described by Moon *et al.* (21). The composition was: 10.29 g/L NaCl, 0.4 g/L KCl, 0.2 g/L $MgSO_4.7H_2O$, 0.06 g/L KH_2PO_4, 0.13 g/L $Na_2HPO_4.12H_2O$, 0.5 g/L $NaHCO_3$, and 2.38 g/L HEPES. The arches were then blotted on tissue and the epithelial layer scraped with a blunt scalpel blade to remove cells. The cells were then suspended in ice-cold Ca^{2+}-free Hank's medium, gently separated by stirring with a magnetic stirrer and filtered through a 100 μm nylon gauze. The cells were collected by centrifugation at 100 × *g* for 5 min.

Ionophore challenge. Glass test tubes (15 mL) used in the incubations were pre-coated with "Sigmacote" prior to use. Gill cells were suspended in modified Hank's medium (containing 2 mM $CaCl_2$) at a concentration of 100 mg wet weight per mL. An aliquot of the suspension was retained for protein determination (22). One mL of whole blood was mixed with an equal volume of modified Hank's medium and both blood and gill cell suspensions were incubated in a shaking water bath at 15°C for

10 min. Calcium ionophore A23187 was added in 5 μL dimethylsulfoxide at a final concentration of 20 μM.

Eicosanoid extraction. After 30 min incubation, the samples were centrifuged at 12,000 × *g* for 2 min and the eicosanoids extracted by the method of Pettitt *et al.* (14).

High-performance liquid chromatography. The profiles of the hydroxy fatty acids and leukotrienes from the gill and blood extracts were determined by reverse phase high-performance liquid chromatography (HPLC) using a Spherisorb 5 μm octadecylsilica (ODS2) column (25 cm × 4.6 mm i.d., Scotlab, Bellshill, Scotland). The chromatographic system was equipped with Waters Model M-45 pumps and 680 Automated Gradient Controller and the effluent was monitored at 280 nm and 235 nm to detect leukotrienes and hydroxy fatty acids, respectively, using a Pye Unicam LC-UV detector. Two isocratic solvent systems were used at a flow rate of 1 mL/min. To separate the monohydroxy acids, solvent A containing methanol/water/acetic acid (80:20:0.05, v/v/v) was used. To separate leukotrienes and monohydroxy acids, solvent B containing acetonitrile/methanol/water/acetic acid/phosphoric acid/ammonia (40:29:30:0.5:0.3:0.2, by vol) was used. Quantitations were based on external standards of 12-HEPE, 12-HETE, LTB_5 and LTB_4. A representative chromatogram of leukotriene and hydroxy fatty acid standards is shown in Figure 1.

Preparation and chromatography of hydroxy acid methyl esters. Methyl esters of standard hydroxy acids and peaks collected from HPLC were methylated using diazomethane as described by Schwartz and Bright (23). The methyl esters were purified using HPLC (Solvent A) and analyzed by gas-liquid chromatography (Philips PU 4500 chromatograph) using a CP Sil 5 column (50 m × 0.32 mm i.d.; Chrompak, Middelberg, The Netherlands). Hydrogen was used as carrier gas and the temperature was programmed from 50°C to 260°C at 4°C/min.

Statistical analysis. Significance of difference ($P<0.05$) between dietary treatments was determined by Student's *t*-test.

RESULTS

The fatty acid compositions of leucocyte phospholipid classes are shown in Tables 3 and 4. In PC from SFO fish there were increases in 18:2n-6, 18:3n-6, 20:2n-6 and 20:3n-6 as compared to FO fish. The SFO fish also showed decreased 20:5n-3, 22:6n-3, total saturated acids and total monoenes. In PE a similar pattern of changes was observed, although 20:4n-6 was also increased in SFO fish as compared to FO fish. In both lipid classes of SFO fish the n-3/n-6 ratio was reduced. In PS of SFO fish the n-6 PUFA were also elevated compared to the FO fish, and 20:5n-3 and 22:6n-3 were reduced. In PI 20:3n-6 and 20:4n-6 were increased in SFO fish but the n-3 HUFA were also elevated. In general, PI was the least altered by dietary lipid and this was reflected by little change in either the n-3/n-6 ratio or the 20:4n-6/20:5n-3 ratio. In PS, as with PC and PE, the n-3/n-6 ratio was decreased and the 20:4n-6/20:5n-3 ratio was increased.

The fatty acid compositions of gill phospholipid classes are shown in Tables 5 and 6. PC from SFO fish showed significantly increased 18:2n-6, 20:2n-6 and 20:3n-6. The SFO fish also had decreased 20:5n-3 and total n-3 PUFA, but increased total PUFA as compared to FO fish. Similar

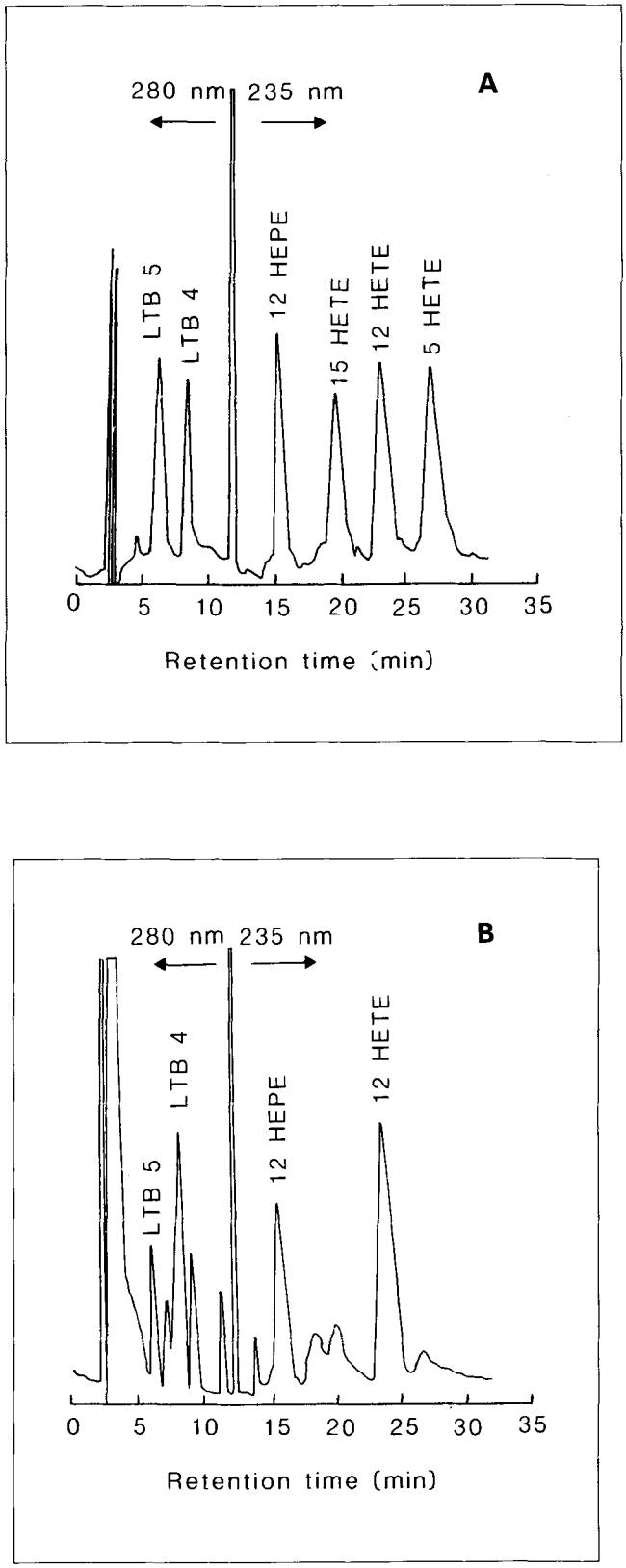


FIG. 1. (A) A typical HPLC chromatogram of leukotriene and hydroxy acid standards separated using solvent system described in Materials and Methods. (B) A typical HPLC chromatogram of lipoxygenase products obtained after stimulation of whole blood with A23187 (20 μM). Experimental conditions are described in Materials and Methods.

TABLE 3

Fatty Acid Compositions of Phosphatidylcholine and Phosphatidylethanolamine from Salmon Leucocytes[a]

Fatty acids (weight %)	PC		PE	
	FO diet	SFO diet	FO diet	SFO diet
Total saturated	34.8	32.6	22.5	18.2
Total monoenoic	25.0	20.6	25.4	14.2
18:2n-6	1.0	8.8	2.4	12.2
18:3n-6	0.2	0.5	0.1	0.6
20:2n-6	0.2	1.6	0.3	2.0
20:3n-6	0.2	1.6	0.2	1.5
20:4n-6	2.8	2.8	2.8	4.2
Total n-6[b]	4.9	15.5	6.5	20.9
18:3n-3	0.2	0.2	0.3	0.2
20:5n-3	8.8	5.1	4.1	2.8
22:5n-3	1.1	0.9	1.0	1.8
22:6n-3	18.6	14.6	29.1	31.1
Total n-3[c]	29.3	20.9	35.5	36.2
Total PUFA	34.2	36.4	42.0	57.0
n-3/n-6	6.0	1.4	5.4	1.7
20:4/20:5	0.3	0.7	0.6	1.5

[a]Results are from pooled leucocyte samples from six salmon.
[b]Includes 22:4n-6 and 22:5n-6.
[c]Includes 18:4n-3 and 20:4n-3.

TABLE 4

Fatty Acid Compositions of Phosphatidylserine and Phosphatidylinositol from Salmon Leucocytes[a]

Fatty acids (weight %)	PS		PI	
	FO diet	SFO diet	FO diet	SFO diet
Total saturated	31.9	34.9	26.7	22.9
Total monoenoic	11.4	7.1	12.5	6.6
18:2n-6	1.0	3.8	1.2	1.4
18:3n-6	<0.1	0.2	0.3	0.2
20:2n-6	<0.1	1.0	0.2	0.3
20:3n-6	<0.1	0.9	0.4	0.8
20:4n-6	1.6	2.7	13.0	17.8
Total n-6[b]	2.8	9.1	15.2	21.1
18:3n-3	0.2	0.3	0.3	<0.1
20:5n-3	2.1	1.7	2.4	2.7
22:5n-3	1.7	1.9	1.8	1.8
22:6n-3	41.8	38.4	25.3	30.4
Total n-3[c]	46.0	42.4	31.7	34.9
Total PUFA	48.7	51.4	47.4	56.0
n-3/n-6	16.7	4.7	2.1	1.7
20:4/20:5	0.8	1.6	5.4	6.6

Footnotes as for Table 3.

changes were apprent in gill PE. In gill PS, 18:2n-6, 20:2n-6 and 20:3n-6 were significantly elevated in SFO fish, while significant decreases were observed in 20:5n-3 and 22:5n-3. Similar changes were observed in gill PI. The n-3/n-6 ratio in all gill phospholipids was reduced in fish given the SFO diet. The mean value of the ratio 20:4n-6/20:5n-3 was generally increased in SFO fish but, due to large standard deviations, the increase was only significant in PS. In the phospholipids from both

TABLE 5

Fatty Acid Compositions of Phosphatidylcholine and Phosphatidylethanolamine from Salmon Gill[a]

Fatty acids (weight %)	PC		PE	
	FO diet	SFO diet	FO diet	SFO diet
Total saturated	40.6 ± 2.3	40.5 ± 1.1	19.1 ± 0.7	21.5 ± 4.1
Total monoenoic	24.9 ± 1.5	22.3 ± 0.9	14.8 ± 1.3	16.2 ± 4.1
18:2n-6	0.6 ± 0.1[b]	7.9 ± 1.0	0.9 ± 0.2[b]	4.8 ± 0.8
18:3n-6	0.2 ± 0.1	0.2 ± 0.2	0.4 ± 0.2	0.2 ± 0.1
20:2n-6	0.1 ± 0.0[b]	1.2 ± 0.2	0.2 ± 0.1[b]	0.9 ± 0.1
20:3n-6	0.1 ± 0.0[b]	1.6 ± 0.3	0.1 ± 0.0[b]	0.7 ± 0.3
20:4n-6	1.4 ± 0.2	2.2 ± 0.9	2.8 ± 0.4	4.2 ± 2.3
Total n-6[c]	2.6 ± 0.4[b]	13.4 ± 0.6	5.0 ± 0.6[b]	11.4 ± 2.6
18:3n-3	<0.1	<0.1	0.1 ± 0.0	<0.1
20:5n-3	4.9 ± 0.6[b]	2.6 ± 0.2	4.3 ± 0.5[b]	2.2 ± 0.2
22:5n-3	0.8 ± 0.3	0.5 ± 0.1	1.2 ± 0.2	0.8 ± 0.3
22:6n-3	15.3 ± 1.3	13.8 ± 0.6	33.7 ± 0.6	26.9 ± 5.2
Total n-3[d]	21.3 ± 2.0[b]	16.9 ± 0.7	39.4 ± 0.6	30.4 ± 5.2
Total PUFA	23.9 ± 2.4[b]	30.3 ± 1.3	44.5 ± 0.4	41.8 ± 7.7
n-3/n-6	8.1 ± 0.5[b]	1.3 ± 0.0	8.0 ± 0.9[b]	2.7 ± 0.3
20:4/20:5	0.3 ± 0.1	0.9 ± 0.4	0.7 ± 0.2	1.9 ± 0.9

[a]Results are % by weight ± standard deviation from three salmon.
[b]Values are significantly different ($P<0.05$).
[c]Includes 22:4n-6 and 22:5n-6.
[d]Includes 18:4n-3 and 20:4n-3.

TABLE 6

Fatty Acid Compositions of Phosphatidylserine and Phosphatidylinositol from Salmon Gills[a]

Fatty acids (weight %)	PS		PI	
	FO diet	SFO diet	FO diet	SFO diet
Total saturated	36.1 ± 0.5[b]	45.8 ± 1.5	36.0 ± 4.8	40.3 ± 4.5
Total monoenoic	15.0 ± 0.7[b]	10.7 ± 1.2	14.6 ± 0.6	14.6 ± 0.3
18:2n-6	0.8 ± 0.3[b]	2.8 ± 0.5	1.6 ± 0.1[b]	3.9 ± 0.4
18:3n-6	0.8 ± 0.1	0.6 ± 0.2	1.0 ± 0.5	0.6 ± 0.2
20:2n-6	0.1[b]	0.9 ± 0.1	<0.1[b]	0.5 ± 0.0
20:3n-6	<0.1[b]	0.9 ± 0.1	<0.4[b]	1.2 ± 0.1
20:4n-6	0.8 ± 0.1	1.1 ± 0.2	9.1 ± 0.8	8.7 ± 2.1
Total n-6[c]	3.3 ± 0.4[b]	6.9 ± 0.3	12.1 ± 0.5	15.4 ± 1.9
18:3n-3	0.1 ± 0.0	<0.1	0.5	0.2 ± 0.1
20:5n-3	1.3 ± 0.3[b]	0.4 ± 0.1	2.5 ± 0.3[b]	1.3 ± 0.1
22:5n-3	1.7 ± 0.1[b]	1.2 ± 0.2	1.0 ± 0.2	0.6 ± 0.1
22:6n-3	27.6 ± 1.0	25.2 ± 1.4	21.5 ± 2.9	17.6 ± 0.8
Total n-3[d]	30.8 ± 1.2	26.9 ± 1.7	25.4 ± 3.3	19.7 ± 0.7
Total PUFA	34.2 ± 1.0	33.8 ± 1.7	37.5 ± 3.8	35.1 ± 2.5
n-3/n-6	9.3 ± 1.4[b]	3.9 ± 0.4	2.1 ± 0.3[b]	1.3 ± 0.2
20:4/20:5	0.7 ± 0.2[b]	2.5 ± 0.7	3.7 ± 0.3	6.9 ± 1.9

Footnotes as described in Table 5.

leucocytes and gills, no changes in the very small amounts of 22:4n-6 and 22:5n-6 detected were observed (data not shown).

The major lipoxygenase products from gill and blood are shown in Tables 7 and 8 and a typical HPLC chromatogram

TABLE 7

Lipoxygenase Products from Gill of Salmon Given Diets Containing Either Fish Oil or Sunflower Oil[a]

Eicosanoid	FO diet (ng/mg protein)	SFO diet (ng/mg protein)
12-HEPE	108.3 ± 7.2[b]	43.1 ± 1.7
12-HETE	54.8 ± 10.9	52.7 ± 7.4
12-HETE/12-HEPE	0.5 ± 0.1[b]	1.3 ± 0.2

[a]Values are mean ± standard error for five fish per treatment.
[b]Values are significantly different (P<0.05).

TABLE 8

Lipoxygenase Products from Blood of Salmon Given Diets Containing Either Fish Oil or Sunflower Oil[a]

Eicosanoid	FO diet (ng/mL blood)	SFO diet (ng/mL blood)
LTB_4	23.2 ± 2.2	18.1 ± 7.4
LTB_5	35.2 ± 10.2[b]	9.6 ± 1.6
LTB_4/LTB_5	0.7 ± 0.2[b]	1.9 ± 0.6
12-HEPE	109.0 ± 25.5[b]	38.1 ± 4.5
12-HETE	185.0 ± 17.3[b]	97.2 ± 14.8
12-HETE/12-HEPE	2.1 ± 0.3	2.7 ± 0.2

[a]Values are mean ± standard error for three fish per treatment.
[b]Values are significantly different (P<0.05).

of the products produced by incubation of blood with A23187 is shown in Figure 1. Peak identity was confirmed by high resolution capillary GC of methyl esters prepared from hydroxy fatty acids recovered from the HPLC separation. No HPLC peaks were detected when blood was incubated with dimethylsulfoxide alone (data not shown).

Gill cells from both dietary treatments produced substantial amounts of both 12-HEPE and 12-HETE. The amount of 12-HETE produced did not vary with dietary n-3/n-6 ratio but the amount of 12-HEPE produced was significantly reduced in fish given SFO. No LTB_4 or LTB_5 were detected in gill cell extracts. A reduction in the dietary n-3/n-6 ratio did not alter production of leucocyte LTB_4, but the production of LTB_5 was significantly reduced in fish given SFO. However, SFO significantly reduced the formation of both 12-HEPE and 12-HETE as compared to leucocytes from fish given FO.

DISCUSSION

The increased levels of 20-carbon n-6 fatty acids present in the individual phospholipids of gill and leucocytes from fish given SFO indicate that both Δ6 and Δ5 desaturation are occurring in these tissues. In gill cells Δ5 desaturase appears less active than Δ6 desaturase. This is confirmed by the accumulation of 20:3n-6 in all phospholipids, whereas 20:4n-6 is only increased in PE. There was a similar accumulation of 20:3n-6 in leucocytes, although 20:4n-6 was also increased in PE, PS and PI. This relatively low Δ5 desaturase activity also was observed in isolated hepatocytes from a related salmonid, the rainbow trout (24).

While trout gill homogenates have a 12-lipoxygenase capable of converting exogenous radiolabeled AA and EPA to their 12-hydroxy metabolites (25), there has been no information on the production of these metabolites from endogenous precursor fatty acids derived from gill phospholipids. This study demonstrates that in Atlantic salmon given a FO containing diet, the major 12-lipoxygenase product from gill cells is EPA-derived 12-HEPE. The reduced production of 12-HEPE in gills of SFO fish corresponds with a reduction of EPA and an increase in the AA/EPA ratio in the gill phospholipids. The ratio of 12-HETE/12-HEPE produced by gill cells and the ratio of the precursor fatty acids in gill PC suggest a possible relationship between the levels of these fatty acids in PC and their metabolism by gill lipoxygenase. A similar relationship has also been suggested by Fischer *et al.* (26) and Takayama *et al.* (27) for mammalian platelets and cultured mammalian umbilical tissue, respectively.

The precise physiological role of 12-HETE is not presently well established, although recent studies have postulated a possible role for this eicosanoid in the control of ion channels (28,29). This role would be particularly important in gills which, along with renal tissue, are responsible for osmoregulation in fish. In general, EPA-derived eicosanoids have less biopotency relative to their AA-derived counterparts (30,31). Therefore, the alteration of 12-HETE/12-HEPE ratio as a result of SFO intake could have a profound effect on gill physiology and function.

The quantity of lipoxygenase products derived from endogenous precursors in salmon gill was very substantial in that the recorded value of approximately 100 ng 12-HEPE/mg tissue protein represents 0.3 mg 12-HEPE/g tissue lipid or 0.7% of total precursor fatty acid metabolized by lipoxygenase. The significance of this high lipoxygenase activity is not clear.

We used whole blood, instead of isolated leucocytes, to study eicosanoid production because this most closely resembled the *in vivo* situation where interactions between different cell types are known to have profound influences on eicosanoid production (32). Studies with rainbow trout have indicated that leucocytes are the only blood cells which are directly capable of eicosanoid production and that no eicosanoids are generated by ionophore-stimulated erythrocytes (14). Salmon given SFO showed decreased production of LTB_5 and, consequently, an increased LTB_4/LTB_5 ratio as compared to those given FO. LTB_4 is an extremely powerful chemotactic agent and is a potent agonist in increasing intracellular calcium (33). EPA-derived LTB_5, however, is up to 30 times less potent than LTB_4 (34). Clearly the changes induced by high n-6 PUFA intake could have significant consequences for immune function.

Both 12-HEPE and 12-HETE production were reduced in fish given SFO even though AA was slightly increased in their phospholipids. However, 20:3n-6 was increased from 1.5% (in FO fish) to 13% (in SFO fish) of the total 20-carbon PUFA in leucocyte total phospholipids (Tables 3 and 4) and this fatty acid, which is also a substrate for lipoxygenase, is known to inhibit production of AA-derived eicosanoids in guinea-pig epidermis (7). In trout, endogenous 20:3n-6 was not utilized directly for prostaglandin production, but it was an effective inhibitor of AA-derived prostaglandin formation (35). Changes in dietary

n-3/n-6 PUFA ratio can increase (36) or decrease (37) the activity of phospholipase A_2 depending on the tissue. A decrease in phospholipase A_2 activity, which would result in less available substrate for lipoxygenase, could explain the decline in both 12-HETE and 12-HEPE production in leucocytes of fish given SFO. Under our experimental conditions, no lipoxygenase products of 22:6n-3 were detected in the eicosanoid extracts from either gill cells or leucocytes. Similar studies with rainbow trout (38) and mammals supplemented with 22:6n-3 (7,39) also found no lipoxygenase-derived products of this fatty acid. While this may be surprising in cells containing large amounts of 22:6n-3, it may reflect a selective conservation mechaism for a fatty acid considered vital in maintaining membrane structure and function (40,41).

In general, therefore, the inclusion of linoleic acid in the diets of salmon results in alterations in the phospholipid fatty acid compositions in gills and leucocytes paralleled by an altered eicosanoid spectrum in these tissues. The biological consequences of these changes remain to be assessed. However, the very high biological activity of AA-derived eicosanoids suggest that caution is required in over-supplementing commercial fish oils with n-6 rich vegetable oils in fish feeds, especially under conditions where the fish are likely to be stressed.

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Effect of Chronic Glucagon Administration on Lipoprotein Composition in Normally Fed, Fasted and Cholesterol-Fed Rats

Catherine Guettet, Najmuddin Rostaqui, Denis Mathé*, Bernard Lecuyer, Nicole Navarro and Bernard Jacotot

Unité de Recherches sur les Dyslipidémies et l'Atherosclérose, INSERM U 32 Hôpital Henri-Mondor, 94010 Créteil, France

Male adult Wistar rats received daily (at 9 a.m. and 5 p.m.) 10 μg of zinc-protamine glucagon by subcutaneous injection for 8 days. Plasma cholesterol levels were decreased by 36% in fed rats, 33% in cholesterol-fed rats and by 55% in fasted rats. Lipoproteins were separated into 22 fractions by ultracentrifugation using a density gradient. Glucagon administration decreased the cholesterol content in all lipoproteins except low density lipoprotein (LDL_1) (1.006–1.040) and very low density lipoprotein (VLDL) from cholesterol-fed rats. The main decrease (−57 to −81%) was observed in 1.050–1.100 g/mL lipoproteins (LDL_2 and HDL_2), which contained a large amount of apo E, while HDL_3 cholesterol was not affected. Triacylglycerol levels were decreased only in chylomicrons and VLDL (−70%) of fed and cholesterol-fed rats, while plasma and lipoprotein triacylglycerol levels were not changed in fasted rats treated with glucagon. In normally fed rats glucagon administration increased by 42% the fractional catabolic rate of [^{125}I]HDL_2 while the absolute catabolic rate appeared to be unchanged. Glucagon seems to be a potent hypolipidemic agent affecting mainly the apo E-rich lipoproteins. Its chronic administration limits lipoprotein accumulation which occurs upon cholesterol feeding.

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Glucagon is known to be a potent hypolipidemic agent. In man and in animals, glucagon administration produces a significant decrease in plasma lipid levels (1–5). However, little is known how lipoproteins are affected by glucagon. In a previous study (6), we showed that chronic administration of glucagon decreased the concentration of cholesterol, triacylglycerol (TG), phospholipids (PL), and proteins, in all lipoproteins except low density lipoprotein (LDL_1) (1.006–1040 g/mL). The lipoprotein fraction isolated between 1.040 and 1.063 g/mL and rich in apo E was most affected by glucagon. The decrease in plasma TG levels was essentially due to a decrease in chylomicron and very low density lipoprotein (VLDL) TG.

In order to obtain insights into the effects of glucagon on plasma lipids, we now separated plasma lipoproteins on a continuous density gradient. The experiments were performed on rats under three nutritional states: fasted, normally fed, or cholesterol-fed. During fasting, the glucagon/insulin ratio increases (7), while the hepatic secretion of VLDL is reduced (8). After ingestion of a cholesterol-rich diet, a moderate hypercholesterolemia appears in rat, characterized by an increase in chylomicrons, VLDL, and LDL_1, while LDL_2 (1.040–1.063 g/mL) and high density lipoprotein (HDL)-cholesterol levels are decreased (9–11).

*To whom correspondence should be addressed.

Abbreviations: ACR, absolute catabolic rate; FCR, fractional catabolic rate; VLDL, very low density lipoprotein; LDL, low density lipoproteins; HDL, high density lipoproteins; TC, total cholesterol; TG, triacylglycerol; PL, phospholipids.

In the present study, glucagon appeared to be effective in decreasing plasma lipid levels under the three nutritional conditions used. Decreased secretion and/or increased catabolism of lipoproteins could explain the effects of glucagon. In order to explore this question, the turnover of HDL_2 (1.063–1.117 g/mL) was studied in control rats and in glucagon-treated rats.

MATERIALS AND METHODS

Animals and diets. Male Wistar rats (from Janvier, 53940 Le Genest, France), weighing about 350 g, were housed individually in an air-conditioned animal house at 25°C with a light-dark cycle of 12 hr (8 a.m.–8 p.m.). Rats were fed *ad libitum* during one month a semi-purified diet containing: sucrose, 53%; casein, 23%; lard, 9.2%; walnut oil, 0.8%; skimmed milk, 4%; salt mixture, 5%; cellulose with vitamins, 2.5%; and yeast, 23.% (12). The cholesterol content of the diet was 0.015%. Three different dietary conditions were studied: rats fed the semi-purified diet and killed with (fasted group, n = 6) or without (normally fed group, n = 6) 24-hr fasting. A third group of rats (cholesterol-fed group, n = 6) received the semi-purified diet enriched with cholesterol (1%) for a period of one month. Ten μg of zinc-protamine glucagon, donated by Novo Industries (Copenhagen, Denmark), were administered subcutaneously twice a day (at 9 a.m. and 5 p.m.) to each rat for 8 days. Control rats were treated identically as were experimental animals, except that saline was injected in place of glucagon.

Rats were killed (at 1 p.m.) by intra-aortic puncture under pentobarbital anesthesia 4 hr after the last injection of glucagon. Blood from each rat was collected in syringes containing 0.3 mL of EDTA (4%) and monoiodoacetamie (4%) for 10 mL of blood and was centrifuged at 2200 × *g* for 20 min at 4°C.

Lipoprotein isolation. Chylomicrons were isolated by overlaying 4 mL of plasma with 2 mL of 1.006 g/mL NaCl solution and centrifuging fro 30 min at 15,000 rpm in a 50 Ti rotor using a Beckman L8-70 ultracentrifuge (Beckman, Gagny, France) at 20°C. Chylomicrons were collected from the meniscus. The infranatant was adjusted to 1.21 g/mL by the addition of solid KBr and centrifuged in a 50 Ti rotor at 40,000 rpm for 40 hr at 15°C. Total lipoproteins collected from the meniscus were incorporated in a density gradient adapted from Redgrave *et al.* (13) and prepared in tubes with saline solutions of densities as follows: 1.5 mL, 1.006 g/mL; 1.5 mL, 1020 g/mL; 2 mL, 1.040 g/mL; 3 mL, 1.063 g/mL; 2 mL of total lipoprotein solution (1.21 g/mL) and 1 mL, 1.25 g/mL. The tubes were centrifuged for 24 hr at 15°C in a SW 41 Ti Beckman rotor at 40,000 rpm. The first 18 fractions (0.5 mL each) were collected from the meniscus of the tubes. A control tube containing only saline solutions was also centrifuged and used to determine by refractometry (refractometer, Atago, Tokyo, Japan) the density of fractions. Each fraction of lipoproteins was assayed for cholesterol content.

The chemical composition of lipoproteins was determined in pooled fractions corresponding to VLDL (d<1.006 g/mL; fractions 1 and 2), LDL_1 (low density lipoproteins of density between 1.006 and 1.040 g/mL; fractions 3 to 6), LDL_2 (low density lipoprotein of density between 1.040 and 1.063 g/mL; fractions 7 to 11) and HDL (high density lipoproteins of density between 1.063 and 1.210 g/mL; fractions 12 to 18). Lipid content of lipoprotein fractions was corrected for loss during ultracentrifugation procedure. The mean recoveries were 92 ± 3, 87 ± 5 and 89 ± 5% for total cholesterol (TC), TG and PL, respectively.

Apolipoprotein analysis. Lipoprotein samples from fractions 7 to 18 were extensively dialyzed against 0.05% EDTA, 0.02% NaN_3, NaCl 0.9%, pH 7.4 at 4°C, and then lyophilized. Each sample was dissolved in a buffer containing Tris HCl 0.02 M and sodium dodecyl sulfate 0.1 M, pH 8.4, and assayed for protein content prior to SDS-PAGE. The separating gels consisted of a discontinuous gradient of 5 and 10% polyacrylamide according to Connelly and Kuksis (14). With this system, delipidation of the sample prior to electrophoresis was unnecessary. Gels were stained with Coomassie Brilliant Blue R 250 (0.05%) and destained in the solution described by Weber and Osborn (15). All gels were scanned with a laser densitometer (LKB 2202 Ultrascan, LKB, Ulis, France) connected to a Delsi Enica (Delsi, Argenteuil, France) 10 integrator. The apolipoprotein bands were identified on the basis of molecular weight as determined by reference to purified protein standards and by comparison to published SDS gel electrophoretograms of rat apolipoproteins (16,17). In assessing the composition of apolipoproteins, no correction was made for the difference in binding of dye to each protein. The actual mass of each apolipoprotein within lipoprotein fractions was estimated as follows: % dye uptake × protein concentration (μg/mL plasma).

HLD turnover studies. HDL_2 (1.063–1.117 g/mL) were isolated by sequential ultracentrifugation in a Beckman 50 Ti rotor at 10°C using a Beckman L8-70 centrifuge. Rat plasma, adjusted to 1.063 g/mL with solid KBr, was centrifuged for 24 hr at 40,000 rpm (10^8 g × min). After the removal of the d < 1.063 g/mL lipoproteins, the subnatant was adjusted to 1.117 g/mL with solid KBr and centrifuged for 48 hr (2×10^8 g × min). HDL_2 were collected and centrifuged again in a NaCl/KBr solution of 1.117 g/mL density. HDL_2 were extensively dialyzed against 0.15M NaCl and 0.04% Na_2 EDTA. They were passed through a 0.22 μm filter (Cera-Labo, Aubervilliers, France). HDL_2 contained apo A-I (50%), apo A-IV (10%), apo E (12%) and apo C (28%).

^{125}I-labelling of HDL_2 was performed according to the method of MacFarlane (18) as modified for lipoproteins (19). After iodination, unbound iodine was removed by chromatography on Sephadex G 25. Labelled HDL contained 220 cpm/ng protein; 96% of total radioactivity was precipitated by trichloroacetic acid (TCA) while less than 4% were present in lipids (Folch lower layer).

Normally fed rats (control and glucagon-treated) received intravenously (jugular vein) 0.1 mL of HDL_2 solution (0.46 mg/mL) at 9 a.m. Blood samples were collected periodically from the tail vein, plasma was separated in an Eppendorf-type centrifuge, and radioactivity was measured in a MR 80 Kontron gamma spectrometer (Kontron, St. Quentin, France). At the end of the experiment (33 hr after [^{125}HDL_2 injection), rats were killed by intraaortic puncture under diethyl/ether anesthesia. Lipoprotein fractions were separated and their radioactivity was measured. The fractional catabolic rate (FCR) was estimated by the method of Matthews (20) with the assumption of a two-compartmental model for the metabolism of apo-HDL. The absolute catabolic rate was determined as FCR × intravascular pool of apo-HDL. Plasma volume was taken as 4.18% of body weight.

Chemical analyses. Cholesterol, phospholipid and triacylglycerol concentrations in plasma and lipoprotein fractions were determined by enzymatic methods using commercial kits (Wako, Biotrol, Paris, France and Boehringer-Mannheim, Mannheim, Germany). Proteins were assayed by the method described by Lowry *et al.* (21) using bovine serum albumin as standard. Samples were extracted with chloroform after color development in order to avoid turbidity due to lipids. Results are expressed as mean ± S.E.M. and statistical differences were analyzed by means of the Student's t-test.

RESULTS

Plasma lipid levels. Plasma concentrations of total cholesterol (TC), triacylglycerol (TG) and phospholipids (PL) in the rats fed the semi-purified diet were respectively 806, 2683 and 1781 mg/L (Table 1). When the rats were fasted, TC, TG and PL concentrations decreased by 20, 73 and 38%, respectively. When the animals were fed the cholesterol-rich diet, plasma lipid levels were slightly increased and reached 1163 mg/L for cholesterol, 4814 mg/L for triacylglycerol and 2014 mg/L for phospholipids.

Chronic glucagon administration significantly decreased plasma cholesterol levels under all three dietary conditions, but the greatest effect was observed in fasted rats (±55%). Plasma phospholipid concentrations were decreased, respectively, by 38%, 37% and 33% in normally fed, fasted and cholesterol-fed rats. Triacylglycerol levels were significantly decreased only in normally fed (−70%) and in cholesterol-fed rats (−69%).

Lipoproteins. The distribution of total cholesterol among the lipoprotein fractions isolated on a continuous density gradient is presented in Figure 1. In control rats, 15% of plasma cholesterol was found in chylomicrons, while 11% was in VLDL, 2% in LDL_1 (1.006–1.040), 14% in LDL_2 (1.040–1.063) and 58% in HDL. The cholesterol distribution was identical in fasted rats, except for the absence of chylomicrons and a lower contribution of VLDL. In control rats fed the cholesterol-rich diet, the lipoprotein profile was markedly changed. The distribution of cholesterol was as follows: chylomicrons, 30%; VLDL, 31.7%; LDL_1, 3.3%; LDL_2, 4.9%; and HDL, 29.9%.

Chronic glucagon administration significantly modified the cholesterol distribution in lipoproteins (Fig. 1). In normally fed rats, glucagon decreased the cholesterol concentration in chylomicrons (−51%), VLDL (−42%), LDL_2 (−57%), and HDL (−28%), but not in LDL_1. It should be noted that heavy HDL fractions (1.125–1.210 g/mL) were not affected by glucagon. In fasted rats the same observations can be made with the geatest effect on lipoproteins isolated between 1.050 and 1.100 g/mL (LDL_2, −81%; HDL_2, −70%). In cholesterol-fed rats the effect of glucagon was moderate on chylomicron cholesterol

TABLE 1

Plasma Concentrations (mg/L) of Cholesterol, Triacylglycerol and Phospholipids in Control Rats (C) and in Rats Injected During Eight Days with Zn-Protamine Glucagon (20 μg/day/rat) (GLU) in Three Nutritional Conditions[a]

Plasma concentrations (mg/L)	Nutritional conditions		
	Fed	Fasted	Cholesterol-fed
Total cholesterol			
C[b]	806 ± 44	643 ± 50[e]	1 163 ± 50[d]
GLU[c]	513 ± 38[d]	289 ± 16[d,g]	783 ± 43[c,g]
Triacylglycerol			
C	2 683 ± 525	881 ± 24[g]	4 814 ± 603[e]
GLU	808 ± 101[d]	700 ± 114	1 520 ± 110[c,g]
Phospholipids			
C	1 781 ± 160	1 121 ± 36[f]	2 014 ± 64
GLU	1 113 ± 91[c]	704 ± 57[c,e]	1 353 ± 23[c]

[a]Results are expressed as mean ± SEM, n = 6 in each group. Significantly different at: b, $P < 0.05$; c, $P < 0.01$; d, $P < 0.001$ for GLU *vs* C; e, $P < 0.05$; f, $P < 0.01$; g, $P < 0.001$ for effects of dietary conditions: fasted rats or cholesterol-fed rats *vs* fed rats in control and glucagon-treated rats.

[b]C, control rats.

[c]GLU, glucagon-injected rats.

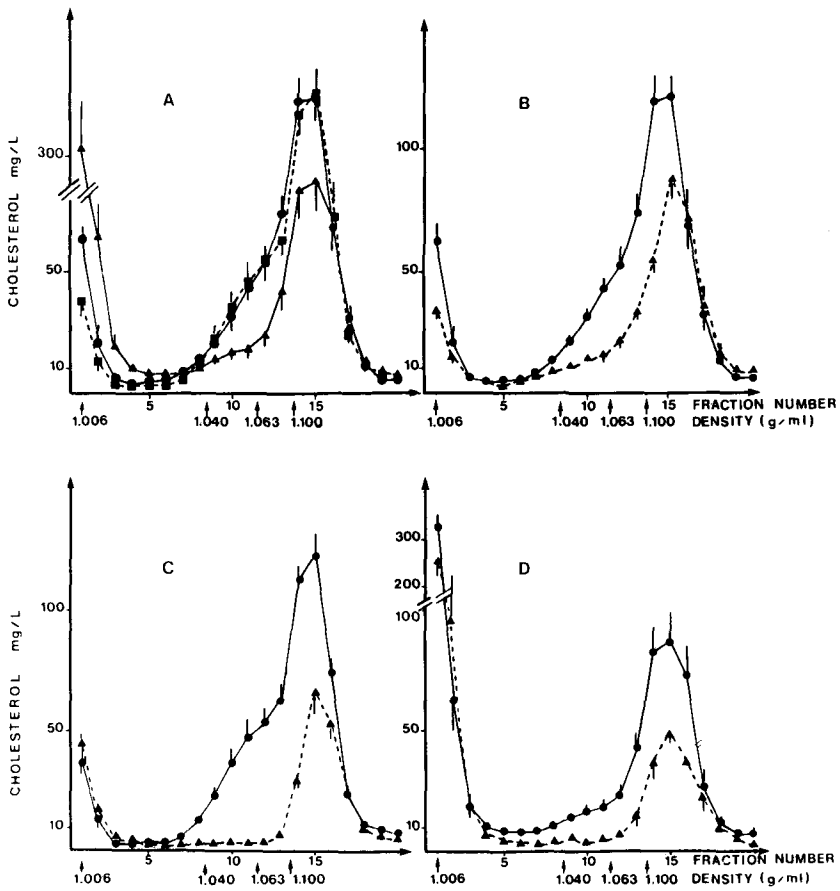


FIG. 1. Cholesterol distribution in 20 lipoprotein fractions from normally fed (●) or fasted (■) or cholesterol-fed (▲) rats (A), and from normally fed (B), fasted (C) or cholesterol-fed (D) control (●) and glucagon-treated rats (▲). Results are expressed as mean ± SEM (n = 6).

(−48%), not significant on VLDL and LDL_1 cholesterol, and marked on LDL_2 (−72%) and HDL (−46%) cholesterol.

The concentrations of lipoprotein constituents are presented in Tables 2 to 4. Independent of the dietary condition, glucagon administration decreased cholesterol concentration of LDL_2 (−57 to − 81%) and HDL (−27 to −59%). Cholesterol levels were decreased in chylomicrons and VLDL only in fed and cholesterol-fed rats. Similarly TG levels were decreased in all lipoproteins of fed and cholesterol-fed rats with great decreases (−70 to 76%) in chylomicrons and VLDL. The relative content of lipids and proteins in the different lipoproteins were not markedly changed either by dietary conditions or by

TABLE 2

Total Cholesterol (TC), Triacylglycerol (TG), Phospholipids (PL) and Protein Concentrations (mg/L) in Lipoproteins from Control Rats (C) and Rats Chronically Injected (8 Days) with Glucagon (20 μg/day/rat) (GLU) Fed *ad libitum*[a]

Concentrations (mg/L)	Lipoproteins				
	CHYLO	VLDL	LDL_1	LDL_2	HDL
Total cholesterol					
C[b]	120 ± 26	85 ± 1	19 ± 3	111 ± 10	470 ± 33
GLU[c]	59 ± 5[b]	49 ± 5[d]	14 ± 2	48 ± 2[d]	341 ± 22[b]
Triacylglycerol					
C	1 301 ± 274	1 295 ± 281	109 ± 20	30 ± 6	39 ± 4
GLU	312 ± 49[b]	380 ± 44[b]	67 ± 16	20 ± 4	28 ± 5
Phospholipids					
C	161 ± 16	291 ± 69	51 ± 14	162 ± 14	1 117 ± 70
GLU	95 ± 16[b]	105 ± 16[b]	26 ± 5	70 ± 12[d]	817 ± 69[b]
Proteins					
C	60 ± 5	116 ± 16	32 ± 4	130 ± 16	1 020 ± 47
GLU	31 ± 4[b]	60 ± 7[c]	16 ± 2[d]	46 ± 6[d]	831 ± 54[b]

[a]Results are expressed as mean ± SEM (C: n = 6; GLU: n = 6). Significantly different at: b = $P < 0.05$; c = $P < 0.01$; d = $P < 0.001$ for GLU *vs* C.

[b]C, control rats.

[c]GLU, glucagon-injected rats.

TABLE 3

Total Cholesterol (TC), Triacylglycerol (TG), Phospholipids (PL) and Protein Concentrations (mg/L) in Lipoproteins from Control Rats (C) and Rats Chronically Injected (8 days) with Glucagon (20 μg/day/rat) (GLU) Fasted 24 hr[a]

Plasma concentrations (mg/L)	Lipoproteins			
	VLDL	LDL_1	LDL_2	HDL
Total cholesterol				
C[b]	40 ± 1	17 ± 11	104 ± 1	483 ± 40
GLU[c]	46 ± 5	13 ± 2	20 ± 3[d]	200 ± 22[d]
Triacylglycerol				
C	710 ± 27	85 ± 27	27 ± 2	58 ± 5
GLU	565 ± 108	51 ± 15	23 ± 4	63 ± 17
Phospholipids				
C	171 ± 27	22 ± 2	91 ± 19	838 ± 36
GLU	133 ± 22	19 ± 7	38 ± 6[b]	513 ± 60[c]
Proteins				
C	119 ± 13	26 ± 3	96 ± 13	1 340 ± 96
GLU	91 ± 16	20 ± 3	65 ± 8	1 120 ± 64

[a]Results are expressed as mean ± SEM (C: n = 6; GLU: n = 6). Significantly different at: b = $P < 0.05$; c = $P < 0.01$; d < $P < 0.001$ for Glu *vs* C.

[b]C, control rats.

[c]GLU, glucagon-injected rats.

glucagon administration. However, it should be noted that cholesterol feeding increased cholesterol content of chylomicrons and VLDL. Glucagon administration decreased the cholesterol content of LDL_2 and HDL of fasted rats, while TG were decreased in chylomicrons, VLDL and LDL_1 of cholesterol-fed rats.

The apolipoproteins of fractions 7 to 18 were determined by PAGE in each fraction pooled from 6 control or 6 glucagon-treated rats fed *ad libitum*. Dye-uptake was proportional to the apolipoprotein content within a range of 0 to 50 μg per gel. The major effect of glucagon administration was a decrease in the apo E content of each fraction from 7 to 18, while the apo A-I content was slightly increased (Fig. 2). Since the protein content of the lipoprotein fractions was decreased, it is clear that the plasma concentrations of apo E were greatly decreased in all

TABLE 4

Total Cholesterol (TC), Triacylglycerol (TG), Phospholipids (PL) and Protein Concentrations (mg/L) in Lipoprotein from Control Rats (C) and Rats Chronically Injected (8 days) with Glucagon (20 μg/day/rat) (GLU) and Fed a Cholesterol-Rich Diet[a]

Plasma concentrations (mg/L)	Lipoproteins				
	Chylos	VLDL	LDL_1	LDL_2	HDL
Total cholesterol					
C[b]	350 ± 55	370 ± 32	39 ± 4	57 ± 14	348 ± 45
GLU[c]	190 ± 38[b]	348 ± 33	46 ± 12	16 ± 3[b]	190 ± 16[c]
Triacylglycerol					
C	2 143 ± 313	2 449 ± 111	116 ± 25	40 ± 5	66 ± 5
GLU	556 ± 146[d]	827 ± 31[d]	98 ± 44	13 ± 2[d]	29 ± 5[d]
Phospholipids					
C	504 ± 66	507 ± 55	65 ± 6	94 ± 21	844 ± 90
GLU	489 ± 57	313 ± 23[c]	53 ± 13	27 ± 3[b]	471 ± 27[c]
Proteins					
C	85 ± 17	301 ± 32	19 ± 3	73 ± 17	737 ± 14
GLU	41 ± 15	216 ± 22	23 ± 8	16 ± 4[c]	550 ± 35[c]

[a]Results are expressed as mean ± SEM (C: n = 6; GLU: n = 6). Significantly different at: b = $P < 0.05$; c = $P < 0.01$; d = $P < 0.001$ for GLU *vs* C.

[b]C, control rats.

[c]GLU, glucagon-injected rats.

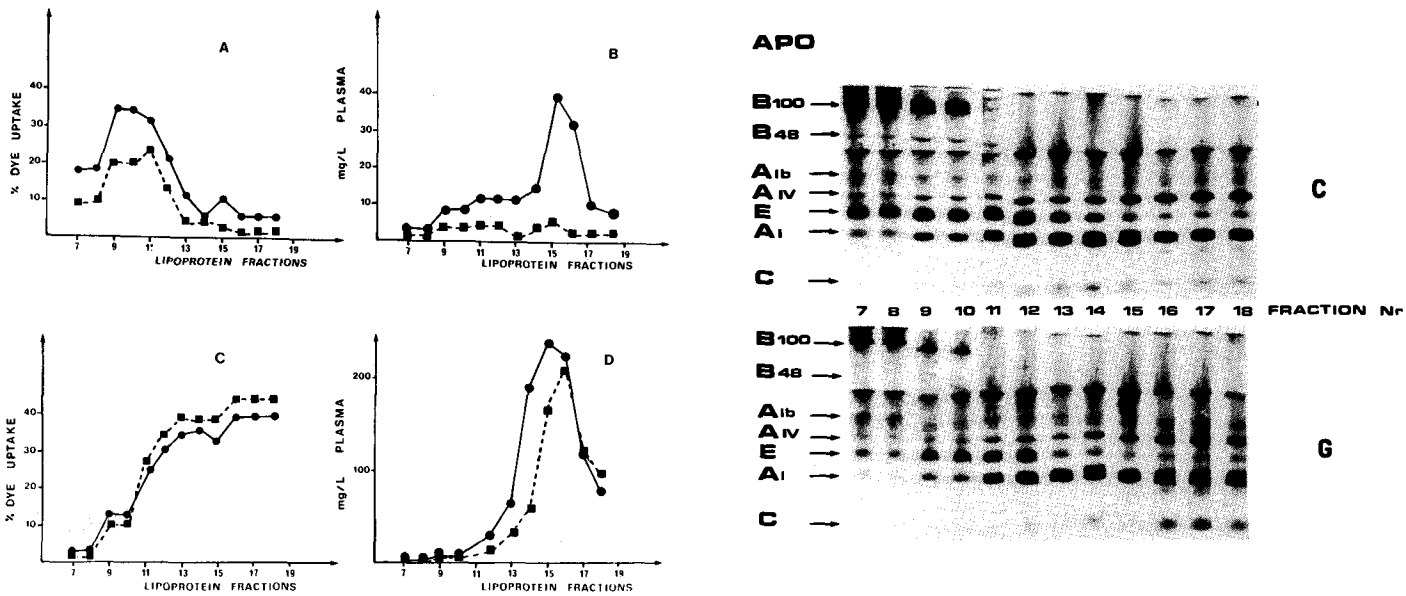


FIG. 2. Photograph of Coomassie stained gels from polyacrylamide gel electrophoresis of lipoprotein fractions 7 to 18 (density range: 1.063–1.210 g/mL) and apolipoprotein distribution among lipoprotein fractions from normally fed control rats (●) and glucagon-treated rats (■). A: % dye uptake and B: estimated concentration of apo E; C: % dye uptake and D: estimated concentration of apo A-I. Each point corresponds to pooled fractions from 6 rats.

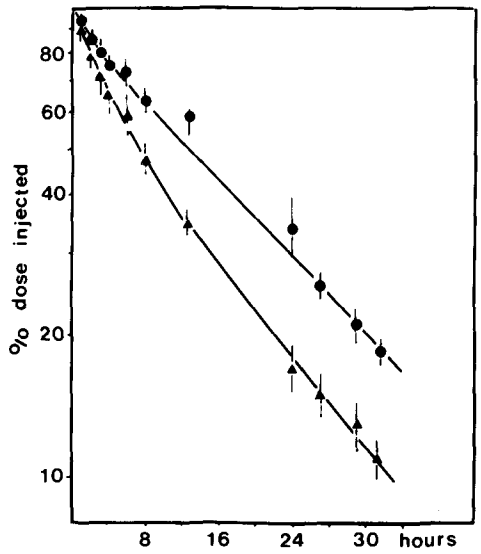


FIG. 3. Decay of plasma radioactivity after intravenous injection of iodine-labelled HDL_2 in normally fed control (●) and glucagon-treated (▲) rats. Results are expressed as mean ± SEM (n = 6).

TABLE 5

Kinetic Parameters: Half-Time ($t_{1/2}$), Relative Proportions of HDL_2 Catabolized in the Slow (C1) and the Rapid (C2) Phase, Fractional (FCR) and Absolute (ACR) Catabolic Rates of [^{125}I]-Labelled HDL_2 Decay from Plasma of Control and Glucagon-Treated Rats[a]

	Plasma	
Kinetic parameters	Control	Glucagon-treated
Slow phase—C1 %	88 ± 2	75 ± 4[b]
($t_{1/2}$ hr)	14.9 ± 0.8	11.9 ± 0.5[b]
Rapid phase—C2 %	12 ± 1	25 ± 4[b]
($t_{1/2}$)	1.4 ± 0.3	2.0 ± 0.6
Fractional catabolic rate ($\%\cdot hr^{-1}$)	5.2 ± 0.4	7.4 ± 0.5[c]
Pool (mg)	17.0 ± 1.2	12.1 ± 1.2[b]
Absolute catabolic rate ($mg\cdot hr^{-1}$)	0.89 ± 0.05	0.90 ± 0.07

[a]Mean ± SEM, n = 6. Significantly different at: b = $P < 0.05$; c = $P < 0.01$.

fractions, fraction 13 to 15 being the most affected (−86%), while apo A-I concentration was decreased in fractions 12 to 16.

Decay of iodine-labelled HDL_2 apolipoproteins in plasma. HDL_2 labelled in the protein moiety were injected into control and glucagon-treated rats. At the end of the experiment, 79% (control) and 74% (glucagon-treated) of the plasma radioactivity was present in HDL. Therefore the decay of plasma radioactivity can be considered as representative of the decay of HDL apolipoproteins. The plasma radioactivity decay was biexponential with an initial rapid phase and a quantitatively more important slow phase (Fig. 3). Kinetic parameters of the decay curves are presented in Table 5. Half-time of the slow phase was decreased in glucagon-treated rats (11.9 ± 0.5 hr) when compared to control rats (14.9 ± 0.8 hr). In contrast, the half-time of the rapid phase did not differ in control and glucagon-treated rats. The fractional catabolic rate (FCR) was increased by 42% in glucagon-treated rats, while the absolute catabolic rate (ACR) appeared to be unchanged.

DISCUSSION

In a previous study, we reported that glucagon, administered twice a day at the dose of 20 μg/day/rat, consistently decreased the plasma cholesterol levels measured 4 hr after the last injection. Body weight and food intake were not markedly changed (5). The effect appeared only after the fifth injection and was not observed after a single injection. Glucagon concentration increased in the plasma for 2 to 3 hr after the injection of glucagon (5). Plasma insulin was also increased but only moderately. Four hours after the last injection, the glucagon/insulin ratio was enhanced two-fold (5). This dose of glucagon is similar to that previously used in man (40 μg/kg/day) (2). As already reported (5,22), glucagon administration significantly decreased plasma lipid levels in fed rats. In the present study the rats were fed a diet enriched in sucrose, a carbohydrate known to increase plasma triacylglycerol concentration (23,24). This diet provided an interesting condition to study the effects of the hypolipidemic hormone glucagon.

Previous fasting of the animals induced a slight decrease in plasma cholesterol and plasma phospholipid concentrations but the distribution among the d > 1.006 g/mL lipoproteins was not modified (see Fig. 1 and compare Table 2 and Table 3 for control rats) as previously reported (17). The main change occurred in triacylglycerol-rich lipoproteins which were absent (chylomicrons), or reduced (VLDL). VLDL-cholesterol (−53%) and VLDL-triacylglycerol (−45%) were decreased by fasting. Ingestion of a diet enriched with cholesterol (1%) raised plasma lipid concentrations. However, the hypercholesterolemia obtained remained moderate as observed by others (9–11), when cholesterol feeding was not associated with hypothyroid drugs or bile acid ingestion. Cholesterol distribution among the lipoprotein fractions isolated by density gradient ultracentrifugation, was greatly modified (Fig. 1A): 62% of plasma cholesterol was carried by chylomicrons and VLDL, while the contribution of LDL_2 and HDL fell to 5 and 30%, respectively.

Glucagon administration decreased plasma cholesterol in these different dietary conditions, but the effect was greater in fasted rats. Triacylglycerol levels were decreased only in fed and cholesterol-fed rats, while no further decrease was observed in fasted rats consistent with what was reported by Byers *et al.* (25).

The three dietary conditions used provide very different situations in regard to the source of the lipoproteins. In fasted rats, most VLDL are synthesized in the liver, while in fed and cholesterol-fed rats a significant amount of VLDL is produced by both intestine and liver. Glucagon administration affected the triacylglycerol-rich lipoproteins differently according to the nutritional state. Cholesterol and triacylglycerol concentrations of chylomicrons were decreased by glucagon in fed and cholesterol-fed animals. Since the absorption coefficient of dietary cholesterol was not markedly decreased by glucagon (5), an increased catabolic rate of these lipoproteins is indicated.

In addition, it should be noted that the decrease observed after glucagon administration was greater for triacylglycerol than for cholesterol, suggesting a specific action of glucagon on the synthesis or the catabolism of lipoprotein triacylglycerol. Glucagon appeared to have no effect on VLDL-cholesterol of cholesterol-fed rats, while triacylglycerol, phospholipid and protein levels were decreased. Consequently the composition of the VLDL was significantly changed. This result suggests that the metabolic fate of some of the cholesterol-rich VLDL produced during cholesterol feeding (26) is differently affected by glucagon administration as we have previously suggested (6). VLDL and LDL_1 (fractions 1 to 6) have not been analyzed for apolipoproteins in this study. We reported previously that relative proportions of apo B_{100} were increased in VLDL and LDL_1 and those of apo C were decreased while the secretion of VLDL-apo B_{100} was enhanced and VLDL-apo_{48} was decreased in glucagon-treated rats (6).

VLDL-cholesterol levels observed in fasted rats were the same as in fed rats treated with glucagon. No additional decrease could be induced by glucagon administration, suggesting that the metabolism of some of the VLDL, particularly those secreted in fasted rats, was not affected by glucagon. A similar conclusion can be drawn concerning plasma TG, since values obtained in fasted rats, and in fasted or fed rats treated with glucagon, were not significantly different.

Separation of lipoproteins by ultracentrifugation on a continuous gradient allowed precise identification of the lipoproteins affected by glucagon administration. The cholesterol concentration of lipoproteins isolated between densities 2.050 and 1.110 g/mL was strikingly decreased in the three nutritional conditions. They correspond to the lightest part of HDL (HDL_2) and to a fraction named LDL_2. In Tables 2 to 4, lipoprotein fractions have been grouped into VLDL, LDL_1, LDL_2 and HDL in order to determine their composition. The lipoproteins of density range 1.006–1.063, currently named LDL, have been separated into 2 fractions: LDL_1 (1.006–1.040) and LDL_2 (1.040–1.063) according to Fidge and Poulis (27). LDL_1 are generally considered to be a product of VLDL lipolysis (27). Glucagon did not significantly affect the plasma concentration of LDL_1, whatever the nutritional state. LDL_2 ($1.040 < d < 1.063$) contain mainly apo E, apo C and apo A and small amounts of apo B (28). This lipoprotein fraction is probably largely composed of apo E-rich HDL (HDL_1). Glucagon administration decreased the concentration of this lipoprotein with the greatest effect in fasted rats. Although apo E-HDL was already decreased in cholesterol-fed rats, glucagon produced an additional decrease. The analysis of apolipoproteins within fractions 7 to 18 showed that glucagon administration decreased the apo A-I concentration in fractions 11 to 16, while the apo E concentration was greatly decreased in all fractions. From this study, it clearly appears that HDL, which contain apo E, are markedly decreased by glucagon administration. This effect can be the result of either a decreased secretion or an increased catabolism, or both. To explore these possibilities, $[^{125}I]HDL_2$ turnover studies were performed in normally fed control and glucagon-treated rats. This fraction (1.063–1.117 g/mL) contained both apo A-I and apo E. The results clearly showed that glucagon administration increased the fractional catabolic rate of HDL_2, while the absolute catabolic rate remained unchanged and the HDL_2 pool was decreased by 30% in glucagon-treated rats. Since the animals were in physiological steady state, it can be concluded that glucagon does not modify the rate of HDL_2 production, but stimulates the catabolic pathways. In cultured hepatocytes, MacKinnon *et al.* (29) showed that an increased catabolism of apo E-rich HDL stimulated bile acid synthesis. Moreover we have reported that chronic glucagon administration enhanced bile acid synthesis (5). Therefore it can be suggested that the decrease in the plasma concentration of LDL_2 and HDL_2 could be the consequence of accelerated turnover of these lipoproteins in glucagon-treated rats.

In conclusion, glucagon appears to be a potent hypolipidemic agent in various nutritional states. Its chronic administration limits lipoprotein accumulation which occurs in cholesterol feeding. Glucagon markedly decreased cholesterol levels in LDL_2 and HDL, while LDL_1 were not affected regardless of the dietary condition. The effects on cholesterol and triacylglycerol content of chylomicrons were also consistent, but the effects on VLDL-lipids varied according to the nutritional state. The relative proportions of cholesterol, triacylglycerol, phospholipid and proteins were virtually unchanged by either dietary conditions or by glucagon administration, suggesting a reduced number of some lipoprotein particles in plasma. However a specific decrease in triacylglycerol content of chylomicrons and VLDL can be noted together with a reduction of plasma apo E concentrations. The hypocholesterolemic action of glucagon can be explained by increased catabolism of HDL rather than decreased secretion. Hypolipidemic effects of glucagon in man have been already reported (1,2,30,31). Hypertriglyceridemic patients with impaired carbohydrate tolerance were those who exhibited the greatest decrease during chronic administration of glucagon (2). In addition normal and hyperlipidemic subjects receiving glucagon had lower esterified cholesterol levels in plasma and lower cholesterol esterification rates than untreated subjects (31). A similar observation was made in rats treated with glucagon (6). Interestingly Eaton *et al.* (32,33) proposed that decreased glucagon activity or a modified insulin/glucagon ratio characterizes genetic or acquired endogenous hyperlipemia, while the hypolipidemic drug clofibrate would modify this ratio. Further examination of the role of glucagon in lipoprotein metabolism would be of interest.

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The Effect of Dietary Zinc Deficiency on the Lipid Composition of the Rat Erythrocyte Membrane

Eric R. Driscoll and William J. Bettger*
Department of Nutritional Sciences, College of Biological Science, University of Guelph, Guelph, Ontario, Canada N1G 2W1

The effect of dietary zinc deficiency in the rat on the lipid composition of the erythrocyte membrane was determined. Weanling male Wistar rats were fed an egg white-based diet containing <1.0 mg Zn/kg diet *ad libitum*. Control rats were either pair-fed or *ad libitum*-fed the basal diet supplemented with 100 mg Zn/kg diet. A zinc refed group was fed the −Zn diet until day 18 and then pair-fed the +Zn diet until day 21. The voluntary feed restriction associated with dietary zinc deficiency resulted in erythrocyte membranes that had depressed phospholipid/protein and elevated cholesterol/phospholipid ratios. Similarly, all feed restricted groups had elevated 22-carbon n-3 polyunsaturated fatty acids (PUFA) and depressed 22-carbon n-6 PUFA concentrations in alkenyl-acyl and diacyl glycerophosphoethanolamine, phosphatidylserine and phosphatidylcholine; they also had depressed 24:2n-6 levels in sphingomyelin. The relative concentrations of phospholipids in the membrane was similar between −Zn and +Zn (*ad libitum*) groups; however, the −Zn group had significantly less phosphatidylserine relative to +Zn (pair-fed) controls.
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Zinc has been proposed to have a critical physiological role in the structure and function of biomembranes (1). Dietary zinc status appears to affect the composition and functional properties of the lipid phase of cell membranes. An interaction of dietary zinc and essential fatty acids has been reported in rats (2) and chicks (3); however, the biochemical basis for this interaction is unknown. Zinc deficiency has been reported to alter fatty acid desaturase activities (4), cholesterol metabolism (5,6) and phospholipid biosynthesis (7,8) in rat tissues. An interaction of zinc and vitamin E has been described in rats (9–11) and chicks (12). Recently, Bray and Bettger (13) have reviewed data suggesting that zinc may function as an antioxidant for specific macromolecules. The "fluidity" of the lipid phase of the liver microsomal fraction (6,14) and from erythrocyte ghosts (15) has been reported to be altered in zinc-deficient rats. In spite of these reports, the effect of dietary zinc deficiency on the detailed lipid composition of a purified plasma membrane fraction has not previously been determined; a preliminary report (16) suggests that there may be substantial alterations in the lipid composition of rat liver plasma membranes from zinc deficient rats.

*To whom correspondence should be addressed.

Abbreviations: ANS, 8-anilinonaphthalene-1-sulfonic acid; FAME, fatty acid methyl ester; FFA, free fatty acids; GPE, glycerophosphoethanolamine; LPC, lysophosphatidylcholine; MCV, mean corpuscular volume; MECL, modified equivalent chain length; PC, phosphatidylcholine; PE, phosphatidylethanolamine; PI, phosphatidylinositol; PUFA, polyunsaturated fatty acids; SPH, sphingomyelin; TLC, thin-layer chromatography; −Zn, zinc deficient; +ZnAL, zinc-adequate, *ad libitum*; +ZnPF, zinc-adequate, pair-fed; +ZnRF, zinc refed.

This study was performed to determine the effect of dietary zinc deficiency on the composition of the major lipids in the rat erythrocyte membrane. Changes in lipid composition will be discussed relative to their potential impact on membrane fluidity, red cell osmotic and peroxidative fragilities and on the interaction of zinc and essential fatty acids.

MATERIALS AND METHODS

Male weanling Wistar rats (43–54 g) were divided into zinc deficient (−Zn), zinc refed (+ZnRF), zinc-adequate, pair-fed (+ZnPF), and zinc-adequate *ad libitum* (+ZnAL) treatment groups and were fed the appropriate diet for three weeks. The basal diet containing <1 μg/g Zn, based on egg white, glucose hydrate and corn oil, has been described in detail (17). Control diets were supplemented to contain 100 μg/g Zn. The +ZnRF group was pair-fed the zinc-adequate diet for the last three days of the feeding period the amount consumed by the −Zn group. For the analysis of plasma lipids and the unsaturation index of total erythrocyte membrane phospholipids, the +ZnRF and +ZnPF groups were fed at 4 hr and the food removed from all groups at 3 hr before blood was taken. For all other analyses, food was removed from all groups at 12 hr before killing. The rats were killed by decapitation after light anesthesia with diethyl ether, and trunk blood was collected into heparinized tubes.

Erythrocyte ghost preparation. Ghosts were prepared according to the method of Steck *et al.* (18) as modified by Avery and Bettger (19) with all steps executed at 4°C or on ice. Briefly, the heparinized blood was centrifuged at 300 × *g* for 10 min and the buffy coat removed. The red cells were washed three times in 145 mM NaCl, 10 mM glucose, 5 mM phosphate buffer at pH 8, then lysed in 5 mM phosphate buffer with protease inhibitors (Sigma, St. Louis, MO) leupeptin (1 μM), pepstatin (1 μM), and phenylmethylsulfonylfluoride (100 μM). The lysate was centrifuged at 20,000 × *g* for 10 min to yield a pellet of red cell membranes. After three washes with lysing solution, a pellet of approximately 1 mL of white ghosts was collected and frozen at −20°C until analyzed.

Thin-layer chromatography. The methods used for lipid analysis have been previously described (20). Lipids were extracted from 40 μL of ghosts according to the method of Bligh and Dyer (21) and the phospholipids were separated using chloroform/methanol/acetic acid/water (50:37.5:3.5:2, by vol) on silica gel 60 thin-layer chromatographic (TLC) plates (Merck, Darmstadt, Germany) that had previously been activated at 75°C for at least 1 hr (22). The lysophosphatidylcholine (LPC) extracted from plasma was separated by TLC into 1- and 2-position species using the basic solvent system chloroform/methanol/ammonium hydroxide/water (45:35:8:4, by vol) (23). Plasma free fatty acids were determined by TLC separation of neutral lipids using heptane/isopropyl ether/acetic acid (60:40:3, by vol) (24). The lipids were visualized by spraying with 0.1% 8-anilinonaphthalene-1-sulfonic acid

(ANS) (25). The phosphatidylethanolamine (PE) spot was exposed to fumes of concentratedhydrochloric acid in a closed system for four minutes to hydrolyze the alkenyl PE leaving lysoPE and diacyl species of PE (26). The PE species were extracted (27) and separated by TLC (22).

Sphingomyelin was transmethylated in 6% sulfuric acid in methanol at 75–80°C for 14 hr (28) with a known quantity of internal standard of 17:0 fatty acid (Nu-Chek-Prep, Elysian, MN). The other phospholipids were similarly transmethylated for 3 hr. The fatty acid methyl esters (FAME) were extracted with hexane and analyzed by gas-liquid chromatography.

Gas-liquid chromatography. FAME samples were dissolved in hexane and separated on a Hewlett-Packard 5890A gas chromatograph (Hewlett-Packard, Palo Alto, CA) using a 30-m DB-255 megabore column (J&W Scientific, Rancho Cordova, CA) at a column temperature of 210°C. FAME were identified by comparing retention times with those of standards (Nu-Chek-Prep), and FAME mass was calculated by integration of peak areas. Unknown fatty acids in sphingomyelin (24:1n-7, 24:2n-6) were first tentatively identified by calculating the Modified Equivalent Chain Length (MECL) (29). Then, argentation chromatography (30) was employed to identify 24:1n-7 and 24:2n-6 as monoenes and dienes, respectively. Plates were spread with a slurry of 8 g of silver nitrate and 50 g of silica gel G (CAMAG D-5) in 90–100 mL of water. After drying for 20 min and activating at 120°C for at least 1 hr, FAME were separated, using chloroform/methanol (99:1, v/v) as solvent, into bands according to the number of double bonds. Dimethyl acetals (16:0, 18:0, 18:1) were tentatively identified by their elution times relative to the corresponding FAME. Dimethyl acetals were separated from FAME by TLC with 1,2-dichloroethane as solvent (20).

Phospholipid, cholesterol, and protein. Phospholipid phosphorus, cholesterol and protein were measured by colorimetric assays. Phosphorus in triplicate aliquots of 40 μL of ghosts was measured by the method of Bartlett (31). Cholesterol from duplicate aliquots of 50 μL of ghosts was measured using modification of the method employed in a kit from Sigma (St. Louis, MO) (32). An aliquot of chloroform lipid extract was dried and 200 μL of ethanol was added and warmed in a heating block to 70–75°C (5–10 seconds). The sample was vortexed for three seconds to dissolve the lipid, 2 mL of reagent was added, and the mixture was vortexed for three seconds. The samples were incubated at room temperature for 18 min before absorbance was measured. Protein was determined according to the method of Markwell *et al.* (33).

Statistics. Data were analyzed by a general linear modeling procedure followed by Tukey's test and a least square means analysis comparing −Zn to +ZnRF and +ZnPF groups. All data were examined to determine whether they represented a normally distributed population by calculation of the Shapiro-Wilk statistic on the residuals. Data were transformed prior to analysis, if necessary, to obtain a normal distribution (34).

RESULTS

After 3 weeks on the dietary regimen, the rats had the following weight gains: −Zn 8.3 ± 0.8 g, +ZnRF 12.7 ± 1.9 g, +ZnPF 29.5 ± 2.1 g, and +ZnAL 127.6 ± 2.3 g. Plasma Zn concentrations were: −Zn 0.26 ± 0.03, +ZnRF 1.30 ± 0.06, +ZnPF 1.22 ± 0.03, and +ZnAL 1.52 ± 0.05 μg/mL. The refeeding regimen reverses plasma Zn concentrations to levels that are equivalent to those of pair-fed controls and causes Zn deficient rats to improve their appetite—all rats consume at least 90% of their feed on the last day within 1 hr. There were no mortalities associated with any dietary treatment.

The reduced feed intake associated with dietary zinc deficiency resulted in a significant lowering of the membrane phospholipid/protein ratio and the unsaturation index of the dimethylacetals derived from total phospholipid. The cholesterol/phospholipid ratio was significantly elevated. Zinc deficiency or the associated decrease in voluntary food intake did not alter the erythrocyte membrane cholesterol/protein ratio, the phosphatidylcholine/sphingomyelin ratio or the unsaturation index of the fatty acids derived from the total phospholipids. Zn deficiency, *per se*, caused a significant decrease in the unsaturation index of the dimethylacetals (Table 1).

Dietary zinc deficiency, and the associated drop in voluntary food intake, did not alter the relative composition of erythrocyte membrane alkenyl acyl glycerophosphoethanolamine (Alkenyl acyl GPE) and (diacyl GPE), total glycerophosphoethanolamine (PE), phosphatidylinositol (PI), phosphatidylcholine (PC) and sphingomyelin (SPH). Dietary zinc deficiency resulted in a significantly lower phosphatidylserine (PS) concentration (% of total) compared to the +ZnPF controls (Table 2).

TABLE 1

Effect of Dietary Zinc Deficiency on Erythrocyte Membrane Phospholipid, Cholesterol Concentration and Fatty Acid Unsaturation Index

Index[c]	Treatment group[a,b]				Standard error[d]
	−Zn	+ZnRF	+ZnPF	+ZnAL	
PL/mg P[e]	0.73^{A}	0.72^{A}	0.75^{A}	0.88^{B}	0.01
chol/mg P[f]	0.61	0.61	0.63	0.62	0.01
chol/PL[g]	0.84^{A}	0.86^{A}	0.84^{A}	0.71^{B}	0.02
PC/SPH[h]	3.4	3.6	4.0	3.9	0.1
UI[i]	4.55	4.75	4.61	4.54	0.04
UI of DMA[j]	0.22^{A}	$0.23^{A,B}$	0.27^{B}	0.36^{C}	0.01

[a] Mean (n=5).

[b] Means that do not share a common superscript capital letter are significantly different by Tukey's ($p<0.05$).

[c] Abbreviations used in the Table: PL, phospholipid; P, protein; chol, cholesterol; PC, phosphatidylcholine; SPH, sphingomyelin; UI, unsaturation index; DMA, dimethylacetal.

[d] Pooled standard error.

[e] μmol Phospholipid (PL)/mg protein. Data transformed (square root) prior to statistical analysis distribution.

[f] μmol Cholesterol (chol)/mg protein. Data analyzed by a nonparametric statistical test (Kruskal-Wallis).

[g] Cholesterol/phospholipid (m/m). Data transformed (hyperbolic tangent).

[h] Phosphatidylcholine/sphingomyelin (m/m).

[i] Unsaturation index of the fatty acid methyl esters from membrane total phospholipids.

[j] Unsaturation index of the dimethylacetals from membrane total phospholipids. (Calculation based on area% of 16:0, 18:0 and 18:1 dimethylacetals).

The reduced food intake associated with dietary zinc deficiency caused a significant reduction of 22:4n-6 and 22:5n-6 and a corresponding increase in 22:5n-3, 22:6n-3 and in the double bond index of membrane alkenyl acyl GPE. Membranes from zinc deficient rats have significantly lower 22:4n-6 and 22:5n-6 compared to pair-fed controls in alkenyl acyl GPE (Table 3). In diacyl GPE, the decreased food consumption associated with dietary zinc deficiency results in a decrease in 22:4n-6 and 22:5n-6 and an increase in 22:5n-3 and 22:6n-3 (Table 4).

The depression of voluntary food intake associated with dietary zinc deficiency results in a decrease of 22:4n-6 and 22:5n-6 and an increase in 22:5n-3 and 22:6n-3 in PS. Dietary zinc deficiency, *per se*, is associated with increased 22:5n-3 and 22:6n-3 (Table 5). In PI, the depression of food intake associated with dietary zinc deficiency results in a decrease in 22:4n-6 and 22:5n-6 (Table 6).

The depression of voluntary food intake associated with dietary zinc deficiency results in a significant decrease in 20:4n-6, 22:4n-6 and 22:5n-6 in erythrocyte membrane PC. Membranes from zinc deficient rats have significantly less total monounsaturated fatty acids and more 20:4n-6, 22:5n-3 and 22:6n-3 than those from +ZnPF controls in PC (Table 7). In SPH, the result of reduced feed intake was a decrease in 24:2n-6 content and double bond index.

TABLE 2

Effect of Dietary Zinc Deficiency on the Mass (mol %) of Erythrocyte Membrane Phospholipid

Phospholipid	Treatment group				Standard error[a]
	−Zn	+ZnRF	+ZnPF	+ZnAL	
Alkenyl acyl GPE	11.9[b]	11.0	11.4	12.8	0.4
Diacyl GPE	8.2	8.4	8.6	9.4	0.3
Total GPE	20.3	19.4	20.2	22.2	0.5
PI	4.8	5.3	5.4	4.4	0.2
PS	12.8[c]	12.9	14.8	13.7	0.3
PC	47.8	49.8	46.6	47.2	0.5
SPH	14.4	12.6	13.1	12.6	0.5

[a]Pooled standard error.

[b]Mean (n=6; +ZnRF group n=5). Data are expressed as the percent of the phospholipids quantitated.

[c]Significantly different than +ZnPF by a least square means analysis ($p<0.05$).

TABLE 3

Effect of Dietary Zinc Deficiency on Fatty Acid Composition (mol %) of Erythrocyte Membrane Alkenyl Acyl Glycerophosphoethanolamine

Fatty acyl	Treatment group				Standard error[a]
	−Zn	+ZnRF	+ZnPF	+ZnAL	
Saturated	5.4[b,c]	3.8	5.2	8.1	0.7
Monounsaturated	3.4	3.7	2.2	6.9	0.7
Polyunsaturated	91.2	92.5	92.6	85.0	1.2
Total n-6	64.8	65.8	67.5	69.6	0.9
18:2n-6	1.2[d,AB]	1.4[B]	0.7[A]	1.9[C]	0.1
20:4n-6	49.7	50.6	49.8	45.6	0.8
22:4n-6	11.6[d,A]	11.5[A]	13.6[A]	17.7[B]	0.3
22:5n-6	2.2[d,A]	2.2[A]	3.2[A]	4.3[B]	0.1
Total n-3	26.4[A]	26.7[A]	25.2[A]	15.4[B]	0.4
22:5n-3	13.1[A]	12.9[A]	12.7[A]	7.5[B]	0.2
22:6n-3	13.3[A]	13.8[A]	12.5[A]	7.8[B]	0.2
DBI[e]	4.08[A]	4.14[A]	4.12[A]	3.70[B]	0.05

[a]Pooled standard error.

[b]Mean (n=6; +ZnRF group n=5).

[c]Means that do not share a common superscript capital letter are significantly different by Tukey's test ($p<0.05$).

[d]Significantly different than +ZnPF by a least square means analysis ($p<0.05$).

[e]DBI, double bond index.

TABLE 4

Effect of Dietary Zinc Deficiency on the Fatty Acid Composition (mol %) of Erythrocyte Membrane Diacyl Glycerophosphoethanolamine

Fatty acyl	Treatment group				Standard error[a]
	−Zn	+ZnRF	+ZnPF	+ZnAL	
Saturated	19.0[b,c]	21.5	15.4	19.5	1.2
Monounsaturated	24.7	24.5	25.6	26.9	0.6
Polyunsaturated	56.3	54.0	58.9	53.6	0.9
Total n-6	47.6	45.1	50.4	48.2	0.8
18:2n-6	12.0	11.0	11.9	11.5	0.3
20:4n-6	32.6	30.8	35.1	31.9	0.5
22:4n-6	1.8[A]	1.9[A]	1.9[A]	2.5[B]	0.1
22:5n-6	0.7[A]	0.8[A]	0.9[A]	1.8[B]	0.1
Total n-3	8.7[A]	8.9[A]	8.5[A]	5.3[B]	0.2
22:5n-3	2.5[A]	2.6[A]	2.6[A]	1.5[B]	0.1
22:6n-3	6.1[A]	6.3[A]	5.9[A]	3.8[B]	0.1
DBI[d]	2.41	2.34	2.52	2.29	0.04

[a]Pooled standard error.

[b]Mean (n=6; +ZnRF group n=5).

[c]Means that do not share a common superscript capital letter are significantly different by Tukey's test ($p<0.05$).

[d]DBI, double bond index.

TABLE 5

Effect of Dietary Zinc Deficiency on the Fatty Acid Composition (mol %) of Erythrocyte Membrane Phosphatidylserine (PS)

Fatty acyl	Treatment group				Standard error[a]
	−Zn	+ZnRF	+ZnPF	+ZnAL	
Saturated	27.0[b,c]	25.7	27.2	27.1	0.5
Monounsaturated	5.4[AB]	4.3[A]	7.4[B]	6.1[A,B]	0.4
Polyunsaturated	67.6	70.0	65.4	66.8	0.9
Total n-6	57.1	59.0	56.4	60.6	0.7
18:2n-6	4.0[AB]	4.1[A]	2.6[A,B]	3.4[B]	0.1
20:4n-6	49.6	51.1	49.1	51.5	0.6
22:4n-6	1.5[A]	1.5[A]	1.4[A]	2.1[B]	0.03
22:5n-6	1.6[A]	1.7[A]	1.8[A]	3.3[B]	0.1
Total n-3	10.5[d,A]	11.1[A]	9.1[B]	6.1[C]	0.2
22:5n-3	1.8[d,A]	1.8[A]	1.5[B]	1.0[C]	0.03
22:6n-3	8.7[d,A]	9.2[A]	7.6[B]	5.2[C]	0.2
DBI[e]	2.88	2.98	2.80	2.81	0.03

[a]Pooled standard error.

[b]Mean (n=6; +ZnRF group n=5).

[c]Means that do not share a common superscript capital letter are significantly different by Tukey's test ($p<0.05$).

[d]Significantly different than +ZnPF by a least square means analysis ($p<0.05$).

[e]DBI, double bond index.

Dietary zinc deficiency was associated with a decrease in 24:1n-9 compared to +ZnPF controls (Table 8).

The depression of voluntary feed intake associated with dietary zinc deficiency results in increased total saturated fatty acids and decreased polyunsaturated fatty acids and 20:4n-6 and 22:4n-6 in plasma PC. Dietary zinc deficiency results in a decrease in 18:2n-6 and an increase in 20:4n-6, 22:6n-3 and in the double bond index compared to +ZnPF controls (Table 9). In plasma SPH, reduced food intake results in a decreased content of 24:1n-9 and 24:2n-6; dietary zinc deficiency results in a increased 24:1n-7 compared to pair-fed controls (Table 10). In plasma free (unesterified) fatty acids (FFA), reduced food intake results in decreased 22:4n-6; zinc deficiency causes reduced 20:4n-6 compared to +ZnPF controls (Table 11).

An analysis of the relative phospholipid content of plasma from rats fed the zinc-adequate diet *ad libitum* yielded the following results: PC 59.5%, SPH 9.1%, LPC 22.8%, PI 6.7%, PE 2.0%, and PS not detectable (n=3).

TABLE 6

Effect of Dietary Zinc Deficiency on the Fatty Acid Composition (mol %) of Erythrocyte Membrane Phosphatidylinositol (PI)

Fatty acyl	Treatment group −Zn	+ZnRF	+ZnPF	+ZnAL	Standard error[a]
Saturated	49.7[b,c,A]	47.3[A,B]	48.1[A,B]	43.3[B]	0.8
Monounsaturated	6.9	9.5	7.2	10.8	0.7
Polyunsaturated	43.4	43.2	44.8	45.9	1.0
Total n-6	41.0	40.6	42.4	43.6	1.0
18:2n-6	7.9	7.4	7.8	6.9	0.2
20:4n-6	31.5	31.5	32.8	33.9	0.9
22:4n-6	0.8[A]	0.7[A]	0.8[A]	1.3[B]	0.03
22:5n-6	0.3[A]	0.3[A]	0.3[A]	0.8[B]	0.02
Total n-3	2.3	2.6	2.4	2.3	0.1
22:5n-3	1.1	1.1	1.0	0.9	0.03
22:6n-3	1.3	1.5	1.4	1.4	0.1
DBI[d]	1.68	1.71	1.74	1.84	0.04

[a] Pooled standard error.

[b] Mean (n=6; +ZnRF group n=5).

[c] Means that do not share a common superscript capital letter are significantly different by Tukey's test ($p<0.05$).

[d] DBI, double bond index.

TABLE 7

Effect of Dietary Zinc Deficiency on the Fatty Acid Composition (mol %) of Erythrocyte Membrane Phosphatidylcholine (PC)

Fatty acyl	Treatment group −Zn	+ZnRF	+ZnPF	+ZnAL	Standard error[a]
Saturated	56.5[b,c]	55.8	57.0	56.9	0.7
Monounsaturated	9.4[d,A]	10.2[A,B]	11.0[B]	10.6[A,B]	0.2
Polyunsaturated	34.1	34.0	32.0	32.5	0.6
Total n-6	31.4	31.7	30.5	31.2	0.6
18:2n-6	19.1[A]	19.0[A]	18.9[A,B]	16.1[B]	0.4
20:4n-6	11.7[d,A]	11.6[A]	10.4[A]	13.5[B]	0.2
22:4n-6	0.3[A]	0.3[A]	0.3[A]	0.5[B]	0.01
22:5n-6	0.4[A]	0.4[A]	0.4[A]	0.9[B]	0.03
Total n-3	2.2[d,A]	2.2[A]	1.6[B]	1.3[B]	0.04
22:5n-3	0.21[d,A]	0.19[A,B]	0.16[B]	0.17[B]	0.01
22:6n-3	2.0[d,A]	2.0[A]	1.4[B]	1.1[B]	0.04
DBI[e]	1.12	1.12	1.04	1.11	0.02

[a] Pooled standard error.

[b] Mean+SEM (n=6; +ZnRF group n=5).

[c] Means that do not share a common superscript capital letter are significantly different by Tukey's test ($p<0.05$).

[d] Significantly different than +ZnPF by a least square means analysis ($p<0.05$).

[e] DBI, double bond index.

TABLE 8

Effect of Dietary Zinc Deficiency on the Fatty Acid Composition (mol %) of Erythrocyte Membrane Sphingomyelin (SPH)

Fatty acyl	Treatment group −Zn	+ZnRF	+ZnPF	+ZnAL	Standard error[a]
Saturated	69.4[b,c,A]	67.5[A]	67.1[A,B]	61.7[B]	0.7
Monounsaturated	18.5[A]	21.0[A,B]	20.9[A,B]	23.1[B]	0.5
24:1n-9	17.8[d,A]	17.9[A]	20.1[A,B]	21.0[B]	0.3
24:1n-7	tr	tr	tr	tr	—
Polyunsaturated (Total n-6)	12.1[A]	11.5[A]	12.0[A]	15.1[B]	0.3
18:2n-6	0.4	0.3	0.1	0.2	0.1
24:2n-6	11.5[A]	11.1[A]	11.8[A]	14.7[B]	0.3
DBI[e]	0.43[A]	0.44[A]	0.45[A]	0.54[B]	0.01
Avg. carbon number	21.13	21.08	21.34	21.40	0.09

[a] Pooled standard error.

[b] Mean (n=6; +ZnRF group n=5).

[c] Means that do not share a common superscript capital letter are significantly different by Tukey's test ($p<0.05$).

[d] Significantly different than +ZnPF by a least square means analysis ($p<0.05$).

[e] DBI, double bond index.

TABLE 9

Effect of Dietary Zinc Deficiency on the Fatty Acid Composition (mol %) of Plasma Phosphatidylcholine (PC)

Fatty acyl	Treatment group −Zn	+ZnRF	+ZnPF	+ZnAL	Standard error[a]
Saturated	42.4[b,c,d,A]	41.4[A]	41.0[A]	37.9[B]	0.2
Monounsaturated	7.2[d,e,A]	10.1[B]	9.7[B]	9.0[B]	0.2
Polyunsaturated	50.4[e,A]	48.4[B]	49.3[A,B]	53.1[C]	0.2
Total n-6	44.5[A]	43.9[A]	45.8[A]	49.1[B]	0.3
18:2n-6	23.8[d,A]	24.9[A,B]	28.7[B]	23.4[A]	0.5
20:4n-6	18.2[A]	15.8[A,B]	14.5[B]	21.8[C]	0.3
22:4n-6	0.71[A]	0.68[A]	0.74[A]	0.98[B]	0.03
22:5n-6	1.5[A,B]	1.8[A,B]	1.3[B]	2.6[A]	0.1
Total n-3	5.8[d,e,A]	4.6[A,B]	3.5[B]	3.9[B]	0.2
22:5n-3	0.37	0.31	0.34	0.41	0.02
22:6n-3	5.5[d,e,A]	4.3[A,B]	3.2[B]	3.5[B]	0.2
DBI[f]	1.74[d,e,A,B]	1.64[B,C]	1.57[C]	1.84[A]	0.01

[a] Pooled standard error.

[b] Mean (n=6).

[c] Means that do not share a common superscript capital letter are significantly different by Tukey's test ($p<0.05$).

[d] Significantly different than +ZnPF by a least square means analysis ($p<0.05$).

[e] Significantly different than +ZnRF.

[f] DBI, double bond index.

The fatty acid composition of the LPC fraction suggested that it was a mixture of LPC containing fatty acid bound in the 1-position (16 and 18 saturated and monounsaturated fatty acids) or 2-position (18 and 20 carbon PUFA). Indeed, the two species were separated by TLC with the putative 2-position LPC species running ahead of the 1-position species. The PC and SPH content of plasma was determined in rats on the dietary treatments. The PC content was 0.92, 1.1, 0.94 and 0.92 μmol/mL (P>0.05) for −Zn, +ZnRF, +ZnPF and +ZnAL, respectively; SPH was 0.13, 0.11, 0.13 and 0.13 (μmol/mL) (P>0.05); and PC/SPH was 7.4^{a}, 10.6^{b}, 7.2^{a} and 7.1^{a} (means that do not share a common superscript letter are significantly different by Tukey's test, P<0.05, pooled standard error = 0.3). The FFA content was as follows: −Zn 0.43^{a}, +ZnRF 0.28^{ab}, +ZnPF 0.14^{b} and +ZnAL 0.41^{a} μmol/mL (P<0.05, pooled standard error = 0.03).

In addition to the major effects of zinc deficiency and feed restriction on the relative content of individual n-6 and n-3 PUFA in erythrocyte membrane and plasma phospholipids, dietary zinc deficiency had distinctive effects on the levels of 20 and 24 carbon n-9/n-7 monoenes in phospholipids and free fatty acids in plasma and in the erythrocyte membrane. For example, the 20:1n-9/20:1n-7 ratio in ghost total phospholipids is 0.374^{a}, 0.471^{ab}, 0.503^{ab} and 0.772^{b} (P<0.05, pooled standard error = 0.04) for −Zn, +ZnRF, +ZnPF and +ZnAL, respectively. Plasma FFA has a 20:1n-9/20:1n-7 ratio of 0.187^{a}, 0.301^{a}, 0.566^{b} and 0.617^{b} (P<0.05, pooled standard error = 0.03), while plasma PC has 0.228^{a}, 0.263^{a}, 0.477^{a} and 0.972^{b} (P<0.05, pooled standard error = 0.03). Similarly, plasma SPH has 22:1n-9/22:1n-7 ratios of 0.125^{a}, 0.256^{a}, 0.303^{a} and 0.64^{b} (P<0.05, pooled standard error = 0.04) while it has a 24:1n-9/24:1n-7 ratio of 6.89^{a}, 8.4^{ab}, 13.1^{bc} and 15.0^{c} (P<0.05, pooled standard error = 0.68). SPH in erythrocyte ghosts had non-detectable levels of 22:1n-7 and 24:1n-7 in all groups.

TABLE 10

Effect of Dietary Zinc Deficiency on the Fatty Acid Composition (mol %) of Plasma Sphingomyelin (SPH)

Fatty acyl	Treatment group −Zn	+ZnRF	+ZnPF	+ZnAL	Standard error[a]
Saturated	$67.5^{b,c,A,B}$	$69.2^{A,B}$	70.1^{A}	65.8^{B}	0.4
Monounsaturated	25.9	24.4	24.3	26.1	0.3
24:1n-9	16.9^{A}	17.3^{A}	17.4^{A}	19.5^{B}	0.2
24:1n-7	$2.6^{d,A}$	$2.1^{A,B}$	$1.5^{B,C}$	1.1^{C}	0.1
Polyunsaturated	6.9^{A}	6.7^{A}	6.6^{A}	8.6^{B}	0.2
18:2n-6	0.37	0.46	0.46	0.24	0.04
24:2n-6	6.4^{A}	5.8^{A}	6.0^{A}	8.1^{B}	0.02
DBI[e]	$0.40^{A,B}$	$0.39^{A,B}$	0.38^{B}	0.44^{A}	0.01
Avg. carbon number	$21.27^{d,A}$	$21.33^{A,B}$	21.66^{B}	21.70^{B}	0.05

[a]Pooled standard error.

[b]Mean (n=6).

[c]Means that do not share a common superscript capital letter are significantly different by Tukey's test (p<0.05).

[d]Significantly different than +ZnPF by a least square means analysis (p<0.05).

[e]DBI, double bond index.

TABLE 11

Effect of Dietary Zinc Deficiency on the Fatty Acid Composition (mol %) of Plasma Free Fatty Acids (FFA)

Fatty acid	Treatment group −Zn	+ZnRF	+ZnPF	+ZnAL	Standard error[as]
Saturated	$29.7^{b,c}$	31.2	29.7	27.1	0.7
Monounsaturated	26.6	28.6	25.0	26.3	0.5
Polyunsaturated	43.7	40.2	45.3	46.6	0.8
Total n-6	$42.3^{A,B}$	38.7^{A}	$44.1^{A,B}$	45.5^{B}	0.8
18:2n-6	35.4^{e}	27.1	32.4	33.9	1.1
20:4n-6	$5.5^{d,e,A}$	$9.7^{A,B}$	10.2^{B}	$9.2^{A,B}$	0.6
22:4n-6	0.70^{A}	0.74^{A}	0.68^{A}	1.17^{B}	0.05
22:5n-6	0.4^{e}	0.8	0.5	0.8	0.1
Total n-3	1.4	1.5	1.2	1.1	0.1
22:5n-3	0.21	0.16	0.12	0.24	0.02
22:6n-3	1.20	1.34	1.10	0.82	0.08
DBI[f]	1.33	1.38	1.44	1.47	0.02

[a]Pooled standard error.

[b]Mean (n=6).

[c]Means that do not share a common superscript letter are significantly different by Tukey's test (p<0.05).

[d]Significantly different than +ZnPF by a least square means analysis (p<0.05).

[e]Significantly different than +ZnRF.

[f]DBI, double bond index.

DISCUSSION

The role of zinc in lipid and fatty acid metabolism and in biomembranes has been reviewed recently (4). It is clear that the effect of dietary Zn deficiency on lipid metabolism in rats depends upon the age and sex of the rat, the composition of the diet and the length and severity of the deficiency. The model for short-term severe Zn deficiency in the rat used in our study is an extension of one of the original models that defined an interaction of Zn and EFA in the rat (2). Extensive data on the feed consumption and eating pattern of rats on this dietary regimen have been described (35,36); a discussion of the results is limited to this model of dietary Zn deficiency.

The lipid composition of the rat erythrocyte membrane is uniquely controlled. Mature erythrocytes are incapable of synthesizing cholesterol, phospholipids or fatty acids from simple precursors. The cholesterol content of the membranes of mature erythrocytes can be increased by assimilation from cholesterol-enriched plasma lipoproteins. The composition of the molecular species of individual phospholipids in mature erythrocytes can be altered by exchange and/or assimilation of PC and LPC from the plasma pool. Mature erythrocytes have a variety of base exchange reactions and phospholipases which could, in theory, modulate the relative concentration of individual phospholipid species within the membrane. Finally, free fatty acids can be taken up from plasma and incorporated into lysophospholipids (37–39). The data in this report must be interpreted with several facts kept in mind. First, though the dynamics of erythrocyte production (hematocrit/reticulocyte count) and the volume of individual red cells (MCV) is dramatically altered by the severe feed restriction that occurs in zinc deficiency in this experimental system (40), the 'age' of the circulating red

cells, as determined by cell density, is not significantly altered (40). Second, dietary zinc deficiency and severe feed restriction apparently have pronounced effects on plasma lipids and lipoprotein composition but this effect is difficult to precisely define because of the lability of the composition of the plasma lipid pool. An additional complication stems from the fact that Zn-adequate, pair-fed rats are 'meal-feeders', an eating pattern which has pronounced effects on lipid metabolism (41,42). However, a consistent finding in rats is that plasma cholesterol concentration is not elevated in dietary Zn deficiency, and, in some cases, is depressed relative to *ad libitum*-fed controls (4,5,43).

The relative concentration of phospholipids in rat plasma, described for *ad libitum*-fed zinc adequate rats, is in close agreement with the values reported by Diagne *et al.* (44) and Nelson (45). In rat serum, the major phospholipids are in descending order: PC > LPC > SPH > PI > PE. PS is virtually non-detectable in plasma, in agreement with the report of Diagne *et al.* (44) and Nelson (45), but in contrast with the report of Cunnane (46), which suggests that PC and PS are the two main phospholipid subclasses in serum. The LPC fraction of rat plasma has a fatty acid composition suggesting that a significant portion of the fraction is 2-position LPC. In fact, the single spot from our acid-TLC solvent plate can be resolved into two spots on an alkali one-dimensional plate. These spots have fatty acid compositions suggestive of 1- and 2-position LPC (data not shown). The phospholipid composition of the rat erythrocyte membrane is in close agreement with that reported by others (44,47). The cholesterol/phospholipid ratio in the erythrocyte membrane from our study is similar to the values reported by most researchers (48–52); however, it is less than that reported by Johanning and O'Dell (53). Our data on phosphatidylcholine fatty acid composition are similar to reported values (48,49, 53–55) and the summation of our data on PE plasmalogen plus PE (other) resembles the fatty acid composition of total PE reported by other workers (49,53–55). Rao *et al.* (54) report very high levels of 22 carbon PUFA in PI, while our data suggest that PI from rat erythrocytes has low levels of 22 carbon PUFA and consists largely of 18:0 and 20:4n-6 in approximately a 1:1 ratio. Our data and those of Rao *et al.* (54) are consistent in suggesting that PS is the phospholipid in erythrocyte membranes that is most enriched with 20:4n-6, containing roughly 50 mole % arachidonic acid. This may imply a special role for arachidonyl-containing PS or for molecular species of PS containing 20-carbon PUFA in both the 1- and 2-position in this membrane. The fatty acid composition we determined for sphingomyelin is similar to that reported by Rao *et al.* (54), but contains far more 24-carbon fatty acids than reported by Innis (49) and Moses and Craig (55). Sphingomyelin in erythrocyte membranes is apparently enriched with 24:2n-6 in the rat and other species when there is a high dietary intake of 18:2n-6 (54,56,57); the function of molecular species of sphingomyelin containing this unusual fatty acid is unknown (56,58–60). The unsaturation index of total erythrocyte membrane phospholipids is similar to the index that can be calculated from the data by Moses and Craig (55).

Dietary zinc deficiency in rats does not cause changes in lipid composition of the erythrocyte membrane that would predict an increased mobility of lipid phase probes. Of the three major indices that have some predictive value of membrane fluidity (Chol/PC, PC/SPH, and UI) (61,62), only the cholesterol/phospholipid ratio is significantly altered compared to *ad libitum*-fed, but not pair-fed, controls. However, in the erythrocyte membrane from Zn-deficient rats the cholesterol/phospholipid ratio is elevated, a condition which would predict a decreased mobility of spin or fluorescent probes. Cholesterol/phospholipid ratios have been reported to be elevated in liver (6,7,16), intestine (7), erythrocyte (53) and testes (6) membrane fractions from zinc-deficient and pair-fed, zinc-adequate control rats. This is consistent with reports of decreased fluidity of the lipid phase in liver microsomal membranes (6,14). The report of increased fluidity in erythrocyte membranes (15) is opposite to the effect predicted by an elevated cholesterol/phospholipid ratio. By contrast, dietary zinc deficiency, *per se*, results in a significantly lower unsaturation index of total membrane dimethylacetals. It does not alter the content of total membrane plasmalogens (data not shown). The impact of these changes is unknown. Recently, it has been demonstrated that plasmalogens may play a unique role in determining membrane fluidity (63). Finally, zinc-deficient and feed restricted rats may have relatively equivalent values of 'total' membrane fluidity but may have a significant alteration of membrane fluidity in the inner and outer layer of the membrane. Erythrocyte membranes are distinctly sided in their phospholipid composition; rat erythrocyte membranes have been reported to have 62–63% of their PC, all of their SPH and 20% of their GPE directed toward the outer surface (64,65). It has been demonstrated that erythrocyte membranes actively maintain this asymmetry by an energy-requiring process and this causes a distinctly lower 'fluidity' in the outer *vs.* the inner membrane leaflet (66,67). In zinc deficient and feed restricted rats, erythrocytes have an increased double bond index in their alkenylacyl GPE (Table 3) and a decreased double bond index (Table 8) in their SPH. This could result in decreased fluidity in the outer layer and an increased fluidity in the inner layer which are 'masked' when measuring total membrane fluidity by various methods. Such an alteration would have an unknown relationship to the alterations in cell size (40) and cell filterability (68) which are attributed to the effect of feed restriction in dietary zinc deficiency.

The changes in lipid composition seen in the erythrocytes from zinc-deficient rats give conflicting predictions about osmotic fragility (69–71) and fragility due to certain hemolysins (71). Osmotic fragility has been inversely correlated to 18:1 content of the total erythrocyte membrane phospholipids in the rat (47,72). In this study we have demonstrated that erythrocytes from zinc deficient rats have a significantly reduced ($P<0.05$) content of 18:1 in total erythrocyte membrane phospholipids; 7.54^{a}, 7.51^{a}, 7.86^{ab} and 8.38^{b} mole % in −Zn, +ZnRF, +ZnPF and +ZnAL rats, respectively. However, the increased osmotic fragility of erythrocytes of zinc-deficient rats has been demonstrated to be reversed by two days of zinc supplementation *in vivo* (53); our data suggest that total phospholipid 18:1 is not increased by zinc supplementation over this time period. An elevated cholesterol/phospholipid ratio has generally been associated with decreased osmotic fragility of erythrocytes (52,73,74). In both this study and another, erythrocytes of zinc-

deficient rats, while having increased osmotic fragility (70,71), have been shown to have significantly elevated cholesterol/phospholipid ratios (53). Finally, erythrocytes from zinc-deficient rats which are also deficient in vitamin E have increased H_2O_2-induced fragility as compared to zinc-adequate, vitamin E-deficient controls (9). Membranes from erythrocytes of zinc-deficient, six-week-old rats are enriched in 22-carbon n-3 PUFA. These fatty acids in phospholipids are likely to be the most extensively peroxidized in a membrane fraction (75,76); vitamin E has been suggested to be more effective in protecting n-6 PUFA from peroxidation than n-3 PUFA (75). The 22-carbon n-3 PUFA content of erythrocyte membranes may be a variable in the observed effects of zinc deficiency on membrane peroxidative fragility (9,77).

The major effects of dietary zinc deficiency on the fatty acids of phospholipids in the erythrocyte membrane can be attributed to the effects of the voluntary drop in food intake associated with the deficiency (78). In short, these effects are dominated by elevated 22-carbon n-3 PUFA and depressed 22-carbon n-6 PUFA relative to *ad libitum*-fed controls. Effects on sphingomyelin, in plasma and erythrocyte membranes, are marked by a depression of the mole % of 24:2n-6. The effect on PC in plasma and red cells is unique, 18:2n-6 is elevated and 20:4n-6 is depressed (53). These general effects can be characterized as an inhibition of the developmental shift to 22-carbon n-6 PUFA from 22-carbon n-3 PUFA in rapidly growing rats fed diets containing 10% corn oil. For example the total phospholipid n-3 PUFA/n-6 PUFA ratio is 2.38 in rat erythrocyte from weanling rats (experimental time zero), while it is 5.20^a in ZnD, 5.08^a in ZnR, 5.81^bin ZnPF and 9.17^c in ZnAL. Similarly, the large effect of feed restriction on the unsaturation index of the total membrane dimethylacetals is 0.17 in time zero, while it is 0.22^a in ZnD, 0.23^{ab} in ZnR, 0.27^b in ZnPF and 0.36^c in ZnAL. Growth inhibition caused by a lack of food intake impairs normal developmental processes associated with the dietary fat. A similar phenomenon may occur with the 20-plus carbon n-9/n-7 monoenes, because of the abundance of 18:1n-9 relative to 16:1n-7 in corn oil. The dampening effect of dietary Zn deficiency on diet-induced changes in tissue fatty acid composition has recently been reported; rats fed fat-free diets have a slower rate of arachidonate depletion and Mead acid build-up when they are zinc-deficient compared to zinc-adequate controls (79). The lack of food intake associated with dietary zinc deficiency resists the influence of dietary fat composition on cell membrane lipid composition. Our data on the fatty acid composition in the erythrocyte membrane and on blood plasma support the concept that zinc deficiency has minimal effects on PUFA distribution in the major phospholipids of the erythrocyte membrane relative to pair-fed controls. However, a relatively normal PUFA distribution may be maintained in erythrocytes of Zn-deficient rats by depleting arachidonic acid in plasma and in other pools (Tables 9 and 11). Refeeding zinc in the diet while limiting food intake for a 3-day period caused no significant change in erythrocyte membrane lipid composition but caused a normalization of the mole % of plasma FFA 20:4n-6 and an elevation of the plasma PC/SPH ratio. The biochemical basis for the interaction of zinc and EFA, in this model of short-term, severe dietary Zn deficiency (2), remains an enigma.

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Effect of Dietary α-Linolenate/Linoleate Balance on Endotoxin-Induced Hepatitis in Mice

Shiro Watanabe* and **Harumi Okuyama**
Department of Biological Chemistry, Faculty of Pharmaceutical Sciences, Nagoya City University, Mizuhoku, Nagoya, 467 Japan

Mice were fed diets with three different ratios of α-linolenate (18:3n-3) to linoleate (18:2n-6), and the severity of hepatitis during endotoxic shock was compared. Dietary enrichment with α-linolenate increased the severity of hepatitis and the mortality induced by lipopolysaccharide (LPS) in combination with D-galactosamine (GalN). Differences in the dietary α-linolenate/linoleate balance were mainly reflected in the levels of arachidonate and eicosapenatenoate in liver phospholipids. Pretreatment of mice with indomethacin was found to also enhance the severity of GalN/LPS-hepatitis. This indicated that cyclooxygenase products of arachidonate may suppress the development of GalN/LPS-hepatitis. The enhancement by high α-linolenate diets was not observed when a lethal dose of LPS in the absence of GalN was given. Our results indicate that there are pathophysiological conditions of endotoxin-induced hepatitis under which cyclooxygenase products of arachidonate play protective roles.
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Bacterial lipopolysaccharide (LPS) is known to cause endotoxic shock in experimental animals; this effect is accompanied by widespread tissue damage (1,2). The circulating levels of prostaglandins and thromboxane are elevated during the development of endotoxic shock, and the pathological changes can be reduced with cyclooxygenase/lipoxygenase inhibitors (3-6). These observations suggested that eicosanoids derived from arachidonate (20:4n-6) stimulated the development of the LPS-induced endotoxic shock.

Several species of rodents are known to be relatively resistant to LPS, but treatment with LPS plus D-galactosamine (GalN) considerably increases their sensitivity to the toxic effect of LPS (7). This GalN/LPS-induced endotoxin shock model is reported to exhibit characteristics common to human fulminant hepatitis (8). In this GalN/LPS model, several lipoxygenase inhibitors were shown to be effective in suppressing the development and lethality of hepatitis (8-10).

Linoleic acid (18:2n-6) and α-linolenic acid (18:2n-3) are desaturated and elongated to form arachidonate (20:4n-6) and eicosapentaenoate (20:5n-3) (EPA), respectively. Both linoleate and α-linolenate compete with each other in the elongation/desaturation systems (11). Both arachidonate and EPA are precursors for eicosanoid synthesis. However, EPA is known to competitively inhibit the incorporation of arachidonate into phospholipids (12) and the conversion of arachidonate into eicosanoids (13,14). In addition, eicosanoids derived from eicosapentaenoate are generally less potent than those derived from arachidonate (13-15). Therefore, the relative proportions of linoleate and α-linolenate in diets could directly affect the proportions of arachidonate and eicosapentaenoate in cellular phospholipids, thereby modulating the eicosanoid balance and resultant cellular physiology (16,17).

The purpose of the present study was to clarify whether or not the dietary α-linolenate/linoleate balance affects the severity of tissue damage during endotoxic shock. A mouse hepatitis model was used in which hepatitis was induced by LPS in the presence and absence of D-galactosamine.

MATERIALS AND METHODS

Animals and feeding protocols. Three-week-old, male C57BL/6 and ICR mice (Sizuoka Laboratory Animal Co., Ltd., Shizuoka, Japan) were randomly divided into three groups and were fed diets with three different ratios of α-linolenate to linoleate (Table 1) for 5-6 weeks and 4-5 weeks, respectively. The test diets were supplemented with: i) α-linolenate rich perilla seed oil; ii) linoleate rich safflower seed oil; or iii) a mixture (65:35, w/w) of perilla oil and safflower oil. The basal diet was prepared by extracting conventional laboratory diet (CE-2, Nippon Clea Co., Tokyo, Japan) with n-hexane by a method commonly used to prepare vegetable oils, and then supplementing the defatted diet with a vitamin mixture (2%, w/w) (Nippon Clea Co., Tokyo, Japan) and the above-mentioned oils (10%, w/w) (18,19). The vitamin mixture supplement contains (per 100 g diet) vitamins A (2400IU), D_3 (480IU), B_1 (3 mg), B_2 (3 mg), B_6 (2 mg), B_{12} (10 μg), E (20 mg), K (0.4 mg), biotin (0.2 mg), dl-pantothenic acid (4 mg), *p*-aminobenzoic acid (20 mg), nicotinamide (20 mg), inositol (30 mg), folic acid (0.4 mg) and choline chloride (600 mg). The validity of using these diets was assessed by examining the reproductive physiology and the growth rates of

TABLE 1

Fatty Acid Composition of Experimental Diets

Fatty acids	High α-linolenate (perilla seed oil)	Mixed diet	High linoleate (safflower seed oil)
	(% of total fatty acid)[a]		
14:0	0.1	0.1	0.1
16:0	7.9	8.1	8.1
18:0	1.8	1.8	2.3
18:1n-9[b]	17.3	15.6	11.8
18:2n-6	17.1	37.0	74.2
18:3n-3	51.7	33.9	1.0
20:4n-6	0.1	0.1	—
20:5n-3	0.7	—	0.7
22:6n-3	0.3	0.3	0.3
18:3n-3/18:2n-6 ratio	3.0	0.9	<0.1

[a]The fatty acid composition was analyzed by gas-liquid chromatography (18,19).

[b]The position of double bond numbered from the methyl terminus is designated as n-9, n-6 or n-3.

*To whom correspondence should be addressed.

Abbreviations: ALT, alanine aminotransferase; EPA, eicosapentaenoate; GalN, D-galactosamine; LPS, lipopolysaccharide; SDH, sorbitol dehydrogenase.

the offspring (20). The diets had peroxide values below 50 meq/kg.

Induction of endotoxic shock and the assessment of hepatitis. In C57B1/6 mice, GalN (Sigma, St. Louis, MO) and LPS (*Salmonella abortus equi*, Sigma) dissolved in physiological saline were injected i.p. at doses of 300 mg/kg and 10 μg/kg, respectively. The severity of the hepatitis was evaluated by measuring alanine aminotransferase (ALT) and sorbitol dehydrogenase (SDH) (21,22) in serum from treated mice 5 hr after injection of GalN/LPS. In ICR mice, 833 mg GalN/kg was required to induce an acute hepatitis by LPS at a dose of 33 μg/kg, and the severity of hepatitis was evaluated 6 hr after the administration of GalN/LPS. Mortality was determined at LPS doses of 0.33, 3.3 and 33 μg/kg in combination with 833 mg GalN/kg in ICR mice fed the test diets. When LPS alone was used, higher doses of LPS (−10 mg/kg) were necessary to induce a significant hepatitis. In this endotoxin shock model, serum ALT activity was measured at 24 hr and the mortality was observed for up to 72 hr after the i.p. injection of LPS. In one experiment, LPS from *Eschericha coli* (055: B5, Difco, Detroit, MI) was also used.

Indomethacin treatment. One hour prior to injection of GalN/LPS, indomethacin (Wako Pure Chemical Co., Osaka, Japan) dissolved in 1/15 M phosphate buffer (pH 8.0) was injected i.p. at doses of 1 and 10 mg/kg into ICR mice (nine-weeks-old) fed a conventional laboratory diet (CE-2, Clea, Tokyo, Japan).

Analysis of fatty acid composition of liver phospholipids. At the end of feeding the test diets, mouse livers were harvested and stored at −80°C until analyzed. The liver was homogenized with chloroform/methanol (1:2, v/v), and the total lipids were extracted according to the method of Bligh and Dyer (23). Phospholipid fractions were separated on Silica Gel thin-layer chromatography (TLC) using a solvent system of chloroform/methanol/acetic acid/water (25:15:4:2, by vol). Phosphatidylethanolamine and phosphatidylcholine were located by spraying with an ethanolic Rhodamine solution (0.03%, w/v) and were recovered from the adsorbent by extracting with chloroform/methanol/6%-ammonium hydroxide (6:5:1, v/v/v). Fatty acids in phosphatidylethanolamine and phosphatidylcholine were transesterified by heating with 5% HCl/methanol (100°C, 1 hr) and were analyzed by gas-liquid chromatography on a capillary column coated with Supelcowax-10 (0.25 mm diameter, 30 m length, Supelco Ltd., Bellefonte, PA). Fatty acid methyl esters were identified by comparing the retention times with those of authentic standards (Supelco). Heptadecanoate (17:0) was used as an internal standard.

Statistical analyses. Statistical analyses of the data were carried out using Student's *t*-test. The values for the serum ALT and SDH activities in mice injected with GalN/LPS were logarithmically transformed and then analyzed, while those in mice injected with LPS alone were directly analyzed. Mortality rates were compared by using Fisher's exact test.

RESULTS

Effect of dietary fatty acids on GalN/LPS-induced hepatitis. Serum ALT and SDH levels of C57B1/6 mice were elevated more than 100- and 85-fold, respectively, at 5 hr after i.p. injection of GalN/LPS (300 mg/kg and 10 μg/kg) (Figs. 1a and 1b) in all the dietary groups. Serum ALT and SDH activities were found to be significantly higher in the high α-linolenate group than in the high-linoleate group ($p<0.01$ and $p<0.05$, respectively). The group fed the mixed diet showed intermediate levels of both enzyme activities, although the differences were not statistically significant. Contrary to C57BL/6 mice, ICR mice did not develop acute hepatitis at a GalN dose of 300 mg/kg, even when the dose of LPS was increased to 1.5 mg/kg (data not shown). However acute hepatitis was induced in ICR mice by injecting a higher dose of GalN (833 mg/kg) in combination with 33 μg LPS/kg. Thus, the responsiveness to GalN/LPS in terms of inducing acute hepatitis was different between the two strains of mice. Nevertheless, similar differences in the severity of GalN/LPS-induced hepatitis were observed among the three dietary groups; the serum ALT activities 6 hr after GalN/LPS injection in ICR mice fed the high α-linolenate diet (1023 ± 1181 U/L, n = 8) and the mixed diet (659 ± 472 U/L, n = 8)

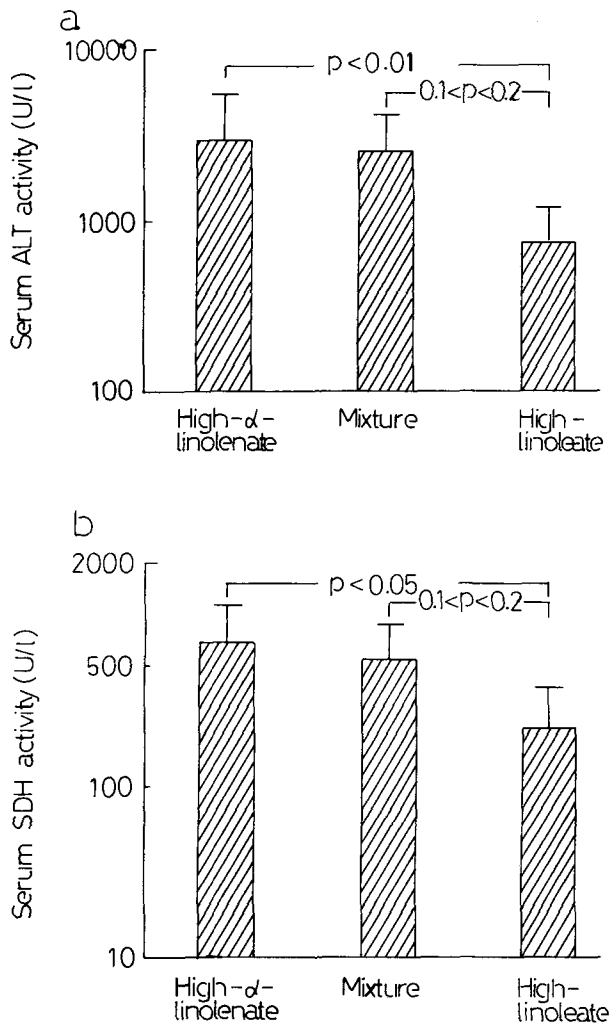


FIG. 1. Serum alanine aminotransferase (ALT) (a), and sorbitol dehydrogenase (SDH) (b), activities in male C57BL/6 mice 5 hr after i.p. injection of GalN/LPS (300 mg/kg and 10 μg/kg). From 8 to 9 mice in each group were used. The serum ALT and SDH activities from untreated mice were below 30 and 10 U/L, respectively. There was no difference in serum ALT and SDH activity in the untreated mice among the dietary groups tested.

were significantly higher than the ALT activity of mice fed the high linoleate diet (236 ± 174 U/L, n = 8, $p<0.05$).

Since diets enriched in α-linolenate increased the severity of GalN/LPS-induced hepatitis, the mortality during the development of hepatitis was also compared among the three dietary groups (Fig. 2). When LPS was injected at doses of 33 and 3.3 μg/kg in combination with 833 mg GalN/kg, more than 80% of the treated mice died within 7 to 12 hr after GalN/LPS injection (Figs. 2a and 2b). However, at an LPS dose of 0.33 μg/kg combined with 833 mg GalN/kg, mice in the high α-linolenate group and the mixture group died more rapidly after GalN/LPS injection than in the high-linoleate group (Fig. 2c). Cumulative mortalities 24 hr after GalN/LPS injection were statistically higher both in the high α-linolenate and the mixed diet groups ($p<0.02$ and $p<0.05$, respectively) than in the high linoleate diet group.

Fatty acid compositions of liver phospholipids. The fatty acid composition of liver phosphatidylethanolamine samples from ICR mice fed the three test diets are shown in Table 2. Differences in the dietary α-linolenate/linoleate ratios did not cause changes in the proportions of unsaturated and saturated fatty acids in the phospholipids or in the liver contents of phosphatidylethanolamine. The α-linolenate/linoleate ratios of diets were, however, reflected in the proportions of n-3 and n-6 polyunsaturated fatty acids in the phospholipids. The most notable differences among the three dietary groups were found in the levels of arachidonate (20:4n-6) and eicosapentaenoate (20:5n-3), with a smaller difference in the proportions of 22:5n-3. The docosahexaenoate (22:6n-3) contents were essentially unchanged by these diets. Similar differences in the fatty acid composition were also found in phosphatidylcholine of liver from mice fed the three test diets (data not shown).

Effect of indomethacin pretreatment of hepatitis induced by GalN/LPS. The findings that dietary enrichment with α-linolenate reduced arachidonate levels in liver phospholipids and accelerated GalN/LPS-induced hepatitis suggested that arachidonate metabolites play protective roles ameliorating the development of GalN/LPS-induced hepatitis. Therefore, we evaluated the effect of pretreatment with indomethacin, a cyclooxygenase inhibitor, on the severity of GalN/LPS-induced hepatitis. Pretreatment

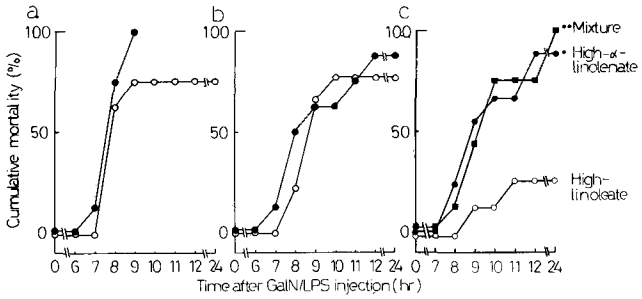


FIG. 2. Mortality of male ICR mice injected with LPS. Eight to nine mice were injected with LPS at doses of (a) 33, (b) 3.3 and (c) 0.33 μg/kg in combination with GalN (833 mg/kg). Cumulative mortalities were not different between the high α-linolenate (●) and the high linoleate (○) groups (a and b). At an LPS dose of 0.33 μg/kg (c), cumulative mortalities in the high α-linolenate group and the mixture group (■) were significantly higher(*, $p<0.02$; and **, $p<0.005$; respectively) than in the high linoleate group.

TABLE 2

Fatty Acid Compositions of Liver Phosphatidylethanolamine from Mice Fed the Test Diets

Fatty acids	High α-linolenate (n=3)	Mixed (n=3)	High linoleate (n=3)
	(% of total fatty acids)[a]		
14:0	0.1 ± 0.1	0.1 ± 0	0.1 ± 0
16DMA	0.3 ± 0.1	0.5 ± 0.1	0.4 ± 0.1
16:0	15.9 ± 0.4	17.5 ± 1.4	15.7 ± 0.7
16:1	0.4 ± 0.1	0.3 ± 0.2	0.3 ± 0.1
18DMA	0.3 ± 0.1	0.2 ± 0.1	0.3 ± 0.1
18:0	23.3 ± 0.9	23.2 ± 1.6	23.5 ± 2.0
18:1	8.3 ± 0.3	6.9 ± 1.2	7.1 ± 0.8
18:2n-6	8.0 ± 1.8	8.2 ± 1.7	8.5 ± 1.1
18:3n-6	0.4 ± 0.1	0.3 ± 0.1	0.4 ± 0.1
18:3n-3	1.5 ± 0.1[b,d]	0.8 ± 0.2[c]	ND
20:0	0.4 ± 0.1	0.4 ± 0.2	0.3 ± 0.2
20:1n-9	0.3 ± 0.2	0.3 ± 0.1	0.2 ± 0.2
20:3n-6	0.8 ± 0.1	1.1 ± 0.1	0.9 ± 0.2
20:4n-6	6.3 ± 0.4[b,d]	12.9 ± 0.9[c]	23.1 ± 1.3
20:4n-3	0.6 ± 0.1[b,d]	ND	ND
20:5n-3	11.8 ± 1.7[b,d]	4.0 ± 1.4[c]	ND
22:4n-6	ND	0.1 ± 0.1[c]	0.4 ± 0.1
22:5n-6	0.4 ± 0.2[b]	0.4 ± 0.4	0.9 ± 0.3
22:5n-3	1.9 ± 0.1[b,d]	1.3 ± 0.1[c]	0.2 ± 0.1
22:6n-3	19.2 ± 0.8[b]	21.5 ± 1.7[c]	17.4 ± 0.5
Unsaturated/ saturated ratio	1.5 ± 0.1	1.4 ± 0.1	1.5 ± 0.1
Total n-6	15.9 ± 2.2[b,d]	23.1 ± 1.3[c]	34.3 ± 1.0
Total n-3	35.0 ± 2.5[b,d]	27.5 ± 2.1[c]	17.6 ± 0.6
Fatty acid content[e]	3.3 ± 0.2	2.9 ± 0.3	2.9 ± 0.9

[a] Fatty acid composition was analyzed by gas-liquid chromatography using a capillary column as described in the text in detail.

[b] $p<0.05$, High α-linolenate *vs.* high linoleate groups.

[c] $p<0.05$, Mixture *vs.* high linoleate groups.

[d] $p<0.05$, High α-linolenate *vs.* mixture groups.

[e] mg Fatty acids/g of liver.

Abbreviations used are: DMA, dimethylacetal, ND, not detected.

of mice fed a conventional laboratory diet with doses of indomethacin of either 1 or 10 mg/kg was found to significantly accelerate the GalN/LPS (833 mg/kg and 33 μg/kg)-induced hepatitis in a dose-dependent manner (Fig. 3a). The mortality induced by a lower dose of LPS (0.33 μg LPS-833 mg GalN/kg) tended to be enhanced by the pretreatment with indomethacin, but the difference was not statistically significant (Fig. 3b). These results were inconsistent with the observation of protective effects of eicosanoid synthesis inhibitors on hepatic damages induced by LPS alone. Therefore, we next examined the effect of the dietary α-linolenate/linoleate balance on hepatitis induced by injection of LPS alone.

By using male ICR mice fed a conventional laboratory diet, the minimum dose of LPS needed to induce 100% death was found to be 10 mg/kg LPS *(Salmonella abortus)* and 20 mg/kg LPS *(Escherichia coli)*. The onset of death was delayed 12 hr beyond the LPS injection. The severity of hepatitis was evaluated 24 hr after the injection of 10 mg/kg of LPS *(S. abortus)*. Only a moderate increase in ALT activities was observed and there was no statistically significant difference among the three dietary groups (Table 3). The mortalities induce by LPS *(S. abortus)* and LPS *(E. coli)* were followed for 72 hr (Fig. 4). No

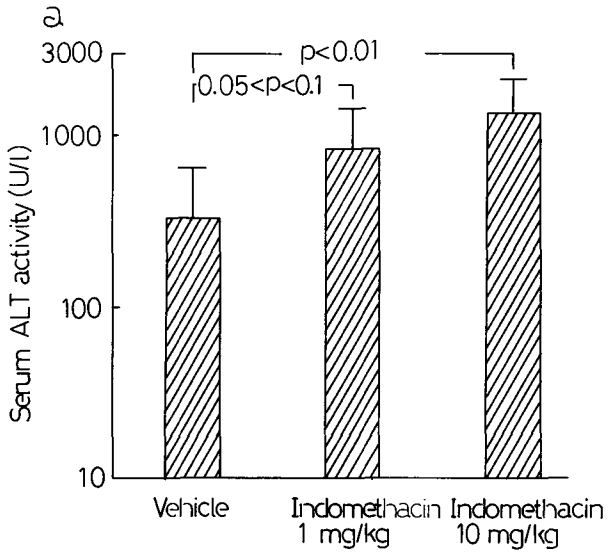


TABLE 3

Serum Alanine Aminotransferase (ALT) Activity 24 hr after i.p. Injection of LPS Alone

Untreated control (6)[a]	LPS-treated groups[b]		
	High α-linolenate group (9)	Mixed (9)	High linoleate group (9)
	Serum ALT activity (U/L)		
26.2 ± 9.7	69.4 ± 45.6[c]	51.6 ± 13.9[e]	44.8 ± 14.4[d]

[a]Two mice each from the three dietary groups were injected with saline.

[b]LPS was injected i.p. at a dose of 10 mg/kg.

[c]$p<0.05$.

[d]$p<0.02$.

[e]$p<0.01$ *vs.* untreated group.

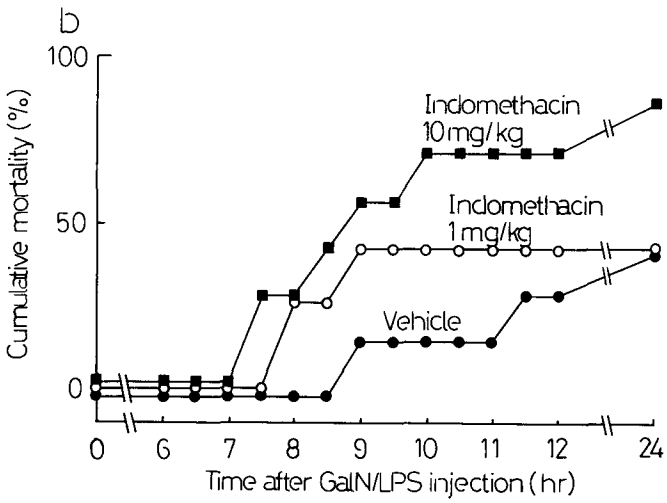


FIG. 3. Effects of pretreatment of mice with indomethacin on GalN/LPS-induced hepatitis. Male ICR mice fed a conventional laboratory diet were injected i.p. with indomethacin (1 and 10 mg/kg, i.p.) 1 hr prior to the injection of GalN/LPS. The serum ALT activity (a) 6 hr after i.p. injection of GalN/LPS (833 mg/kg and 33 μg/kg) in the high dose group (10 mg indomethacin/kg, n=6) was significantly higher ($p<0.01$) than in the vehicle group (n=7). However, statistically significant difference was not observed between the low dose group (1 mg indomethacin/kg, n=6) and the vehicle group ($0.05 < p < 0.1$). Mortalities (b) in the low dose (n=7) and high dose (n=7) groups tended to be higher than in the vehicle group (n=7), but the differences were not statistically significant.

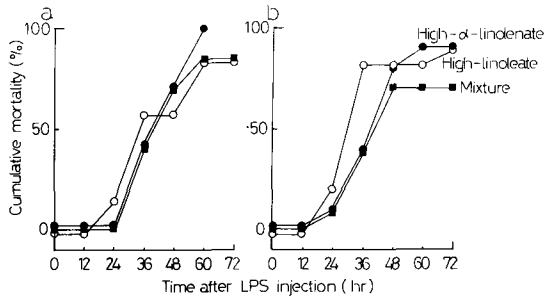


FIG. 4. Mortality of male ICR mice fed the test diets after i.p. injection of LPS alone. Male ICR mice fed the test diets were injected i.p. with LPS from *S. abortus* (a) or *E. coli* (b) at doses of 10 or 20 mg/kg, respectively. There was no difference in the mortality rates among the three dietary groups.

difference was found in the mortality rates of the three dietary groups when LPS *(S. abortus)* was used. With LPS *(E. coli)*, the patterns of the mortality rates were generally similar to those induced with LPS from *S. abortus*, but the rate was slightly higher in the high-linoleate group than in the other two dietary groups at 36 hr after the injection of LPS *(E. coli)* ($0.05<p<0.01$).

DISCUSSION

In general, dietary n-3 fatty acids suppress the production of eicosanoids from n-6 fatty acids; moreover, 20 carbon n-3 fatty acids are themselves converted to eicosanoids with relatively lower physiological activities. In the present experiments, dietary enrichment with α-linolenate was shown to exert relatively little effect on LPS *(S. abortus)*-induced hepatitis and a slight suppressive effect on LPS *(E. coli)*-induced hepatitis (Fig. 4); these same effects are seen when mice are pretreated with low doses of aspirin (4).

Elevated responsiveness of mice to LPS toxicity induced by GalN treatment results from enhancement of host susceptibility to tumor necrosis factor (TNF) (24), a known crucial mediator of LPS toxicity (25,26). TNF production in macrophages is stimulated by the 5-lipoxygenase products of arachidonate, the leukotrienes (27). In contrast, prostaglandin E_2, one of the major cyclooxygenase products of macrophages, is reported to be an endogenous suppressor of macrophage TNF production (28), and plays protective roles in target organs (29). Furthermore, the prostaglandin I_2/thromboxane A_2 balance is known to affect the severity of endotoxic shock (4). Thus, the balance of cyclooxygenase and lipoxygenase products of arachidonate appears to affect the severity of hepatitis.

Both dietary enrichment with n-3 fatty acids and indomethacin pretreatment enhanced the severity of GalN/LPS-induced hepatitis in mice (Figs. 1–3), which suggests that these treatments suppressed the production of protective eicosanoids, such as prostaglandin E_2 and I_2, relatively more than that of stimulatory eicosanoids, such as leukotrienes. Consistent with this interpretation is the

observation that feeding with fish oils enriched in n-3 fatty acids suppressed prostaglandin E_2 production effectively, but not leukotriene B_4 production, in mouse peritoneal macrophages, leading to the enhancement of the footpad anaphylactic response (30). Another study also showed that fish oil supplement affected B_4/B_5 ratios of leukotrienes in macrophages (31). In fact, the protective effects of prostaglandin E_2 or E_1 on viral hepatitis have been shown both in mice (32) and in humans (29). Very recently, we demonstrated that α-linolenate rich diet increased LPS-induced TNF production in mouse peritomeal macrophages (33).

Recent studies in our laboratory have revealed beneficial effects of feeding high α-linolenate diets in preventing several kinds of chronic diseases in animals (18,34-38). However, as shown in the present study, feeding a high α-linolenate diet had a detrimental effect in GalN/LPS-induced hepatitis. Although the applicability of GalN/LPS-induced hepatic damage in mice to human diseases is not certain, these results establish that there are pathophysiological conditions under which eicosanoids derived from n-6 fatty acids play protective roles.

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Study on the Lipid Composition of Aging Fischer-344 Rat Lymphoid Cells: Effect of Long-Term Calorie Restriction

Serge Laganiere[1] and **Gabriel Fernandes***
Department of Medicine, The University of Texas Health Science Center at San Antonio, San Antonio, Texas 78284-7874

Long-term calorie restriction (LCR) is widely known to increase the survival rate of laboratory rodents and appears to retard the aging and senescence process. The present study was undertaken in Fischer-344 male rats maintained on *ad libitum* (AL) or LCR (40% less food intake than AL starting at 6 weeks of age). Age-associated changes in the proliferative response of lymphoid cells to mitogenic stimuli were studied in relation to alterations in the fatty acid composition of adherent and non-adherent-enriched subpopulations of spleen cells. Increases in spleen cell long-chain highly unsaturated fatty acids (20:4, 22:4 and 22:5) were accompanied by decreases in linoleic acid (18:2) in aging AL-fed rats. However, LCR stabilized levels of 18:2 and prevented the rise in highly unsaturated fatty acids. In addition, LCR markedly modulated the fatty acid profiles of thymocytes and bone marrow cells. A 70% decline in concanavalin A (Con A) stimulated [^{3}H]thymidine uptake of spleen cells from AL animals was normalized by LCR. Splenic reduced glutathione (GSH), a potential modulator of the mitogenic response, was unaffected by age and nutritional regimen. Thus, normalization of lymphoid cell fatty acid composition by LCR parallels the preservation of mitogenic responsiveness to Con A.
***Lipids 26,* 472–478 (1991).**

Long-term calorie restriction (LCR) is a powerful nutritional intervention that increases both the mean and maximum lifespan of laboratory rodents. LCR retards, attenuates or abolishes the age-related decline of many immunological (1–3) and physiological functions (4,5) as well as autoimmune disease and malignant diseases (6,7). The underlying mechanisms for prolongation of longevity by calorie restriction are still unknown. Recent reports have emphasized that LCR can influence age-associated changes in neuroendocrine hormones, free radicals, gene expression and several immune functions. Previous observations have demonstrated that LCR-induced delay in age-related loss of immune function correlates with preservation of lymphoid cell proliferative responsiveness in various strains of mice and rats (3,8,9).

Reactivity of lymphoid cells can be affected by dietary fatty acid composition (10–13). Therefore, we hypothesized that the age-related decline in immune response may be associated with age-related alterations in the lymphoid cell subsets and their composition of fatty acids and that LCR can reverse age-related immune deficiency by modulation of both Interleukin-2 (IL-2) receptors and IL-2 production (14,15). Our hypothesis is also based primarily on our recent finding that LCR can prevent age-related changes in subcellular membrane lipid composition of liver tissue (16). The present detailed studies show effects of diet and aging on lymphoid cell fatty acid composition, mitogenic response and GSH levels. Implications of age-related changes in membrane lipids and the role of LCR in maintaining immune function throughout the lifespan are further discussed in this paper.

MATERIALS AND METHODS

Animals and diet. Male specific-pathogen-free Fischer-344 rats (Charles River Lab, Portage, MI), 28 ± 2 days of age were maintained in a barrier facility. They were housed singly in plastic cages with wire mesh floors suspended on the Hazelton-Enviro Rack System (Hazelton Systems, Inc., Aberdeen, MD). The procedures for monitoring and operating the barrier facility and composition of the diets (Table 1) have been reported in detail previously (5).

All weanling rats were fed Diet A (Ralston-Purina, St. Louis, MO) *ad libitum* (AL) until six weeks of age. Afterwards, only rats belonging to the AL-fed group were continued on Diet A until they were sacrificed. Calorie restricted rats (LCR) were provided Diet B in daily allotments, 60% of the mean amount of diet consumed by the AL-fed group cohort, which was measured regularly from 6 weeks until 18 months of age. Thereafter, the caloric intake of the LCR rats was maintained at the 18-month level of intake. Food intake, body weight, organ weight and lifetime caloric intake did not differ significantly from previous reports (5). Diet B was identical to Diet A, except that the concentration of dietary supplements (vitamin, minerals and choline, Ralson Purina) was modified in order to provide both groups of rats with the same intake. Dextrin concentration was adjusted to meet the restricted caloric requirements. Both AL-fed and LCR rats were provided their daily food allotment about 1 hr before the start of the dark phase of a 12-hr light/12-hr dark

TABLE 1

Composition of Diets[a]

Dietary components	Diet A (%)	Diet B (%)
Casein (vitamin-free)	21.00	21.00
Sucrose	15.00	15.00
Dextrin	43.65	39.55
Corn oil	10.00	10.00
Mineral mix	5.00	7.64
Vitamin mix	2.00	3.33
Choline chloride	0.20	0.33
DL-methionine	0.15	0.15
Solka-Floc	3.00	3.00

[a]The concentration of the vitamin, mineral mixes and choline chloride in Diet B was modified to provide a daily intake that is the same as the mean intake of the rats fed on Diet A.

[1]Present Address: Department of Pharmacology and Experimental Therapeutics, Loyola University Chicago, 2160 First Avenue, Maywood, IL 60153.

*To whom correspondence should be addressed at the Department of Medicine, The University of Texas Health Science Center at San Antonio, 7703 Floyd Curl Drive, San Antonio, TX 78284-7874.

Abbreviations: AL, *ad libitum*; Con-A, concanavalin A; FBS, fetal bovine serum; GSH, glutathione; IL-2, Interleukin-2; LCR, long-term calorie restriction; PBS, phosphate-buffered saline.

cycle in order to maintain their circadian-based normal feeding pattern throughout the study.

Lymphoid cell isolation. Rats under anesthesia were sacrificed by exsanguination. Spleen and thymus were aseptically collected only from those rats which were visibly tumor-free. Connective tissue was removed and single cell suspensions were prepared. Briefly, the minced tissue was gently homogenized in complete RPMI-containing medium at room temperature and the cell suspension was centrifuged at 100 × *g* and washed three times in RPMI. The cell suspension free of debris was placed on plastic Petri dishes, that had been coated previously with 10% fetal bovine serum (FBS), and was incubated for 2 hr at 37°C, and the non-adherent cell fraction was obtained by washing with warm (37°C) phosphate-buffered saline (PBS). Adherent spleen cells were scraped off and washed in cold PBS or RPMI at 4°C. To obtain a bone marrow-enriched fraction, femurs were removed and cleaned, and the bone cavity was tightly fitted to a needle and slowly flushed with cold PBS. Large clumps of cells were further dispersed through a 27-gauge needle, and cells were washed as described before. For each cell fraction, red blood cells were lysed using Tris/ammonium chloride solution.

Assessment of mitogenic response. Concanavalin A (Con-A) in appropriate serial dilutions, was used to measure *in vitro* mitogenic responses of splenocytes in microtiter plates as described previously (15).

Glutathione assessment. Total and reduced glutathione (GSH) were assayed spectrophotometrically by the enzymatic method of Anderson (17) and by the chemical method of Moron *et al.* (18), respectively.

Fatty acid analysis. Lipids were isolated by chloroform/methanol (2:1, v/v) extraction according to the procedure of Folch *et al.* (19). Extracted lipids were redissolved in chloroform/methanol (2:1, v/v). Fatty acid methyl esters were obtained by transesterification with methanolic HCl (90°C, 2 hr) (Alltech-Applied Science, Deerfield, IL). The esters were extracted and then redissolved in hexane and analyzed on a Hewlett-Packard Model 5880A gas chromatograph equipped with a fused silica megabore column (DB-225, J&W Scientific, Folsom, CA). Oven temperature was 205°C, the flame ionization detector was set at 250°C, and helium served as carrier gas at a flow rate of 9 mL/min. Peaks were identified by comparison of retention times with those of fatty acid standards (Nu-Check-Prep, Elysian, MN).

Data analysis. All data were compared by using multiple analyses of variance. Post-hoc analysis of individual group means was performed by the Duncan multiple-range test.

RESULTS

The body weights of the AL-fed and LCR animals are presented in Figure 1. LCR rats maintained a significantly lower body weight compared to AL animals throughout their lifespan. Although calories were restricted by 40%, LCR rats consumed between 0.13 and 0.15 kcal/g body weight/day after 6 months of age, which was very close to the amount of food consumed by the AL-fed rats (4,5). Therefore, it is of interest to analyze the possible relationship between immune function and the fatty acid

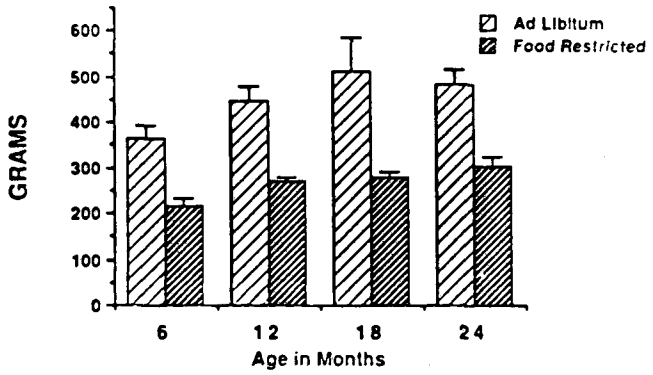


FIG. 1. Effect of aging and LCR on the body weights of rats.

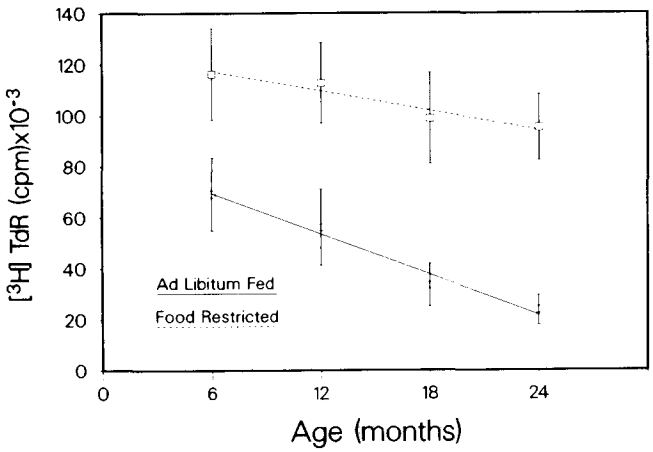


FIG. 2. Spleen cells from *ad libitum* and food restricted rats of various age groups were cultured at a concentration of 5 × 10^6 cells/mL for 48 hr in the presence of various levels of Con-A and pulsed with tritiated thymidine for a further 16 hr before harvest and thymidine uptake was determined. The results reported here are CPM obtained for optimal doses of Con-A (2.5 mg/mL) after subtracting background counts of medium alone, which ranged from 2000–4500 CPM.

composition of lymphoid cells from LCR rats and AL-fed controls (4,5).

The splenocyte mitogenic response to Con-A in aging rats is illustrated in Figure 2. The proliferative response decreased linearly ($r=0.59$, $p<0.005$) in AL-fed rats between the ages of 6 and 24 months. Approximately 70% of the proliferative response was lost during this age interval. In contrast, the slight reduction in Con-A-induced incorporation of [^{3}H]thymidine in LCR rat splenocytes was not statistically significant from 6 to 24 months of age (Fig. 2).

The effect of LCR on whole spleen fatty acid composition was first analyzed in animals of both dietary groups at 12 and 18 months of age and the results are presented in Table 2 and Figure 3. Spleen cell fatty acids were affected by calorie restriction in both age groups. Specifically, linoleic acid (18:2) was higher, whereas polyunsaturated fatty acyls such as arachidonic (20:4), docosatetraenoic (22:4) and docosapentaenoic (22:5) acids were lower in LCR rats as compared to AL rats (ANOVA

$p<0.01$, <0.05, <0.05 and <0.01, respectively). The saturated fatty acids 16:0 and 18:0 were also significantly lower in LCR rats than in AL-fed rats at 12 and 18 months of age ($p<0.05$ and <0.01, respectively.).

These findings were further investigated in isolated splenic lymphoid cells obtained from rats aged 6, 12, 18 and 24 months. Data pertaining to non-adherent spleen cells are presented in Table 3. None of the saturated fatty acids were modulated either by age or by calorie restriction. However, percent composition of 18:2, 20:4, 22:4 and 22:5 were found to be significantly affected by age in the lymphocyte-enriched fraction of aged AL-fed rats. Linoleic acid content decreased between 12 and 24 months of age, whereas 20:4, 22:4 and 22:5 levels increased steadily during this period in AL-fed animals ($p<0.01$, <0.01, <0.01 and <0.001, respectively). These age-related changes in fatty acid composition were completely prevented by LCR. Accordingly, linoleic acid was higher at 18 and 24 months ($p<0.01$) and 20:4 was significantly lower in the lymphocyte-enriched fraction of 24-month-old LCR rats. Levels of 22:4 and 22:5 remained significantly lower in LCR rats after six months of age.

Fatty acids of the adherent cells were also altered by age. The data presented in Table 4 and Figure 4 show that 18:1 and 18:2 progressively decreased from 6 to 24 months ($p<0.005$ and $p<0.001$, respectively) and that 22:4 ($p<0.01$) and 22:5 ($p<0.005$) levels increased steadily between 12 and 24 months of age in the AL-fed rats. Although 18:2 was also decreased with age in the LCR rats, levels of this fatty acid were consistently higher than in AL-fed rats, at all ages studied (ANOVA, $P<0.05$). Arachidonic acid content was significantly lower in LCR rats at 12, 18 and 24 months of age (ANOVA, $p<0.025$). However, no significant diet-related differences in 22:4 or 22:5 were noted during this age interval.

Interestingly, the fatty acid composition of bone marrow cells was not markedly altered by age in the AL-fed rats, as described in Table 5. On the other hand, except for 16:0, all of the major fatty acids were strikingly modulated by long-term calorie restriction. Higher levels of oleic and linoleic acids were found in LCR rat bone marrow cells at all ages tested (ANOVA, $p<0.01$). Also, 18:0, 20:4, 22:4 and 22:5 were all significantly lower in LCR rats at all ages tested (ANOVA, $p<0.01$, <0.005, <0.01, <0.005, respectively) and differences in contents were more accentuated at 24 months of age.

Data pertaining to thymocyte fatty acids are reported in Table 6 and Figure 5. Proportions of 18:1 and 18:2 were dramatically increased with age in both dietary groups,

TABLE 2

Effect of Long-Term Calorie Restriction on the Fatty Acid Composition of Spleen from Aging Rats[a]

Fatty acid	12 months		24 months	
	AL	LCR	AL	LCR
16:0	21.98 ± 1.45	20.43 ± 0.03	24.20 ± 1.30	19.94 ± 0.90
16:1	1.05 ± 0.10	1.00 ± 0.08	1.15 ± 0.10	0.816 ± 0.09
18:0	16.08 ± 0.31	14.43 ± 0.46	17.29 ± 0.35	15.54 ± 0.25
18:1	9.97 ± 0.20	10.45 ± 0.38	10.76 ± 0.36	10.59 ± 0.38
18:2	9.99 ± 0.13	11.98 ± 0.40	9.78 ± 0.12	11.10 ± 0.50
20:2	1.65 ± 0.10	1.84 ± 0.11	1.12 ± 0.10	2.18 ± 0.15
20:4	19.89 ± 0.59	7.98 ± 0.61	19.49 ± 0.51	15.79 ± 0.53
22:4	3.63 ± 0.23	3.04 ± 0.08	4.15 ± 0.35	3.30 ± 0.14
22:5	1.03 ± 0.10	0.64 ± 0.07	1.38 ± 0.10	0.62 ± 0.08
22:6	0.97 ± 0.34	1.72 ± 0.50	0.43 ± 0.02	0.64 ± 0.20

[a]Values represent mean ± S.E.M. of 4–5 independent observations. Results are expressed as percent composition. AL, *ad libitum* fed rats; LCR, long term calorie restricted rats. Diet effect (two-way ANOVA): 16:0, 20:4, 22:4 ($p<0.05$); 18:0, 18:2, 22:5 ($p<0.01$). Age effect: non-significant.

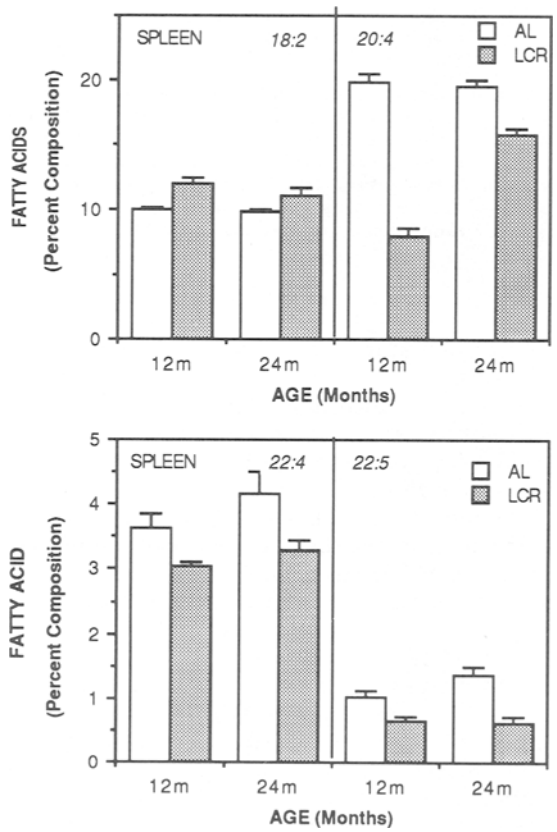


FIG. 3. Effect of age and LCR on the fatty acid composition of spleens of 12- and 24-month-old Fischer-344 rats.

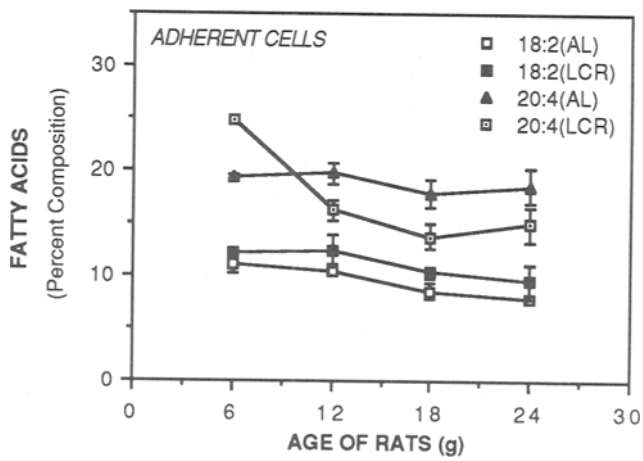


FIG. 4. Effect of LCR on 18:2 and 20:4 composition of splenic adherent cells in 6- to 24-month-old Fischer-344 rats.

TABLE 3

Effect of Long-Term Calorie Restriction on the Fatty Acid Composition of Splenic Non-Adherent Cells from Aging Rats[a]

Fatty acid	6 months		12 months		18 months		24 months	
	AL	LCR	AL	LCR	AL	LCR	AL	LCR
16:0	19.12 ± 1.28	20.63 ± 0.98	20.72 ± 0.65	19.98 ± 0.68	20.36 ± 0.93	18.85 ± 0.44	18.75 ± 1.12	20.71 ± 1.31
16:1	1.42 ± 0.24	1.26 ± 0.20	1.22 ± 0.27	0.87 ± 0.04	0.98 ± 0.06	0.93 ± 0.21	0.66 ± 0.04	0.71 ± 0.07
18:0	13.16 ± 0.58	16.15 ± 1.50	15.08 ± 0.65	15.19 ± 0.47	15.47 ± 0.56	15.92 ± 0.25	15.29 ± 0.75	15.72 ± 1.46
18:1	10.03 ± 1.04	10.05 ± 0.50	10.22 ± 0.39	10.07 ± 0.350	9.86 ± 0.24	10.18 ± 0.44	9.83 ± 0.51	11.00 ± 0.29
18:2	9.54 ± 1.48	9.94 ± 0.63	10.24 ± 0.76	10.34 ± 0.22	7.85 ± 0.13	10.34 ± 0.36	8.07 ± 0.16	9.22 ± 0.36
18:3	0.16 ± 0.04	0.00	0.70 ± 0.01	0.10 ± 0.02	0.10 ± 0.01	0.11 ± 0.02	0.82 ± 0.03	0.00
20:2	1.37 ± 0.53	2.39 ± 0.19	1.48 ± 0.10	1.75 ± 0.28	1.33 ± 0.15	1.87 ± 0.12	1.45 ± 0.20	0.97 ± 0.92
20:4	19:36 ± 0.67	20.26 ± 1.18	19.03 ± 0.69	19.12 ± 1.08	20.78 ± 0.25	20.26 ± 1.46	21.57 ± 1.05	16.75 ± 1.63
22:4	3.59 ± 0.18	3.75 ± 0.50	3.61 ± 0.31	3.16 ± 0.48	4.65 ± 0.24	4.05 ± 0.18	6.39 ± 1.11	4.24 ± 1.13
22:5	0.97 ± 0.06	0.78 ± 0.08	0.92 ± 0.05	0.70 ± 0.08	1.41 ± 0.12	0.72 ± 0.06	2.40 ± 0.47	0.82 ± 0.20
22:6	0.57 ± 0.07	0.56 ± 0.15	0.82 ± 0.08	0.33 ± 0.01	0.44 ± 0.05	0.56 ± 0.23	0.71 ± 0.08	0.55 ± 0.12

[a]Values represent mean ± S.E.M. of 5–6 independent observations. Results are expressed as percent composition. AL, *ad libitum* fed rats; LCR, long-term calorie restricted rats. Age effect in 12- to 24-month-old AL-fed rats (one-way ANOVA): 18:2, 20:4, 22:4 ($p<0.01$); 22:5 ($p<0.01$); 22:5 ($p<0.001$). Age effect in LCR rats: non-significant. Diet effect (two-way ANOVA and multiple-range test): 18:2 (at 18 and 24 months, $p<0.01$): 20:4 (at 24 months, $p<0.025$); 22:4 (from 12 to 24 months, $p<0.025$); 22:5 (at 12 months, $p<0.025$; at 18 and 24 months, $p<0.001$).

TABLE 4

Effecct of Long-Term Calorie Restriction on the Fatty Acid Composition of Splenic Adherent Cells from Aging Rats[a]

Fatty acid	6 months		12 months		18 months		24 months	
	AL	LCR	AL	LCR	AL	LCR	AL	LCR
16:0	15.37 ± 2.06	16.50 ± 2.47	17.86 ± 1.58	20.37 ± 1.62	16.90 ± 1.10	15.31 ± 1.90	18.44 ± 1.30	20.54 ± 1.75
16:1	0.94 ± 0.18	0.48 ± 0.03	1.01 ± 0.16	1.35 ± 0.35	1.06 ± 0.16	2.08 ± 0.72	1.12 ± 0.20	1.39 ± 0.03
18:0	15.57 ± 0.82	20.25 ± 1.76	15.63 ± 0.72	16.09 ± 0.75	16.56 ± 0.81	15.16 ± 1.30	15.46 ± 0.81	16.30 ± 0.76
18:1	12.89 ± 1.31	13.51 ± 0.80	10.54 ± 0.75	10.83 ± 1.92	9.77 ± 0.73	9.82 ± 0.77	9.00 ± 0.56	11.95 ± 2.20
18:2 (6)	11.17 ± 0.96	12.23 ± 0.43	10.50 ± 0.55	12.39 ± 1.60	8.58 ± 0.67	10.36 ± 0.49	7.78 ± 0.46	9.46 ± 1.60
20:2 (6)	2.01 ± 0.45	4.22 ± 1.70	1.92 ± 0.50	2.68 ± 0.53	1.55 ± 0.16	1.96 ± 0.40	±0.25 ± 0.25	±0.20 ± 0.20
20:4 (6)	19.32 ± 0.25	24.70 ± 1.14	19.74 ± 1.00	16.22 ± 1.22	17.83 ± 1.21	13.73 ± 2.60	18.52 ± 1.65	14.96 ± 2.60
22:4 (6)	3.77 ± 0.49	6.95 ± 0.27	3.40 ± 0.15	3.55 ± 0.18	4.33 ± 0.53	3.12 ± 0.59	5.29 ± 0.35	6.76 ± 0.09
22:5 (3)	0.96 ± 0.12	1.62 ± 0.18	1.04 ± 0.09	0.89 ± 0.15	1.48 ± 0.42	0.69 ± 0.09	1.90 ± 0.30	0.86 ± 0.11
22:6 (3)	0.96 ± 0.14	0.00	0.66 ± 0.10	0.00	0.00	1.10 ± 0.37	0.00	0.00

[a]Values reprsent mean ± S.E.M. of 5–6 independent observations. Results are expressed as percent composition. AL, *ad libitum* fed rats; LCR, long-term calorie restricted rats. Age effect in Al-fed rats (one-way ANOVA): 18:1, 18:2, (from 6 to 24 months, $p<0.005$): 22:4, 22:5 (from 12 to 24 months, $p<0.01$); 20:4, non-significant. Age effect in LCR rats: 18:2 (from 12 to 24 months, $p<0.05$). Diet effect (two-way ANOVA): 18:2 (from 6 to 24 months, $p<0.05$): 20:4 (from 12 to 24 months, $p<0.025$); 22:4, 22:5, non-significant.

TABLE 5

Effect of Long-Term Calorie Restriction on the Fatty Acid Composition of Bone Marrow Cells from Aging Rats[a]

Fatty Acid	6 months		12 months		18 months		24 months	
	AL	LCR	AL	LCR	AL	LCR	AL	LCR
16:0	21.28 ± 0.59	19.91 ± 1.06	19.86 ± 1.10	20.01 ± 0.34	20.78 ± 0.54	20.07 ± 0.36	18.51 ± 1.83	20.49 ± 0.75
16:1	1.54 ± 0.12	2.50 ± 0.23	1.17 ± 0.11	1.36 ± 0.13	1.38 ± 0.08	1.27 ± 0.06	0.97 ± 0.15	1.54 ± 0.24
18:0	12.49 ± 0.33	10.80 ± 1.11	13.60 ± 0.56	12.60 ± 0.70	12.88 ± 0.70	13.25 ± 1.11	16.21 ± 0.06	10.71 ± 1.00
18:1	16.54 ± 0.53	21.55 ± 0.70	13.37 ± 0.80	16.05 ± 1.25	15.57 ± 1.36	15.80 ± 1.46	13.86 ± 0.42	18.06 ± 2.00
18:2	16.13 ± 0.68	19.54 ± 1.03	13.16 ± 0.80	16.50 ± 1.26	14.80 ± 1.25	16.63 ± 1.30	13.38 ± 0.46	18.43 ± 2.80
18:2	0.23 ± 0.01	0.26 ± 0.04	0.25 ± 0.05	0.25 ± 0.07	0.37 ± 0.10	0.26 ± 0.06	0.07 ± 0.05	0.33 ± 0.08
20:2	1.09 ± 0.05	0.74 ± 0.04	0.96 ± 0.16	0.93 ± 0.04	0.88 ± 0.09	1.08 ± 0.12	1.30 ± 0.09	0.56 ± 0.06
20:4	15.50 ± 0.42	11.75 ± 0.70	18.43 ± 1.23	15.75 ± 1.00	15.70 ± 1.23	15.10 ± 1.58	19.83 ± 0.37	9.53 ± 0.60
22:4	2.72 ± 0.10	1.90 ± 0.09	2.88 ± 0.14	2.40 ± 0.13	2.41 ± 0.26	2.45 ± 0.26	3.26 ± 0.27	1.85 ± 0.24
22:5	0.87 ± 0.03	0.65 ± 0.03	1.03 ± 0.08	0.71 ± 0.03	1.10 ± 0.08	0.68 ± 0.03	1.90 ± 0.20	0.49 ± 0.07
22:6	0.51 ± 0.13	0.35 ± 0.04	0.51 ± 0.10	0.33 ± 0.02	0.77 ± 0.23	0.19 ± 0.01	0.42 ± 0.03	1.25 ± 0.43

[a]Values reprsent mean ± S.E.M. of 5–6 independent observations. Results are expressed as percent composition. AL, *ad libitum* fed rats; LCR, long-term calorie restricted rats. Age effect in AL-fed rats (one-way ANOVA): 22:5 (from 6 to 24 months, $p<0.001$); all other fatty acids, non-significant. Diet effect (two-way ANOVA from 6 to 24 months): 18:0, 18:1, 18:2, 22:4 ($p<0.01$); 20:4, 22:5 ($p<0.005$).

TABLE 6

Effect of Long-Term Calorie Restriction on the Fatty Acid Composition of Thymocytes from Aging Rats[a]

Fatty Acid	6 months		12 months		18 months		24 months	
	AL	LCR	AL	LCR	AL	LCR	AL	LCR
16:0	22.56 ± 0.43	22.18 ± 0.58	22.27 ± 1.03	20.65 ± 1.50	22.05 ± 2.26	20.80 ± 1.84	26.81 ± 1.68	22.37 ± 1.16
16:1	1.58 ± 0.17	1.69 ± 0.21	2.20 ± 0.35	2.07 ± 0.33	1.52 ± 0.21	2.05 ± 0.34	2.31 ± 0.23	2.39 ± 0.36
18:0	15.41 ± 0.53	14.95 ± 0.61	8.76 ± 1.73	11.22 ± 1.33	9.10 ± 1.20	10.47 ± 1.95	6.16 ± 0.05	8.99 ± 0.63
18:1	12.66 ± 0.54	12.28 ± 0.43	22.12 ± 2.10	18.73 ± 1.14	22.66 ± 0.06	22.39 ± 2.25	26.85 ± 1.46	26.23 ± 1.47
18:2	8.57 ± 0.46	8.12 ± 0.32	22.65 ± 2.66	16.57 ± 1.10	23.90 ± 1.92	20.16 ± 2.43	25.52 ± 1.70	20.01 ± 1.60
18:3	0.00	0.00	0.22 ± 0.06	0.26 ± 0.05	0.21	0.68 ± 0.00	0.15 ± 0.01	0.40 ± 0.13
20:2	2.46 ± 0.31	2.06 ± 0.04	0.61 ± 0.10	1.44 ± 0.18	0.91 ± 0.26	0.57 ± 0.10	0.02 ± 0.01	0.00
20:4	20.47 ± 1.00	19.31 ± 1.06	8.22 ± 2.10	11.81 ± 0.94	6.98 ± 2.31	3.14 ± 0.48	3.34 ± 0.06	3.44 ± 0.93
22:4	1.89 ± 0.16	2.00 ± 0.18	1.52 ± 0.30	1.71 ± 0.26	2.95 ± 1.17	0.76 ± 0.10	1.15 ± 0.07	0.83 ± 0.24
22:5	0.51 ± 0.06	0.45 ± 0.03	0.47 ± 0.10	0.51 ± 0.20	0.83 ± 0.07	0.34 ± 0.13	0.47 ± 0.02	0.35 ± 0.04
22:6	0.00	0.00	0.12 ± 0.03	1.36 ± 0.39	0.53 ± 0.04	0.16 ± 0.07	0.28 ± 0.04	0.24 ± 0.05

[a]Values represent mean ± S.E.M. of 5–6 independent observations. Results are expressed as percent composition. AL, *ad libitum* fed rats; LCR, long-term calorie restricted rats. Age effect in AL-fed rats (one-way ANOVA from 6 to 24 months): 18:0, 18:1, 18:2, 20:2, 20:4 ($p<0.001$). Age effect in LCR rats: 18:0, 18:1, 18:2, 20:2, 20:4, 22:4 ($p<0.001$). Diet effect (two-way ANOVA from 12 to 24 months): 18:0, 18:2 ($p<0.005$).

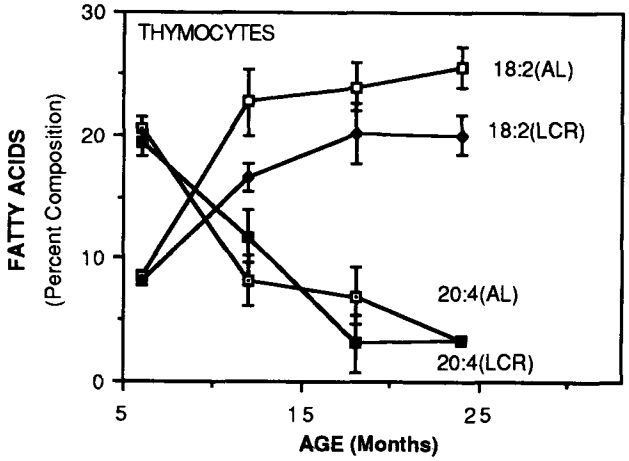


FIG. 5. Influence of LCR on 18:2 and 20:4 levels in thymocytes of 3- to 24-month-old Fischer-344 rats.

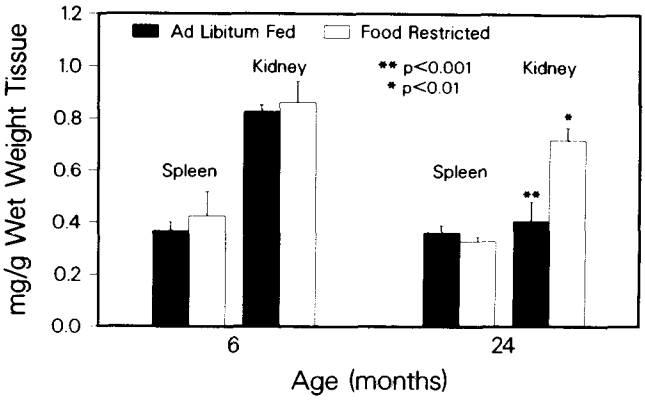


FIG. 6. Effects of age and food restriction on glutathione reductase activity in spleen and kidney tissues obtained from 6- and 24-month-old Fischer-344 male rats maintained on *ad libitum* and food restricted dietary regimens.

whereas 18:0, 20:2 and 20:4 were decreased (one-way ANOVA, $p<0.001$ for each fatty acid). No age-related change in proportions of 22:4 or 22:5 were observed in AL-fed rats, although 22:4 was significantly decreased with age in LCR rats. Diet-related changes in 16:0, 18:0, 18:1 and 20:4 failed to reach significance. However, 18:2 content was significantly lower in thymocytes of 12-, 18- and 24-month-old LCR rats.

Because reduced GSH modulates blastogenesis, we decided to measure GSH levels in the whole spleen supernatant fraction. The data presented in Figure 3 indicate that reduced GSH concentration, which was measured at 6 and 24 months of age in both dietary groups, remained constant at approximately 0.35 mg/g wet weight of spleen. This finding was not replicated in the kidney as shown in Figure 6. Reduced GSH concentration was lowered by 50% in the kidney supernatant of 24-month-old AL-fed rats. In contrast, reduced GSH was only slightly lowered ($p>0.05$) in LCR rat kidney. These results strongly suggest that regulation of spleen GSH concentration is well sustained in aged Fischer-344 male rats as opposed to the aging kidney in the same rats.

DISCUSSION

Our results confirm and extend previous observations that aging alters fatty acid distribution in organs and cells. Our studies of major lymphoid organs indicate that, except in thymocytes, the aging effect showed the following pattern of change: an increase in long chain highly unsaturated fatty acids concomitant with losses in linoleic acid levels. LCR counteracted the age-related loss of 18:2 in spleen cells and bone marrow cells. Therefore, long-term calorie restriction stabilized the fatty acid content of aging animals, and forestalled the loss of mitogenic responsiveness in splenocytes of Fischer-344 rats. Our results confirm the findings of Cheung and Richardson (20), who described the loss of immune function with age in Fischer-344 rats. Hence, alterations of splenocyte fatty acids may underlie the modulation of the response to mitogens in aging rats.

This postulate is in agreement with the paradigm that immune cell reactivity is directly influenced by the enrichment of lymphoid cell membranes with particular fatty acids (20,21). Specifically, membrane enrichment in linoleic acid was associated in various instances with immunosuppression (22–24), as seen in dietary-supplemented multiple sclerosis patients (21,25), experimental allergic encephalomyelitis (26) and rejection of skin allografts (23). Immunosuppression is most likely to occur through the subsequent increase in the content of membrane phospholipid arachidonic acid, which usually results from high linoleic acid supplementation (27). Arachidonic acid is the principal desaturated-elongated derivative of the essential fatty acid, linoleic acid. It is released from membrane phospholipids by the action of phospholipase A_2 (28,29), and is then taken up as precursor for the 2 series prostaglandins (PG) (30).

The PG suppresses blastogenesis, lymphokine production and cytotoxicity (31), especially in the spleen, which is a major site for the production of immunologically active PG (32). In the aging AL-fed rats, increasing membrane content of 20:4 may augment precursor availability for PG release, which may interfere with mitogenic responsiveness of non-adherent spleen cells. Conversely, maintenance of adequate 20:4 content in aging LCR rat lymphoid cells appears to regulate the synthesis of PG, and thereby prevent or delay the fall in splenocyte immune responsiveness.

Most importantly, modulation of cellular fatty acids by LCR was most prominent in the aging bone marrow. The mechanism underlying this finding is not clear, but the bone marrow is the major source of all lymphoid cells for distribution to the organism. Thus, changes in the fatty acid composition of bone marrow lymphoid precursors may be one of the mechanisms by which LCR regulates the lipid composition of mature lymphoid cell subsets, particularly the populations which reside in the spleen.

Because of the strikingly similar trend in the changes reported previously in liver subcellular membrane fatty acids (15,33) and the present results that desaturation of 18:2 into long-chain polyunsaturated derivatives is accelerated with age, this may be a general phenomenon that is inhibited efficiently by chronic calorie restriction. It has been shown earlier that serum 18:2 concentration decreased with age in AL-fed rats (34). Long-term calorie restriction has also been found to alter the profile of serum fatty acids in aging Fischer-344 rats and to reduce the loss of serum 18:2 levels with age (34). The effects of long-term calorie restriction on the fatty acid composition of lymphoid cells is of particular interest since approximately the same amount of food is consumed per gram of body weight by both the dietary groups throughout their lifespan (5,7). The present study further supports the evidence that age-related changes in serum fatty acids are also reflected in the lymphoid cell fatty acid composition, and this finding is in agreement with previous reports (21,25,35).

The marked increase in 18:2 content observed after six months of age for thymus gland reflects the gradual accumulation of adherent cells and surrounding fat tissue which is rich in 18:1 and 18:2 that accompanies the early onset of involution and degeneration of this organ (36). Interestingly, the age-related increase in 18:2 was significantly blunted in LCR rats, in agreement with the previous view that either the function of the thymic tissues or its age related involution may have been forestalled by long-term protein and/or calorie restriction (37–39).

Although the role of reduced GSH in immune activity is still unresolved (40), an increase in lymphocyte proliferation to mitogen stimulation has been reported after enhancing intracellular GSH levels (41). Furthermore, Chaplin and Wedner (42) have shown that depletion of intracellular GSH inhibited [^{3}H]thymidine incorporation and lymphocyte blastogenesis. Therefore, we speculated that possibly higher levels of splenic reduced GSH might influence the sustained proliferative response of aging LCR rats (Fig. 1). A previous report also showed that calorie restriction was able to blunt the age-related loss of reduced GSH in liver (43). Our data suggest that reduced GSH alone probably may not account for diminished lymphoid responsiveness to Con A as lymphoid cells from LCR and AL animals had similar levels of reduced GSH, but markedly different mitogenic responsiveness. However, in the present study, a significant age-related loss of kidney GSH was noted for AL rats which was markedly prevented by calorie striction (Fig. 6). Indeed, caloric restriction is known to reduce renal disease both in Fischer-344 rats as well as in short-lived autoimmunity prone mice (5–7). It appears that reduced GSH may play a role in modulating the renal disease, but more defined studies are required to confirm such a possibility in caloric restricted animals.

In conclusion, our study has provided new evidence that lymphoid cell membrane fatty acids are characteristically altered with age and offers clues regarding one of the possible mechanisms by which long-term calorie restriction prevents the age-dependent decline in lymphoid cell immune reactivity. Calorie restriction may prevent or forestall the increase of suppressor PG production in adherent cell populations. Another consideration (possibly related) is that LCR rats show a higher rate of induction for high affinity IL-2 receptors than AL-fed rats (15). The changes in fatty acid composition measured in this study for non-adherent cells may also relate to preservation of IL-2 receptors and their functions (14,15).

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METHODS

Headspace Gas Chromatography of Volatile Lipid Peroxidation Products from Human Red Blood Cell Membranes

E.N. Frankel* and **A.L. Tappel**
Department of Food Science and Technology, University of California, Davis, California 95616

An improved headspace capillary gas chromatographic (GC) method was developed to measure the oxidative susceptibility of human red blood cell (RBC) membranes. This method analyzed volatile peroxidation products of both n-6 (hexanal and pentane) and n-3 (propanal) polyunsaturated fatty acids. Oxidative susceptibility tests were standardized by incubating in a sealed 10-mL headspace bottle 0.25 or 1 mL of human RBC membrane in 40 mM phosphate buffer for 1 hr at 37°C with a mixture of Fe^{++}, ascorbic acid and H_2O_2. Sodium dodecyl sulfate increased significantly the amount of hexanal measured by headspace GC. By this standard headspace method, in one series of red blood cell membranes (RBCM) samples a four-fold variation in oxidative susceptibility was observed in RBCM from blood freshly drawn from six healthy subjects. In another series of RBCM samples a sixteen-fold variation in oxidative susceptibility was noted in frozen RBCM from blood freshly drawn from five healthy subjects. Correlation between hexanal formation and polyunsaturated fatty acids (PUFA) depletion provided good evidence that under these standard conditions hexanal is exclusively derived from the oxidation of arachidonic acid. Hydroperoxides of arachidonic acid are more readily formed and decomposed than those of linoleic acid in the presence of Fe^{++}, ascorbic acid and H_2O_2 to produce hexanal as the main product that can be readily analyzed by headspace GC. This method may provide a useful tool to study susceptibility toward lipid peroxidative damage in human RBC membranes.
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Many *in vitro* studies indicate that free radical stress can damage hemoglobin (1,2), membrane lipids, and proteins (3–5). Polyunsaturated fatty acids (PUFA) and oxidative susceptibility vary in human red blood cells (RBC) in health and disease. Lipids of red blood cell membranes (RBCM) vary in fatty acid patterns with dietary fat (6). The susceptibility of RBC to lipid peroxidation increases by deficiencies in vitamin E (3), in glucose-6-phosphate dehydrogenase (7,8), or by the presence of abnormal hemoglobin (2,5,9). The genetic disease sickle cell anemia lowers the vitamin E content of the RBCM and increases the susceptibility of RBCM to lipid peroxidation (10–13). Neonatal erythrocytes undergo significant lipid peroxidation apparently because of lower levels of vitamin E in the plasma, deficiency of glutathione peroxidase, and increased levels of PUFA and cellular hemoglobin (14).

*To whom correspondence should be addressed.

Abbreviations: AA, ascorbic acid; AAPH, 2,2′-azo*bis*(2-aminidopropane) dihydrochloride; EDTA, ethylenediaminetetraacetic acid; FA, total fatty acids; GC, gas chromatography; PUFA, polyunsaturated fatty acids; RBC, red blood cells; RBCM, red blood cell membranes; SDS, sodium dodecyl sulfate; TBA, thiobarbituric acid; TBARS, thiobarbituric acid reactive substances.

Oxidative stress in RBC was induced by H_2O_2 in sodium azide to inhibit catalase (15), by sodium nitrite causing the formation of methaemoglobin (16), by adding mixtures of $FeSO_4$ and ethylenediaminetetraacetic acid (EDTA) (17), *tert*-butyl hydroperoxide (1), *tert*-butyl hydroperoxide in the presence of hemoglobin (18), cumene hydroperoxide in the presence of hemin-Fe^{3+} (19), and Fe^{2+}, ascorbate and H_2O_2 (20). PUFA in the lipids of human and rat RBCM were oxidized exclusively in the presence of an azo initiator, 2,2′-azo*bis*(2-aminido-propane) dihydrochloride (AAPH), after an induction period during which α-tocopherol was depleted (21). Oxidation of RBC with AAPH resulted in hemolysis, which developed when the tocopherol concentration fell below a critical low level (22). The final oxygen uptake correlated better with PUFA than the initial oxygen uptake in RBCM of cord, mother and healthy adults (23).

Measurements of the susceptibility of RBC toward oxidative stress were based on determinations of thiobarbituric acid reactive substances (TBARS) (15,24), fluorescent products (19,25), volatile hydrocarbons (16,17), and oxidized protein products (26,27). Better methods are needed to measure oxidative stress (28). The popular thiobarbituric acid (TBA) test is not only strongly influenced by reaction conditions, but the results obtained by different modifications of the test in human serum can vary as much as ten-fold (29). Because of lack of specificity, Halliwell and Gutteridge (30) do not recommend the TBA test for human biological material.

To elucidate how lipid oxidation products act in the complex mechanism of lipid repair in RBCM, a more specific assay than the TBA test would be desirable. Recently a rapid headspace gas chromatographic (GC) method was described for the determination of hexanal as an important volatile decomposition product of n-6 PUFA peroxidation in rat liver samples (31). This method was used to study effects of dietary lipids and vitamin E on *in vitro* lipid peroxidation in rat liver and kidney (32) and on *in vivo* lipid peroxidation in rats induced by iron (33).

The present paper describes an improved headspace capillary GC method for the determination of volatile peroxidation products of n-6 PUFA (pentane and hexanal) and n-3 PUFA (propanal). This method was used to determine the susceptibility of human RBCM to lipid peroxidation.

MATERIALS AND METHODS

Materials. Hexanal and propanal used as standards for the headspace GC analyses were purified by passing a 0.5-mL sample of each (Aldrich Chemical Co., Milwaukee, WI) through a Waters Sep-Pak silica cartridge (Waters Associates, Milford, MA) and sealing it in a serum bottle under nitrogen. Ferrous sulfate (10 mM $FeSO_4$, Fisher Scientific, Fairlawn, NJ) solutions used as oxidant were prepared fresh daily. Ascorbic acid (0.1 M) and hydrogen

peroxide (0.1 M, Fisher Scientific) solutions were stored at 4°C.

Red blood cell membranes (RBCM). Samples of freshly drawn and outdated packed blood cells were obtained from the Sacramento Blood Center (Sacramento, CA). The method of Burton *et al.* (34) was used to prepare white or pink RBCM. For some preparations, the final concentration of each sample was equalized to the same protein content by dilution with 40 mM phosphate buffer. All analyses of volatile oxidation products and fatty acids were based on the initial volume of packed blood cells and expressed per mL RBC. Final RBCM solutions were stored either at 0°C for oxidative susceptibility assays made within 24 hr, or stored at −5°C for assays made after 24 hr.

Headspace capillary GC method. A procedure was developed to determine hexanal, propanal and pentane within 6 min, using a Perkin-Elmer Sigma 3B gas chromatograph with an H-6 headspace sampler (Norwalk, CT), a capillary DB-1701 column (30 m × 0.32 mm, 1 μm thickness, J & W, Folsom, CA) heated isothermally at 80°C. The GC conditions were: He linear gas velocity, 20 cm/sec (He pressure gauge of 60 psi); splitless injector temperature, 180°C; and detector temperature, 200°C.

Samples of 0.25 or 1.0 mL RBCM (containing 0.9 to 2.1 mg protein/mL RBC) diluted in 40 mM phosphate buffer and standard solutions of hexanal were measured into special headspace 10-mL bottles sealed with silicone rubber Teflon caps with a crimper, and incubated in a water-bath shaker at 37°C for 60 min. After incubation, the bottles were retained with an insulated holder, inserted manually through the stationary injection needle in the headspace sampler, and pressurized for 30 sec before injection.

A method was standardized in which pentane, propanal and hexanal were separated with respective retention times of 1.18, 1.42 and 5.10 min. A fourth peak of retention time of 2.90 min, which corresponded to pentanal, was detected in highly oxidized samples of RBCM. Standards of 20 and 30 nmol hexanal in 1 mL 40 mM phosphate buffer were analyzed daily with each group of experimental samples. A linear standard curve was obtained with a series of headspace bottles containing 2.5 to 12.5 nmol hexanal per mL 40 mM phosphate buffer. Regression analysis gave correlation coefficients of 0.98, $p < 0.005$, for the increase in GC peak areas with hexanal concentration. Standards of 20 nmol and 30 nmol propanal were analyzed in 1 mL 40 mM phosphate buffer. Standards of 20 and 30 nmol pentane, made up in 95% ethanol/water (1:1, v/v), were analyzed in 1 mL 40 mM phosphate buffer.

Lipid analyses. Total lipids were extracted with ethanol, hexane, and sodium dodecyl sulfate (SDS) (35). An aliquot of the lipids was methylated with acetyl chloride (36) in the presence of tridecaheptadecanoin added as internal standard. Fatty acid methyl esters were separated by GC (Hewlett-Packard model 5890, Avondale, PA, equipped with a flame ionization detector) with a polar DB-225 capillary column (50% cyanopropylphenyl, J & W, 30 m × 0.25 mm) and identified by comparison with authentic standards (NuChek Prep, Elysian, MN).

Other determinations. The protein content of RBCM samples was estimated by the method of Bradford (37). Hemoglobin was determined in fresh RBCM by the hemochrome method of Fuhrhop and Smith (38) and values ranged between 0.2 and 0.8 mmol/mg protein, averaging 0.4 (n = 7).

RESULTS

The previously described headspace GC method for hexanal in rat liver samples (31) was improved by using a special headspace attachment with a stationary injection system to obviate the use of gas-tight syringes, which are prone to leakage after extensive use. A more polar column was also used to separate the early peaks and to resolve pentane and propanal. This improved method was first applied to different samples of human RBCM obtained from outdated packed cells. Lipid peroxidation was induced by various amounts of Fe^{++}. In contrast to previous results obtained with rat liver tissues (31), addition of ascorbic acid to RBCM decreased the catalytic effect of Fe^{++} (Table 1). At concentrations of 10 and 50 mM, ascorbic acid alone also decreased the amount of volatiles compared to the control. Although the relative amounts of pentane and pentanal produced were much smaller than the amount of hexanal, they correlated well with the corresponding amounts of hexanal formed ($r = 0.94$ and 0.91, respectively). As expected, the correlation coefficient between hexanal and propanal was lower ($r = 0.80$).

The combination of Fe^{++}, ascorbic acid and H_2O_2 was very effective in inducing lipid peroxidation in RBCM. A typical gas chromatogram of a sample of RBCM oxidized with Fe^{++}, ascorbic acid and H_2O_2 is shown in Figure 1. The GC peak preceding pentane was not identified, but was found in all samples including blanks and may be due to some atmospheric contaminant.

The addition of excessive ascorbic acid decreased the amount of peroxidation. The ratio of Fe^{++}, ascorbic acid and H_2O_2 was optimized. At constant concentrations of

TABLE 1

Susceptibility of Red Blood Cell Membranes (RBCM) to Lipid Peroxidation with Fe^{++} and Ascorbic Acid (AA) Measured by Headspace Capillary Gas Chromatography[a]

Treatment[b]	Pentane	Propanal	Pentanal	Hexanal
	(nmol/mL RBC)			
Control	1.4	0	0	9.4
+ 50 μM Fe	6.4	3.9	3.9	28
+ 50 μM Fe + 25 mM AA	1.5	1.9	0	11
+ 2.5 mM AA	1.4	1.6	1.3	11
+ 10 mM AA	1.2	1.1	1.1	8.7
+ 50 mM AA	1.9	0.6	0	7.6
+ 100 μM Fe	1.7	1.9	1.3	12
+ 250 μM Fe	2.8	2.4	2.8	21
+ 500 μM Fe	4.4	3.0	3.0	23
+ 500 μM Fe + 2.5 mM AA	2.9	2.5	2.7	17
+ 500 μM Fe + 10 mM AA	2.0	3.2	2.1	13
+ 500 μM Fe + 50 mM AA	1.0	1.9	1.1	9.2

[a] Volatiles determined after incubation for 1 hr at 37°C. Values represent the mean of two determinations.

[b] RBCM was prepared from outdated packed cells, frozen and thawed before the oxidative susceptibility test. This sample contained 1.28 mg protein/mL RBC. Fe^{++} was added as ferrous sulfate.

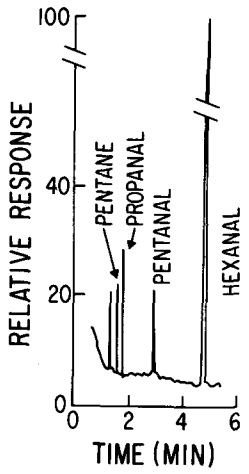


FIG. 1. Capillary gas chromatogram of a 1-mL sample of red blood cell membrane (containing 1.6 mg protein/mL RBC) in 40 mM phosphate buffer after incubation 1 hr at 37°C in the presence of 0.1 mM Fe^{++}, 0.25 mM ascorbic acid, and 0.5 mM H_2O_2.

ascorbic acid (1 mM) and H_2O_2 (2 mM), hexanal formation increased with Fe^{++} concentration up to a maximum between 0.4 and 0.5 mM (Fig. 2). Hexanal formation showed a sharp maximum at 1 mM ascorbic acid, and decreased at concentrations of 2 and 4 mM. At constant levels of ascorbic acid (1 mM) and Fe (0.4 mM), hexanal formation also increased with H_2O_2 concentration. To minimize the H_2O_2 concentration, a molar ratio of Fe/ascorbic acid/H_2O_2 of 1:2.5:5 was chosen for studies to test the susceptibility of RBCM to lipid peroxidation under standardized conditions.

Copper salts were compared with iron salts as catalysts for the oxidation of RBCM. In the presence of 0.2 mM metal, 0.5 mM ascorbic acid and 1 mM H_2O_2, three times more hexanal was produced with $FeSO_4$ than with $CuSO_4$ (Table 2). In the presence of 0.2 mM metal and 0.5 mM ascorbic acid, twice as much hexanal was formed with $FeSO_4$ as with $CuSO_4$. Therefore, Fe^{++} is a better catalyst than Cu^{+} for the oxidation of RBCM as measured by hexanal formation.

The peroxidation of RBCM under different conditions was investigated to optimize a method to estimate biological susceptibility to peroxidative damage. One sample of frozen RBCM was treated with various concentrations of Fe^{++}/ascorbic acid/H_2O_2. A linear relationship was obtained between hexanal formation and Fe^{++}/ascorbic acid/H_2O_2 concentrations varying from 0.05 to 0.4 mM Fe^{++}, 0.1 to 1 mM ascorbic acid, and 0.25 to 2.0 mM H_2O_2. Plots of hexanal formation *versus* incubation time at 37°C were initially linear and the slope decreased after 1 to 2 hr.

Other factors investigated included the effect of phosphate buffer concentrations and surfactants. No significant difference was noted in hexanal formation between 2.5 and 40 mM phosphate, and the use of a 40 mM phosphate buffer was adopted. Tween-20 and sodium dodecyl sulfate (SDS) were tested as surfactants in the oxidation of RBCM. In the presence of 0.5 and 1% Tween-20, a small increase in hexanal formation was observed with 0.2 mM Fe^{++}, 0.5 mM ascorbic acid and 1 mM H_2O_2, but a significant decrease in hexanal was noted with 0.4 mM Fe^{++}, 1 mM ascorbic acid and 2 mM

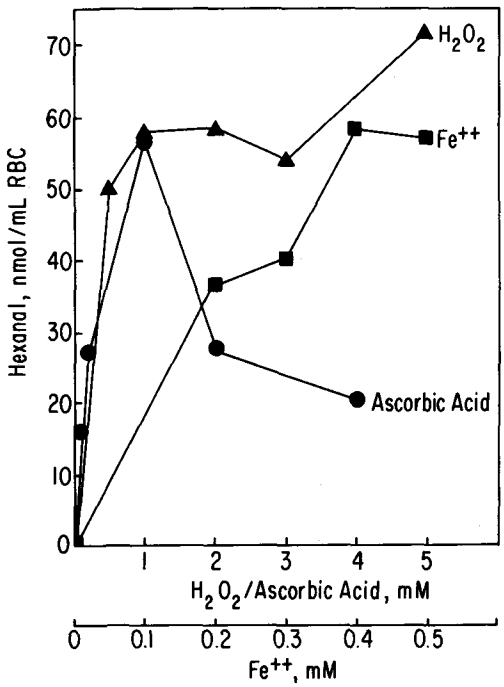


FIG. 2. Effect of Fe^{++}, ascorbic acid and H_2O_2 on the susceptibility of red blood cell membranes to hexanal formation measured by headspace capillary gas chromatography (see Table 2, footnote a). The Fe^{++} concentration was varied in the presence of 1 mM ascorbic acid and 2 mM H_2O_2. The ascorbic acid concentration was varied in the presence of 0.4 mM Fe^{++} and 2 mM H_2O_2. The H_2O_2 concentration was varied in the presence of 0.4 mM Fe^{++} and 1 mM ascorbic acid.

TABLE 2

Susceptibility of Red Blood Cell Membranes (RBCM) to Lipid Peroxidation with Fe^{++} or Cu^{++}, Ascorbic Acid (AA) and H_2O_2 Measured by Headspace Capillary Gas Chromatography[a]

Metal[b] (mM)	AA (mM)	H_2O_2 (mM)	Pentane	Propanal	Pentanal	Hexanal
			(nmol/mL RBC)			
0	0	0	0	0	0	0
0.2 Fe	0.5	1	4.0	1.0	4.0	38
0.2 Cu	0.5	1	4.0	0.6	2.0	13
0.2 Fe	0.5	0	4.0	0.7	2.0	21
0.2 Cu	0.5	0	2.5	0.6	1.0	11

[a] Volatiles determined after incubation for 1 hr at 37°C. Values represent the mean of two determinations. RBCM was prepared from outdated packed cells, and thawed before the oxidative susceptibility test. This sample contained 2.8 mg protein/mL RBC.

[b] Fe^{++} added as $FeSO_4$; Cu^{++} added as $CuSO_4$.

H_2O_2. With 0.4 mM Fe^{++}, 1 mM ascorbic acid and 2 mM H_2O_2, hexanal formation increased with increasing concentrations of SDS up to a maximum of 1.8 mM. The amount of hexanal formed in a sample of RBCM increased 2.6-fold by the addition of 1.8 mM SDS in the presence of 0.2 mM Fe^{++}, 0.5 mM ascorbic acid and 1 mM H_2O_2, and 1.8-fold in the presence of 0.4 mM Fe^{++}, 1 mM ascorbic acid and 2 mM H_2O_2.

METHODS

The oxidative susceptibility of RBCM was tested in samples prepared from freshly drawn blood of six healthy subjects. Final preparations were kept at 0°C overnight and analyzed by headspace GC within 24 hr. Lipid and protein analyses were later carried out in frozen aliquots of the same samples. Total fatty acid contents varied from 2.9 to 4.4 μmol/mL RBC, and the protein contents varied from 1.1 to 2.1 mg/mL RBC. Because of this variation in protein content, hexanal values were expressed in nmol/mg protein. In the presence of a standard mixture of Fe^{++}/ascorbic acid/H_2O_2, the formation of hexanal varied about four-fold from 26 to 108 nmol/mg protein. All fresh RBCM samples produced no volatiles in the absence of Fe^{++}, ascorbic acid and H_2O_2.

Analyses of fatty acid composition of fresh RBCMs show significant variations in linoleic acid, arachidonic acid, PUFA, and total fatty acids (Fig. 3). Percent conversion of total PUFA to hexanal varied from 2.4 to 6.2. Hexanal values correlated significantly with initial total fatty acids, arachidonic acid and PUFA ($p < 0.01$ to 0.02). The correlation between hexanal and linoleic acid was not significant. It was concluded therefore that hexanal is derived mainly from oxidation of arachidonic acid.

In another experiment, RBCM were prepared on the same day as the blood was drawn from five healthy subjects. The final concentration of each sample was equalized on the basis of protein content determined on the freshly prepared RBCM. The RBCM were frozen and stored at −5°C for 24 hr before headspace GC analyses. Changes in fatty acid composition were also determined after oxidation as an alternate method to measure lipid peroxidation in RBCM. Duplicate samples were incubated under the same conditions, and fatty acids were analyzed in the lipid extracts of RBCM samples stored at −5°C after oxidation. In the presence of a mixture of 0.1 mM Fe^{++}, 0.25 mM ascorbic acid and 0.5 mM H_2O_2, and 1.8 mM SDS, the formation of hexanal varied about sixteen-fold, from 6.5 to 113 nmol/mg protein (Table 3). All RBCM samples produced no volatiles in the absence of Fe^{++}, ascorbic acid and H_2O_2. Analyses for pentane and pentanal correlated well with those for hexanal (results not shown) with respective $r = 0.97$ and 0.97 ($p < 0.005$). The correlation ($r = 0.70$) of propanal with hexanal was not significant (Table 4).

Analyses of fatty acid composition of frozen RBCM showed significant variations in linoleic acid, arachidonic

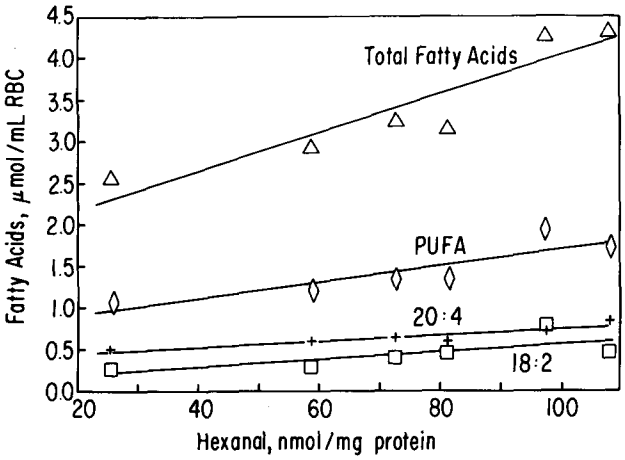


FIG. 3. Relation between hexanal formation and fatty acid composition of red blood cell membranes (RBCM) oxidized in the presence of Fe^{++}/ascorbic acid/H_2O_2 (0.4 mM:1 mM:2 mM). RBCM were prepared from freshly drawn blood from healthy subjects and stored 12 hr at 0°C. Fatty acids were determined by gas chromatography using triheptadecanoin as internal standard. The following correlation coefficients (r) were calculated (n = 6) for hexanal *vs* initial fatty acids: total fatty acids, 0.92 ($p < 0.01$), 20:4, 0.91 ($p < 0.01$), PUFA, 0.87 ($p < 0.02$), and 18:2, 0.73 (N.S.).

TABLE 3

Susceptibility of Frozen Red Blood Cell Membranes (RBCM) from Freshly Drawn Blood to Lipid Peroxidation with Fe^{++}, Ascorbic Acid and H_2O_2[a]

RBCM No.	Sex	Age	Total fatty acids (FA) μmol/mL RBC	18:2 μmol/mL RBC	20:4 μmol/mL RBC	PUFA μmol/mL RBC	Hexanal[b] nmol/mL RBC	Hexanal[b] nmol/mg protein	Hexanal[b] % of PUFA
1	M	54	2.7	0.18	0.65	1.2	39	42	3.5
2	M	53	3.3	0.28	0.69	1.5	51	55	3.8
3	F	63	4.2	0.30	1.0	1.9	105	113	6.0
4	F	48	2.6	0.16	0.61	1.1	33	36	3.2
5	F	51	1.9	0.12	0.37	0.69	6.3	6.5	0.93

Regression analysis (n=5)[c]

	r	$p <$
Hexanal *vs* initial FA	0.99	0.002
Hexanal *vs* initial 20:4	0.99	0.001
Hexanal *vs* initial PUFA	0.98	0.002
Hexanal *vs* initial 18:2	0.89	0.03

[a] RBCM was prepared from freshly drawn blood from healthy subjects and stored 24 hr at −5°C. Final dilution was on the basis of a protein content of 0.93 mg/mL RBC. Fatty acids were determined by gas chromatography using triheptadecanoin as internal standard.

[b] Hexanal was determined by headspace capillary gas chromatography after incubation of RBCM for 1 hr at 37°C in the presence of Fe/ascorbic acid/H_2O_2 (0.1 mM:0.25 mM:0.5 mM) and 1.8 mM SDS. Values represent the mean of two determinations.

[c] Hexanal in nmol/mg protein, fatty acids in μmol/mL RBC.

TABLE 4

Changes in Polyunsaturated Fatty Acids After Oxidation of Red Blood Cell Membranes (RBCM) with Fe^{++}, Ascorbic Acid and H_2O_2[a]

RBCM No.	−18:2	−20:4	−PUFA	−18:2	−20:4	−PUFA
	(wt %)			(μmol/mL RBC)		
1	−1.1	2.5	−6.6	−0.031	0.070	−0.18
2	−1.1	2.7	4.9	−0.036	0.088	0.16
3	−1.4	7.1	6.8	−0.057	0.30	0.29
4	−3.1	1.4	−0.76	−0.080	0.036	−0.020
5	−1.1	−1.6	−3.5	−0.020	−0.030	−0.067

Regression analysis (n=5)[b]

	r	p, <
Hexanal *vs* −20:4	0.99	0.001
Hexanal *vs* −PUFA	0.76	N.S.
Hexanal *vs* −18:2	0.37	N.S.

[a] See footnote a in Table 3.

[b] See footnotes b and c in Table 3.

acid, PUFA, and total fatty acids (Table 3). Total fatty acid contents varied from 1.9 to 4.2 μmol/mL RBC. Conversion of PUFA to hexanal varied from 0.93 to 6.0%. Highly significant correlations were calculated for hexanal *versus* initial total fatty acids, arachidonic acid, and PUFA ($p < 0.001$ to 0.002). The correlation between hexanal and linoleic acid was less significant ($p < 0.03$).

Hexanal expressed per milligram protein was also correlated with changes in different PUFA after oxidation (Table 4). An excellent correlation was calculated between hexanal formation and decrease in 20:4 ($p < 0.001$). The correlation ($r = 0.76$) between hexanal formation and decrease in PUFA was not significant. No correlation was found between hexanal and decrease in 18:2. These results provide good evidence that under the standard conditions used for oxidative susceptibility, hexanal is exclusively derived from the oxidation of arachidonic acid.

DISCUSSION

A large variety of conditions have been used to investigate lipid peroxidation in RBC and RBCM (1,15,16,19,21,22). Such terms used in the literature as oxidative stress, antioxidant capacity, lability or susceptibility to lipid peroxidation or to autoxidative breakdown, are often difficult to interpret because a large variation in experimental conditions and methods has been used to determine the extent of peroxidation. Under the assay conditions of the TBA test, TBARS generated reflect not only the extent of lipid peroxidation but also the amounts of peroxide and aldehyde precursors (39,40), which are also related to the susceptibility of the tissue to peroxidation. The determination of TBARS in biological tissues can thus also be interpreted as a measure of susceptibility against oxidative damage.

In this paper a headspace GC method was developed to estimate oxidative susceptibility in RBCM, which are good models to study peroxidative damage. The commonly used mixture of Fe^{++} and ascorbic acid produced low levels of volatile lipid peroxidation products as measured by this assay (Table 1). However, the addition of H_2O_2 to this mixture significantly increased the amount of hexanal (Fig. 2). A mixture of Fe^{++}, ascorbic acid and H_2O_2 produced significant amounts of hexanal as measured by this headspace technique. These results are in agreement with the literature that active oxygen species are generated by iron in the presence of both reducing and oxidizing agents. These conditions promote the formation of OH^- radicals by the Fenton reaction (30,41). The addition of 1.8 mM SDS increased about four-fold the amount of hexanal formed from RBCM at two levels of Fe^{++}/ascorbic acid/H_2O_2 concentrations. As a powerful surface-active compound, SDS may increase the surface of the phospholipid mixed micelles. The resulting increased accessibility of the PUFA lipid substrate to the metal catalyst and oxygen would accelerate hydroperoxide formation and decomposition to hexanal. In combination with ascorbic acid and with or without H_2O_2, Fe^{++} was a better catalyst than Cu^{++} for the peroxidation of RBCM as measured by hexanal formation (Table 2).

By this standard headspace GC method, in one series of RBCM samples a four-fold variation in oxidative susceptibility was observed in fresh RBCM from blood freshly drawn from six healthy subjects. In another series of RBCM samples, a sixteen-fold variation in oxidative susceptibility was noted in frozen RBCM from blood freshly drawn from five healthy subjects. Correlation between hexanal formation and PUFA depletion provides good evidence that under the standard conditions of the headspace assay, hexanal is exclusively derived from oxidation of arachidonic acid. Previous reports (21,22) showed that all PUFA decreased when RBC ghosts were oxidized in the presence of AAPH, and arachidonic acid was consumed faster than linoleic acid. Under the conditions used in this study, the Fe^{++}-ascorbic acid/H_2O_2 catalyst is therefore more selective for the oxidation of arachidonic acid than is the free radical initiator AAPH. In the presence of an azo initiator, the oxidizability of arachidonic acid is 2.8 times greater than that of linoleic acid (42). Therefore, in the presence of Fe^{++}-ascorbic acid/H_2O_2 catalyst used in this study, the hydroperoxides of arachidonic acid are more readily formed and decomposed than those of linoleic acid to produce hexanal as the main volatile product that can be readily analyzed by headspace GC.

The headspace assay described in this paper may have potential application to study the susceptibility of RBCM toward oxidative damage resulting from pathological conditions. This headspace oxidative susceptibility assay has advantages over many other assays in permitting the specific analysis of multiple products of lipid peroxidation. In nutritional studies, this assay should permit the study of how dietary polyunsaturated lipids affect the relative amounts of n-6 (hexanal and pentane) and n-3 (propanal) PUFA peroxidation. However, some applications of this assay may be limited in stable systems because of the relatively high concentrations of Fe^{++} required and the method may not be sensitive to minor changes in antioxidants and oxidation products.

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Incorporation of Lipids Labeled with Various Fatty Acids into the Cytoskeleton of Aggregating Platelets

M.A. Packham*, M.A. Guccione, N.L. Bryant and A. Livne[1]
Department of Biochemistry, University of Toronto, Toronto, Ontario, Canada M5S 1A8

Earlier studies showed that during the first 20 to 25 seconds of aggregation induced by thrombin (0.1 U/mL) or adenosine diphosphate (ADP) (2 μM) of rabbit or human platelets prelabeled with [^{3}H]palmitic acid, labeled lipid became associated with the cytoskeleton (isolated after lysis with 1% Triton X-100, 5 mM EGTA [ethylene glycol-*bis*-(β-aminoethyl ether)*N,N,N',N'*-tetraacetic acid] in the presence of 0.5 mM leupeptin and 50 mM benzamidine). In comparison with labeled lipid in intact platelets, the labeled lipid that was associated with the cytoskeleton was enriched in phospholipids and ceramide. To determine whether these effects were specific for lipids labeled with palmitic acid, we studied rabbit platelets in which lipids had been labeled by incubation of the platelets with pairs of ^{14}C- or ^{3}H-labeled palmitic, stearic, arachidonic, and linoleic acids. Examination of the distribution of label among the lipid classes of intact platelets showed that phospholipids contained most of the label. Under the conditions of limited, thrombin-induced aggregation used, labeled lipids were not lost from the platelets and the distribution of label among the lipid classes was essentially unchanged. There were major differences in the incorporation of labeled lipids into the cytoskeleton. The greatest incorporation (2.1 to 2.8% of the label in the platelets) was observed with palmitic acid-labeled lipids; by direct comparison, only 44% as much of the label of stearic acid-labeled lipids, 21% as much of the label of linoleic acid-labeled lipids, and only 6% as much of the label of arachidonic acid-labeled lipids was incorporated into the cytoskeleton. Thus the pool of phospholipid that is readily labeled with arachidonic acid appears to be selectively excluded from the cytoskeleton. Also noteworthy is the 4- to 5-fold enrichment of the cytoskeleton with labeled ceramide; an average of 16% of the label from stearic acid in the cytoskeleton was in ceramide. We suggest that ceramide and phospholipids that are readily labeled with saturated fatty acids are selectively incorporated into the cytoskeleton during the early stages of aggregation and may be specifically associated with the points of contact between platelets.
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In earlier studies (1,2), we found that during the early stages of platelet aggregation induced by thrombin or adenosine diphosphate (ADP), labeled phospholipids and ceramide were selectively incorporated into the cytoskeleton of both human and rabbit platelets that had been prelabeled with [^{3}H]palmitic acid. Cytoskeleton from aggregating platelets had 1.5 to 8% of the label of intact platelets, whereas the cytoskeleton of unactivated platelets had only 0.03% (1). The association of labeled lipids with the cytoskeleton appeared to be related to the number of sites of contact between platelets and was dependent on aggregation, but not on the release of granule contents. Most of the label that became associated with the cytoskeleton during limited aggregation was in phospholipids, mainly in phosphatidylcholine (PC). The distribution of label among the phospholipids in the cytoskeleton was similar to that in intact platelets, but the small amounts of label in triacylglycerol and cholesteryl ester (as a percentage of the total label in lipids) were reduced in the cytoskeletons (2). In contrast, the label in ceramide was enriched 3-fold in the cytoskeletons. This was particularly pronounced with rabbit platelets which contained a higher percentage of the label in ceramide than did human platelets.

To determine whether some of these effects are specific for lipids that are readily labeled with palmitic acid, we have carried out experiments with rabbit platelets labeled with the following pairs of fatty acids: [^{3}H]palmitic acid and [^{14}C]stearic acid; [^{14}C]palmitic acid and [^{3}H]arachidonic acid; [^{14}C]stearic acid and [^{3}H]arachidonic acid; and [^{3}H]palmitic acid and [^{14}C]linoleic acid.

MATERIALS AND METHODS

Materials. Albumin fraction V was from ICN Immunobiologicals (Lisle, IL); bovine thrombin was from Parke Davis (Detroit, MI); ADP, ethylene glycol-*bis*-(β-aminoethyl ether)*N,N,N',N'*-tetraacetic acid (EGTA), palmitic acid, leupeptin, benzamidine, and lipids (standards, including ceramide III) were from Sigma Chemical Co. (St. Louis, MO). Ceramide (natural mixture, bovine) was from Serdary (London, Ontario, Canada); petroleum hydrocarbon (80–100°C) was from BDH Chemicals (Toronto, Ontario, Canada); Triton X-100 was molecular biology grade, ultra pure reagent, and was obtained from International Biotechnologies (New Haven, CT). Soluene-350 and Hionic-fluor were purchased from Packard Instruments (Mississauga, Ontario, Canada). Apyrase (adenosine triphosphate diphosphohydrolase, EC 3.6.1.5.) was prepared from potatoes by the method of Molnar and Lorand (3) and used as described previously (4). Labeled palmitic acid ([9,10-^{3}H], 30 Ci/mmol or [1-^{14}C], 56 mCi/mmol), [5,6,8,9,11,12,14,15-^{3}H(N)]arachidonic acid (100 Ci/mmol) and Aquasol were obtained from Du Pont, Canada (NEN Products, Lachine, Quebec, Canada). [1-^{14}C]Stearic acid (57.5 mCi/mmol) was from ICN Radiochemicals (Irvine, CA). [1-^{14}C]Linoleic acid (54.7 mCi/mmol) was from Amersham Canada (Oakville, Ontario, Canada). Labeled and unlabeled fatty acids were dissolved in ethanol.

Preparation of suspensions of platelets. Suspensions of washed platelets from rabbits were prepared from blood anticoagulated with acid-citrate-dextrose according to the method of Ardlie *et al.* (5,6). Platelets were labeled at a platelet count of approximately 500,000/μL. Preliminary

*To whom correspondence should be addressed.
[1] Present address: Department of Biology, Ben-Gurion University of the Negev, Beer-Sheva, Israel.

Abbreviations: ADP, adenosine diphosphate; EGTA, ethylene glycol-*bis*(β-aminoethyl ether)*N,N,N',N'*-tetraacetic acid; PA, phosphatidic acid; PC, phosphatidylcholine; PE, phosphatidylethanolamine; PI, phosphatidylinositol; PS, phosphatidylserine; TLC, thin-layer chromatography; UV, ultraviolet.

experiments were done to determine the conditions that gave the greatest incorporation of labeled fatty acids into the platelet lipids; these conditions differed for each pair of labeled fatty acids.

[^{3}H]Palmitic acid and [^{14}C]stearic acid labeling. Platelets were labeled in the third washing fluid (modified Tyrode solution, pH 7.35, with no added Ca^{2+}, no albumin, but with apyrase). For each milliliter of platelet suspension, 1 μL of unlabeled palmitic acid and 1 μL of [^{3}H]-palmitic acid were added to give a final concentration of 3.6 μM, and 2 μL of [^{14}C]stearic acid was also added to give a final concentration of 3.6 μM. The mixture was incubated for 30 min at room temperature and then albumin was added to give a final concentration of 0.35%.

[^{14}C]Palmitic acid and [^{3}H]arachidonic acid labeling. Platelets were labeled in the second washing fluid (modified Tyrode solution, pH 6.5, with no albumin). For each milliliter of platelet suspension, 2 μL of [^{14}C]palmitic acid was added (final concentration 3.6 μM) and after 15 min of incubation at 37°C, albumin was added to give a final concentration of 0.35%. Lastly, [^{3}H]arachidonic acid (2 μL/mL of platelet suspension, final concentration 0.002 μM) was added and the incubation was continued for 30 min.

[^{14}C]Stearic acid and [^{3}H]arachidonic acid labeling. Platelets were labeled as above, but [^{14}C]palmitic acid was replaced with [^{14}C]stearic acid.

[^{3}H]Palmitic acid and [^{14}C]linoleic acid labeling. Platelets were labeled with [^{3}H]palmitic acid (1 μL/mL of platelet suspension, final concentration 0.18 μM) for 15 min at 37°C in the second washing fluid, as described above. [^{14}C]Linoleic acid was mixed with 3.5% albumin before addition to the platelet suspension to give final concentrations of 3.6 μM linoleic acid and 0.35% albumin. Incubation was continued for 30 min.

After labeling, the platelets were recovered by centrifugation and resuspended for aggregation studies at a platelet count of 300,000/μL in a modified Tyrode-albumin solution containing 1.8 mM Ca^{2+}, and apyrase (pH 7.35). Samples of intact platelets for radioactivity determinations and lipid extraction were obtained by centrifugation.

Aggregation and isolation of cytoskeletons. At least 60 min elapsed from the end of the incubation until the platelets were aggregated and the cytoskeletons prepared. Aggregation was measured at 37°C by recording the changes in light transmission through 1-mL samples of platelet suspension stirred at 1100 rpm in a cuvette in an aggregometer (Payton Associates, Scarborough, Ontario, Canada). No aggregation (0%) is defined as the lowest point of the light transmission curve after addition of the aggregating agent, and 100% as light transmission after addition of Triton X-100-EGTA (see below). The aggregating agents used were ADP (2 μM) and thrombin (0.1 U/mL). The amounts of all materials added to the platelet suspensions are expressed as final concentrations after all additions. Aggregation was stopped at the same extent of change in light transmission (approximately 25%, which occurred between 20 and 25 sec) by the addition of a lysis solution containing 10% Triton X-100, 50 mM EGTA, leupeptin, and benzamidine, pH 7.35, to give final concentrations of 1% Triton, 5 mM EGTA, 0.5 mM leupeptin and 50 mM benzamidine. The material that is insoluble in this medium is commonly defined as cytoskeleton (7). Cytoskeletons were isolated and washed by the methods of Phillips *et al.* (8) and Burn *et al.* (9), and radioactivity was determined as described previously (1,2). To assess the extent of incorporation of labeled lipids into the cytoskeleton that formed upon aggregation, the amounts of ^{3}H and ^{14}C in the cytoskeletons were determined and expressed as a percentage of the total cpm in ^{3}H and ^{14}C in the platelet pellets.

Lipid extraction. Lipids were extracted from intact platelets (obtained from 1-mL samples of platelet suspension) and from ten combined platelet cytoskeletons (each prepared from 1 mL samples of platelet suspension) by a modification of the method of Bligh and Dyer (10), as described earlier (11).

Thin-layer chromatography (TLC). Lipids were separated by one- and two-dimensional chromatography on thin-layer plates (TLC-plates precoated SIL G-50, 0.5 mm with fluorescent indicator, Brinkmann, Rexdale, Ontario, Canada). The solvent used for separation of phospholipids in one dimension was Solvent I, *i.e.* chloroform/methanol/acetic acid/water (90:40:12:2, by vol). For two-dimensional separation of neutral lipids and glycolipids from each other and from phospholipids, Solvent II, *i.e.* petroleum hydrocarbon/diethyl ether/acetic acid (120:25:1.5, v/v/v) was used in the first dimension and Solvent III, *i.e.* chloroform/methanol/acetic acid (95:5:5, v/v/v), was used in the second dimension. Non-radioactive standards were run with the radioactive samples so that the positions of the various phospholipids could be visualized by ultraviolet (UV) light or after exposure to iodine vapor. These standards were: phosphatidylcholine (PC), phosphatidylethanolamine (PE), phosphatidylserine (PS), phosphatidylinositol (PI), phosphatidic acid (PA), sphingomyelin, ceramide type III, a natural mixture of ceramides, monoacylglycerol, diacylglycerol, triacylglycerol and cholesteryl ester. Squares of the silica layer containing the respective lipid fractions were scraped from the plates into counting vials containing Aquasol. The approximate average numbers of dpm determined by liquid scintillation counting for each radioisotope in the samples of the lipid extracts applied to the chromatographic plates are shown in Table 1. The very low incorporation of arachidonic acid-labeled lipids into the cytoskeletons of aggregating platelets is responsible for the low number of dpm of arachidonic acid-labeled

TABLE 1

Approximate Average Number of dpm in Lipid Extracts Applied to Chromatographic Plates

Fatty acid used to label platelet lipids	dpm in lipid extract of 5×10^8 intact platelets[a]	dpm in lipid extract of 10 cytoskeletons (from 5×10^9 platelets)[b]
[^{3}H]Palmitic	100,000	13,000
[^{14}C]Palmitic	90,000	9,000
[^{14}C]Stearic	90,000	5,000
[^{3}H]Arachidonic	160,000	1,000
[^{14}C]Linoleic	2,000	150

[a] Lipid extracts of intact, unstimulated platelets in 1-mL samples of platelet suspension.

[b] Lipid extracts of cytoskeletons from ten 1-mL samples of platelet suspension after aggregation for 20 to 25 sec with 0.1 U/mL thrombin.

lipids that could be applied to the chromatographic plates. The extremely low uptake of [^{14}C]linoleic acid into the intact platelets, and the low incorporation of linoleic acid-labeled lipids into the cytoskeleton prevented reliable detailed analysis of individual cytoskeletal lipids labeled with linoleic acid.

Analysis of data. Means ± S.D. are shown. Paired t-tests were used to calculate the significance of differences. Differences were considered to be significant when $P < 0.05$.

RESULTS

The labeling pattern of the lipids of intact platelets was determined after the platelets had been isolated from their suspending medium by centrifugation so that any radioactivity in the medium was not included. Table 2 shows the distribution of the labels among the lipids separated in two dimensions by solvent Systems II and III. Nearly all of the label of arachidonic acid and linoleic acid appeared in phospholipids, and in contrast to the saturated fatty acids, less than 1% of labeled arachidonic acid was in the ceramide fraction and less than 2% in triacylglycerol. The percentages of labeled linoleic acid in ceramide and triacylglycerol were even lower (Table 2). The unstimulated platelets contained very little free, labeled fatty acids (Table 2).

Table 3 shows data for the major phospholipid classes in which the distribution of the labels from the four different labeled fatty acids are compared. A higher proportion of labeled stearic acid than labeled palmitic acid appeared in PE and PS/PI when these labeled fatty acids were used together at the same concentration to label the platelets. There was more labeled material from the saturated fatty acids than from the unsaturated fatty acids at the solvent front. (The solvent front contains the label of triacylglycerol, diacylglycerol, monoacylglycerol, free fatty acids, cholesteryl ester, ceramides, cardiolipin and cerebrosides (12).)

During the limited aggregation (20 to 25 sec) of the platelets with 0.1 U/mL of thrombin, there was no detectable loss of radioactivity from labeled palmitic acid or labeled stearic acid into the suspending medium. In four experiments with rabbit platelets in which this measurement was made for [^{14}H]arachidonic acid, a mean of 1.3 ± 1.3% of the label was lost from the platelets upon aggregation, but by paired difference analysis with control samples, this loss was not statistically significant. In four experiments in which platelets had been labeled with both [^{14}C]stearic acid and [^{14}H]arachidonic acid, there were no significant differences between unstimulated platelets and intact, thrombin-stimulated platelets, in the distributions of either of the labels among the lipid classes (Table 4). We showed previously (2) with labeled palmitic acid that the distribution of label among the lipid fractions was unchanged during limited aggregation in response to thrombin.

TABLE 2

Intact, Unstimulated Rabbit Platelets: Distribution of Label (%) Among Lipids[a]

Lipid	[^{14}C]Palmitic acid (3.6 μM)	[^{14}C]Stearic acid (3.6 μM)	[^{3}H]Arachidonic acid (0.002 μM)	[^{14}C]Linoleic acid (3.6 μM)
Phospholipids	86.5 ± 4.2	88.4 ± 4.5	94.0 ± 3.6	93.6 ± 5.3
Ceramide	2.1 ± 0.8	2.3 ± 0.8	0.6 ± 1.0	0.2 ± 0.2
Triacylglycerol	5.1 ± 1.6	4.8 ± 4.0	1.9 ± 1.4	0.8 ± 0.7
Diacylglycerol	1.0 ± 0.4	1.1 ± 0.6	0.8 ± 0.7	0.7 ± 0.5
Free fatty acid	2.7 ± 2.6	1.7 ± 1.4	0.6 ± 0.7	0.5 ± 0.6
	(n = 6)	(n = 6)	(n = 12)	(n = 4)

[a]Lipids were separated in two dimensions with Solvents II and III. Column headings indicate the type of labeled fatty acid used to label the platelet lipids. In the incubation of platelets with labeled fatty acids, [^{14}C]palmitic acid or [^{14}C]stearic acid was paired with [^{3}H]arachidonic acid; [^{3}H]palmitic acid was paired with [^{14}C]linoleic acid. Values are means ± S.D. of the percentages of the label applied to the chromatographic plates.

TABLE 3

Intact, Unstimulated Rabbit Platelets: Distribution of Label (%) Among the Phospholipids[a]

Lipid	[^{14}C]Palmitic acid (3.6 μM)	[^{14}C]Stearic acid (3.6 μM)	[^{3}H]Arachidonic acid (0.002 μM)	[^{14}C]Linoleic acid (3.6 μM)
PC	53.1 ± 5.2	35.8 ± 7.1	55.6 ± 7.7	77.1 ± 0.9
PS/PI	6.0 ± 3.0	10.8 ± 4.9	11.9 ± 3.1	7.2 ± 1.5
PE	20.1 ± 4.3	40.0 ± 3.8	21.5 ± 7.2	10.9 ± 1.7
Sphingomyelin	3.9 ± 1.5	2.2 ± 1.9	4.1 ± 1.1	1.4 ± 0.4
Solvent front	18.7 ± 7.9	11.3 ± 3.6	7.0 ± 1.6	3.1 ± 1.5
	(n = 5)	(n = 4)	(n = 7)	(n = 4)

[a]Phospholipids were separated by Solvent I. Column headings indicate the type of labeled fatty acid used to label the platelet lipids. The solvent front contained label associated with triacylglycerol, diacylglycerol, monoacylglycerol, free fatty acid, cholesteryl ester, ceramide, cardiolipin and cerebroside (12). In the incubation of platelets with labeled fatty acids, [^{14}C]palmitic acid or [^{14}C]stearic acid was paired with [^{3}H]arachidonic acid; [^{3}H]palmitic acid was paired with [^{14}C]linoleic acid. Values are means ± S.D. of the percentages of the label applied to the chromatographic plates.

In experiments with two differently labeled fatty acids, there were major differences among the labeled lipids in their incorporation into the cytoskeleton isolated after lysis with 1% Triton X-100, 5 mM EGTA, 0.5 mM leupeptin and 50 mM benzamidine. Upon aggregation of the platelets to the extent of 20 to 25% with 0.1 U/mL of thrombin, the incorporation of the label into the cytoskeleton was highest when the lipids had been labeled with palmitic acid (2.1 to 2.8%) (Table 5). In contrast, only 1.2% of lipids labeled with stearic acid became associated with the cytoskeleton during this limited aggregation (Table 5). By direct comparison, the incorporation of lipids labeled with stearic acid was 44% of the incorporation of lipids labeled with palmitic acid. The incorporation of lipids labeled with arachidonic acid into the cytoskeleton was much less (Table 5); by direct comparison with palmitic acid-labeled lipid, only 6% as much arachidonic acid-labeled lipid was incorporated; by direct comparison with stearic acid-labeled lipids, only 19% as much arachidonic acid-labeled lipid was incorporated. The incorporation of linoleic acid-labeled lipid was 21% of the incorporation of palmitic acid-labeled lipid. When the platelets were aggregated with 2 μM ADP, similar differences were found between palmitic acid-labeled lipid and arachidonic acid-

TABLE 4

Comparison of Intact, Unstimulated Platelets and Intact Platelets Aggregated with Thrombin: Distribution of Label (%) Among Lipids[a]

	[^{14}C]Stearic acid		[^{3}H]Arachidonic acid	
Lipid	Unstimulated	Aggregated with thrombin	Unstimulated	Aggregated with thrombin
Phospholipids	88.4 ± 5.3	88.8 ± 5.9	97.0 ± 0.9	93.6 ± 5.5
Ceramide	2.6 ± 2.2	2.2 ± 0.3	0.1 ± 0.1	0.4 ± 0.3
Triacylglycerol	6.5 ± 3.9	6.2 ± 4.0	1.8 ± 0.9	3.0 ± 2.2
Diacylglycerol	0.9 ± 0.3	1.0 ± 0.7	0.5 ± 0.1	1.1 ± 1.2
Free fatty acid	0.8 ± 0.4	0.6 ± 0.3	0.2 ± 0.1	0.5 ± 0.6

[a]Platelets were aggregated with 0.1 U/mL thrombin for 20 to 25 sec. Platelet lipids had been labeled by incubation of the platelets with [^{14}C]stearic acid (3.6 μM) and [^{3}H]arachidonic acid (0.002 μM). Values are means ± S.D. of the percentages of the label applied to the chromatographic plates (n = 4). By paired difference analysis, there were no significant differences between the values for unstimulated platelets and platelets aggregated with thrombin.

TABLE 5

Comparisons of Incorporation of Labeled Lipids into the Cytoskeleton of Rabbit Platelets Aggregated by Thrombin or ADP[a]

Aggregating agent	Pairs of fatty acids used to label platelet lipids	n	Label in cytoskeleton (percent of label in intact, unstimulated platelets)	P
Thrombin	[^{3}H]Palmitic (3.6 μM)	12	2.7 ± 0.8	<0.001
	vs [^{14}C]stearic (3.6 μM)		1.2 ± 0.4	
Thrombin	[^{14}C]Palmitic (3.6 μM)	22	2.1 ± 1.3	<0.001
	vs [^{3}H]arachidonic (0.002 μM)		0.1 ± 0.2	
Thrombin	[^{14}C]Stearic (3.6 μM)	11	1.2 ± 0.6	<0.001
	vs [^{3}H]arachidonic (0.002 μM)		0.2 ± 0.2	
Thrombin	[^{3}H]Palmitic (0.18 μM)	16	2.8 ± 0.8	<0.001
	vs [^{14}C]linoleic (3.6 μM)		0.6 ± 0.4	
ADP	[^{14}C]Palmitic (3.6 μM)	6	1.2 ± 0.8	<0.02
	vs [^{3}H]arachidonic (0.002 μM)		0.0 ± 0.0	
ADP	[^{14}C]Stearic (3.6 μM)	9	1.4 ± 0.4	<0.001
	vs [^{3}H]arachidonic (0.002 μM)		0.3 ± 0.3	

[a]Platelets were aggregated with 0.1 U/mL thrombin or 2 μM ADP for 20 to 25 sec. Platelet lipids had been labeled by incubation of the platelets with pairs of labeled fatty acids. Values are means ± S.D.

TABLE 6

Cytoskeletons of Rabbit Platelets Aggregated with Thrombin: Distribution of Label (%) Among Lipids[a]

Lipid	[^{14}C]Palmitic acid (3.6 μM)	[^{14}C]Stearic acid (3.6 μM)	[^{3}H]Arachidonic acid (0.002 μM)
Phospholipids	79.2 ± 9.5	70.8 ± 14.2	93.5 ± 3.2
Ceramide	9.1 ± 3.2	16.0 ± 5.8	3.4 ± 2.3
Triacylglycerol	2.6 ± 2.8	4.1 ± 6.1	1.0 ± 1.5
Diacylglycerol	1.3 ± 1.2	2.9 ± 2.4	0.2 ± 0.6
Free fatty acids	2.4 ± 3.8	4.9 ± 4.6	1.4 ± 1.3
	(n = 6)	(n = 4)	(n = 5)

[a]Platelets were aggregated with 0.1 U/mL thrombin for 20 to 25 sec. Column headings indicate the type of labeled fatty acid used to label the platelet lipids. In the incubation of platelets with labeled fatty acids, palmitic acid was paired with [^{14}C]stearic acid or [^{3}H]arachidonic acid; [^{14}C]stearic acid was paired with [^{3}H]palmitic acid or [^{3}H]arachidonic acid; [^{3}H]arachidonic acid was paired with [^{14}C]palmitic acid or [^{14}C]stearic acid. Values are means ± S.D. of the percentages of label applied to the chromatographic plates.

TABLE 7

Paired Difference Analysis of Enrichment of Cytoskeletons with Labeled Ceramide[a]

	Percentage of label in ceramide		
	Palmitic acid	Stearic acid	Arachidonic acid
Intact platelets	2.4 ± 1.2	3.6 ± 1.3	0.5 ± 0.4
Cytoskeleton	9.7 ± 3.2	16.0 ± 5.8	3.4 ± 2.3
P	<0.001	<0.02	<0.05
Fold enrichment	4.0	4.4	7.1
	(n = 5)	(n = 4)	(n = 5)

[a]To obtain cytoskeletons, platelets were aggregated with 0.1 U/mL thrombin for 20 to 25 sec. Column headings indicate the type of labeled fatty acid used to label the platelet lipids. In the incubation of platelets with labeled fatty acids, palmitic acid was paired with [^{14}C]stearic acid or [^{3}H]arachidonic acid; [^{14}C]stearic acid was paired with [^{3}H]-palmitic acid or [^{3}H]arachidonic acid; [^{3}H]arachidonic acid was paired with [^{14}C]palmitic acid or [^{14}C]stearic acid. Values are means ± S.D. Paired difference analysis (two-tailed) between intact platelets and cytoskeletons.

labeled lipid, and between stearic acid-labeled lipid and arachidonic acid-labeled lipid (Table 5).

Table 6 shows the distributions of the labels among the lipids in platelet cytoskeletons following aggregation for 20 to 25 sec with 0.1 U/mL thrombin. In comparison with Table 2, the most noteworthy difference between the distributions in intact, unstimulated platelets and the distributions in the cytoskeletons was the enrichment of the cytoskeletons with labeled ceramide. Table 7 shows that on average, the cytoskeletons were enriched 4-fold with palmitic acid-labeled ceramide and 4.4-fold with stearic acid-labeled ceramide. Although a low percentage of the lipids labeled with arachidonic acid was found in the cytoskeletons in comparison with lipids labeled with the saturated fatty acids, and the percentage of arachidonic acid-labeled ceramide in intact platelets was only 0.6%, arachidonic acid-labeled ceramide appeared to be enriched in the cytoskeletons. However, the dpm in arachidonic acid-labeled ceramide was so low that the calculation of enrichment of the cytoskeletons (7-fold) can be only approximate. The percentage of linoleic acid-labeled ceramide in the intact platelets and in the cytoskeletons was too low to permit determination of whether or not enrichment occurred in the cytoskeletons. However, as in the intact platelets, nearly all of the label of linoleic acid in the cytoskeletons was in phospholipids (data not shown).

Labeled triacylglycerol of intact, unstimulated platelets was largely excluded from the cytoskeletons (Tables 2 and 6), as observed previously for platelets labeled with palmitic acid (2). The difference in the percentage of label in palmitic acid-labeled triacylglycerol between intact platelets and cytoskeletons was significant ($P < 0.05$, n = 6, paired difference analysis).

Table 8 shows the distribution of labeled phospholipids in the cytoskeletons. In comparison with intact platelets (Table 3), there was less label from palmitic acid in PS/PI in the cytoskeletons ($P < 0.005$) and less label in PE in the cytoskeletons ($P < 0.005$) (n = 5, paired difference analysis). There were no significant differences for stearic acid-labeled phospholipids (n = 3). For arachidonic acid-labeled lipids, the only significant difference between intact platelets and the cytoskeletons was the lower amount of label in PC in the cytoskeletons ($P < 0.05$) (n = 5).

TABLE 8

Cytoskeletons of Rabbit Platelets Aggregated with Thrombin: Distribution of Label (%) Among the Phospholipids[a]

Lipid	[^{14}C]Palmitic acid (3.6 μM)	[^{14}C]Stearic acid (3.6 μM)	[^{3}H]Arachidonic acid (0.002 μM)
PC	45.7 ± 9.9	24.8 ± 5.7	42.8 ± 11.3
PS/PI	4.5 ± 3.0	9.4 ± 2.3	9.0 ± 9.1
PE	15.9 ± 5.2	23.7 ± 9.5	24.0 ± 8.3
Sphingomyelin	5.7 ± 3.3	4.5 ± 3.9	4.9 ± 4.9
Solvent front	28.1 ± 15.8	35.5 ± 9.1	20.2 ± 6.2
	(n = 5)	(n = 3)	(n = 5)

[a]Platelets were aggregated with 0.1 U/mL thrombin for 20 to 25 sec. Column headings indicate the type of labeled fatty acid used to label the platelet lipids. In the incubation of platelets with labeled fatty acids, [^{14}C]palmitic acid was paired with [^{3}H]arachidonic acid; [^{14}C]stearic acid was paired with [^{3}H]arachidonic acid; [^{3}H]arachidonic acid was paired with [^{14}C]palmitic acid or [^{14}C]stearic acid. Values are means ± S.D. of the percentages of label applied to the chromatographic plates.

DISCUSSION

These results show that during the early stages of platelet aggregation induced by a low concentration of thrombin or ADP, lipids that are readily labeled with palmitic acid are preferentially incorporated into the cytoskeleton. In all the experiments, platelet lipids had been labeled by incubation of the platelets with two differently labeled fatty acids. Direct comparison of palmitic acid-labeled lipids with stearic acid-labeled lipids showed that only 44% as much stearic acid-labeled lipid as palmitic acid-labeled lipid became associated with the cytoskeleton of platelets aggregating under the influence of thrombin, 21% as much linoleic acid-labeled lipid, and only 6% as much arachidonic acid-labeled lipid. Thus, in contrast to the lipids that are labeled with the saturated fatty acids (particularly with palmitic acid), very little lipid that is readily labeled with arachidonic acid is associated with the cytoskeleton during the initial stages of aggregation. Burn *et al.* (9) reported that under their conditions of thrombin stimulation of platelets, "the binding of lipid components to the cytoskeleton of activated platelets labeled with palmitic acid is six times higher than that of control platelets labeled with arachidonic acid." The finding that the pool of phospholipid which is readily labeled with arachidonic acid is largely excluded from the cytoskeleton of aggregating platelets is of interest in relation to an early report of Bills *et al.* (13) that only 30% of the PC in human platelets is readily labeled with arachidonic acid. It appears that very little of this pool is incorporated into the cytoskeleton, in contrast to the pool(s) of phospholipids that are labeled with palmitic and stearic acids. This selective incorporation into the cytoskeleton of aggregating platelets of what must be different pools of phospholipid has not been emphasized previously. It may be related to the known preponderance of saturated fatty acids at position 1 of phospholipids and the preponderance of unsaturated fatty acids (particularly arachidonic acid) at position 2 (14).

In general, the extent of incorporation of labeled phospholipids into the cytoskeleton did not depend on the phospholipid class. The lower percentage of arachidonic acid-labeled PC in the cytoskeletons (in comparison to the percentage in intact platelets) is unlikely to have been caused by the loss of labeled arachidonic acid from PC as free fatty acid due to the action of phospholipase A_2 because we found that the mean 1.3% loss of label into the suspending fluid of intact platelets upon thrombin stimulation was not statistically significant. It should be emphasized that our conditions of limited aggregation with a low concentration of thrombin are probably responsible for the lack of mobilization of arachidonic acid. Stearic acid-labeled lipids in intact platelets were also not changed by aggregation.

The low percentage of arachidonic acid-labeled lipid in the cytoskeleton (compared to palmitic acid-labeled lipid or stearic acid-labeled lipid) was also observed when the platelets were aggregated with ADP. Unlike thrombin, ADP does not cause the release of granule contents from rabbit platelets, nor induce the mobilization of arachidonic acid for the formation of thromboxane A_2 (15). Thus the observations are related to aggregation *per se*, rather than to the more extensive reactions that occur when platelets are stimulated with high concentrations of thrombin.

In earlier studies (1,2) we established in several ways that the incorporation of the label of palmitic acid into the cytoskeleton does not represent the nonspecific trapping of membrane fragments due to incomplete lysis by Triton X-100. This previous conclusion is further supported by the observations in the present experiments that although most of the label of all the fatty acids was in phospholipids, there were major differences in percentages of the labeled lipids that were incorporated into the cytoskeleton.

The distribution of the label of palmitic acid among the phospholipid classes of intact, unstimulated platelets in the present experiments differs slightly from that reported previously (2). This difference appears to be related to the changes in the labeling conditions that were necessary to obtain optimum uptake of the other labeled fatty acids with which palmitic acid was paired (data not shown). These differences do not affect the direct comparisons between the distribution of the label in intact, unstimulated platelets and in the cytoskeleton.

The most noteworthy finding was the enrichment of the cytoskeleton with labeled ceramide; this enrichment was particularly pronounced with stearic acid-labeled ceramide. An average of 16% of the labeled stearic acid in the cytoskeletons was in ceramide. We had noted previously the enrichment of palmitic acid-label in the ceramides of the cytoskeleton (2). Although very little labeled arachidonic acid was detected in ceramide in intact platelets, ceramide labeled with arachidonic acid was higher in the cytoskeleton.

In earlier experiments (1), we found that incorporation of palmitic acid label into the cytoskeleton of rabbit and human platelets increased exponentially as aggregation proceeded and decreased exponentially during deaggregation, and we suggested that the extent of incorporation was related to the number of points of contact between platelets. It is tempting to speculate that ceramide and phospholipids that are readily labeled with saturated fatty acids may be specifically associated with these sites of contact.

Schick (16) suggested that certain lipids may anchor the cytoskeleton to the plasma membrane and a number of investigators have reported non-covalent associations of lipids with cytoskeletal proteins (17). Phospholipids and ceramide that are readily labeled with saturated fatty acids may thus play a part in the involvement of the cytoskeleton in the early stages of platelet aggregation.

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Effect of 3-Hydroxy-3-methylglutaryl Coenzyme A Reductase Inhibition on Human Gut Mucosa

Roger L. Gebhard*, Stephen L. Ewing, Linda A. Schlasner, Donald B. Hunninghake and William F. Prigge

Departments of Medicine and Laboratory Medicine and Pathology, VA Medical Center, Minneapolis, Minnesota 55417 and University of Minnesota, Minneapolis, Minnesota 55455

Mevalonic acid is an important biochemical intermediate in cholesterol synthesis and other processes involved in cell replication. 3-Hydroxy-3-methylglutaryl coenzyme A (HMG-CoA) reductase is the enzyme which catalyzes mevalonic acid synthesis. To determine whether a potent competitive inhibitor of this enzyme, the drug simvastatin, may have an adverse effect on enterocyte cell replication and cholesterol metabolism, small intestinal biopsies from nine hypercholesterolemic subjects were obtained before and during treatment with simvastatin as a lipid-lowering agent. Histologic review of biopsies in a blinded manner detected no change in ratio of villous length to crypt length or in mitotic index which might indicate altered cell replication. Similarly, no significant change in measured activity of HMG-CoA reductase activity was observed. In spite of the high exposure of jejunal mucosal cells to this potent competitive inhibitor of a key enzyme, no adverse effect on growth could be detected.

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3-Hydroxy-3-methylglutaryl coenzyme A (HMG-CoA) reductase is the rate-determining enzyme of cholesterol synthesis (1,2). The enzyme catalyzes formation of mevalonate, an important precursor not only for cholesterol, but also for ubiquinone, dolichols and isopentanyl adenine (3). All of these mevalonate products are involved in cell replication (3–5). Competitive inhibitors of HMG-CoA reductase are an important new class of drugs with potent cholesterol lowering activity. Such drugs include lovastatin (formerly mevinolin), simvastatin, and pravastatin.

In tissue culture experiments, competitive inhibitors of HMG-CoA reductase have been shown to prevent cell growth and replication (5–7). This inhibition of growth can be reversed by providing mevalonate (the enzyme product) to the cells (6,7). Reductase inhibitors *in vitro* and *in vivo* have also been shown to induce HMG-CoA reductase levels within cells in response to activity inhibition (8–10). Gut mucosal cells are rapidly replicating cells, having a renewal time of 4–6 days (11). Gut enterocytes are also the portal of entry for therapeutic reductase inhibitors, so these cells are exposed to a high drug concentration. For these reasons, gut mucosa may be particularly susceptible to any adverse effects of reductase inhibitors on cell growth and reductase enzyme inhibition. Stange *et al.* (12) have reported evidence for decreased enterocyte differentiation when gut mucosal explants were cultured in the presence of compactin.

*To whom correspondence should be addressed at GI Section (111D), VA Medical Center, One Veterans Drive, Minneapolis, MN 55417.

Abbreviations: HMG-CoA, 3-hydroxy-3-methylglutaryl coenzyme A; LDL, low density lipoprotein.

The focus of the present study was to evaluate the effect of one potent reductase inhibitor on mucosal histology and HMG-CoA reductase activity. A subset of hypercholesterolemic patients participating in larger clinical trials of simvastatin (synvinolin; Merck, Sharp, and Dohme, West Point, PA), which have previously been reported (13,14), underwent pre- and post-treatment small bowel biopsy for histologic measurements and for determination of mucosal HMG-CoA reductase activity. Simvastatin is structurally similar to lovastatin and is about twice as potent as lovastatin for competitive inhibition of reductase enzyme *in vitro* (15).

METHODS

The study protocols were approved by human subjects committees at the University of Minnesota and the Minneapolis VA Medical Center, and informed consent was obtained from each subject. Type IIa and IIb hypercholesterolemic subjects entering two lipid-lowering drug trials were asked to undergo studies of bile lipids and/or small bowel mucosa before and during their treatment, and a subgroup of subjects volunteered (13,14). Following overnight fast, these volunteers for the intestinal intubation substudy underwent duodenal intubation for collection of gallbladder bile stimulated by 10% protein hydrolysate solution, followed by mucosal biopsy with the same Carey capsule-tipped biopsy tube. Results of the gallbladder bile studies have been previously reported (16). Mucosal biopsy samples were briefly rinsed in ice-cold saline and, if size permitted, divided into two pieces. If sufficient tissue was available, one portion was oriented with villous side up on filter paper and placed in formalin for subsequent histologic studies. A second portion (or the entire sample, in the event of small biopsies) was placed in sucrose/phosphate buffer, homogenized, and processed as previously described for measurement of HMG-CoA reductase activity in the whole mucosal homogenate (17). Total reductase activity was expressed as pmol mevalonate formed per mg mucosal protein per minute. Assays were done in duplicate and were shown to be linear with respect to protein.

Initial mucosal samples were obtained while subjects were treated only with American Heart Association Step I diet for at least four weeks. A second sample was obtained after subjects had been treated with this diet plus 7–22 wk of therapy with a study drug in accordance with the protocol of the broader cholesterol-lowering studies (13,14). Once the drug study code had been broken, pre- and post-treatment HMG-CoA reductase values for the nine subjects treated with 20 or 40 mg per day of simvastatin were compared. For histologic studies, the formalin-fixed tissue samples from seven simvastatin-treated subjects with both pre- and post-treatment biopsies were sectioned and stained with hematoxylin and eosin for histologic evaluation by standard criteria (18,19).

Criteria included villous/crypt ratio, villous surface cell morphology, number of mitoses/500 crypt cells, and lamina propria cellularity. Histologic sections were coded and independently evaluated in random sequence by two observers, a pathologist with special interest in GI histology and a gastroenterologist with experience in GI histopathology. The observers were not aware of the sequence for the paired biopsies.

RESULTS

All subject volunteers for intubation studies were males with a mean age of 56 years. Mean ± SE serum total cholesterol fell from 332 ± 15 mg/dL to 214 ± 11 mg/dL for the nine simvastatin subjects during the treatment period, while low density lipoprotein (LDL) cholesterol fell from 250 ± 15 mg/dL to 144 ± 14 mg/dL. These changes are consistent with our previous report (16). Individual subject data for intestinal HMG-CoA reductase activity are shown in Figure 1. Mucosal HMG-CoA reductase activity for these nine subjects prior to simvastatin treatment was 2.0 ± 0.3 pmol mevalonate formed/mg protein/min (mean ± SE). Mucosal reductase activity after 7-22 wk of simvastatin therapy (but at least 8 hr after last oral drug dose) was also 2.0 ± 0.3 pmol mevalonate formed/mg protein/min. Thus, there was no change in measured total reductase activity with simvastatin therapy. Values for five subjects were numerically greater during treatment, while values for four subjects were numerically lower during treatment, but all differences were minor.

Histologic assessment of jejunal biopsies from seven subjects having paired samples, as well as three subjects with only one sample, was independently completed by two reviewers without knowledge of the randomization pattern. A high degree of concordance existed for the grading by the reviewers. Interobserver variability for villous-to-crypt length ratio was within one unit difference for 94% of biopsies, mitotic index was within one mitosis/500 cells for 81% of biopsies, and inflammatory infiltrate rating was within one unit for 100% of biopsies. No differences existed for direction of change in biopsy pairs. Thus, ratings by the two reviewers were averaged for final tabulation.

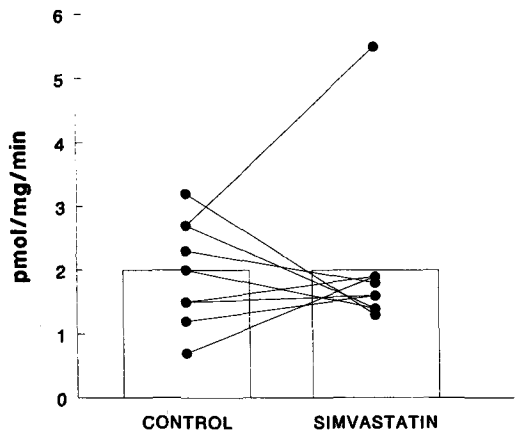


FIG. 1. HMG-CoA reductase activity for individual subjects before (control) and during oral treatment with simvastatin.

There were no significant histologic changes related to simvastatin treatment. All biopsies, pre- and post-treatment, demonstrated normal morphology of columnar cells lining the mucosa. The cellular infiltrate in the lamina propria consisted of the usual plasma cells and lymphocytes in normal numbers (all were graded 0-1 on a scale of 0-5 for increase in cellular density). No pattern of change occurred during treatment with the HMG-CoA reductase inhibitor. Villous/crypt length ratio and mitotic index pre- and post-treatment for each subject are shown in Figure 2 and Figure 3. No pattern of change in these features of mucosal regeneration occurred during inhibitor therapy, with equal numbers of pairs falling above and below the lines of identity. Furthermore, there were no correlations

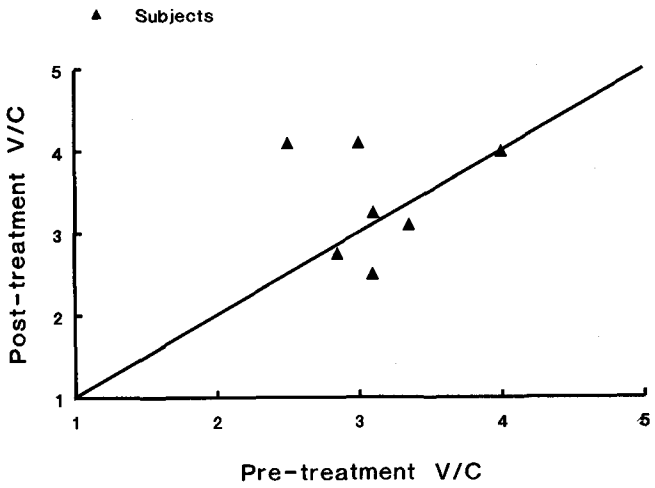


FIG. 2. Villous-to-crypt length ratios in jejunal biopsies from seven subjects before and after chronic simvastatin treatment. Individual values pre- and post-treatment are the mean of measurements from two observers blinded as to origin of specimens. No pattern of deviation from the line of identity is apparent.

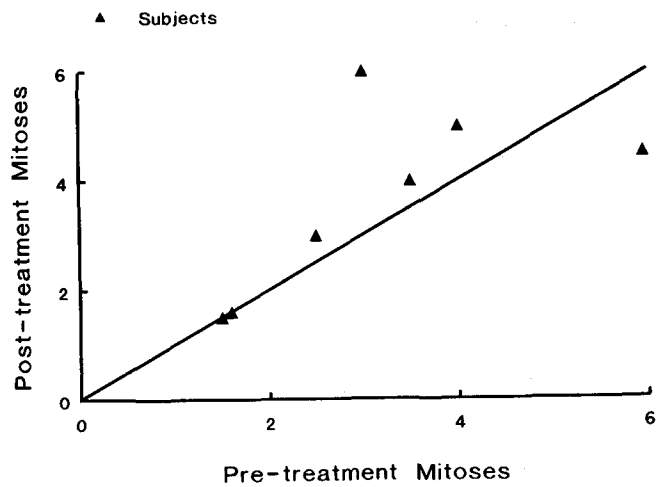


FIG. 3. Mitotic index (mitoses per 500 crypt cells) in jejunal biopsies from seven subjects before and after chronic simvastatin treatment. Individual values pre- and post-treatment are the mean of measurements from two observers blinded as to origin of specimens. No pattern of deviation from the line of identity is apparent.

between change in reductase activity or absolute level of post-treatment reductase activity and either villous/crypt ratio change or mitotic index change. Finally, three subjects had unpaired biopsies (two pretreatment and one post-treatment) for evaluation. Values for villous/crypt ratio, mitotic index, and inflammation for these samples were quite consistent with those of the paired samples.

DISCUSSION

In this study, simvastatin was clearly quite effective in reducing levels of serum cholesterol. Virtually all of the reduction occurred in the LDL cholesterol fraction. Simvastatin, similar to the other reductase inhibitors, is absorbed through the mucosal cells of the small intestine, and its metabolites are excreted into bile (20). In spite of high enterocyte exposure to this potent drug, and in spite of prior experience with the effects of reductase inhibitors on deoxyribonucleic acid synthesis and cell replication (5–7,21), no adverse histologic changes in gut mucosa morphology were observed. A significant decrease in enterocyte replication would be expected to result in consistently diminished crypt mitotic index and decreased villous/crypt length ratio. A shortened life span for mature enterocytes would be expected to result in increased crypt mitotic index and decreased villous/crypt length ratio, as seen in celiac sprue. Absence of either of these changes in a consistent manner suggests that this reductase inhibitor does not produce an adverse effect on enterocyte replication, in spite of results of *in vitro* studies (12).

Treatment with HMG-CoA reductase inhibitors has been shown to induce the quantity of enzyme mass and activity, after removal of the drug, in rat liver (9,22), human liver (23), human mononuclear leukocytes (10), fibroblasts (8) and rat and canine ileum (22). Our current finding, that measured HMG-CoA reductase activity in human jejunal homogenate is not induced by simvastatin, is consistent with our previous findings for lovastatin in human jejunal homogenates (17) and mouse jejunal mucosa microsomes (24). It is also consistent with the lack of effect on observed parameters of cell replication.

There are several possible explanations for the failure to measure jejunal enzyme induction by reductase inhibitors. Since activity was measured in whole mucosal homogenate, it is possible that simvastatin or an active metabolite remained present in the homogenate and served to inhibit measured enzyme activity. Prior studies with lovastatin suggested this possibility, since inhibitory activity was observed in some post-treatment homogenate samples (17). Similarly, enzyme induction in human mononuclear leukocyte microsomes was associated with a reduction in effective cholesterol synthesis *in vivo*, so that inhibitory drug activity was able to overcome enzyme induction in this circumstance (10). Studies reported in mice, however, measured reductase activity in microsomal pellets and also failed to show apparent enzyme induction of measured enzyme activity (24). Thus, it is also possible that jejunal mucosa may differ from other tissues, including rat ileum, in its ability for HMG-CoA reductase induction by enzyme inhibitors. Species differences are one possibility. Alternatively, parent drug absorbed by jejunal enterocytes may be less active intramucosally than metabolites following hepatic metabolism (20). Since biopsies were obtained at least 8 hr after last drug dose, it is also possible that any acute drug effect on this enzyme with known rapid turnover may have been too transient to have been detected. Finally, the previous study which did report enzyme induction in rat ileum (22) used proportionally much larger drug doses in rats (0.075% of diet weight) and in dogs (180 mg/kg/d).

ACKNOWLEDGMENTS

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Effect of Acylation Stimulating Protein on the Triacylglycerol Synthetic Pathway of Human Adipose Tissue

Zivart Yasruel, Katherine Cianflone, Allan D. Sniderman, Mark Rosenbloom, Mark Walsh and Miguel A. Rodriguez*

The McGill Unit for the Prevention of Cardiovascular Disease, McGill University, Royal Victoria Hospital, Montreal, Quebec, Canada H3A 1A1

Acylation stimulating protein (ASP) is a 14 kDa plasma protein which causes *in vitro* triacylglycerol synthesis in human adipocytes and fibroblasts to increase substantially. ASP was found to stimulate human adipose tissue microsomal glycerophosphate acyltransferase and diacylglycerol acyltransferase activities by 23% and 90%, respectively. However, phosphatidate phosphohydrolase activity showed no increase in activity, nor did microsomal acyl-CoA synthetase activity. Moreover, ASP did not decrease the apparent K_m of diacylglycerol acyltransferase (DGAT), but rather increased its apparent V_{max} suggesting direct interaction of ASP with DGAT. *Lipids 26,* 495–499 (1991).

We have recently reported the purification and partial characterization of acylation stimulating protein (ASP) from human plasma (1). ASP is a 14 kDa protein which stimulates triacylglycerol synthesis in human adipocytes (300% stimulation) and skin fibroblasts (170% stimulation) (1,2). Indeed, ASP appears to be the most potent *in vitro* stimulant yet described of triacylglycerol synthesis in human adipocytes. Beyond this, there is now evidence ASP may play an important role *in vivo* in the normal response to an oral fat load in that the higher the plasma ASP, the more rapid the clearance of triacylglycerol from plasma (3). Moreover, a reduced response of fibroblasts to ASP in terms of triglyceride synthesis appears to be characteristic of a subgroup of patients with hyperapobetalipoproteinemia (4,5).

In preliminary experiments with cell-free systems, ASP has been shown to stimulate fatty acid incorporation into triacylglycerol in the microsomal fraction of human adipose tissue (2). Since ASP is internalized after binding to a specific receptor in the cell membrane (5), this observation suggests that it may exert its physiological effect by interacting intracellularly with the enzymes involved in fatty acid metabolism. Therefore, in this study we have determined the effect of ASP on the *in vitro* activity of glycerolipid synthetic enzymes.

MATERIALS AND METHODS

Palmitoyl-CoA, oleic acid, diolein, L-α-glycero-3-phosphate, sodium phosphatidate (from egg yolk), ATP, CoA and bovine serum albumin (BSA) (essentially fatty acid free) were obtained from Sigma (St. Louis, MO). [9,10-^{3}H]Oleic acid (9 Ci/mmol), [2-^{3}H]L-glycerol-3-phosphate (10.6 Ci/mmol) and [1-^{14}C]palmitoyl-CoA (58 Ci/mol) were from New England Nuclear (Boston, MA).

ASP was purified from human plasma as previously described (3). Adipose tissue was obtained at surgery from obese patients undergoing gastroplasty, and the corresponding microsomal and cytosolic fractions were prepared by differential centrifugation of the homogenized tissue (6,7).

All enzyme assays were performed in duplicate at 37°C. Our assay of acyl-CoA synthetase activity is a modification of the method described by Bar-Tana *et al.* (8). An aliquot of microsomal preparation (from 1–5 μg protein) was incubated with 2.5 mM ATP, 0.5 mM CoA and 18 μM [^{3}H]oleic acid (46 Ci/mol, New England Nuclear) in 250 μL of 0.1 M Tris/HCl, pH 7.5, containing 2mM DTT and 10 mM $MgCl_2$. The tritiated fatty acid was delivered first in 2-propanol to the empty glass tube followed by evaporation of the solvent under an N_2 stream. After 1-min incubation at 37°C, 1 mL of Dole's reagent (2-propanol/heptane/1 N H_2SO_4, 40:10:1, v/v/v) was added to stop the reaction. Following addition of 0.3 mL H_2O and 0.6 mL heptane and vortexing, the lower phase was washed three times with heptane and an aliquot of 250 μL was transferred to a vial containing 7.5 mL Scintiverse II (Fisher Scientific Co., Montreal, Canada) for counting in a Beckman LS-1800 beta counter (Beckman Instruments, Inc., Irvine, CA).

Microsomal glycerophosphate acyltransferase activity was determined by measuring the incorporation of 1 mM [^{3}H]glycero-3-phosphate into butanol-soluble products in the presence of oleic acid, ATP and CoA. The incubation mixture had essentially the same composition as previously, except that 1 mM [^{3}H]glycerophosphate (2.5 Ci/mol) was added, and oleic acid was not radioactive. At the indicated times, the reaction was stopped with 0.75 mL of butanol saturated with water. The butanol phase was washed three times with 1 mL of water saturated with butanol. An aliquot of 400 μL was counted in Scintiverse. When resolved by thin-layer chromatography (TLC), neutral lipids (diacylglycerol and triacylglycerol) accounted for 4% of the extracted radioactivity, whereas phosphatidate and lysophosphatidate accounted for about 95%.

Phosphatidate phosphohydrolase (PAPH) activity was assayed by measuring the rate of phosphate release from exogenous phosphatidate (9). Aliquots of microsomes (about 10 μg protein) were incubated for 1 hr in 0.5 mL of 25 mM tris-maleate, pH 6.5, containing 1 mM DTT, 5 mM $MgCl_2$, and 0.8 mg/mL of sodium phosphatidate. The reaction was stopped with 100 μL of 60% trichloracetic acid and 10 μL of 30 mg/mL BSA. After 20 min at 0°C, the protein was centrifuged down and a 400 μL aliquot from the supernatant was removed for phosphate determination (10). Under these conditions, the apparent K_m of the enzyme for phosphatidate was 50 μg/mL.

*To whom correspondence should be addressed at the Biochemistry Department, University of Ottawa, 31 George Glinski, Ottawa, Ontario K1N 6N5.

Abbreviations: ASP, acylation stimuating protein; BSA, bovine serum albumin; DGAT, diacylglycerol acyltransferase; DTT, dithiothreitol; FABP, fatty acid binding protein, GPAT, glycerophosphate acyltransferase; PAPH, phosphatidate phosphohydrolase; TLC, thin-layer chromatography.

Microsomal diacylglycerol acyltransferase was assayed by measuring the rate of esterification of the acyl-CoA formed *in situ* with exogenous diacylglycerol. The incubation mixture was the same as in the acyl-CoA synthetase assay, but 30 nmol of diolein were delivered in 2 μL of acetone at the beginning of an 8-min incubation. Following addition of 1 mL of Dole's reagent, the top phase was washed three times with 1 mL of 2-propanol/heptane (4:1, v/v) and 1 mL of 0.05% KOH, and a 500-μL aliquot was counted in Scintiverse. When the extracted lipids were resolved by TLC on silica gel, about 90% of the radioactivity migrated with the triacylglycerol fraction and 5% with the diacylglycerol fraction.

Protein concentrations were measured by the dye-binding method of Bradford (11), using BSA as standard. Neutral lipids were resolved by TLC on silica gel developed with hexane/diethyl ether/acetic acid (85:15:1, v/v/v).

Kinetic parameters were determined by non-linear regression to the Michaelis Menten equation by using the program Enzfitter (Biosoft, Cambridge, U.K.).

RESULTS

ASP effect on microsomal acyl-CoA synthetase. We first investigated the effect of ASP on microsomal acyl-CoA synthetase from human adipose tissue. As shown in Figure 1, the initial rate of acyl-CoA formation declined rapidly with time and after 15 min incubation approached a plateau level of acyl-CoA formation corresponding to about 40% of the added [^{3}H]oleic acid. This plateau level increased with the amount of microsomes in the incubation medium and likely reflected product inhibition of the enzyme. Addition of up to 60 μg/mL of ASP to the assay medium did not result in any significant change in either the rate of oleoyl-CoA synthesis: 112 ± 16 (SEM) nmol/min/mg *vs* 115 ± 19 nmol/min/mg for the control (averages of six independent experiments), or the plateau levels. This was confirmed by measuring the amount of acyl-CoA formed at 1- and 15-min incubation in the presence of increasing amounts of ASP (not shown). Under our experimental conditions, microsomal acyl-CoA synthetase showed an apparent K_m for oleic acid of about 5 μM, and thus the experiment of Figure 1 was performed at a saturating concentration of fatty acid (18 μM). However, when the experiment was repeated with 5 μM oleic acid, a similar result was obtained.

ASP effect on microsomal GPAT. Microsomal glycerophosphate acyltransferase activity (GPAT) was determined by measuring the esterification of [^{3}H]glycero-3-phosphate with *in situ* formed oleoyl-CoA. The initial rate of GPAT activity at 18 μM oleate (9.1 ± 0.24 nmol/min/mg) was more than ten times lower than the rate of acyl-CoA formation; thus no lag is observed in Figure 2. Addition of ASP (60 μg/mL) to the incubation medium resulted in a slight but significant increase of glycerophosphate incorporation into lipids. The stimulation of 23% ± 7.8% (SEM) with 10-min incubation was significant (n=5, $p<0.05$ paired *t*-test). The effect was concentration dependent and reached a plateau at 30 μg/mL ASP. No change in the level of GPAT stimulation by ASP

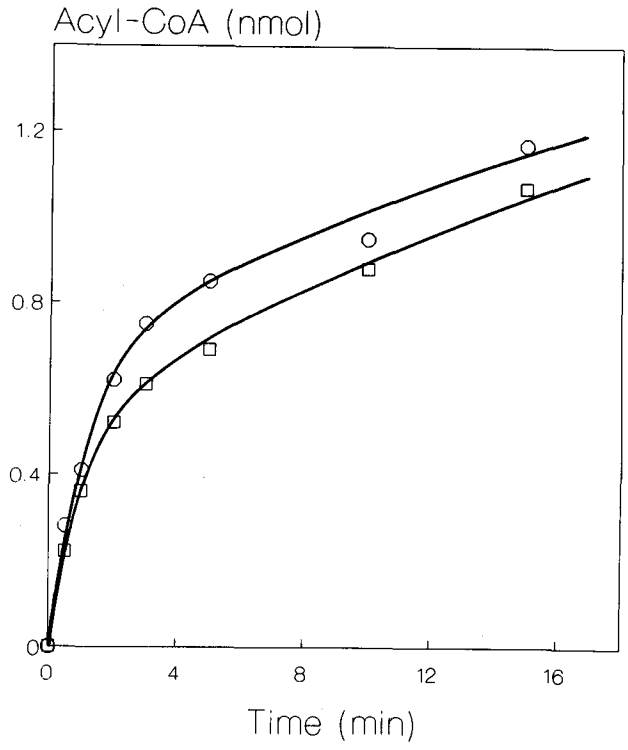


FIG. 1. ASP effect on acyl-CoA synthetase activity. Human adipose tissue microsomes (10 μg/mL) were incubated with (□) and without (○) 60 μg/mL ASP under the conditions described for acyl-CoA synthetase assay. At indicated times, 250 μL aliquots were removed and their [^{3}H]oleoyl-CoA content determined.

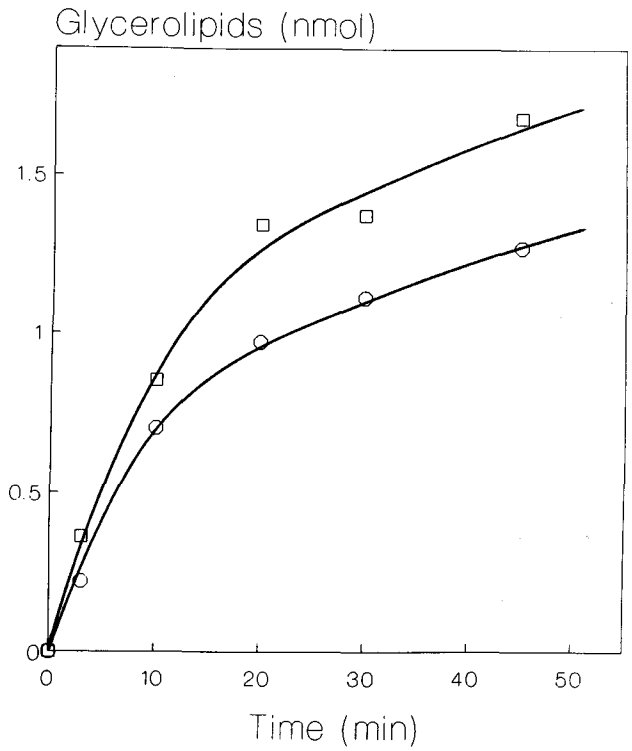


FIG. 2. ASP effect on glycerophosphate acyltransferase. Human adipose tissue microsomes (8 μg) were assayed for glycerophosphate acyltransferase activity in the presence (□) and absence (○) of 60 μg/mL ASP.

was observed in the range of oleate concentration from 1 to 36 μM (below saturation of the enzyme). A similar degree of stimulation was observed when fatty acid, CoA and ATP were substituted by 20 μM oleoyl-CoA in the assay.

ASP effect on PAPH. We then investigated the effect of ASP on phosphatidate phosphohydrolase activity by using exogenous phosphatidate delivered as an aqueous emulsion. ASP was found not to increase the rate of phosphate release (19.8 ± 1.4 nmol/min/mg *vs* 20.2 ± 0.5 nmol/min/mg in the absence of ASP) at high phosphatidate concentration (0.8 mg/mL). Similarly, ASP did not change the activity of cytosolic PAPH, whose specific activity was about 2.5 times lower than the microsomal activity (data not shown).

By contrast, at a non-saturating concentration of phosphatidate (33 μg/mL), ASP inhibited PAPH activity by 40 ± 6% (n=6, $p<0.01$, paired *t*-test). The effect was dose dependent and plateaued at about 20 μg/mL ASP.

Using a different approach, we measured the effect of ASP on the glycero-3-phosphate dependent synthesis of neutral lipids. In these experiments we proceeded as for the DGAT assay, but diolein was replaced with 1 mM glycero-3-phosphate. In the presence of 0.2 mg/mL cytosolic fraction, the rate of incorporation of oleic acid into neutral lipids was 0.6 ± 0.2 (SEM) nmol/min/mg. Upon addition of 80 μg/mL ASP that rate increased by 43 ± 12% (SEM) (n=6, $p<0.05$, paired *t*-test).

ASP effect on DGAT. Under our experimental conditions, microsomes from human adipose tissue catalyzed the esterification of oleic acid with diolein in the presence of ATP and CoA at an average rate of 5.0 ± 0.25 nmol/min/mg (SEM) (13 determinations). Addition of ASP resulted in an increase in the rate of triacylglycerol synthesis (Fig. 3a). The effect is dependent on the concentration of ASP, reaching a plateau of maximal stimulation at about 80 μg/mL of ASP (Fig. 3b). The average maximal stimulation observed in 12 experiments with different preparations of microsomes and ASP was 90 ± 10% (SEM) ($p<0.001$ paired *t*-test). A similar degree of stimulation was observed when fatty acid, CoA and ATP were substituted by 20 μM oleoyl-CoA in the incubation medium. Substitution of 6 μM ASP (84 μg/mL) by 20 μM BSA did not stimulate diacylglycerol acyltransferase activity; in fact, addition of BSA to the incubation medium prevented stimulation by ASP (results not shown).

To investigate whether the ASP effect was due to improved delivery of substrates or to increased specific activity of the enzyme, we followed the fatty acid and diacylglycerol dependence of DGAT activity in the presence and absence of ASP (Fig. 4). Stimulation was observed at all oleate concentrations assayed in the range of 1-36 μM (Fig. 4a) and at all diolein concentrations in the range of 15-250 μM (Fig. 4b). The results were analyzed by non-linear regression to the Michaelis Menten equation in order to obtain the apparent kinetic parameters for both substrates (Table 1). At 120 μM diolein, the apparent V_{max} for oleate saturation (7.2 ± 0.36 nmol/min/mg) (SEM) was increased by 100% in the presence of ASP. No significant change was observed for the apparent K_m for oleate. A similar degree of stimulation (150%) by ASP was observed for the V_{max} corresponding to diolein saturation in the presence of 18 μM oleate, even though the microsomal preparation for this experiment showed

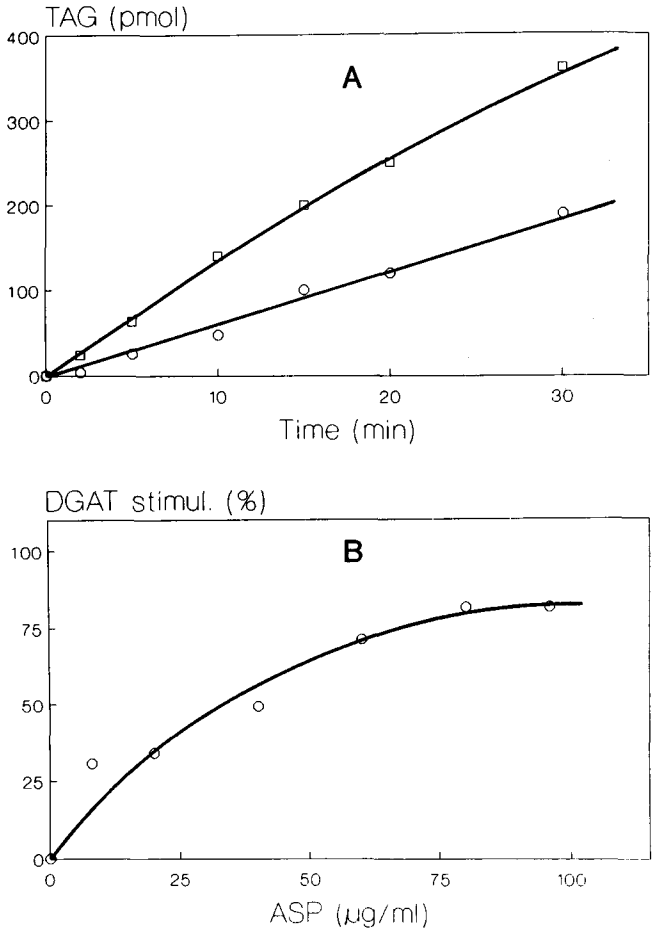


FIG. 3. ASP effect on diacylglycerol acyltransferase activity. A) Microsomes (1.7 μg) were assayed for incorporation of [^{3}H]oleic acid into triacylglycerol (TAG) in the presence (□) and absence (○) of 80 μg/mL ASP as described for the DGAT assay. B) Microsomes (6 μg) were assayed for DGAT activity in the presence of increasing amounts of ASP and the results expressed as percentage stimulation over the control rate (3.2 nmol/min/mg).

TABLE 1

ASP Effect on DGAT Kinetic Parameters

Substrate	Parameter[a]	Control	ASP	p[b]
Oleate[c]	V_{max} (nmol/min/mg)	7.2 ± 0.36	14.5 ± 1.3	<0.001
	K_m (μM)	8.3 ± 1.1	10.7 ± 2.1	n.s
Diolein[d]	V_{max} (nmol/min/mg)	3.2 ± 0.53	8.0 ± 1.1	<0.01
	K_m (μM)	51 ± 25	58 ± 24	n.s

[a] Apparent kinetic parameters for the experimental conditions described in the legend of Figure 4.

[b] For *t*-test.

[c] Three independent experiments were performed with variable concentrations of oleate as described in the legend of Figure 4a. Non-linear regression to the Michaelis Menten equation was computed for each experiment. Average values ± SEM are given.

[d] Results in Figure 4b were analyzed by non-linear regression as above. Fitted parameters ± standard error are given.

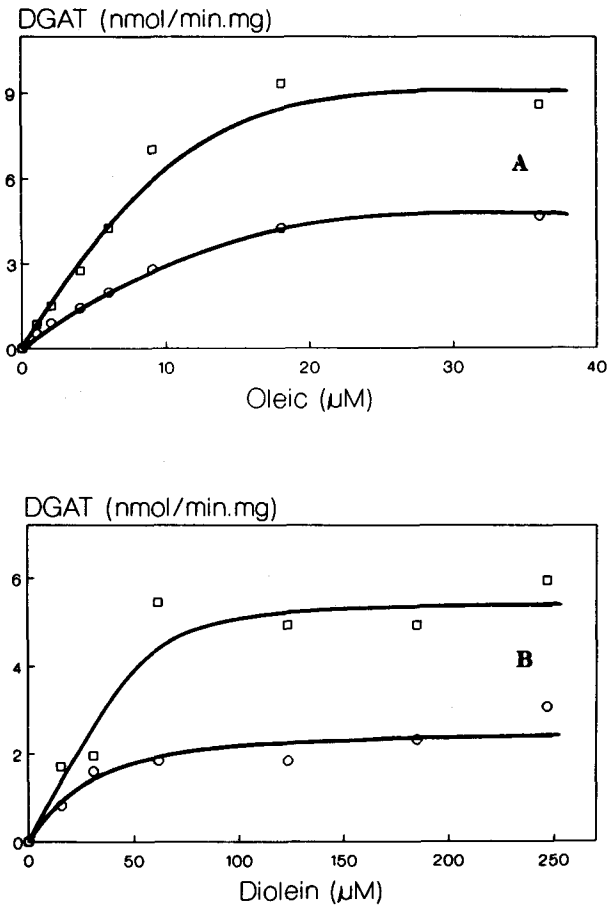


FIG. 4. Substrate dependence of ASP effect on DGAT. A) Microsomes (4 μg) were assayed for diacylglycerol acyltransferase activity with 0.12 mM diolein and different oleic acid concentrations in the presence (□) and absence (○) of 80 μg/mL ASP. B) Microsomes (5 μg) were assayed for diacylglycerol acyltransferase activity with 18 μM oleic acid and different diolein concentrations in the presence (□) and absence (○) of 60 μg/mL ASP. Results were corrected for activity with no diolein added.

lower DGAT activity. By contrast, estimates of K_m for diolein showed no significant change in the presence of ASP.

DISCUSSION

A number of cytosolic proteins, in particular fatty acid binding protein (FABP) have been reported to stimulate *in vitro* triacylglycerol synthesis in microsomes (12–16), and it has been proposed that FABP stimulates *in vivo* lipid synthesis by enhancing delivery of fatty acid and/or acyl-CoA to the lipid synthetic enzymes in the endoplasmic reticulum (17).

ASP also stimulates triacylglycerol synthesis in adipocytes but, in contrast to FABP, does so in intact cells (1,2). Because ASP is internalized after binding specifically to the cell membrane (5), and because the degree to which ASP stimulates triacylglycerol synthesis is proportional to the extent of its binding and internalization (5), and because ASP stimulates triacylglycerol synthesis in broken cell preparations (2), we have considered the hypothesis that it may be the internalized ASP which is the active metabolic agent. If so, it seemed likely that ASP would affect one or more of the enzymes involved in microsomal triacylglycerol synthesis.

Since at present there is no consensus as to which enzyme controls the rate limiting step in adipose tissue triglyceride synthesis, it was decided to investigate all the enzymes involved with the exception of monoacylglycerophosphate acyltransferase. Monoacylglycerophosphate acyltransferase was not assayed because of the difficulty involved in assaying this enzyme, and because it has been established that GPAT controls the rate limiting step in phosphatidate synthesis in microsomes.

Two possibilities were considered—either that ASP was acting to increase delivery of fatty acid or acyl-CoA to the microsomal enzymes as has been shown for FABP, or that it was directly affecting enzyme activity. The assays, therefore, were designed to avoid any fatty acid or acyl-CoA delivery system such as BSA or cytosolic proteins since, in their absence, any enzyme activity stimulated by ASP could then be analyzed for an effect on K_m, which would suggest an effect on substrate delivery. No such effects were found.

Microsomes readily take up oleic acid, and in the presence of ATP and CoA synthesize oleoyl-CoA at a much higher rate than can be utilized by the triglyceride synthetic enzymes. Therefore, a relatively constant pool of acyl-CoA is created which can be used for the assay of GPAT and DGAT activities. ASP has no significant effect either on acyl-CoA synthetase initial rate or plateau level at both saturating and non-saturating fatty acid concentrations.

The slight, but statistically significant, effect of ASP on GPAT activity (23%) is independent of oleate concentrations in the range of 1–36 μM, a concentration range below saturation of the enzyme. This suggests an increase in the intrinsic GPAT activity rather than increased delivery of *in situ*-formed oleoyl-CoA. A complete study of the effect of ASP on the kinetic parameters of the enzyme was prevented by the difficulty of achieving saturation of the enzyme with oleate and by the small net changes observed.

The inhibitory effect of ASP on PAPH activity with exogenous phosphatidate at subsaturating concentration may not reflect a specific effect on the enzyme, but rather interference with the delivery of substrate to the membrane vesicles. Indeed, when we used *in situ*-formed phosphatidate from acyl-CoA and glycerophosphate, the rate of neutral lipid (di- and triacylglycerol) formation was significantly increased by 40%. This stimulation cannot be fully accounted for by the 23% stimulation of GPAT activity.

The clearest effect of ASP was observed in the assay of DGAT activity with exogenous diacylglycerol. ASP doubles the maximal activity of the enzyme at saturating concentrations of both oleate and diolein, suggesting that it increases the intrinsic DGAT activity rather than improving substrate delivery. Indeed, the K_m of the enzyme for both substrates are not significantly changed by ASP.

The percent stimulation of DGAT was independent of fatty acid or diacylglycerol concentration, but was dependent on ASP concentration and shows saturation. Thus, ASP could be a cofactor of DGAT similar to the way in which apo-CII is a cofactor of lipoprotein lipase. Other less specific mechanisms could also explain our results. For

instance, modification of membrane structure resulting in conformational changes of enzymes or inhibition of phosphatidate and triglyceride lipase(s) could occur.

Whether the observed effect on DGAT fully explains the mechanism of action of ASP is not clear since maximal stimulation in broken cell preparations is less than that in intact cells. Also, we have as yet to establish that intact ASP is present in adipocytes. Nevertheless, we believe that the present data constitute further evidence that ASP may play a fundamental role in fatty acid metabolism in humans.

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Effect of Fish Oil Supplementation on the Excretion of the Major Metabolite of Prostaglandin E in Healthy Male Subjects

Aldo Ferretti[a,*], Joseph T. Judd[a], Rachel Ballard-Barbash[b], Padmanabhan P. Nair[a], Philip R. Taylor[b] and Beverly A. Clevidence[a]

[a]Lipid Nutrition Laboratory, Beltsville Human Nutrition Research Center, ARS-USDA, Beltsville, Maryland 20705 and [b]Division of Cancer Prevention and Control, National Cancer Institute, NIH, DHHS, Bethesda, Maryland 20892

We investigated the effect of fish oil supplementation on the synthesis of prostaglandin E (PGE) *in vivo* by measuring the excretion of its catabolite, PGE-M, in 24-hr urine by gas chromatography/mass spectrometry. Forty healthy male volunteers (24–57 years of age) consumed a controlled basal diet providing 40% of energy from fat (P/S ratio about 0.8:1), 130 mg/1000 kcal cholesterol, and a minimum of 22 mg/day of α-tocopherol (α-T), for three experimental periods lasting a total of 28 weeks. During period 1 (10 weeks) the diet was supplemented with placebo (PO) capsules (15 × 1 g/day) consisting of a blend of fats approaching the fatty acid profile of the basal diet. This was followed by a second 10-week period during which the subjects received 15 × 1 g/day capsules of fish oil concentrate (FOC). During period 3 (8 weeks) they continued the 15 g/day intake of FOC but received an additional 200 mg/day of α-T. PO and FOC capsules contained 1 mg α-T/g fat as antioxidant. A 14% reduction of PGE-M excretion was observed after 10 weeks of FOC supplementation (period 2), compared to an identical period of placebo supplementation (period 1), P=0.009. PGE-M excretion during the last week of period 3 was not significantly different from that at the end of period 2. The reduction in PGE synthesis in response to the relatively high marine oil supplementation was large in many subjects participating in this study.
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A protective effect of ω3 polyunsaturated fatty acids (PUFA) against atherothrombotic disorders has been established reasonably well from studies with humans and animal models (1–5). The underlying biochemical mechanisms of this protective action are not entirely understood, but a major role has been attributed to effects on ω6 fatty acid metabolism with resultant changes in the profile of products of arachidonate cyclooxygenation (5,6). Considerable work has been done to evaluate the effects of ω3 PUFA on thromboxane (TXA_2) and prostacyclin (PGI_2) synthesis (4,6–10). This is justified in view of the preeminent roles of these eicosanoids in vascular biology. It is a reasonable assumption, however, that in addition to thromboxane and prostacyclin, other eicosanoids may also respond to dietary manipulations of adequate magnitude.

*To whom correspondence should be addressed at USDA, ARS, Lipid Nutrition Laboratory, Bldg. 308, Rm. 122, BARC-EAST, 10300 Baltimore Avenue, Beltsville, MD 20705-2350.

Abbreviations: α-T, α-tocopherol; EPA, eicosapentaenoate; FOC, fish oil concentrate; GC/MS, gas chromatography/mass spectrometry; PGE, prostaglandin E; PGE-M, 11α-hydroxy-9,15-dioxo-2,3,4,5,20-pentanor-19-carboxyprostanoic acid; PGI_2, prostacyclin; PO, placebo oil; P/S, ratio dietary linoleate to saturated fatty acids; PUFA, polyunsaturated fatty acids; TXA_2, thromboxane A_2.

Prostaglandin E_2 (PGE_2) has been characterized as the most common and most potent of mammalian prostaglandins. It is clearly involved in mechanisms of blood pressure regulation (11,12), tumorigenesis (13–15), and immune response modulation (16–18). Notwithstanding these critical roles, surprisingly little is known about the capacity of dietary fish oil to modulate PGE_2 synthesis in humans *in vivo* and attendant physiologic consequences (4).

The present study was designed to determine if some changes in physiological variables attributed to fish oil consumption might be explained by altered PGE biosynthesis. Based on the ubiquity and potency of PGE, we expected this work to help identify other, yet uncovered, effects of fish oil consumption. We quantified the effect of a relatively high supplementation of a marine oil concentrate on prostaglandin E biosynthesis, by measuring the urinary excretion rate (μg/24 hr) of 11α-hydroxy-9,15-dioxo-2,3,4,5,20-pentanor-19-carboxyprostanoic acid (PGE-M). This compound is commonly regarded (19,20) as an index of systemic production of E-series prostaglandins (E_2+E_1).

MATERIALS AND METHODS

Subjects. Forty non-smoking male subjects, 24–57 years of age (mean 37.6 ± 8.8, SD), were selected among volunteers from the area surrounding the Beltsville Agricultural Research Center (Beltsville, MD). They were screened by a preliminary medical questionnaire to eliminate those with complicating health problems and unusual dietary habits. Regular users of dietary supplements (especially vitamin E) and of excessive quantities of alcohol were also excluded. Potential subjects were examined by a physician, and by hematological and chemical screening of blood and urine. Those with plasma α-tocopherol (α-T) levels outside of the normal range were excluded. Body weights were between 90 and 120% of the desirable weights as given in the 1983 Metropolitan Life Insurance tables. Aspirin, aspirin-containing drugs and other anti-inflammatory drugs were not allowed during the study. Occasional use of tylenol was the only permitted analgesic. Antibiotics and other medications taken during the study under a physician's direction were evaluated for possible effects on study parameters. All procedures were approved by the Human Studies Committees of the National Institutes of Health and the Georgetown University School of Medicine.

Controlled diets. The basal diet for the controlled dietary periods was designed to reflect the typical dietary pattern of Beltsville area volunteers as determined in earlier studies, except that the fat level of the diet was reduced to allow isoenergetic supplementation with 15 g/day placebo oil (PO) or fish oil concentrate (FOC) without permitting energy from fat to exceed 40% of total. Cholesterol intake was about 130 mg/1000 kcal. All nutrients

for which food data are available were provided by the diets in amounts to meet the Recommended Dietary Allowances (21). Caloric intake was adjusted in increments of 400 kcal to maintain constant body weights throughout the study. The contents of long-chain (C_{20}, C_{22}, and C_{24}) ω3 fatty acids were minimized in the basal diet by excluding fish from the menus, while vitamin E content was minimized by excluding highly fortified foods from the diet.

A fourteen-day menu cycle provided variety and helped maintain acceptability of the diets. During the controlled diet periods, breakfast and supper were consumed in the Beltsville Human Nutrition Research Center, Human Studies Facility (HSF), Monday through Friday. A box lunch was provided at breakfast time. Weekend and holiday meals were pre-packaged in the HSF for home consumption and used the same menus as other days of the menu cycle. No foods other than those provided by the study were permitted, and alcohol consumption was not allowed during the controlled diet periods.

Lipid supplementation/placebo oil (PO). The placebo consisted of a blend of commonly available fats (corn oil, beef tallow, lard) approaching the fatty acid profile of the basal diet, and was encapsulated in one-gram amounts with one mg α-T per capsule as antioxidant. PO was prepared and encapsulated by Hoffmann-La Roche, Inc. (Nutley, NJ), and was indistinguishable in external appearance from the fish oil concentrate.

Fish oil concentrate (FOC). The ω3 fatty acid supplement (ROPUFA®) was an anchovy oil derivative prepared in one batch sufficient for the duration of the study. FOC was prepared by Hoffmann-La Roche by a distillation/deodorization process which increases the ω3 fatty acid content to about 50%, compared to about 30% for most commercial fish oil supplements. FOC was also provided as hard gelatin capsules containing one gram of fat and one mg α-T. Table 1 shows the main fatty acid constituents of the placebo and fish oil supplements. Both supplements were administered to participants at the rate of 15 g/day, 7 capsules at breakfast and 8 capsules at supper.

Experimental protocol. Prior to supplementation, the subjects were on a self-selected diet for two weeks while baseline measurements were taken. This was followed by the controlled diets described above for a total of 28 weeks divided into three periods according to the supplement given: Period 1 (ten weeks) 15 g/day PO; Period 2 (ten weeks) 15 g/day FOC; Period 3 (eight weeks) 15 g/day FOC plus 200 mg/day α-tocopherol in two equal doses. Dieticians observed the subjects as they consumed the oil capsules and the vitamin E supplements. No attempt was made to provide a placebo for the vitamin E supplement. The estimated daily intake of nutrients for the three periods was (in percent of energy): fat, 40%; carbohydrate, 45%; and protein, 15%. α-Tocopherol intake from natural foods was at least 22 mg. Table 2 shows the estimated daily fatty acid intakes during the controlled-diet periods.

Urine collection. Three consecutive 24-hr urines were collected during the last week of each period. Samples were collected in silanized glass bottles and kept on ice during the collection period. After the 24-hr collections were completed, the volumes were measured, 2%-portions of each 24-hr collection were pooled and either analyzed immediately or stored at −22°C.

Measurement of PGE-M. Analyses of 11α-hydroxy-9,15-dioxo-2,3,4,5,20-pentanor-19-carboxyprostanoic acid (PGE-M) were done on 20-mL aliquots of the 72-hr pools prepared as described above. This enabled us to assess the mean daily excretion of PGE-M during the 72-hr period. Procedures and instruments were described previously by Ferretti *et al.* (22). When prolonged urine storage was unavoidable, the PGE-M values were adjusted to compensate for decay according to a first-order rate constant determined in our laboratory (23). PGE-M excretion rates are expressed as μg/24 hr.

TABLE 1

Principal Fatty Acid Constituents of Placebo and Fish Oil Supplements

Fatty acids	g/100 g Supplement	
	Placebo	Fish oil
14:0	2.1	4.9
16:0	21.8	9.3
16:1ω7	2.4	6.5
18:0	13.3	1.4
18:1ω9	36.4	5.4
18:2ω6	13.7	1.9
18:3ω3	0.4	1.0
18:4ω3	ND[a]	4.6
20:1ω9	0.4	0.4
20:4ω6	0.2	1.4
20:4ω3	ND	1.1
20:5ω3	ND	30.2
22:5ω3	ND	2.5
22:6ω3	ND	13.1
Saturated[b]	38.4	16.5
Monounsaturated[b]	42.8	15.7
Polyunsaturated[b]	14.4	63.6
ω6 Fatty acids	13.9	3.7
ω3 Fatty acids	0.4	52.5
Ratio ω3/ω6 Fatty acids	~0.0	14.2

[a]None detected.

[b]Including minor constituents.

TABLE 2

Estimated Daily Fatty Acid Intake on Controlled Diets[a] (g/day at 2800 kcal)

Fatty acids	Supplement	
	Placebo	Fish oil
Total saturated (S)	33	29
Palmitic (16:0)	20	18
Stearic (18:0)	9	7
Total monounsaturated (M)	46	42
Oleic (18:1ω9)	44	39
Total polyunsaturated (P)	27	33
Total ω3 polyunsaturated	2	10
α-Linolenic (18:3ω3)	2	2
Eicosapentaenoic (20:5ω3)	0	5
Docosahexaenoic (22:6ω3)	0	2
Total ω6 polyunsaturated	25	23
Linoleic (18:2ω6)	25	23

[a]Including contributions from indicated supplement.

Statistical analysis. Twenty-four-hr PGE-M excretion rates and lipid intakes were evaluated by paired *t*-tests with the computer methodology of the Statistical Analysis System (SAS Institute, Inc., Cary, NC). All P values less than 0.05 were considered statistically significant.

RESULTS

Four subjects were withdrawn entirely from the study because they developed health conditions that required administration of interfering prescription drugs. This left 36 subjects for analysis. Period 3 PGE-M excretion data from one subject (No. 35) were disregarded because of a health problem that required prescription drugs during period 3, and period 3 PGE-M data from another subject (No. 36) were disregarded because of non-adherence to study protocol. The data were analyzed by analysis of variance. There were no significant carryover effects. A paired *t*-test was therefore utilized for comparison between diets as planned in the study design.

TABLE 3

PGE-M Urinary Excretion Rates (μg/24 hr) During the last Week of the Indicated Diet Periods

Subject	Diet period 1	Diet period 2	Diet period 3	%Reduction[a]
1	3.79	3.44	3.36	9.2
2	30.83	13.11	26.05	57.5
3	13.15	11.67	6.40	11.3
4	11.48	21.91	7.92	−90.8
5	9.44	15.64	21.39	−65.7
6	14.71	13.09	7.63	11.0
7	8.05	6.09	8.64	24.3
8	19.62	12.49	17.13	36.3
9	12.71	9.07	8.53	28.6
10	11.06	11.03	9.35	0.3
11	5.93	4.85	4.23	18.2
12	23.18	9.85	5.68	57.5
13	52.28	38.54	40.96	26.3
14	14.48	12.58	14.75	13.1
15	11.12	2.78	16.47	75.0
16	6.09	5.66	6.35	7.1
17	18.61	13.65	12.64	26.7
18	5.04	4.45	4.33	11.7
19	12.76	10.04	12.95	21.3
20	10.84	5.53	3.83	49.0
21	8.00	7.78	7.43	2.7
22	8.24	8.24	9.03	0.0
23	4.52	4.01	3.85	11.3
24	60.92	58.98	48.64	3.2
25	4.61	4.84	4.35	−5.0
26	17.49	13.96	14.22	20.2
27	41.14	29.31	38.56	28.8
28	16.89	16.00	12.16	5.3
29	11.93	8.21	8.17	31.2
30	12.33	9.79	8.07	20.6
31	10.78	10.57	8.69	1.9
32	10.16	11.29	12.03	−11.1
33	7.76	6.06	8.23	21.9
34	13.44	13.05	12.28	2.9
35	6.55	6.92	UD[b]	−5.6
36	24.93	15.94	UD[b]	36.1

[a]Reduction calculated as period 1 minus period 2.
[b]Data not recorded (see Results).

The mean PGE-M urinary excretions (μg/24 hr) during the last week of periods 1, 2 and 3 were 15.41 ± 2.12 (SEM) (N=36), 12.51 ± 1.78 (N=36) and 12.77 ± 1.85 (N=34), respectively. Paired *t*-tests indicated a significant reduction of PGE-M excretion during period 2, compared to that of period 1 [difference = 2.90 ± 0.89 (SEM) μg/24 hr, P=0.002]. The reduction based on individual contributions was 14% (P=0.009). Excretion rates of each subject for the three feeding periods are shown in Table 3. PGE-M excretion during the last week of period 3 was not significantly different from that at the end of period 2.

DISCUSSION

PGE-M excretion rates during periods 1 and 2 provide clear evidence that fish oil supplementation inhibits *in vivo* production of E-series prostaglandins in most individuals. PGE synthesized in the kidneys and seminal vesicles are excreted virtually unmetabolized, thus they escape measurement with our methodology. The rationale for disregarding their contribution to total synthesis has been discussed (24). PGE-M excretion reflects PGE production at sites easily accessible to systemic clearance. In a study of much shorter duration (four weeks), Knapp and FitzGerald (4) reported a non-significant trend toward lower PGE-M values in a group of eight volunteers receiving 50 mL/day of fish oil. In a rat study, Hornstra and Stegen (25) demonstrated a diet-induced shift in the ratio monoic to dioic prostaglandin metabolites. Although our work is concerned with the synthesis and metabolism of PGE alone—and not of the aggregate PGE + PGF, as is the paper by Hornstra and Stegen (25)—it is still possible that our results were similarly influenced by the dietary manipulation, if indeed the metabolic shift observed in rats also occurs in humans.

Research by other investigators on the effect of α-tocopherol on arachidonate cyclooxygenation has produced conflicting results. In our study, a large vitamin E supplement was provided during period 3. Vitamin E was given to counteract the effects of oxidative stress (26) from prolonged intake of several grams of ω3 PUFA and to determine if a large daily dose of vitamin E has any measurable effect on PGE synthesis when other dietary parameters were held constant. Two-hundred mg/day of α-T had no significant effect on PGE-M excretion.

In view of the high simultaneous intake of linoleate (25 g/day), the magnitude of PGE-M excretion rate reduction (14%) during FOC supplementation is, in our opinion, remarkable. The relevant issue we have to address is: What is the biological impact of changes in PGE production of the magnitude measured in this study? The synthesis of PGE is not localized in a specific tissue as is the case with thromboxane A_2 (platelets) and most of prostacyclin (vascular endothelium). The biological functions of PGE are manifold depending on the tissue where the synthesis takes place; hence the physiologic consequences of reduced PGE production are not as obvious or predictable as those of a reduced synthesis of TXA_2 and PGI_2.

In a recent pilot study on long-term fish oil supplementation Ferretti *et al.* (27) demonstrated that, in addition to a reduction of renal PGE_2 synthesis in absolute terms, the pre-supplementation (renal) synthetic ratio PGE_3/PGE_2=O changed to PGE_3/PGE_2>O following fish oil administration. Thus, in this specific case, to assess the

effect of marine oil on renal physiology as modulated by arachidonate metabolism we must consider not only the effects of reduced PGE_2 synthesis but also the different biological activity of PGE_3 as compared to that of PGE_2. In view of the central role of PGE_2 in renal physiology (28), we wondered whether a shift in the PGE_3/PGE_2 ratio might have a detrimental effect on renal function (27). This is an issue of obvious concern which has been expressed by other investigators, expecially in reference to subjects with impaired renal function (4). Presumably, similar shifts in the PGE_3/PGE_2 synthetic ratio take place concurrently in other tissues.

Present knowlege of PGE biology strongly suggests that, in addition to renal function, the impact of PGE modulation is most likely to be manifested in the cardiovascular (11,12,29-32) and immune (16,17,33-38) systems, and possibly in tumorigenesis (13,15,39-41). Therefore, it is desirable to assess the long-term effects of a diet-induced reduction of PGE biosynthesis. Perhaps the most critical question that must be answered is: What is the extent of dietary lipid manipulation necessary to influence the eicosanoid system to a clinically significant degree? We also need to know more about the biological activity of PGE_3 *vis-à-vis* that of PGE_2.

In conclusion, the results of this study, if considered in conjunction with the regulatory roles attributed to prostaglandin E in critical areas of human physiology, suggest that prolonged use of fish oil supplements should not be incorporated into dietary recommendations until the pharmacology of marine lipids is better understood.

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Metabolism of n-3 Polyunsaturated Fatty Acids by the Isolated Perfused Rat Liver

Zu Jun Zhang[1], Henry G. Wilcox, Marshall B. Elam, Lawrence W. Castellani and Murray Heimberg*
Departments of Pharmacology and Medicine, The University of Tennessee, Memphis, Memphis, Tennessee 38163

The hepatic metabolism of oleic acid and n-3 fatty acids (eicosapentaenoic acid, EPA and docosahexaenoic acid, DHA), and secretion of very low density lipoprotein (VLDL) were studied in isolated perfused rat livers from normal chow fed male rats. The basal perfusion medium contained 30% bovine erythrocytes, 6% bovine serum albumin (BSA), and 100 mg/dL glucose, in Krebs-Henseleit bicarbonate buffer (pH 7.4), which was recycled through the liver for 2 hr. Individual fatty acids (EPA, DHA or oleic acid), as complexes with 6% BSA, or albumin alone, were infused at a rate of 70 μmol/hr. When any of these fatty acids was infused at this rate, the ambient concentration in the medium was maintained at 0.3–0.4 μmol/mL, indicative of similar hepatic rates of uptake for each fatty acid (*i.e.*, approximately 6 μmol/g liver/hr). When fatty acid was not infused, the ambient free fatty acid level was 0.16 μmol/mL. The concentrations of infused free fatty acids increased appropriately in the perfusion medium; however, with infusion of EPA, DHA, or oleate, the concentrations of perfusate palmitate and linoleate were the same as when fatty acid was not infused. Additionally, the perfusate concentration of oleate in the free fatty acid fraction was not affected by infusion of EPA and DHA. These data indicate a constant outflow of endogenous fatty acid unaffected by the presence of the exogenously supplied fatty acid.

The net secretion rate of VLDL lipids and protein was stimulated by infusion of oleate, whereas when EPA was infused, secretion rates were lower and similar [except for VLDL cholesterol (C), which was greater] to those occurring when fatty acid was not provided. DHA stimulated the secretion of VLDL triacylglycerol (TG), phospholipid (PL) and C to a similar rate, as did oleate, but secretion of VLDL cholesteryl ester (CE) and protein was lower and similar to that with EPA. VLDL and hepatic TG and CE were enriched with the infused fatty acids, compared to experiments without fatty acids, as determined by gas chromatography. Enrichment of PL, however, was significant only in liver upon infusion of EPA. The formation of $^{14}CO_2$ and perchloric acid soluble products from [1-^{14}C]EPA, considered separately, did not differ statistically from that obtained with [1-^{14}C]oleate, although the mean values were higher with [1-^{14}C]EPA. However, the sum of oxidation products derived from EPA was significantly greater than that from oleate. Incorporation of [1-^{14}C]EPA into TG and CE, but not into PL, was lower as compared to that from [1-^{14}C]oleate. These lower rates of incorporation of [1-^{14}C]EPA into VLDL lipids therefore paralleled the mass fatty acid enrichment patterns. It may be concluded that EPA is used to a similar extent as oleate for synthesis of PL, but is a poorer substrate for synthesis of TG. The reduced output of newly synthesized (radioactive) PL reflected the lower hepatic output of VLDL. Since hepatic uptake of EPA, DHA or oleate was identical, utilization of EPA for TG synthesis was less than that of oleate or DHA. Furthermore, utilization of endogenous fatty acids for TG synthesis and secretion of the VLDL was reduced in the presence of EPA. The decreased TG synthesis resulted in reduced formation of VLDL for transport of TG from the liver. These effects taken together with an apparently increased oxidation of EPA provide substantial evidence for a decrease in formation of VLDL and transport of TG, PL, C and CE into the circulation in response to EPA. DHA, however, appears to be an adequate substrate for TG synthesis and stimulates VLDL secretion. The reduced transport of CE may reflect lower selectivity of DHA by acyl-CoA; cholesterol acyltransferase for CE formation.

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Several studies describing the hypolipidemic effects of fish diets, or dietary supplements of fish oil, on concentrations of blood lipids and lipoproteins have been published. Fish oils contain significant quantities of eicosapentaenoic acid (EPA, 20:5) and docosahexaenoic acid (DHA, 22:6), n-3 polyunsaturated fatty acids, which may be the primary components responsible for the reduction of plasma lipids. The ingestion of fish oils has been reported to reduce plasma concentrations of triacylglycerol (TG), very low density lipoprotein (VLDL), and cholesterol in humans (1,2) and in animals (3–5). Suppression of hepatic synthesis of TG and apolipoprotein (apo) B after ingestion of fish oil has been observed in humans (6), in cultured human hepatoma cells (Hep G2) (7), in perfused rat liver (8,9) and in cultured rat hepatocytes (10) and may be the principal mechanism by which the n-3 fatty acids lower plasma levels of TG and VLDL. Parks *et al.* (11), however, did not observe any suppression of apo B synthesis, although hepatic VLDL lipid synthesis and secretion were reduced in perfused livers from non-human primates (African green monkeys) which had been maintained for 2.5 years on a diet containing fish oil (11). The results were compared to livers from animals fed a lard enriched diet (11). More recently, Homan *et al.* (12), in studies with Hep G2 cells concluded that both oleate and EPA were equally effective for TG synthesis, while oleate was a better substrate for PL synthesis. Nevertheless, the release of newly formed lipids was lower by 20–40% in response to EPA. Interestingly, Wong *et al.* (13) also observed in Hep G2 cells that EPA was inhibitory to TG synthesis, but that DHA was not. However, both n-3 fatty acids were inhibitory to apo B synthesis resulting in depressed VLDL

[1]Present Address: Royal Victoria Hospital, Montreal, Quebec, Canada.

*To whom correspondence should be addressed at Department of Pharmacology, The University of Tennessee, Memphis, The Health Science Center, School of Medicine, 874 Union Avenue, 115 Crowe Building, Memphis, Tennessee 38163.

Abbreviations used: ACAT, acyl-CoA:cholesterol acyltransferase; apo, apolipoprotein; BSA, bovine serum albumin; C, cholesterol (unesterified); CE, cholesteryl esters; EPA, eicosapentaenoic acid (20:5); DHA, docosahexaenoic acid (22:6); FA, fatty acid; FFA, free (unesterified) fatty acid; GC, gas chromatography; PL, phospholipid; TG, triacylglycerol; VLDL, very low density lipoprotein.

secretion. A similar result was obtained by Lang and Davis (14) in studies with rat hepatocytes, except that they did not observe EPA to be inhibitory to TG synthesis.

We studied the effects of EPA and DHA on hepatic lipid metabolism and the formation and secretion of VLDL by isolated perfused livers from normal chow fed rats, a system more suitable for studies of VLDL secretion. This was done to evaluate differences in metabolism of n-3 fatty acids reported by others using perfused livers or liver cells in tissue culture. We observed that EPA was a poorer substrate for synthesis of TG and secretion of VLDL than was oleic acid when the fatty acids were provided to the liver at a modest level (70 μmol/hr for 2 hr). However, under identical conditions, the incorporation of DHA into TG and the secretion of VLDL occurred to an extent comparable to that of oleic acid, suggesting TG synthesis and secretion are not inhibited by DHA, as appears to be the case with EPA. A preliminary report of some of these studies has appeared (15).

MATERIALS AND METHODS

Materials. EPA (>99% purity) was obtained from Cayman Chemical Co. (Ann Arbor, MI). DHA (>95% purity) and oleic acid (>99% purity) were obtained from NuCheck Prep (Elysian, MN). Purity of these fatty acids was confirmed by gas chromatography, as described below. [1-^{14}C]Oleic acid was purchased from NEN (Boston, MA) and [1-^{14}C]-EPA from Amersham Corp. (Arlington Heights, IL). Bovine serum albumin (BSA; fraction V) was obtained from Sigma Chemical Co. (St. Louis, MO). The BSA was delipidated with methanol as described previously (16). Biocount and hyamine hydroxide were obtained from Research Products, Inc. (Elk Grove, IL). All other chemicals were reagent grade.

Animals. Male Sprague-Dawley rats were obtained from Harlan, Inc. (Indianapolis, IN). Animals (225–250 g) were housed for 1–3 weeks with free access to Purina Rodent Chow and water before being used in these studies.

Liver perfusions. Livers were perfused in a recycling system *in vitro*, as described previously (17,18), for 2 hr with a basal medium (80 mL) consisting of washed bovine erythrocytes (30%) and purified (16) bovine serum albumin (6%) in Krebs-Henseleit bicarbonate buffer, pH 7.4, gassed continuously with 95%5%-O_2/CO_2. After 20 min equilibration, a pulse of 12 μmol fatty acid, as a complex with albumin (or albumin alone in buffer) was added. During the 2-hr perfusion, the complex of fatty acid (oleic acid, EPA or DHA) with BSA (6%), or albumin alone, in Mg^{++} and Ca^{++} free buffer (19) was infused at a rate of 11.7 mL/hr (70 μmol fatty acid/hr). The molar ratio of free fatty acid/BSA in the complex was 6.9. During perfusion, this ratio was reduced to 0.8 because of free fatty acid (FFA) uptake. When albumin was infused alone, the amount of free fatty acid associated with the delipidated albumin was negligible (0.3 μmol/g), resulting in an infusion rate of only 0.2 μmol FFA/hr. In some of the experiments, 1 μCi [1-^{14}C]EPA or [1-^{14}C]18:1 was included in the medium as an initial pulse and 2 μCi infused/hr for the 2-hr experiment.

Analyses. Samples of perfusate were taken hourly for chemical analysis. A 60-mL aliquot of the final perfusate, after sedimentation of the erythrocytes, was obtained for isolation of VLDL (19). Lipids were extracted from aliquots of perfusate plasma and VLDL with chloroform/methanol (2:1, v/v) using 20-mL solvent/mL sample (16). At the end of each perfusion experiment, the liver was flushed with 60 mL ice-cold 0.9% NaCl, blotted and weighed. Samples were minced and homogenized in a Polytron homogenizer with 20 volumes of chloroform/methanol (2:1, v/v)/g liver. Lipid classes were isolated by thin-layer chromatography (TLC) from $CHCl_3$/CH_3OH extracts of liver, perfusate, and VLDL (16). Appropriate bands scraped off the plate were analyzed chemically for TG (20), PL (21), C or CE (22), and FFA (23). When radioactive fatty acids were used, an aliquot of whole perfusate was added to an equal volume of 15% perchloric acid. Incorporation of [1-^{14}C]oleic acid and [1-^{14}C]EPA into acid soluble products, an indication of soluble oxidative metabolites, was determined on aliquots of the perchloric acid soluble material of perfusate samples by liquid scintillation spectrometry (24). Radioactive CO_2 generated during the perfusions was trapped by passing the gas through a series of three traps containing 10% KOH (w/v) (25). Contents of the traps wre changed after each hr and combined for analysis. $^{14}CO_2$ was determined on filter paper strips saturated with hyamine hydroxide (25) after release of the CO_2 from the KOH solution with HCl.

When the radiolabeled fatty acids were used, bands of PL, TG, and CE isolated from liver and VLDL extracts were scraped directly from TLC plates into vials for determination of radioactivity. All measurements of radioactivity were made with a Beckman LS3150 counter (Beckman Instruments, Fullerton CA) using Biocount scintillation fluid. Data were obtained as DPM, calculated from the H number quench curve efficiency correction program of the instrument.

Gas chromatography (GC) analyses of fatty acid methyl esters (16,26) were carried out with a Varian 3300 system (Varian Associates, Palo Alto, CA) using a column of SP2340 (16). Methanolic BF_3 was used to prepare the methyl esters of the fatty acids (16). Integration of peak areas was carried out with a Model 4290 Varian Integrator. Eluted fatty acid methyl esters up to DHA were included in the total area analyzed (equal to 100%). The percentage of each fatty acid, therefore, is expressed in relation to this area total. The specific radioactivity of perfusate oleate and EPA was calculated from chemical and radioactivity analyses of perfusate FFA and GC analysis of that fraction.

Protein content of dialyzed VLDL was estimated by the method of Lowry *et al.* (27) as modified by Markwell *et al.* (28) for elimination of turbidity with SDS.

Statistics. All data are shown as means ±SE. Data were analyzed using one-way ANOVA. The statistical significance of differences between the means of groups was established by a protected Fisher least significant difference test (PLSD) and significance is reported for levels of $p<0.05$.

RESULTS

The concentrations of perfusate FFA remained essentially constant during the experiments; they were 0.47 ± 0.05 (n=11), 0.33 ± 0.03 (n=9) and 0.34 ± 0.05 (n=4) μmol/mL during infusion of oleate, EPA or DHA, respectively. Means were calculated from the average concentration at the second and fourth hr in each separate experiment.

When albumin alone was infused, the ambient concentration of FFA was lower, 0.16 ± 0.02 (n=5) μmol/mL, but relatively constant, presumably resulting from a small, outward flow of FFA from the liver (29). The rates of uptake by the liver of EPA, DHA or oleic acid were not different, 5.3 ± 0.4 (n=5), 5.8 ± 0.4 (n=3) and 5.9 ± 0.1 (n=5) μmol/g liver/hr, respectively. The absolute concentrations of perfusate palmitate and linoleate were not significantly affected by the infusion of oleate, EPA or DHA, although percentage composition varied (Table 1). Similarly, the concentration of oleate was not affected when either EPA or DHA was infused, compared to that when exogenous fatty acid was not provided.

The concentrations of hepatic lipids measured at the end of the 2 hr perfusion with oleic acid, EPA, or DHA were not different from that when fatty acid was not provided (data not shown). The average values for a total of 34 of the experiments are 2.44 ± 0.21, 25.1 ± 1.6, 4.24 ± 0.28 and 0.86 ± 0.09 μmol/g liver for TG, PL, C, and CE, respectively.

When EPA was infused, secretion of all VLDL lipids, except for cholesterol, as well as of protein was reduced when compared to oleic acid and, except for cholesterol which was greater, was similar to the secretion observed when fatty acid was not infused (Table 2). The stimulation of VLDL cholesterol output may reflect specific, unusual requirements for the surface of the particle (see below). Unexpectedly, infusion of DHA stimulated secretion of VLDL TG, C and PL (Table 2) to the same extent as did oleic acid; output of VLDL CE was less than that with oleate, and was not different from that when fatty acid was not infused. Secretion of VLDL protein was

TABLE 1

Composition and Concentration of Perfusate Free Fatty Acid[a]

Infused FFA	Analyzed perfusate FFA					
	16:0	18:0	18:1	18:2	20:5	22:6
	% of Total FFA					
None (3)	30.7 ± 0.6	9.6 ± 0.9	32.0 ± 1.5	15.3 ± 2.0	1.2 ± 0.1	1.7 ± 0.4
18:1 (5)	10.8 ± 2.2[c]	5.9 ± 1.1[c]	71.7 ± 3.1[c]	2.9 ± 1.4[c]	0.1 ± 0.1	0.8 ± 0.5
20:5 (5)	14.1 ± 1.4[c]	7.9 ± 0.2	14.7 ± 1.3[c,d]	6.6 ± 0.8[c,d]	43.4 ± 1.7[c,d]	4.3 ± 1.7
22:6 (3)	16.9 ± 1.6[c,d]	13.8 ± 1.7[c,d,e]	15.6 ± 1.9[c,d]	7.1 ± 0.7[c,d]	1.0 ± 0.2[e]	37.0 ± 6.0[c,d,e]
	nmol/mL[b]					
None (3)	52 ± 5	16 ± 2	54 ± 3	26 ± 5	2 ± 1	3 ± 1
18:1 (5)	60 ± 12	33 ± 6[c]	414 ± 38[c]	16 ± 7	1 ± 1	4 ± 3
20:5 (5)	44 ± 13	33 ± 3[c]	62 ± 9[d]	27 ± 4	179 ± 13[c,d]	17 ± 7
22:6 (3)	64 ± 21	52 ± 4[c,d,e]	59 ± 5[d]	28 ± 5	4 ± 1[e]	147 ± 38[c,d,e]

[a] Values are means ±SEM. Only the values for the major fatty acids are reported. After each group the number of observations is given in parentheses. Fatty acids were infused for 2 hr at a rate of 70 μmol/hr into the medium perfusing isolated livers from normal male rats. An initial pulse of 12 μmol of fatty acid was added to the medium. "None" refers to experiments in which the albumin solution was infused, but which contained no free fatty acid.

[b] The data were derived from the product of the "% of Total FFA" and the average of the concentration of the total FFA (nmol FFA/mL) at the first and second hr in each separate experiment. These values are given in the Results section of the text.

[c] $p<0.05$ *vs.* None.

[d] $p<0.05$ *vs.* 18:1.

[e] $p<0.05$ *vs.* 20:5.

TABLE 2

Hepatic Output of VLDL Lipid and Protein[a]

Infused FFA	TG	PL	C	CE	Protein
	nmol/g liver/2 hr				ng/g liver/2 hr
None (6)	292 ± 32	100 ± 19	40 ± 4	44 ± 5	899 ± 104
18:1 (14)	548 ± 22[b]	168 ± 10[b]	102 ± 7[b]	96 ± 8[b]	1987 ± 148[b]
20:5 (12)	282 ± 18[c]	114 ± 9[c]	68 ± 5[b,c]	48 ± 4[c]	1282 ± 131[c]
22:6 (5)	582 ± 26[b,d]	192 ± 14[b,d]	84 ± 18[b]	56 ± 2[c]	1350 ± 106[c]

[a] Values are means ±SEM. Values in parentheses represent the number of observations. Experimental perfusion conditions are as described in the legend to Table 1 and in the Materials and Methods section.

[b] $p<0.05$ *vs.* None.

[c] $p<0.05$ *vs.* 18:1.

[d] $p<0.05$ *vs.* 20:5.

stimulated by infusion of oleate, but not by DHA or EPA. The secretion of total TG in the whole perfusate was similar to that for VLDL TG (85% of that in perfusate in all experiments) (data not shown).

Incorporation of [1-^{14}C]EPA, infused at 70 μmol/hr, into VLDL TG, PL and CE was also less than with [1-^{14}C]-oleic acid (Table 3). Incorporation into hepatic TG and CE, but not into PL, was reduced for EPA, even though concentrations of hepatic lipids were not different when either oleic acid or EPA were infused (see above). Although the formation of radioactive oxidation products measured as incorporation into $^{14}CO_2$ and into perchloric acid, soluble products tended to be greater from EPA than with oleate, the mean values were not statistically different. A comparison of total oxidation products, however, indicated that conversion from EPA was statistically greater.

Under the experimental conditions described, the molar ratios of the surface lipids of VLDL produced from oleate, DHA or endogenous sources only, relative to TG + CE (core lipids) were not different from one another, indicating similarity in particle size (Table 4). The higher ratio in experiments with EPA suggests a population of smaller particles. The relationship between PL and C of the surface was similar for VLDL produced by DHA or endogenous sources, but higher than that for 18:1 and 20:5. Except for DHA infusion, from which less CE was secreted, the ratio of TG/CE was constant. Although apo B *per se* was not determined in these experiments, it is evident that differences seem to exist between the surface protein and the core lipid contents. Particularly, a greater proportion of protein (as with the surface lipids) is required to form VLDL with EPA, whereas relatively less protein is required for the particles formed in response to DHA. Alterations in the amount of VLDL lipids secreted resulted primarily from differences in numbers of VLDL particles formed and secreted, although specific differences in size and composition of the VLDL also must have contributed to the quantity of lipid secreted.

The degree to which the infused fatty acids were incorporated into VLDL and hepatic TG was also evaluated by GC (Tables 5 and 6). Included in these Tables are calculated amounts of the various fatty acids present in TG. These latter data provide a better appreciation of the utilization of fatty acids of exogenous and endogenous origin for TG synthesis. When FFA was not infused, the fatty acid compositions of hepatic and VLDL TG were quite similar. When FFA was infused, the percentage of the exogenous fatty acid in VLDL TG was considerable and always exceeded that of the liver (Tables 5, 6; refs. 16,30). It is probable that utilization of the endogenous pool of fatty acid was diminished when EPA was supplied exogenously, since the amount of palmitate and linoleate present in the secreted VLDL TG was 40–60% of that present when only endogenous fatty acid was available for synthesis (Table 5). This was also the case with stearate

TABLE 3

Incorporation of [1-^{14}C]Fatty Acid into Esterified Lipids of VLDL and Liver, and into Oxidation Products[a]

	Infused FFA	
	18:1	20:5
Lipids	μmol fatty acid/g liver/hr	
VLDL		
TG	0.800 ± 0.068	0.350 ± 0.026[b]
PL	0.036 ± 0.010	0.008 ± 0.002[b]
CE	0.016 ± 0.002	0.006 ± 0.002[b]
Liver		
TG	4.184 ± 0.408	2.598 ± 0.112[b]
PL	3.014 ± 0.288	3.128 ± 0.164
CE	0.358 ± 0.042	0.096 ± 0.008[b]
Oxidation products	μmol/g liver/2 hr	
CO_2	1.748 ± 0.240	2.114 ± 0.056
$HClO_4$ soluble fraction	3.956 ± 0.632	5.942 ± 0.242
Total	5.704 ± 0.834	8.056 ± 0.266[b]

[a]Values are means ±SE. Experimental details are given in Table 1, except that either [1-^{14}C]oleate or [1-^{14}C]EPA was also present. Data were calculated from total incorporated radiolabel, based on constant perfusate specific activity, 28,700 DPM/μmol and 27,000 DPM/μmol for 18:1 and 20:5 respectively, determined from DPM in perfusate FFA and GC analysis for 18:1 or 20:5.

[b]$p<0.05$ *vs.* 18:1.

TABLE 4

Relationships among VLDL Constituents[a]

Infused FFA	PL+C/ TG+CE	PL/C	TG/CE	Protein/ TG+CE
	μmol/μmol			μg/μmol
None (6)	0.42 ± 0.06	2.64 ± 0.59	7.18 ± 1.06	274.6 ± 31.6
18:1 (14)	0.43 ± 0.02	1.75 ± 0.16[b]	6.34 ± 0.60	323.8 ± 33.0
20:5 (12)	0.56 ± 0.04[b,c]	1.72 ± 0.13[b]	6.15 ± 0.52	398.7 ± 40.7[b]
22.6 (5)	0.43 ± 0.06	2.76 ± 0.66[c,d]	10.44 ± 0.82[b,c,d]	217.5 ± 23.5[d]

[a]Values are means ±SEM, and derived from data presented in Table 2. Numbers in parentheses are the number of observations.

[b]$p<0.05$ *vs.* None.

[c]$p<0.05$*vs.* 18:1.

[d]$p<0.05$ *vs.* 20:5.

TABLE 5

Fatty Acid Composition of VLDL TG[a]

Infused FFA	Analyzed fatty acid							
	16:0	16:1	18:0	18:1	18:2	20:4	20:5	22:6
	% of Total TG fatty acid							
None (5)	26.7 ± 1.4	1.2 ± 0.7	2.9 ± 1.8	24.3 ± 1.0	24.0 ± 1.2	3.5 ± 0.3	3.1 ± 0.1	8.3 ± 0.3
18:1 (8)	16.8 ± 0.3[c]	3.4 ± 0.3[c]	1.9 ± 0.3	46.7 ± 0.8[c]	13.8 ± 0.7[c]	2.3 ± 0.2[c]	1.8 ± 0.2	4.2 ± 0.3
20:5 (10)	16.5 ± 0.8[c]	1.4 ± 0.5[c]	1.8 ± 0.6	17.2 ± 0.6[c,d]	16.6 ± 1.5[c]	3.2 ± 0.1	26.4 ± 1.7[c,d]	4.7 ± 0.6
22:6 (4)	13.0 ± 0.9[c,d,e]	1.4 ± 0.5[c]	2.6 ± 0.9	14.2 ± 0.7[c,d,e]	17.5 ± 1.0[c]	2.5 ± 0.1[e]	5.0 ± 1.6[e]	36.8 ± 4.4[c,d,e]
	nmol/g liver/2 hr[b]							
None (5)	236 ± 22	11 ± 7	28 ± 9	217 ± 22	214 ± 24	32 ± 6	29 ± 4	74 ± 8
18:1 (8)	281 ± 19	57 ± 7[c]	31 ± 5	784 ± 55[c]	230 ± 18	38 ± 5	30 ± 4	75 ± 6
20:5 (10)	127 ± 7[c,d]	14 ± 5[d]	12 ± 3[d]	133 ± 7[d]	131 ± 15[c,d]	26 ± 4	211 ± 24[c,d]	38 ± 6
22:6 (4)	223 ± 18[d,e]	24 ± 10[d]	43 ± 15[e]	255 ± 18[d,e]	303 ± 30[c,d,e]	43 ± 2[e]	84 ± 27[e]	698 ± 98[c,d,e]

[a]Values are means ±SEM. After each group the number of observations is given in parentheses. Experimental conditions are given in the legend to Table 1 and in Materials and Methods. Only values for the major fatty acids are reported.

[b]The data represent TG fatty acids secreted/g liver in 2 hr in VLDL and are the products of individual values for TG output (Table 3) and the % fatty acid in this table.

[c]$p<0.05$ *vs.* None.

[d]$p<0.05$ *vs.* 18:1.

[e]$p<0.05$ *vs.* 20:5.

TABLE 6

Fatty Acid Composition of Hepatic TG[a]

Infused FFA	Analyzed fatty acid							
	16:0	16:1	18:0	18:1	18:2	20:4	20:5	22:6
	% of Total TG fatty acid							
None (5)	27.2 ± 1.3	0.7 ± 0.6	1.3 ± 0.7	26.0 ± 0.4	24.1 ± 1.0	2.5 ± 0.3	3.2 ± 0.6	6.9 ± 0.4
18:1 (8)	21.3 ± 0.5[c]	2.6 ± 0.6	1.8 ± 0.2	38.8 ± 0.9[c]	20.0 ± 1.4	2.0 ± 0.1	1.4 ± 0.2	2.0 ± 0.1[c]
20:5 (10)	23.7 ± 1.9	1.4 ± 0.5	1.7 ± 0.6	23.9 ± 0.9[d]	19.7 ± 1.2	3.3 ± 0.6	12.3 ± 1.1[c,d]	5.0 ± 0.6[d]
22:6 (4)	19.8 ± 0.3[c]	1.9 ± 0.6	2.0 ± 0.7	20.5 ± 0.3[c,d,e]	20.8 ± 1.0	2.0 ± 0.1	3.9 ± 0.2[e]	21.6 ± 2.6[c,d,e]
	nmol/g liver/2 hr[b]							
None (5)	1520 ± 100	40 ± 3	70 ± 40	1450 ± 70	1390 ± 90	140 ± 20	180 ± 40	380 ± 20
18:1 (8)	1600 ± 170	180 ± 40	130 ± 20	2930 ± 340[c]	1500 ± 190	150 ± 20	100 ± 10	350 ± 40
20:5 (10)	1710 ± 160	120 ± 50	140 ± 50	1750 ± 160[d]	1450 ± 150	240 ± 40	920 ± 130[c,d]	370 ± 60
22:6 (4)	1600 ± 210	150 ± 60	170 ± 60	1700 ± 250[d]	1700 ± 250	160 ± 20	310 ± 40[e]	1790 ± 360[c,d,e]

[a]Values are means ±SEM. After each group the number of observations is given in parentheses. Perfusion conditions are as presented in the legend to Table 1 and the Materials and Methods section. Only values for the major fatty acids are reported.

[b]The data are nmol fatty acids in hepatic TG after perfusion derived from actual individual data (see Results section) and "% fatty acid" in this Table.

[c]$p<0.05$ *vs.* None.

[d]$p<0.05$ *vs.* 18:1.

[e]$p<0.05$ *vs.* 20:5.

and oleate, but the assigned level for significance was not attained.

The stimulated output of TG by oleate is accounted for by increased oleate and palmitoleate content only. However, the stimulated output by DHA is represented by significant increase in output of linoleate as well as DHA in the secreted TG. Compared to perfusion without the addition of exogenous fatty acids, hepatic TG was not enriched with "endogenous" fatty acids, *e.g.*, palmitate, palmitoleate, stearate, linoleate, or arachidonate, when oleate, EPA, or DHA was infused. Enrichment of the TG occurred only with the infused fatty acid (Table 6).

Compared to hepatic TG, the composition of hepatic PL, under our experimental conditions, showed a very small but statistically significant enrichment only by EPA when it was infused (Table 7). The percentage fatty acid composition of VLDL and hepatic CE is shown in Table 8. Enrichment of CE by infused oleate was about two-fold,

TABLE 7

Fatty Acid Composition of VLDL and Hepatic PL[a]

Infused FFA	Analyzed fatty acids						
	16:0	18:0	18:1	18:2	20:4	20:5	22:6
	% of Total VLDL PL fatty acid						
None (5)	19.6 ± 1.4	16.6 ± 4.6	11.1 ± 3.2	15.5 ± 1.5	17.8 ± 2.5	3.0 ± 0.6	7.6 ± 0.2
18:1 (8)	22.8 ± 0.6	18.5 ± 2.9	12.6 ± 0.7	15.5 ± 1.5	18.5 ± 0.5	1.2 ± 0.4	6.0 ± 0.1
20:5 (10)	21.8 ± 0.3	18.3 ± 2.2	11.1 ± 0.4	16.3 ± 2.0	16.6 ± 0.5	2.7 ± 0.4	7.8 ± 1.9
22:6 (4)	20.2 ± 5.0	20.1 ± 2.2	8.6 ± 0.9	15.1 ± 1.5	19.0 ± 2.0	1.9 ± 0.1	9.7 ± 1.9
	% of Total hepatic PL fatty acid						
None (5)	17.0 ± 1.6	19.4 ± 0.9	7.7 ± 0.3	17.2 ± 0.2	23.5 ± 0.4	1.3 ± 0.2	7.3 ± 0.2
18:1 (3)	18.7 ± 0.2	18.4 ± 0.5	9.6 ± 0.7	16.5 ± 0.9	20.1 ± 0.5	1.9 ± 0.3	7.8 ± 0.3
20:5 (3)	17.6 ± 0.9	18.9 ± 1.7	8.3 ± 0.9	15.6 ± 1.0	22.0 ± 0.8	3.2 ± 0.6[b,c]	8.3 ± 0.3
22:6 (3)	17.5 ± 0.6	21.5 ± 0.4	7.1 ± 0.1	16.4 ± 0.6	21.7 ± 0.2	2.2 ± 0.1	8.2 ± 0.4

[a]Values are means ±SEM. The number of observations are in parentheses. Experimental details are given in Table 1 and the Materials and Methods section. Only the values for the major fatty acids are reported.
[b]$p<0.05$ *vs.* None.
[c]$p<0.05$ *vs.* 18:1.

TABLE 8

Fatty Acid Composition of VLDL and Hepatic Cholesteryl Ester[a]

Infused FFA	Fatty acid						
	16:0	18:0	18:1	18:2	20:4	20:5	22:6
	% of Total fatty acids						
None							
VLDL	34.0	8.6	27.6	9.8	6.4	2.8	4.0
Liver	48.5	9.7	20.4	8.6	2.7	0.9	1.8
18:1							
VLDL	11.0	5.0	51.4	10.2	4.8	4.4	1.7
Liver	31.0	8.7	35.8	9.8	3.0	0.9	1.8
20:5							
VLDL	15.0	13.2	28.9	8.2	ND	27.9	2.3
Liver	37.8	8.6	25.0	10.2	4.2	3.0	1.2
22:6							
VLDL	13.1	34.7	15.0	12.1	5.4	1.8	6.8
Liver	40.9	8.9	21.2	10.5	5.5	2.0	3.1

[a]Values are averages of two separate determinations. Experimental details are as presented in the legend to Table 1 and in the Materials and Methods section. Only the values for the major fatty acids are reported.
ND = not detected.

while that for EPA was ten-fold in VLDL and three-fold in the liver. Enrichment by DHA was much less in that only a 50% increase was obtained.

DISCUSSION

Alterations in rates of synthesis, secretion, or clearance of the VLDL may account for the decreased concentrations of plasma lipids in humans (1,2) and animals (3–5) when relatively large amounts of the n-3 fatty acids become available. The reduction in the utilization of preformed endogenous fatty acids for synthesis of TG, an important stimulus to VLDL secretion, or inhibition of *de novo* synthesis of fatty acid might account for reduction of VLDL secretion by the liver. Since plasma concentrations of VLDL will vary with rates of clearance, as well as secretion, more rapid clearance of VLDL particles enriched with n-3 fatty acids might also account for decreased plasma concentrations of VLDL.

Hepatic formation and secretion of VLDL is dependent on the rate of TG synthesis which is stimulated by fatty acids and is a saturable phenomenon (31,32). Furthermore, the particle size and lipid composition of VLDL is a function of the rate of TG synthesis, and the structure of the fatty acids from which the TG is derived (16,30). In general, rates of TG secretion are greater and larger VLDL particles are formed when monounsaturated and polyunsaturated fatty acids are the substrates for TG synthesis,

rather than saturated fatty acids. Regardless of fatty acid structure, larger particles are formed and secreted at high rates of TG synthesis. We reported previously that less TG was secreted by the perfused liver as the number of double bonds increased, in a series of 18 carbon atom fatty acids (18:3<18:2<18:1<18:0) (30). These observations have been confirmed in studies with isolated hepatocytes (7), although in a later liver perfusion study from our laboratory such an absolute relationship was less apparent (16). The VLDL formed in response to fatty acids containing 1 or 2 double bonds was larger and, therefore, required relatively less surface lipid (PL and free C) per mole TG to stabilize the particle for transport than did VLDL particles synthesized principally from saturated fatty acids (16). The particles assembled when saturated fatty acids predominated in the TG contained relatively more surface components (C, PL and apoproteins), indicating that they were smaller than when mono- or polyunsaturated fatty acids predominated. Therefore, depending on the rate of TG synthesis and the structure of the fatty acid from which TG is derived, the same amount of TG may be transported in fewer, but less dense, larger particles, or in more numerous, but smaller, denser particles. In our present study, differences in lipid output in VLDL appeared to reflect the number of VLDL particles secreted, rather than differences in the size of the particles. The size was not correlated with the TG secretion rate, perhaps because TG synthesis rates were not maximal and did not exceed the availability of some potentially limiting component (*e.g.*, cholesterol), as we suggested (33). A limitation of availability of cholesterol for VLDL formation may occur when TG synthesis is stimulated to some as yet undefined level, or when preformed hepatic C (as CE) is depressed. The fatty acid composition of the synthesized and secreted VLDL TG affected the lipid composition and size of the particle in these experiments only modestly, even though TG ranged in composition from about 25–50% monounsaturated fatty acid, and up to to 37% polyunsaturated n-3 fatty acid.

Dietary TG is an important source of plasma free fatty acids. We reported previously (34) that the composition of the circulating free fatty acid pool reflects that of the ingested TG fatty acids, which may differ significantly from adipose tissue TG fatty acids. Long periods of time on diets of specific fatty acid composition are necessary for adipose tissue TG fatty acids to reflect the composition of dietary fatty acids (34). The immediate question pertinent to this study is whether a substantial increase in the availability of n-3 fatty acids to liver when an individual consumes large amounts of fish, or supplements of fish oil, significantly affects VLDL formation, secretion, and composition. Experiments with the isolated perfused liver by Wong *et al.* (9) demonstrated that the basal secretion of TG in the absence of exogenous FFA was diminished in livers from rats fed chow with fat content rich in fish oil. A similar finding was derived from *in vivo* studies of roosters fed fish oil (3). Studies with cultured hepatocytes from fish oil fed rats produced similar observations. In hepatocytes from normal chow fed rats, both EPA and DHA were reported to be poorer substrates for TG synthesis than was oleic acid (35,36); EPA, however, was incorporated more readily into PL than was 18:1 (7). In our studies, we observed similar amounts of oleate and EPA incorporated into PL, as was suggested by lack of enrichment of PL and similarity of PL mass compared to basal levels (Table 7) or incorporation of labeled fatty acid (Table 4). In perfused livers from fish oil-fed rats, lipogenesis from ^3H_2O was less than that by livers from safflower oil-fed rats, while in hepatocytes, EPA and DHA depressed the synthesis of TG from [^{3}H]glycerol (7). These authors suggested that EPA depressed TG synthesis *via* inhibition of acyl-coenzyme A:1,2-diacylglycerol acyltransferase (35). Wong and Marsh (8) reported recently that the secretion of TG by livers from chow fed rats perfused with DHA or EPA was less than that of livers perfused with oleate or without fatty acid. Wong and Marsh (8) also noted that VLDL apoprotein synthesis and hepatic phosphatidate phosphohydrolase activity was decreased by DHA or EPA, compared to infusion of either oleate or omission of exogenous fatty acid (8). This latter observation could, in part, explain the decrease in TG synthesis from EPA or DHA (8). In a later study using Hep G2 cells, EPA, but not DHA, was found to be inhibitory of TG synthesis (13).

The hepatic uptake of fatty acids in our studies was similar for EPA, DHA, and oleate. Our data support previous observations of the reduction of TG synthesis and VLDL TG secretion from EPA, but also indicate that DHA was similar to oleate as a substrate for TG synthesis and VLDL TG secretion, when examined in an experimental system in which VLDL formation and secretion is a substantial reflection of the integrated metabolic processes occurring in the liver as a whole. These data emphasize that the inhibitory influence of EPA results from its being a poor substrate for TG synthesis at lower concentrations, as measured by the lower enrichment of hepatic and VLDL TG by this fatty acid, as compared with oleate. Additionally, EPA may exert an inhibitory influence on the incorporation of *de novo* synthesized fatty acid into TG.

We did not observe any statistically significant difference in the metabolism of EPA and oleate to CO_2 or oxidation products individually, although the mean values of either were 25% greater for EPA than for oleate. Total conversion, however, was statistically higher for EPA; therefore, hepatic oxidation of EPA may, indeed, exceed that of oleate, and thus contribute to the decreased synthesis of TG through a relative limitation in availability of EPA.

Since in our hands, the measured effects of DHA were basically not different than oleate, we did not continue to examine effects of DHA in this study. In this regard, our studies do differ from those of other workers using hepatocytes (35,36) or perfused rat liver (8), who observed that DHA reduced VLDL secretion. The reasons for these differences are presently unclear. Interestingly, it has been proposed that there is a distinct correlation between apo B synthesis and CE availability, *i.e.*, that CE synthesis is more a stimulus to apo B synthesis than TG (37). As noted in the present study, DHA stimulated TG synthesis and secretion to a similar extent as did oleate, but VLDL CE and protein output were depressed compared to that of oleate.

In support of previous reports from this laboratory (16,30), the exogenously supplied DHA, EPA and oleate enriched the VLDL TG to a greater extent than hepatic TG with the specific infused fatty acid. The current and previous data are indicative of the slower turnover of the

total hepatic TG compared to that transported in VLDL (*i.e.*, the existence of a separate metabolic pool of TG). When it is considered that a substantial portion of the secreted nascent VLDL is taken up by the liver and metabolized (19), the existence of at least two pools of TG in the liver which are not in rapid equilibrium (*e.g.*, storage and metabolic pools) becomes even more evident. Enrichment of PL by the infused fatty acid was insignificant, and differences between hepatic and VLDL PL were not apparent. This would suggest that any putative metabolic pool of PL, separate from the large membrane and/or storage pool of PL, equilibrates much more rapidly with the secreted VLDL PL pool, than do the pools of TG.

As shown in Table 5, when oleate was infused, the amount of oleate secreted in TG was 0.784 μmol/g liver/2 hr. A value similar to this was obtained from radioactive data (0.800 μmol 18:1/g liver/2 hr) (Table 4) suggesting little conversion of 18:1 to other long chain fatty acids incorporated into VLDL-TG. A similar comparison of data for EPA, 0.200 *vs.* 0.350 μmol EPA/g liver/2 hr (Table 5 and Table 4, respectively), suggests that EPA is converted to other fatty acids and incorporated into VLDL TG. Comparable calculations for hepatic TG could not be interpreted similarly, since both fatty acids were present in the relatively large pool of TG in the liver prior to the perfusion experiments.

Fish oil is enriched with similar amounts of DHA and EPA (20–30%, total), but the proportion of each may differ depending on the source, compared to the negligible amounts in most vegetable oils. Oleic acid is also present in considerable quantiatites in fish oils, and in amounts equal to or exceeding that of the n-3 fatty acids. Perhaps the hypolipidemic effect of fish oil diets requires chronic treatment with major modification of the composition of plasma FFA and hepatic TG, prior to any significant change in output and composition of the VLDL, and enrichment by EPA predominating.

In summary, it appears that the reduced secretion of VLDL in the presence of relatively low substrate amounts of EPA (0.3–0.4 μmol EPA/mL medium) resulted, in part, from decreased TG synthesis because of low specificity of the substrate for esterification to TG, reduced utilization of endogenous fatty acids for TG synthesis and, in part, from increased oxidation of EPA. In contrast to TG synthesis, EPA and oleate appear to be incorporated into PL to similar degrees. DHA, however, was esterified efficiently to form TG, which was transported in VLDL at a rate comparable to that resulting from infusion of oleate.

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Sesamin Is a Potent and Specific Inhibitor of Δ5 Desaturase in Polyunsaturated Fatty Acid Biosynthesis

Sakayu Shimizu[a,*], Kengo Akimoto[a,1], Yoshifumi Shinmen[a,1], Hiroshi Kawashima[a,2], Michihiro Sugano[b] and Hideaki Yamada[a]

[a]Department of Agricultural Chemistry, Kyoto University, Sakyo-ku, Kyoto 606 and [b]Department of Food Science and Technology, Kyushu University, Higashi-ku, Fukuoka 812, Japan

Incubation with sesame oil increases the mycelial dihomo-γ-linolenic acid content of an arachidonic acid-producing fungus, *Mortierella alpina*, but decreases its arachidonic acid content [Shimizu, S., K. Akimoto, H. Kawashima, Y. Shinmen and H. Yamada (1989) *J. Am. Oil Chem. Soc. 66*, 237–241]. The factor causing these effects was isolated and identified to be (+)-sesamin. The results obtained in experiments with both a cell-free extract of the fungus and with rat liver microsomes demonstrated that (+)-sesamin specifically inhibits Δ5 desaturase at low concentrations, but does not inhibit Δ6, Δ9 and Δ12 desaturases. Kinetic analysis showed that (+)-sesamin is a noncompetitive inhibitor (K_i for rat liver Δ5 desaturase, 155 μM). (+)-Sesamolin, (+)-sesaminol and (+)-episesamin also inhibited only Δ5 desaturases of the fungus and liver. These results demonstrate that (+)-sesamin and related lignan compounds present in sesame seeds or its oil are specific inhibitors of Δ5 desaturase in polyunsaturated fatty acid biosynthesis in both microorganisms and animals.
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Sesame seed oil, which has long been used as an edible oil, contains considerable amounts of sesamin and related lignan compounds (1,2). Little is known about the biological activity of these compounds, except that some of them show potent antioxidative activity (3). During our studies on the biosynthesis of C_{20} polyunsaturated fatty acids (PUFA) in *Mortierella alpina* and related filamentous fungi (4–11), we found that the mycelial dihomo-γ-linolenic acid (DGLA or 20:3) content of *M. alpina* drastically increases, while arachidonic acid (AA or 20:4) decreases upon incubation with sesame oil. This phenomenon was explained by specific inhibition of the conversion of DGLA to AA, *i.e.*, inhibition of Δ5 desaturation (7,8). To clarify the mechanism by which sesame oil exerts this inhibitory effect, we searched for factors that could be responsible for this effect. We report here that (+)-sesamin and related lignan compounds, which are commonly present in sesame oil, are potent and apparently specific inhibitors of mammalian and microbial Δ5 desaturases involved in PUFA biosynthesis.

MATERIALS AND METHODS

Chemicals. Crude and refined oils from unroasted sesame seeds were obtained from Kitamura Shokai Co. (Osaka, Japan). [1-^{14}C]Stearic acid (18:0) (2.07 GBq/mmol), [1-^{14}C]-oleic acid (18:1) (1.92 GBq/mmol), [1-^{14}C]linoleic acid (18:2) (1.89 GBq/mmol) and [2-^{14}C]DGLA (2.13 GBq/mmol) were purchased from Amersham (Buckingham, U.K.). (+)-Episesamin and (+)-sesaminol were prepared from refined sesame oil as described previously (3). Sesamol was obtained from Aldrich (Milwaukee, WI). All other reagents used in this work were obtained as described previously (4,5,9).

Microorganism and cultivation. The fungus *Mortierella alpina* 1S-4 (AKU 3998) (4,5) was grown aerobically for 7 days at 28°C in a 10-mL Erlenmeyer flask containing 2 mL of a basal medium (4% glucose and 1% yeast extract, pH 6.0), unless otherwise stated.

Extraction and determination of mycelial fatty acids. Fungal mycelia were harvested by suction filtration, washed with 50 mL water and then dried overnight at 100°C. Transmethylation of the fatty acids in the mycelia, extraction of the fatty acid methyl esters and analysis by gas-liquid chromatography were carried out as described previously (5,6,9).

Fractionation of sesame oil by high-performance liquid chromatography (HPLC). An acetone-extractable non-oil fraction of crude oil was prepared as described previously (7). The non-oil fraction (1.5 g) was dissolved in chloroform (*ca.* 3 mL) and the resultant solution (150 μL each) was subjected to HPLC on a reversed phase $10C_{18}$ Cosmosil packed column (20 × 250 mm; Nakarai Tesque, Kyoto, Japan). The chromatograph was operated with a mobile phase of methanol/water (67:33, v/v) at a flow rate of 9.9 mL/min and the effluent was monitored at 205 nm. Six fractions of 100 mL each were collected and concentrated under reduced pressure. The same separation was repeated 20 times.

Assay of inhibitors of Δ5 desaturation reaction with M. alpina *culture.* Fractions from 1 of the 20 HPLC runs were used for the assay. Each of the concentrated fractions was dissolved separately in 150 μL of chloroform, and 40 μL of the resultant chloroform solution was added to the sterilized basal medium. The assay of inhibitors of the Δ5 desaturation reaction was carried out by monitoring the changes in the fatty acid profile of *M. alpina* grown in the basal medium supplemented with each of the concentrated fractions as described above (see also Fig. 1).

Isolation of Δ5 desaturase inhibitors. The other fractions obtained from the remaining 19 HPLC runs were used for the isolation of the inhibitors. (+)-Sesamin was obtained from the fractions corresponding to fraction 4 in Figure 1 by repeated crystallization from ethanol until the compound was homogenous as judged by thin-layer chromatography (TLC) (yield from 1.5 g of the non-oil fraction, 137 mg; *Anal.* Calcd for $C_{20}H_{18}O_6$: C, 67.79; H, 5.12%; Found: C, 67.86; H, 5.20%; mp, 123.2–123.9°C; MS (direct inlet, 200°C) *m/z* (%): 354 (45, M^+), 323(5), 203(19), 178(12), 162(8), 149(100), 135(42), 122(25), 103(13), 89(6),

*To whom correspondence should be addressed.

[1]Present address: Laboratory of Microbial Science, Institute for Fundamental Research, Suntory Ltd., Mishima-gun, Osaka 618, Japan.

[2]On leave from Suntory Ltd.

Abbreviations: AA or 20:4, arachidonic acid; DGLA or 20:3, dihomo-γ-linolenic acid; HPLC, high-performance liquid chromatography; MS, mass spectrometry; NMR, nuclear magnetic resonance; PUFA, polyunsaturated fatty acids; TLC, thin-layer chromatography; 18:0, stearic acid; 18:1, oleic acid; 18:2, linoleic acid.

65(15), 39(10); $[\alpha]_D^{25}$ (c=1, $CHCl_3$), +64.7°; ^1H NMR δ ($CDCl_3$): 3.05 ppm (*m*, 2H), 3.87 (*dd*, *J*=4.0 and 8.5Hz, 2H), 4.24 (*dd*, *J*=6.0 and 8.5Hz, 2H), 4.74 (*d*, *J*=4.0Hz, 2H), 5.96 (*s*, 4H), 6.85 (*m*, 6H). In a similar manner, (+)-sesamolin was isolated from the fractions corresponding to fraction 5 in Figure 1 (yield, 70 mg; *Anal.* Calcd for $C_{20}H_{18}O_7$: C, 64.86; H, 4.90%; Found: C, 64.93; H, 4.97%; mp: 93.5°C; MS (direct inlet, 200°C) *m/z* (%): 370 (12, M^+), 233(36), 203(43), 173(3), 149(24), 135(100), 115(12), 81(41), 65(11), 52(7), 39(7); $[\alpha]_D^{25}$ (c=1, $CHCl_3$), +213°; ^1H NMR δ ($CDCl_3$): 2.95 ppm (*m*, 1H), 3.30 (*dd*, *J*=9.0 and 16.5Hz, 1H), 3.64 (*dd*, *J*=7.5 and 9.0Hz, 1H), 3.96 (*dd*, *J*=1.0 and 9.0Hz, 1H), 4.13 (*dd*, *J*=6.0 and 9.0Hz, 1H), 4.40 (*d*, *J*=7.5Hz, 1H), 4.44 (*t*, *J*=9.0Hz, 1H), 5.50 (*s*, 1H), 5.94 (*d*, *J*=18.5Hz, 4H), 6.50 (*dd*, *J*=3.5 and 8.5Hz, 1H), 6.62 (*d*, *J*=2.5Hz, 1H), 6.71 (*d*, *J*=8.5Hz, 1H), 6.78 (*dd*, *J*=8.0Hz, 1H), 6.82 (*dd*, *J*=1.5 and 8.0Hz, 1H), 6.88 (*d*, *J*=1.5Hz, 1H).

Preparation of cell-free extract of M. alpina. This was done as described previously (5).

Preparation of rat liver microsomes. Male Wistar rats maintained on a standard pelleted diet for eight weeks were killed and the livers were immediately removed and rinsed in cold homogenizing medium (12). They were homogenized in four volumes of the same medium in a Teflon pestle homogenizer and then centrifuged at 10,000 × *g* for 20 min at 4°C. Microsomes were recovered by centrifuging the supernatant at 100,000 × *g* for 1 hr at 4°C. The pellet was resuspended in the homogenizing medium to give a protein concentration of 30 mg/mL. The sample was stored at −80°C till use.

Assay of desaturase activities. The cell-free extract of *M. alpina* was assayed for Δ9, Δ12, Δ6 and Δ5 desaturase activities using labeled 18:0, 18:1, 18:2 and 20:3 as substrates, respectively. The standard reaction mixture contained, in 1.0 mL, 250 μmol of sucrose, 100 μmol of potassium phosphate buffer (pH 7.4), 40 μmol of NaF, 150 μmol of KCl, 4 μmol of *N*-acetyl-L-cysteine, 0.3 μmol of nicotinamide, 15 μmol of $MgCl_2$, 7.5 μmol of ATP, 0.4 μmol of CoA, 1.5 μmol of NADH, 100 μmol of fatty acid (18:0, 18:1, 18:2 or 20:3 containing 3.7 KBq of the respective labeled fatty acid) and the cell-free extract (1.0 mg protein). The reactions were carried out at 28°C for 30 min and terminated by the addition of 5 mL of ethanol and 1 mL of 4N KOH. Each of the resultant mixtures was saponified at 60°C for 30 min. After addition of 3 mL of water, 100 μg of the corresponding unlabeled substrate and product fatty acids, and 1 mL of 6N HCl, the fatty acids were extracted twice with *n*-hexane (5 mL each). The procedures for the preparation of fatty acid methyl esters and silica gel/silver nitrate TLC, and the quantitative analysis of radioactive fatty acid methyl esters were as described previously (13). The liver microsomes were assayed for Δ9, Δ6 and Δ5 desaturases. Each incubation mixture contained, in 1 mL, 250 μmol of sucrose, 100μmol of potassium phosphate buffer (pH 7.4), 45 μmol of NaF, 150 μmol of KCl, 1.5 μmol of glutathione, 0.5 μmol of nicotinamide, 5 μmol of $MgCl_2$, 7.5 μmol of ATP, 0.4 μmol of CoA, 1.5 μmol of NADH, microsomal suspension (1.2 mg protein) and 100 μmol of the desired labeled fatty acid as described above. The incubations were carried out for 10 min (except as stated) at 37°C. Other conditions were the same as those for the assays with the *M. alpina* cell-free extract.

Other methods. TLC on lignan compounds was performed on a $60F_{245}$ silica gel plate (Merck, Darmstadt, Germany) with chloroform/diethyl ether (9:1, v/v) as a solvent. Fractions were localized under an ultraviolet lamp. Protein concentrations were determined by the method of Bradford (14). Mass spectra were recorded on a Hitachi M-80B instrument at 70eV; ^1H NMR spectra were recorded on a Nicolet 360-MHz instrument with tetramethylsilane as internal standard.

RESULTS

Isolation and identification of Δ5 desaturase inhibitors. Two major fractions (Rf=0.65 and 0.81) were detected by TLC of the non-oil fraction together with several minor ones. The separation of the non-oil fraction into six fractions by preparative HPLC (see Fig. 1) and TLC of each of the six fractions demonstrated that the compounds corresponding to Rf=0.65 and 0.81 were present in fractions 4 and 5, respectively. The inhibitor assays as judged by the change of mycelial fatty acid profile of *M. alpina* showed that only fraction 4 caused an increase in mycelial DGLA content and a decrease in AA content, as shown in Figure 1. The mycelial DGLA/AA ratio was 0.51. By contrast, no significant change in mycelial fatty acid profile was observed on cultivation with the remaining five fractions. In each case, the ratio was only about 0.09. The compound isolated from fraction 4 was identified as (+)-sesamin based on the data of MS, ^1H NMR, optical rotation and melting point in comparison to literature values

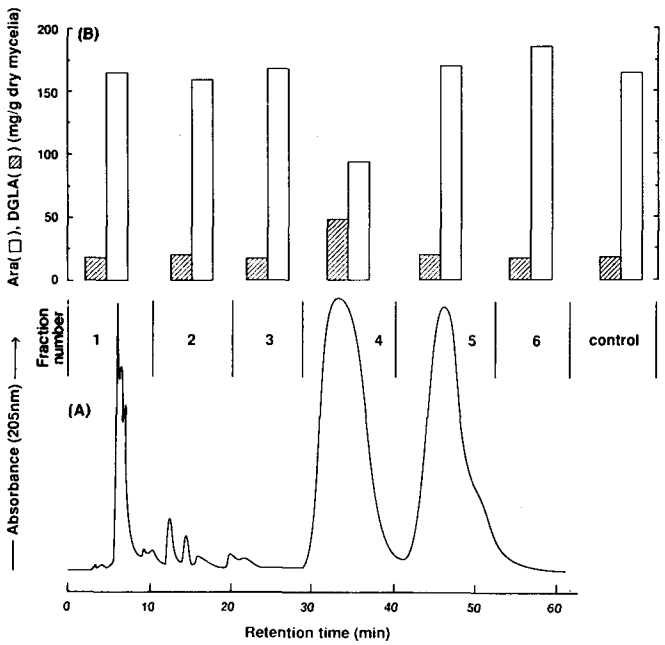


FIG. 1. Separation of the factor(s) which cause(s) the increase in mycelial DGLA content and the decrease in AA content from an acetone-extractable non-oil fraction of crude sesame oil by HPLC. *M. alpina* was grown in the basal medium supplemented with each of the fractions obtained on HPLC as described under Materials and Methods. (A) Elution profile of the acetone-extractable non-oil fraction of sesame oil at 205 nm. The numbers in the Figure indicate fraction numbers. (B) Production of DGLA and AA by the fungus grown in a medium supplemented with the concentrated fractions. Control represents no supplementation.

(3,15-17). When *M. alpina* was grown in the basal medium supplemented with 0.1 mg of (+)-sesamin under the conditions described in Materials and Methods, the mycelial content of DGLA reached 51 mg/g dry mycelia (DGLA/AA ratio, 1.45). The compound from fraction 5 was identified as (+)-sesamolin based on the analytical data in comparison with literature values (17). No significant change in mycelial fatty acid profile was observed when *M. alpina* was grown in the basal medium supplemented with 0.03-0.5 mg of (+)-sesamolin.

Specific inhibition of fungal and rat liver Δ5 desaturases by sesamin. The above results strongly suggest that sesamin (or its metabolites) was incorporated into the mycelia during growth of the fungus and caused specific inhibition of the Δ5 desaturation reaction, which resulted in a marked increase in mycelial DGLA content and a decrease in AA content. We measured the activities of several desaturases involved in the biosynthesis of AA in the presence or absence of sesamin using both cell-free extracts of the fungus and rat liver microsomes as enzymes. Figure 1 shows that sesamin specifically inhibits only Δ5 desaturase in both the microbial and rat systems. The concentrations inducing 50% inhibition were 5.6 μM (0.002 mg/mL) and 75 μM (0.002 mg/mL) for microbial and rat enzymes, respectively. By contrast, no significant decrease with sesamin was observed for Δ6, Δ9 and Δ12 desaturases in the fungal extract (Fig. 2A). Rat liver Δ6 and Δ9 desaturases also were not sensitive to sesamin (Fig. 2B).

The rat enzyme was studied in more detail to clarify the sesamin-induced inhibition of Δ5 desaturation. Double reciprocal plots of reaction velocity against DGLA concentration gave convergent lines that intersected at the *abscissa* for various sesamin concentrations. This result indicates that the inhibition proceeded noncompetitively (Fig. 2C). The K_i values estimated by Cornish-Bowden (18) was 115 μM.

Inhibition of Δ5 desaturation by other sesamin-related compounds. Table 1 compares the effects of several sesamin-related compounds (see Fig. 3 for chemical structures) on desaturase activities with those of sesamin. Sesamolin, which did not cause any increase in mycelial DGLA content and decrease in AA content (see Fig. 1), also inhibited only Δ5 desaturase, when it was incubated with either fungal extract or liver microsomes. Episesamin and sesaminol isolated from refined sesame oil also showed specific inhibition of Δ5 desaturase. The inhibitory effects on the Δ5 desaturase in either *M. alpina* cell-free extract or rat liver microsomes of these compounds were in the order of sesamin > sesamolin > sesaminol > episesamin. However, no significant inhibition was observed with sesamol, which does not carry the 3,7-dioxabicyclo[3.3.0]-octane ring.

DISCUSSION

The present study demonstrated that (+)-sesamin and related lignans specifically inhibit Δ5 desaturase in PUFA biosynthesis. This inhibitory activity seems to be unrelated to the known antioxidative activity of sesamin related lignans because only the compound carrying a hydroxyl group at the 6 position of the methylenediox-

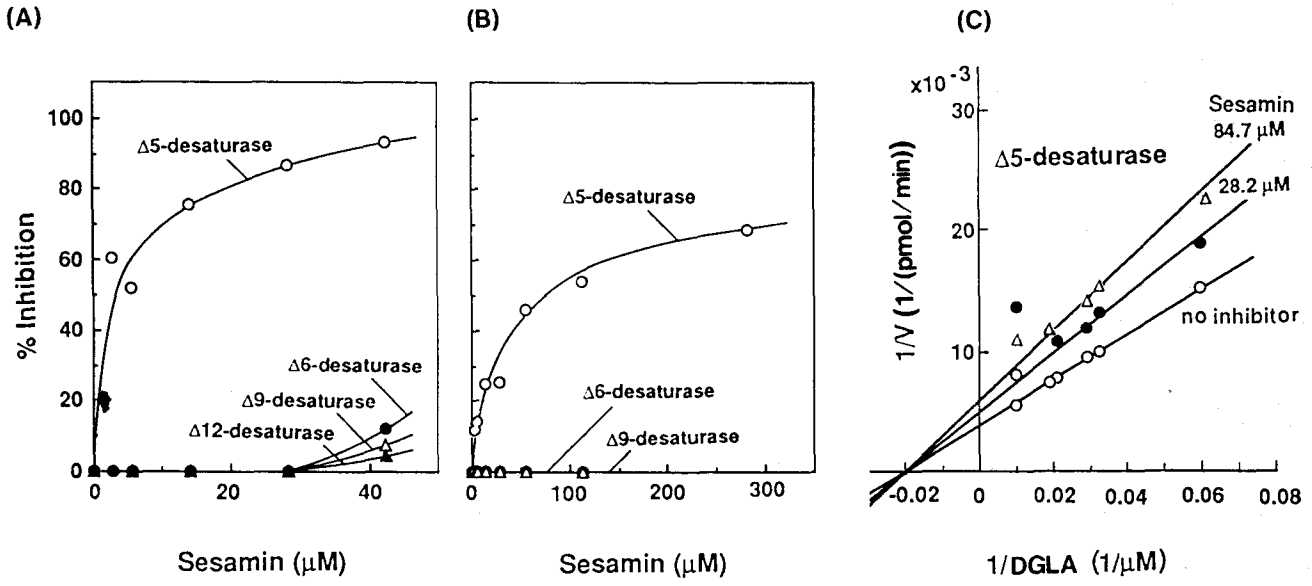


FIG. 2. The effect of sesamin concentration on various desaturase activities of *M. alpina* cell-free extract (A) and rat liver microsomes (B and C). (C) shows double reciprocal plots for Δ5 desaturase activities in the presence (●,△) or absence (○) of sesamin. Assay of desaturases were carried out under the conditions described in Materials and Methods, except that the indicated amount of sesamin was present in the incubation mixtures in (A) and (B) and concentrations of substrate (DGLA) and sesamin were changed as indicated in (C). In (A) and (B), the values without sesamin were taken as 0% inhibition (see Table 1 for absolute values) and each point represents percentage of inhibition. All values are means of three independent assays (standard deviation, within ±10%).

TABLE 1

Specific Inhibition of Fungal and Rat Liver Δ5 Desaturases by Sesamin-Related Compounds[a]

Compound[b] added	Desaturase activity (pmol/min/mg protein)						
	M. alpina				Rat liver		
	Δ9	Δ12	Δ6	Δ5	Δ9	Δ6	Δ5
None	10.1	8.16	18.2	21.2	37.2	15.0	116
Sesamin	10.5	9.27	19.1	2.80	34.9	16.4	71.8
Episesamin	10.3	8.40	18.2	5.20	36.1	17.5	92.1
Sesaminol	10.1	8.37	18.4	4.67	36.0	15.4	87.9
Sesamolin	9.93	8.77	17.7	3.97	37.7	18.6	86.6
Sesamol	9.86	8.10	17.2	16.8	34.3	14.7	114

[a]Desaturase activities were measured as described under Materials and Methods except that each of the indicated compounds were added and incubations with the liver microsomes were carried out for 30 min. All the compounds were present at 28 or 85 μM in the reaction mixtures with *M. alpina* extract or rat liver microsomes, respectively. Values are means of three independent assays (standard deviation, within ±7%).

[b]See Figure 3 for chemical structures.

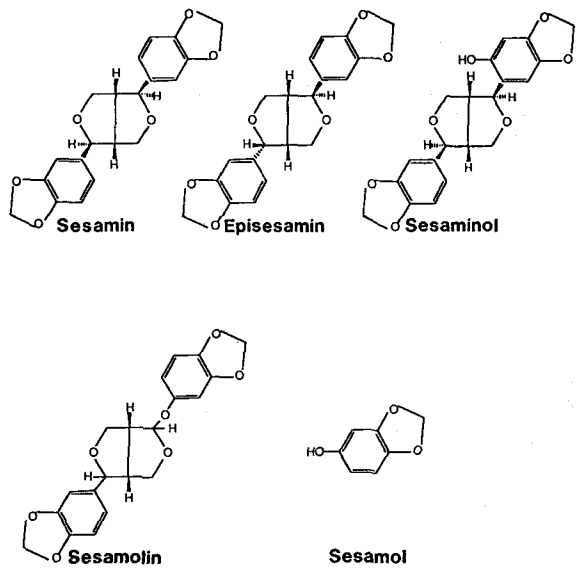


FIG. 3. Chemical structures of sesamin and related compounds.

yphenyl moiety (*i.e.*, sesaminol, episesaminol and sesamol) exhibit antioxidative activity, and sesamin itself is inactive as an antioxidant (1–3). The synergistic activity of sesamin for some insecticides, such as pyrethrin, has been suggested to be due to inhibition of the cytochrome P-450 oxygenase system catalyzing degradation of the insecticides (2). Since fatty acid desaturases are also of the oxygenase family, there may be some relation between the inhibitory activity on Δ5 desaturase and the synergistic activity. The finding that sesamol, which lacks the dioxabicyclo[3.3.0]octane ring, does not show any inhibitory activity suggests that this ring (or some other bulky substituent equivalent to the ring) is necessary for the activity. The inability of sesamolin to cause the increase in DGLA production and the decrease in AA production in the fungus *M. alpina* might be due to degradation of the lignan during the growth of the fungus.[3]

Our study is the first to demonstrate the occurrence of desaturase inhibitors in nature. Several unnatural desaturase inhibitors have been synthesized; most of them are analogs of fatty acids and they rather nonspecifically inhibit any of the desaturases involved in PUFA biosynthesis. For example, several *trans*-octadecenoic acids are competitive inhibitors of Δ9, Δ6 and Δ5 desaturases of rat liver microsomes (13,19). By contrast, sesamin or related lignans inhibit only Δ5 desaturase noncompetitively. This suggests that the inhibition induced by sesamin differs from those previously reported (13,19). The enzyme may have a site different from the substrate binding site, which shows high affinity toward sesamin.

The enzyme assay system used here involves acylation of the substrate with CoA, and the rates of the desaturation reactions measured here are the results of at least two sequential reactions, acylation and desaturation. Since the acylation step is not the rate-limiting step in rat liver microsomes and a single enzyme is responsible for the activation of all desaturase substrates used here (13,20), we can exclude the possibility that sesamin inhibited the acylation reaction.

The present results suggest that sesamin alters *in vivo* metabolism and composition of PUFA. Nutritional experiments are in progress in our laboratories.

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Acylation of Lyso Platelet-Activating Factor by Splenocytes of the Rainbow Trout, *Oncorhyncus mykiss*

G.L. Pool, B. Samples, M.R. Turner and R.H. Lumb*
Mountain Aquaculture Research Center, Western Carolina University, Cullowhee, North Carolina 28723

In mammalian systems, platelet-activating factor, 1-*O*-alkyl-2-acetyl-*sn*-glycero-3-phosphocholine, (PAF) is rapidly inactivated by a deacetylation/reacylation system that produces 1-*O*-alkyl-2-acyl-*sn*-glycero-3-phosphocholine which is highly enriched in arachidonic acid. There is some evidence that n-3 fatty acids may have an impact on this system in humans but the nature of this impact is unclear. In rainbow trout, n-3 fatty acids are known to be essential dietary components which are derived through the food chain. Substantial quantities of n-3 fatty acids are found in trout membrane phospholipids. We show here that in sharp contrast to mammalian cells, trout cells acylate lyso platelet-activating factor, alkyl-GPC, 1-*O*-alkyl-2-lyso-*sn*-glycero-3-phosphocholine, (lyso-PAF) with a high degree of specificity for n-3 fatty acids. When [^{3}H]lysoPAF was incubated with these cells, only three molecular species of alkylacylglycerophosphocholine were produced, and 92% contained n-3 fatty acids. Since isolated membranes yielded similar results, it appears that the acylation proceeds *via* a coenzyme A-independent transacylase as found in mammalian systems. *Lipids 26,* 517–520 (1991).

Recent findings demonstrate that a variety of lipid mediators play a major role in eliciting cellular responses to various stimuli. Platelet-activating factor (PAF, 1-*O*-alkyl-2-acetyl-*sn*-glycero-3-phosphocholine), diradylglycerols, inositol triphosphate, and eicosanoids (prostaglandins, leukotrienes and lipoxins) all have important biological activities and all are derived from the products of membrane phospholipid hydrolysis. Membrane phospholipid metabolism must therefore play a central role in signal transduction systems, and the diversity of phospholipid molecular species present may in part reflect the functional requirements of these systems. It is quite clear that the type of mediator produced is highly dependent upon the molecular species of phospholipid selected as precursor; what is less clear is how the selection is achieved.

Arachidonic acid, the precursor of the eicosanoids, can be released from several phospholipids, and one mechanism appears to involve hydrolytic cleavage of the *sn*-2 acyl group of 1-*O*-alkyl-2-acyl-*sn*-glycero-3-phosphocholine (alkylacyl-GPC) which is enriched in arachidonate in polymorphonuclear leukocytes (1). This enzymatic activity simultaneously releases lyso platelet-activating factor (lysoPAF, 1-*O*-alkyl-2-lyso-*sn*-glycero-3-phosphocholine) which can be acetylated to form PAF or acylated in a coenzyme A-independent manner to yield alkylacyl-GPC. In mammalian cells this latter activity shows a high degree of specificity for arachidonate (2), implying a link between PAF and eicosanoid metabolism. In fact, the biosynthesis of PAF is frequently (1), but not always (3), accompanied by the generation of eicosanoids.

More recently, health benefits in man have been associated with the intake of dietary fish oil containing n-3 fatty acids (4,5). These fatty acids may in fact be a requirement in human diet (6), but the mechanisms involved have remained largely obscure. The n-3 fatty acids have unambiguously been shown to be essential in rainbow trout (7). We have previously demonstrated that cells of the rainbow trout synthesize PAF (8) and that the alkylacyl-GPC fraction contains primarily n-3 fatty acids and little arachidonate (9). We have now examined the specificity of the acylation reaction by trout splenocytes and report here that lysoPAF is acylated with a high degree of specificity for n-3 fatty acids from endogenous sources in a coenzyme A-independent fashion both *in vivo* and *in vitro*.

MATERIALS AND METHODS

Cell preparation. Rainbow trout were obtained from hatchery-reared stock populations and maintained in laboratory flow-through culture tanks. Spleens were removed and minced with scissors in RPMI 1640 (Sigma Chemical Co., St. Louis, MO) buffered with HEPES at pH 7.2. In some experiments the minced preparations were stirred for one hr at 4°C to increase cell yield and viability. The cells were filtered through cheesecloth and nylon mesh (150 μ) and then centrifuged at 350 × *g* for 8 min. The erythrocytes were lysed by hypotonic washing and the recovered splenocytes suspended in RPMI 1640. Cells were counted with a hemocytometer and viability determined by Trypan blue exclusion. To prepare membrane fractions, the cells were disrupted using a tissue grinder or by nitrogen cavitation (10) in Tris/HCl buffer containing 0.25 M sucrose and 1 mM EDTA, pH 7.4. The homogenates were centrifuged at 1000 × *g* for 10 min to remove unbroken cells and nuclei and the supernatants recentrifuged at 100,000 × *g* for 60 min to obtain the membrane fraction. The membrane pellets were resuspended in 50 mM Tris/HCl pH 7.1 using a small Dounce homogenizer.

Incubations. Intact cells or isolated membranes were incubated with 1-*O*-[^{3}H]hexadecyl-*sn*-glycero-3-phosphocholine (a generous gift of Dr. R. L. Wykle, The Bowman Gray School of Medicine, Winston-Salem, NC) at 15–20°C in plastic containers. The radiolabeled substrate had a specific radioactivity of 56 Ci/mmole and was added to the incubation mixtures in 10 μL of ethanol. At the end of the incubation periods, lipids were extracted according to the method of Bligh and Dyer (11) and kept under nitrogen at −20°C until analyzed.

Analysis of products. Lipids were analyzed by thin-layer chromatography (TLC) on silica gel plates (Brinkmann

*To whom correspondence should be addressed.

Abbreviations: alkylacyl-GPC, 1-*O*-alkyl-2-acyl-*sn*-glycero-3-phosphocholine; DHA, docosahexaenoate; HPLC, high-performance liquid chromatography; lysoPAF, lyso platelet-activating factor, alkyl-GPC, 1-*O*-alkyl-*sn*-glycero-3-phosphocholine, 1-*O*-alkyl-2-lyso-*sn*-glycero-3-phosphocholine; PAF, platelet-activating factor, 1-*O*-alkyl-2-acetyl-*sn*-glycero-3-phosphocholine; PC, phosphatidylcholine; PUFA, polyunsaturated fatty acids; TLC, thin-layer chromatography.

Instruments Co., Westbury, NY) using chloroform/methanol/acetic acid/water (50:30:8:5, by vol) as solvent. Absorbant bands corresponding to phosphatidylcholine (PC) and lysophosphatidylcholine were eluted and radioactivity determined by liquid scintillation counting. The PC fraction was hydrolyzed with phospholipase C (*B. cereus*, Sigma), and benzoate derivatives were formed from the resulting diradylglycerols as reported previously (8,12). Subclasses were separated by normal phase high-performance liquid chromatography (HPLC) using a 4.6 × 250 mm Econosphere 5 μ Si column (Alltech Associates, State College, PA) eluted with cyclohexane/diethyl ether/acetic acid (97:3:0.07, v/v/v). Molecular species were separated by reverse phase HPLC on a 4.6 × 250 mm Microsorb C18 column (Rainin Instruments, Woburn, MA) eluted isocratically with acetonitrile/isopropanol (65:35, v/v) and monitored at 230 nm. Identification of molecular species was accomplished by comparison with authentic standards kindly supplied by Dr. R. L. Wykle. All HPLC solvents were from Burdick and Jackson (Muskegon, MI).

RESULTS

Incubation of trout splenocytes in the presence of 1-*O*-[^{3}H]hexadecyl-GPC resulted in the incorporation of radiolabel into a fraction which migrated with authentic phosphatidylcholine (PC) in thin-layer chromatography (TLC). Incorporation was time-dependent and resulted in decreased recovery of radiolabel in the alkyl-GPC fraction (Fig. 1). Maximum incorporation was about 70% after 2 hr of incubation. More than 90% of the radioactivity was recovered from the alkyl-GPC and alkylacyl-GPC regions of TLC plates. The labeled "PC fraction" was isolated by TLC; following phospholipase C treatment and benzoylation, more than 90% of the radioactivity eluted with the alkylacyl-glycerobenzoate subclass in normal phase HPLC. Incorporation of alkyl-GPC into alkylacyl-GPC was completely eliminated by heating the cells to 60°C for 15 min while treatment of the cells with 1 mM *N*-ethylmaleimide for 15 min resulted in a 70% loss of incorporation. The incorporation was not appreciably altered by complete loss of cell viability through treatment with sodium azide (Table 1).

When the alkylacyl-glycerobenzoate fraction that was recovered from normal phase HPLC was applied to reverse-phase HPLC, the elution pattern showed radioactivity in only three peaks (Fig. 2A). The molecular species corresponding to these peaks were identified using authentic diradylglycerol benzoate standards prepared from 1-*O*-hexadecyl-2-docosahexaenoyl-*sn*-glycero-3-phosphocholine (16:0-22:6n-3), 1-*O*-hexadecyl-2-eicosapentaenoyl-*sn*-glycero-3-phosphocholine (16:0-20:5n-3) and 1-*O*-hexadecyl-2-arachidonoyl-*sn*-glycero-3-phosphocholine (16:0-20:4n-6). Upon addition of these standards to the diradylglycerol benzoates derived from the [^{3}H]alkylacyl-GPC produced by the cell preparations, virtually all of the radioactivity co-eluted with the standards. Peak identification was confirmed by hydrogenation of the standards and labeled products in the presence of palladium oxide followed by reverse-phase HPLC. The radiolabel co-eluted with the hydrogenated standards at 130 min (16:0-20:0) and 180 min (16:0-22:0) with evidence for the presence of some partially-hydrogenated products.

When the isolated membrane fraction was incubated with 1-*O*-[^{3}H]hexadecyl-GPC, the pattern of incorporation was similar to that observed in intact cells (Fig. 2B). While the amount of eicosapentaenoate-containing species was the same, slightly more arachidonate-containing species were produced (Table 2). Thus the n-3 containing molecular species dropped slightly from 92% to 84%.

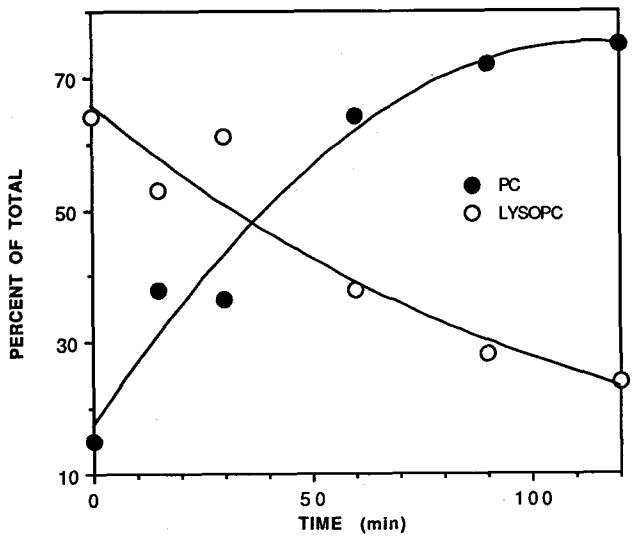


FIG. 1. Time course of the disappearance of 1-*O*-[^{3}H]alkyl-*sn*-3-phosphocholine (lysoPAF) and the appearance of [^{3}H]alkylacyl-GPC. Cells were incubated for the indicated times and terminated by lipid extraction. Precursor and product were separated by TLC; small amounts of radioactivity chromatographed in other areas of the TLC plate.

DISCUSSION

The metabolism of polyunsaturated fatty acids (PUFA) and the membrane phospholipids with which they are associated is recognized as an important process in cell regulation. In some mammalian cells there is evidence for a tight coupling between the metabolism of PAF and

TABLE 1

Inhibition of Acylation of LysoPAF by Treatment with Heat, *N*-Ethylmaleimide or Sodium Azide

Treatment[a]	Percent radioactivity in PC
Control	40.4
Heat (60°C, 15 min)	0.0
N-Ethylmaleimide[b] (1 mM, 15 min)	12.5
Sodium azide[c] (1%, 60 min)	35.0

[a]Each incubation contained 0.5 μCi 1-*O*-[^{3}H]alkyl-*sn*-glycero-3-phosphocholine.

[b]Cells (1.65×10^6) were preincubated for 15 min at 20°C with *N*-ethylmaleimide before addition of label.

[c]Cells (5×10^7) were treated for 60 min until Trypan blue showed complete loss of viability before addition of label.

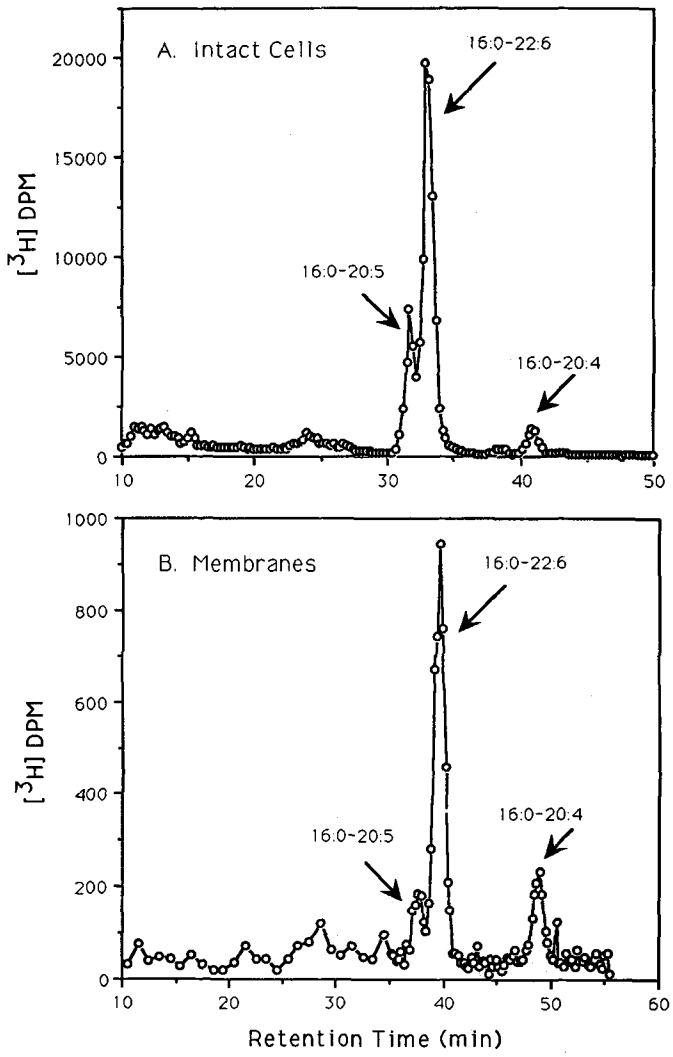


FIG. 2. Reverse-phase HPLC elution profiles of the 1-*O*-[^{3}H]alkyl-acyl-glycerobenzoates. Intact cells or membrane fractions from disrupted cells were incubated with [^{3}H]lysoPAF for 60 min. The PC fraction was isolated by TLC, derivatized to the glycerobenzoates, and the alkylacyl-glycerobenzoate fraction was isolated by normal phase HPLC. This fraction was then chromatographed on a Microsorb C18 column (4.6 × 250 mm) with acetonitrile/isopropanol (65:35, v/v) as the mobile phase. Peak identification was by cochromatography with authentic standards and by analysis of the products of palladium oxide-catalyzed hydrogenation.

TABLE 2

Specificity of Acylation of LysoPAF by Trout Splenocytes and Isolated Membranes

	% Distribution ± S.E.M.	
Molecular species	Intact cells	Membrane
16:0-20:5n-3	22.1 ± 1.28	14.0 ± 0.32
16:0-22:6n-3	69.7 ± 2.97	70.1 ± 2.17
16:0-20:4n-6	8.1 ± 1.70	15.8 ± 2.45

arachidonic acid (1,13). There is also evidence that some eicosanoids act synergistically with PAF (14,15). In other mammalian systems, however, it appears that the two mediator families are independently regulated (3). Some studies point toward other subclasses of phospholipids, such as alk-1′-enyl-2-acyl-*sn*-glycero-3-phosphoethanolamine, as a major store of n-6 PUFA (16) and precursors of other phospholipid mediators (17). The precursors of eicosanoids as well as of PAF can be released on hydrolysis of membrane-associated alkylacyl-GPC (13). Thus the nature of the acyl moiety in the *sn*-2 position can be crucial in determining how a cell responds to a stimulus. Associated with the generation of lipid mediators is the production of lysophospholipids, which, because of their detergent properties, cannot be allowed to accumulate; several lysophospholipid acylation systems which can remove these compounds have been described in mammalian cells. One acylation system uses acyl-coenzyme A as acyl donor while another system is independent of coenzyme A and catalyzes a direct transfer of an acyl moiety from a donor phospholipid to the acceptor lysophospholipid (2,18–20). The latter transacylation system in mammals appears to have a high degree of selectivity for arachidonoyl-containing donor species, implicating this system in stimulus-coupled lipid mediator generation.

Arachidonate may not be the only precursor of bioactive eicosanoids in mammals. Evidence of a cardiovascular health benefit in man derived from dietary n-3 fatty acids has been reported (4,5), but the reasons for this effect are not understood. There are reports that dietary n-3 fatty acids may be essential in man (6); it has been clearly demonstrated that docosahexaenoate (DHA) is required for normal growth and development in humans, particularly with respect to the growth of the brain and eye (21). DHA has been shown to accelerate differentiation of HL-60 cells (22) and to activate protein kinase C in sponges (23). Lipoxygenase products have been shown to be produced from DHA in human platelets (24) and canine retina (25), but the functions of these metabolites are unclear.

Certainly, mammalian cells will incorporate (26,27) and utilize (28) n-3 fatty acids when they are available. When $P388D_1$ cells were supplemented with n-3 fatty acids, the acids were selectively incorporated into the ether-linked phosphatidylethanolamines (27). When monkeys were fed a fish oil supplemented diet, much of the 20:5n-3 was found in the phosphatidylethanolamine fraction; interestingly, neutrophils from these animals preferentially acylated lysoPAF with 20:5 as compared to control cells which utilized predominantly 20:4 (27). It has also been demonstrated that 20:5n-3 stimulates PAF synthesis in 20:4-depleted granulocytic HL-60 cells, but synthesizes less PAF than cells supplemented with 20:4 (29). Sperling *et al.* (30) found that in human monocytes, containing normal levels of 20:4, 20:5 supplementation inhibits PAF synthesis. Thus while 20:4 plays a major role in the reacylation of PAF in mammalian cells, 20:5 appears to have an impact on this system when it is available. We have shown here that in rainbow trout cells endogenous 22:6n-3 and 20:5n-3 are the major fatty acids used in the acylation of lysoPAF, representing more than 90% of the alkylacyl-glycerophosphocholine produced.

The role of n-3 fatty acids in mammals has remained unclear, perhaps obscured by the predominance of arachidonate, whereas in the rainbow trout n-3 essentiality has been documented (7). The concentration of n-3 fatty acids is enriched in the ether-linked subclasses of phosphatidylcholine and phosphatidylethanolamine (9), while

arachidonic acid (20:4n-6) is almost entirely associated with diacyl phosphatidylinositol (9). Tissues of the rainbow trout have been shown to synthesize PAF (8) and thus provide a model for studying the metabolism of lipid mediators in organisms that balance the utilization of n-3 and n-6 fatty acids differently than is known to occur in mammals. We have shown that trout splenocytes and an isolated membrane fraction from these splenocytes acylate alkyl-GPC with a high degree of specificity for n-3 fatty acids. This acylation appears to be independent of cellular energy as it is unaffected by treatment of the cells with sodium azide. The process is coenzyme A-independent and shows sensitivity to N-ethylmaleimide in a manner similar to that system in mammals. The reported observations that trout cells generate oxygenated products from n-3 fatty acids (31–37) and also show physiological responses to them (38), together with the specific n-3 acylation of lysoPAF reported here, defines a basis on which PAF and PUFA metabolism may be linked. Rainbow trout thus offer a good model system for obtaining a better understanding of that interface.

ACKNOWLEDGMENT

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Alterations of Hepatic Acetyl-CoA Carboxylase by 2,3,7,8-Tetrachlorodibenzo-*p*-dioxin

J.M. McKim, Jr.[a], K. Marien[a] H.W. Schaup[b] and D.P. Selivonchick[a,*]

[a]Department of Food Science and Technology and [b]Department of Biochemistry and Biophysics, Oregon State University, Corvallis, Oregon 97331

The effect of 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD) on acetyl-CoA carboxylase (ACC) activity and synthesis was examined. Male Wistar rats received a single *i.p.* injection of TCDD (53 μg/kg), and nine days later body weight, liver weight, hepatic lipid, ACC activity and mass were determined and compared to pair-fed controls. Body weights of TCDD-treated animals decreased, while liver weights increased resulting in an increase in liver to body weight ratios. ACC activity was decreased by 65%, however sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and Western analysis using a biotin specific probe revealed that ACC protein levels were not appreciably changed. In addition, there was a large increase in exogenous lipid material in TCDD-treated livers as determined by osmium tetroxide staining. These data suggest that the decrease in ACC activity may be due to direct inhibition of the enzyme by negative allosteric interactions with free fatty acids released from adipose tissue that subsequently accumulate in liver.

Lipids 26, 521–525 (1991).

The chlorinated dioxin 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD), a contaminant formed during the production of chlorophenoxy herbicides, is a toxic environmental and occupational pollutant (1–3). TCDD toxicity, coupled with its persistence in organisms and in the environment, have made this compound the focus of extensive investigations aimed at identifying the mechanism(s) that account for its toxicity (4–14). These studies have produced valuable information on the physiological and biochemical effects of TCDD, but they have not revealed a clear molecular mechanism(s) for its toxicity.

The most obvious reoccurring physiological and toxicological change observed in animals exposed to TCDD is the "wasting syndrome". Weight loss in TCDD-treated animals is greater than in pair-fed controls (6,10,11). This implies that decreased food consumption alone is insufficient to account for the accelerated weight loss and death in exposed animals. The mechanism by which TCDD induces mortality may be indirectly linked to the wasting syndrome *via* alterations in lipid metabolism. Animals exposed to TCDD also show alterations in thyroid and insulin levels. These hormones control a wide range of metabolic responses, including changes in lipid and carbohydrate metabolism (6,7,9–11,15).

Large amounts of TCDD accumulate in the liver after oral or intraperitoneal administration (16). A dose dependent decrease in the *de novo* synthesis of fatty acids in the liver of TCDD-treated (1–20 μg/kg) Wistar rats has been reported (17). In contrast, Sprague-Dawley rats receiving a single high dose of TCDD (125 μg/kg) show an increase in hepatic fatty acid synthesis (18). A molecular mechanism that explains these observations remains to be determined.

In all organisms examined to date, *de novo* synthesis of fatty acids requires acetyl-CoA carboxylase (ACC) [EC 6.4.1.2] and fatty acid synthase (FAS) [EC 2.3.1.85]. Other enzymes, such as pyruvate carboxylase (PC) [EC 6.4.1.1] and malic enzyme [EC 1.1.1.40] that shunt carbon into citrate may be an important factor in the stress response (19–21). For example, in TCDD stressed rats the level of malic enzyme increases (22). In plants ACC is the target of several chlorophenoxy herbicides (23–25). These authors suggested that plant death was linked to inhibition of ACC that caused a decrease in acyl lipid and membrane synthesis.

Here we report the effects of TCDD on the activity and mass of ACC in rats. In animals exposed to TCDD, the activity of ACC was drastically reduced, but the apparent mass of ACC remained unchanged. The amounts of FAS and PC also appear to decrease in treated animals.

MATERIALS AND METHODS

Chemicals. 2,3,7,8-Tetrachlorodibenzo-*p*-dioxin, presolubilized in nonane at 50 μg/mL, was purchased from Cambridge isotope Laboratories (Woburn, MA). [^{14}C]$NaHCO_3$ was obtained from Amersham, Inc. (Arlington Heights, IL). Polyacrylamide (99% pure), protein molecular weight standards, 1-methyl-2-pyrrolidinone (MPO), Tween-20, avidin-HRP, HRP-developer and Zeta Probe membrane were purchased from BioRad (Richmond, CA).

Preparation of TCDD for injection. All procedures were performed under a fume hood in a contained area. Double layer gloves were worn when handling TCDD and all contaminated disposable materials were placed in biohazard bags and incinerated. Nondisposable glassware was rinsed with toluene and then washed with soap and water. Solutions for injection were prepared by adding 2.0 mL of the TCDD solution to 10 mL of corn oil in a 50-mL glass beaker. The mixture was stirred under a fume hood for 48 hr in order to insure uniform mixing and to allow complete evaporation of the nonane carrier. A fraction of the corn oil/TCDD solution was removed and analyzed by gas chromatography to determine the TCDD concentration.

Animals. Male Wistar rats weighing 140–160 g were purchased from Simonsen Laboratories (Gilroy, CA) and housed individually in wire bottom cages. The animal room was maintained at 22°C at 50–60% humidity with a 12-hr light/dark cycle.

Diets and TCDD treatment. The animals were paired according to weight, and separated into two groups with eight animals in each group. They were fed Wayne Rodent Blox lab chow (Wayne Pet Food, Chicago, IL) *ad libitum* for five days after their arrival to the laboratory. On the

*To whom correspondence should be addressed.

Abbreviations: ACC, acetyl-CoA carboxylase; FAS, fatty acid synthase; MPO, 1-methyl-2-pyrrolidinone; PC, pyrivate carboxylase; SDS-PAGE, sodium dodecyl sulfate-polyacrylamide gel electrophoresis; TCDD, 2,3,7,8-tetrachlorodibenzo-*p*-dioxin.

sixth day, eight animals were injected *i.p.* with 53 μg/kg TCDD in 1 mL of corn oil and placed on a pair-feeding regime. The pair-fed controls were injected *i.p.* with corn oil only. Five days after injection, TCDD-treated and pair-fed control animals were fasted for 48 hr and then fed standard rat chow diet for an additional 48 hr. No difference in food intake between control and experimental animals was observed. This dietary procedure is the same as that described by Lakshman *et al.* (26) that ensures optimal conditions for lipogenic enzyme activity.

Tissue preparation. Animals were weighed before decapitation. Livers were removed immediately and weighed, and portions were taken for histopathological analysis. To obtain sufficient material for biochemical analyses, two liver homogenates were combined from the pair-fed and TCDD-treated animals to give 4 samples (N = 4). The samples were placed into ice-cold buffer (Tris-HCl, 20 mM, pH 7.4.; mannitol, 0.3 M; EDTA, 2 mM; dithiothreitol, 1 mM; and phenylmethylsulfonyl fluoride, 0.2 mM) and homogenized in a blender by pulsating (5–10 seconds) at high speed for ~30 seconds (27). The homogenate was filtered through gauze to remove fibrous material. The filtrates were centrifuged for 10 min at 20,000 × *g* at 4°C. The supernatant was removed and centrifuged again at 100,000 × *g* for 1 hr at 4°C. Portions of the supernatant were used for enzyme activity assays. The remaining supernatant was aliquoted and stored in 10% glycerol at −70°C.

Histopathologic preparation. Tissue samples for pathologic analysis were placed in Bouin's fixative (23.3% formaldehyde, 4.8% acetic acid, and 0.7% picric acid) for two days. The samples were stained with osmium tetroxide prior to being embedded in paraffin. With this stain lipids appear as black spots (28).

Activity assays. Cytosolic ACC was assayed as previously described (29). The method is a modification of that reported by Allred and Roehrig (30). The final reaction mixture (0.5 mL) contained: Tris-acetate, 70 mM, pH 7.5; potassium acetate, 120 mM; reduced glutathione, 4 mM; potassium citrate, 10 mM; ATP, 1 mM; bovine serum albumin, 0.6 mg/mL; acetyl-CoA, 0.35 mM; magnesium acetate, 10 mM; and [^{14}C]$NaHCO_3$, 7 mM (0.25 μCi). Blanks were assayed without protein and without substrate. Pyruvate carboxylase (PC) activity was assayed as described by Ballard and Hanson (31). The reaction was started with addition of protein and incubated 2–3 min for ACC and 10 min for PC at 37°C. Reactions were stopped with 0.05 mL of concentrated HCl. Unincorporated label was removed by drying samples (0.2 mL) of the reaction mixture at 95°C for 30 min. Samples were resolublized with 0.2 mL distilled water and counted in Aqueous Counting Scintillant fluid (Amersham).

Polyacrylamide gel electrophoresis of cytosolic protein. Cytosolic proteins were separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE), as previously described (32).

Western blotting and avidin-horse radish peroxidase stain for biotin. Protein (200 μg) was separated according to size by SDS-PAGE as described. Following electrophoresis the gel was cut in half; one section was stained with Coomassie Blue and the other was equilibrated in transfer buffer (Tris/HCl 2.5 mM; and glycine 192 mM pH 8.3) for 30 min at room temperature to swell the gel and remove excess SDS. The gel was then put in the transfer cassette and placed on two pieces of 3 MM filter paper and a Scotch pad. Zeta Probe® membrane (BioRad), presoaked in transfer buffer, was then put on the anode (+) side of the gel. Ice-cold buffer (3.5 L) was added and the proteins were transferred in a BioRad Trans-Blot apparatus at 50 V (~0.560 amps) for 6.5 hr. After transfer, the membrane was incubated with 100 mL of borate Tween blocking buffer ($Na_2B_4O \times 10\ H_2O$; 0.05 M; NaCl 0.5 M; 5% MPO; 0.2% Tween-20, pH 9.3) for 10 min; the incubation was repeated two times. The membrane was then washed three times with 100 mL Tris-Tween buffer (0.02 M Tris; 0.5 M NaCl, pH 7.5; 0.2% Tween-20; and 5.0% MPO) for 5 min. Avidin-HRP was prepared by adding 150 μL of premixed avidin-HRP (BioRad) to 100 mL of the Tris-Tween buffer. The membrane was put in the avidin-HRP solution and then placed on a rotating shaker for 1.5 hr at room temperature. Excess avidin-HRP was removed by washing two times with 100 mL Tris-Tween buffer and two more times with 100 mL Tris-buffered saline (0.02 M Tris; 0.5 M NaCl, pH 7.5). The samples were visualized using HRP-developer (60 mg developer in 20 mL ice-cold methanol, 0.015% H_2O_2 in 100 mL distilled water for 30 min). Development was stopped by soaking in distilled water.

RESULTS

Rats given a single *i.p.* injection of (53 μg/kg) TCDD showed a weight loss relative to control animals at the end of the nine-day period. Liver enlargement was apparent visually and the liver weight to body weight ratios increased from approximately 4 to 6% (Table 1). The observed increase in liver mass is associated with a dramatic increase in the lipid content of TCDD treated *vs.* control livers (Fig. 1, A and B) as judged by lipid specific histological evaluation of the status of livers from TCDD-treated animals.

ACC specific activity was suppressed 65% in TCDD exposed animals (Table 1). To assess the effects of TCDD on the actual amount of ACC present in liver samples, equal amounts of cytosolic protein from control and TCDD-exposed livers were analyzed by SDS-PAGE (Fig. 2). The location of ACC on the gel was determined using purified rainbow trout hepatic ACC as a standard (M_r = 230,000) (lane 3). The trout ACC has been shown previously to have the same biochemical and electrophoretic properties as rat hepatic ACC (29). Initially it appeared from Figure 2 that the 230,000 dalton protein, putatively ACC, had dramatically decreased in TCDD-treated (lane 1) *vs.* pair-fed animals (lane 2). However, carboxylases are characterized by the presence of a covalently linked biotin moiety. If the observed decrease in ACC activity was due to reduced ACC protein levels, then the biotin content of the 230,000 dalton band should be reduced proportionately. A western transfer of a gel comparable to that shown in Figure 2 was done. The blot was incubated with avidin-HRP, which has a high binding affinity for biotin. This enabled visualization of all the biotin-containing proteins present in the soluble fraction. Figure 3 shows a typical blot. This experiment was done four times with no indication of differences between amounts of ACC in control and in experimental animals. The amount of ACC protein (M_r = 230,000) was equivalent in TCDD (lane 4) to that observed in control preparations (lane 3). We have

TABLE 1

Effects of 2,3,7,8-Tetrachlorodibenzo-*p*-Dioxin on Animal Body Weight, Liver Weight and Acetyl-CoA Carboxylase (ACC) Specific Activity[a]

Group	(N)	Body wt.	Liver wt.	Liver wt. Body wt. × 100	ACC activity (U/mg) %	Inhibition	Total protein (mg/g liver)
Control (corn oil only)	(8,6)	228.1 ± 17.5	9.30 ± 1.64	4.0	.40 ± .07	0.0	34.8 ± 2.90
Experimental (53 μg/kg TCDD)	(8,6)	202.0 ± 13.7[b]	12.02 ± 1.71[b]	6.0	.14 ± .02[c]	65.0	30.8 ± 3.13

[a]Statistical analysis was by Student paired *t*-test; all values are means ± S.D. (U) equals nmoles of malonyl-CoA produced per min at 37°C. Activity is expressed as per mg total protein. ACC activity has been corrected for acid stable products other than malonyl-CoA.

[b]$p<0.01$.

[c]$p<0.001$.

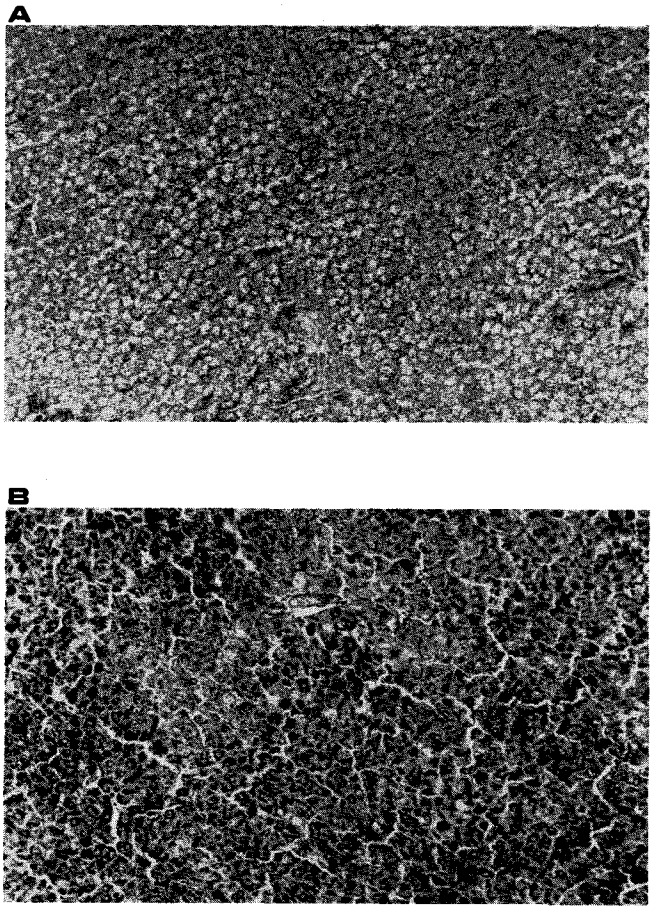


FIG. 1. Photomicrographs (250x) of control (A), and 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD)-treated (B), liver stained with osmium tetroxide to visualize fat. The large black areas apparent in (B) represent fat deposits.

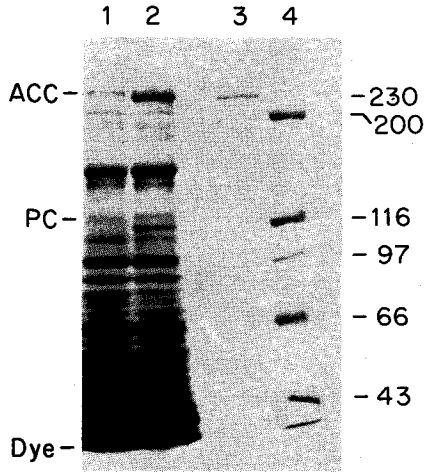


FIG. 2. SDS-PAGE analysis (5% polyacrylamide) of cytosolic proteins. Lane 1, 200 μg TCDD treated liver cytosolic protein. Lane 2, 200 μg of control liver cytosolic protein. After electrophoresis for 4 hr at 21 mA the gel was stained with Coomassie Blue. Acetyl-CoA carboxylase (ACC) and pyruvate carboxylase (PC) bands are identified. Lane 3 is an avidin-Sepharose purified rainbow trout liver ACC, which has been shown to have essentially the same biochemical and electrophoretic properties as rat liver ACC (ref. 29). Lane 4 contains BioRad high molecular weight standards ($\times 10^{-3}$). Samples were fractionated on an SDS-PAGE (18 cm × 16.5 cm × 0.2 cm) consisting of a 3% stacking gel and a 5% resolving gel at 21 mA constant current for 4 hr at 4°C. Gels were stained overnight with Coomassie Blue and destained with 50% methanol, and 10% acetic acid.

no explanation for the slight change in migration observed between ACC protein in lanes 3 and 4. The higher molecular weight form of ACC that is visible using the avidin-HRP probe appears as doublet. The doublet was present in all of the control and experimental crude cell preparations. Although this higher molecular weight band has been reported by several authors, its exact source has not been clearly defined (23,33). It is important to note, however, that in all control and TCDD samples analyzed in this study, there was no visible difference between the high and low molecular weight forms. The data in Figure 3 suggest that reduced staining intensity for the putative ACC observed for TCDD-treated animals in Figure 2, lane 1, was due to co-migration of ACC with one or more protein(s). FAS has been shown to co-migrate with ACC on low-percentage acrylamide gels such as those used in this work (21,34). Tentatively, we believe that a reduction in the amount of FAS may account for the reduced Coommassie Blue staining intensity observed in Figure 2.

On the western blots, a smaller biotin-containing protein (M_r = 125,000) was observed (Fig. 3). It was reproducibly present in lower amounts in TCDD-treated animals (lane 4) relative to control animals (lane 3). The mitochondrial protein pyruvate carboxylase (PC) has a molecular weight of 130,000 daltons, and the presence of

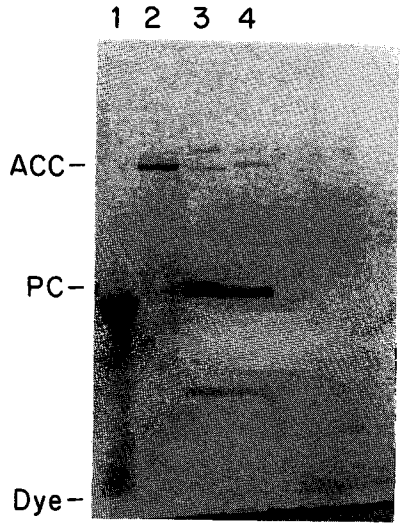


FIG. 3. Western transfer and biotin specific staining of protein with avidin-HRP. Lane 1 is a prestained β-galactosidase (M_r = 116,000) standard obtained from Diversified Biotech (Newton Centre, MA). Lane 2 is 3 μg purified rainbow trout liver ACC. Lane 3, 200 μg cytosolic protein from control liver. Lane 4, 200 μg cytosolic protein from TCDD liver.

this enzyme in preparations such as those employed here has been previously established (31,35). Activity assays were done to establish a relationship between the gel data and PC activity. These assays revealed the presence of PC in our normal preparations; however, PC specific activity in preparations from TCDD-treated animals was reduced. The average value for control-fed animals and for TCDD animals was 10.5 nmol/min/mg protein and 4.0 nmol/min/mg/protein, respectively. Previous work by others using these procedures has shown that this carboxylase appears in the supernatant fractions (27); we presume that mitochondria were disrupted. We attribute the reduction in activity to decreased amounts of PC in TCDD-treated animals. Tentatively, this conclusion is supported by SDS-PAGE analysis (Fig. 2, lanes 1 and 2), western blots analyzed with a biotin carboxylase specific probe (Fig. 3, lanes 3 and 4), and PC specific activity assays.

DISCUSSION

Animals given TCDD showed a reduction in body weight after nine days as compared to pair-fed controls. Hepatomegaly was also observed in TCDD-treated animals, with liver weight to body weight ratios increasing relative to controls. The increase in liver weight was due principally to lipid accumulation in the liver (Fig. 1). TCDD is known to increase the activity of hormone sensitive lipoprotein lipase activity in adipose tissue. This leads to an increase in the levels of serum fatty acids (33,36). Serum fatty acids accumulate in the liver and are converted principally to triacylglycerols. We observed that TCDD had a suppressive effect (65% inhibition) on ACC, a key enzyme in *de novo* synthesis of fatty acids. The actual mass of the enzyme is nevertheless comparable to that observed in control animals (Fig. 3). This suggests that post-translational regulation is the initial response to TCDD, which is most likely due to the accumulation of acyl-CoA fatty acids. These fatty acids are known negative effectors of acetyl-CoA carboxylase activity (37).

Fatty acid synthase (FAS) is the only protein that is known to co-electrophorese with ACC on low-percentage acrylamide gels (21,34). Both ACC and FAS activities are greatly increased following a fasting/refeeding protocol as used in the present study. The increased activity of FAS is most likely due to increased translational efficiency that produces more enzyme (38,39). Lakshman *et al.* (26) have shown that FAS activity is greatly reduced by TCDD. Our data strongly suggest that the reduction in FAS activity is a consequence of decreasd amounts of the enzyme, possibly due to a reduction in translation. This interpretation is based upon our preliminary observation that the primary staining component of the 230,000 dalton band in Figure 3 is FAS, and therefore it is this protein that decreases in TCDD-treated animals (Fig. 2, lane 1).

A second biotin-containing protein was detected in the western transfer/avidin-HRP procedure. This 125,000 dalton protein has the same molecular weight as that reported for PC. PC enzymatic activity was present in our preparations. The PC enzymatic activity was at least ten times greater than ACC activity, but the activity was greatly decreased in TCDD treated animals. Based on molecular weight, biotin content and enzyme activity, we conclude that the 125,000 dalton band in Figures 2 and 3 is PC. PC is a gluconeogenic enzyme. Our observations are consistent with recent data showing TCDD-mediated decreases in two gluconeogenic enzymes (phosphoenolpyruvate carboxykinase and glucose-6-phosphatase) in exposed rats (40).

Clearly ACC and PC are suppressed in the presence of TCDD. We also have indirect evidence of TCDD suppressive effects on FAS. The observed inhibitory effects on ACC enzymatic activity and mass reductions of FAS are significant. A diminution in *de novo* fatty acid biosynthesis could alter membrane synthesis, structure and function through aberrant incorporation of salvaged fatty acids into triacylglycerols and phospholipids. Such a response in terms of phospholipids may alter cellular ion flux and effective membrane/protein interactions. This possibility is supported by a recent report showing a substantial increase in the influx of exterior Ca^{2+} into TCDD-treated thymocytes (13).

The data presented here complement previous studies that have examined the *in vivo* regulation of ACC as well as the effects of TCDD on hormones, fat disposition and enzyme activities. We suggest that post-translational regulation of ACC is the initial response to TCDD exposure. It has been shown that ACC is regulated rapidly and specifically by phosphorylation of specific sites within the enzyme. The kinase responsible for this phosphorylation is AMP-phosphokinase (AMP-PK) and it is activated by nanomolar amounts of fatty acyl-CoA *via* a kinase-kinase cascade (41–43). Under normal circumstances, the negative allosteric affects of fatty acyl-CoA on ACC may be negligible due to the presence of acyl-binding proteins and low levels of free fatty acyl-CoA (44). However, the dramatic increase in hepatic lipids caused by TCDD exposure may saturate these binding proteins, and in combination with the AMP-PK allow the fatty acyl-CoA to exert their effect on ACC both allosterically and by

activation of the AMP-PK, resulting in the phosphorylation of ACC. We propose that the reduction in ACC activity without loss of protein may be due initially to an increase in phosphorylation and/or allosteric inhibition of ACC by fatty acyl-CoA.

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Differential Effects of Ursodeoxycholic Acid and Ursocholic Acid on the Formation of Biliary Cholesterol Crystals in Mice

Kiyohisa Uchida*, Takashi Akiyoshi, Hirotsune Igimi, Haruto Takase, Yasuharu Nomura and Shouichi Ishihara

Shionogi Research Laboratories, Shionogi & Co., Ltd., Fukushima-ku, Osaka 553, Japan

The preventive effect of 3α,7β,12α-trihydroxy-5β-cholanoic acid (ursocholic acid) and ursodeoxycholic acid on the formation of biliary cholesterol crystals was studied in mice. Cholesterol crystals developed with 80% incidence after feeding for five weeks a lithogenic diet containing 0.5% cholesterol and 0.25% sodium cholate. When 0.25% ursocholic acid or ursodeoxycholic acid was added to the lithogenic diet, the incidence as well as the grade (severity) of the gallstones were reduced. Plasma and liver cholesterol levels were decreased by ursodeoxycholic acid but not by ursocholic acid. Gallbladder cholesterol and phospholipid levels were decreased by both bile acids. The biliary bile acid level was decreased by ursocholic acid but not by ursodeoxycholic acid. After feeding ursocholic acid, its level in the bile was about 25% and the levels of cholic acid and β-muricholic acid decreased. Fecal sterol excretion was not changed by ursocholic acid, but was increased by ursodeoxycholic acid. After feeding ursocholic acid, fecal excretion of deoxycholic acid, cholic acid, and ursocholic acid increased. No differences were found between mice, with or without gallstones, in plasma and liver cholesterol levels, biliary phospholipid and bile acid levels, fecal sterol and bile acid levels, and biliary and fecal bile acid composition. The results suggest that the lower incidence of crystal formation after treatment with ursocholic acid is probably by a different mechanism than with ursodeoxycholic acid. In the mouse model, ursodeoxycholic acid exerts its effect at least partially, by decreasing cholesterol absorption. Ursocholic acid is well absorbed and excreted into bile and transformed into deoxycholic acid by the intestinal microflora in mice.
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Biliary cholesterol crystals are produced in mice that are fed excess cholesterol and cholic acid (1–3). Crj:ICR (Charles River Japan, Kanagawa, Japan) male mice develop cholesterol crystals with an incidence of 70% after being fed a diet containing 0.5% cholesterol and 0.25% sodium cholate for 5–10 weeks (4). When a high cholesterol diet without added sodium cholate was fed to mice, no crystals formed (1,4). In addition, no crystals developed (6) when cholic acid was replaced with chenodeoxycholic acid, which is less effective in enhancing cholesterol absorption (5). Diabetic mice have a markedly enlarged pool of cholic acid and develop cholesterol gallstones even when fed cholesterol alone (4). These results suggest that the mechanism by which cholesterol crystals form in gallbladder bile seems to be dependent on cholesterol absorption in the gut.

Ursodeoxycholic acid is well known as an oral gallstone solubilizer and recently 3α,7β,12α-trihydroxy-5β-cholanoic acid (ursocholic acid, 7β-hydroxy-isocholic acid, or 7β-cholic acid) has been studied for the same purpose, because it is the 7β-hydroxyepimer of cholic acid, possessing a 7β-hydroxyl group as well as ursodeoxycholic acid (7–11). This bile acid is reported to resemble cholic acid but not ursodeoxycholic acid in its response to biliary bicarbonate excretion and has a low cytotoxicity (7). Ursocholic acid is also reported to be well absorbed by the intestine and excreted into the bile without biotransformation in the liver, and it decreases biliary excretion of cholesterol and phospholipids, thus decreasing bile cholesterol saturation (8–11). The critical micellar concentration is higher for this bile acid than for cholic acid (12), which suggests that ursocholic acid has a low micelle-forming capacity (8). In the present study, we examined whether ursocholic acid has the same effect as ursodeoxycholic acid on biliary cholesterol crystal formation in mice.

MATERIALS AND METHODS

ICR strain male mice, six-weeks-old, obtained from Charles River Japan (Crj:ICR), were kept in an air-conditioned room (25 ± 1°C, 50–60% humidity) lighted 12 hr a day (8:00 a.m. to 8:00 p.m.). An ordinary powder diet (CA-1, Clea Japan Inc., Tokyo, Japan) was provided as a basal diet. The lithogenic diet was prepared by adding 0.5% cholesterol and 0.25% sodium cholate to the basal diet. The composition of the basal diet was as follows: 25.5% protein, 4.0% lipids, 53.5% carbohydrate, 4.0% fiber, 7.0% ash, and 6.0% water. The content of cholesterol was about 0.1%.

The test compounds ursocholic acid (>99.5%, Shionogi Research Laboratories, Osaka, Japan) and ursodeoxycholic acid (≥98%, Tokyo Kasei Kogyo Co., Ltd., Tokyo, Japan) were added (0.25% each) to the lithogenic diet. The animals were given *ad libitum* tap water and the diets, utilizing a powder diet feeding apparatus (Natsume Seisakusho Co., Ltd., Tokyo, Japan) for five weeks. Two-day feces were collected during the last week of the experiments.

Before being killed, the mice were fasted for 5 hr and the gallbladder was removed under sodium methylhexabital anesthesia (125 mg/kg, i.p.). Blood was then withdrawn from the abdominal aorta and the liver was removed.

The gallbladder was visually examined for cholesterol crystals under a dissecting microscope. The severity of crystal formation was classified from Grade 0 (cholesterol content <10 mg/mL), I(11–20), II (21–30), III (31–40), IV (41–50) to Grade V (>50), as reported previously (4).

The gallbladder was crushed in ethanol with a glass rod and biliary lipids were extracted by refluxing for 10 min at 80–90°C (4,13). After filtration, a portion was evaporated to dryness under a stream of nitrogen and the residue was hydrolyzed in 2 mL of 1.25 N NaOH solution at 120°C for 6 hr. Cholesterol was extracted from the NaOH solution with diethyl ether; then the bile acids were extracted with diethyl ether after the solution had been

*To whom correspondence should be addressed.

Abbreviations: GLC, gas-liquid chromatography; TFA, trifluoroacetate; UCA, ursocholic acid; UDCA, ursodeoxycholic acid.

acidified with 2 N HCl (14). Cholesterol was determined by gas-liquid chromatography (GLC), with a GC-7A chromatograph (Shimadzu, Kyoto, Japan), using a 1.5% SE-30 column (15). The bile acids were converted into trifluoroacetate (TFA) methyl ester derivatives and subjected to GLC on 1.5% QF-1 and 1.5% AN-600 columns (4,15,16), though TFA derivatives are not well suited for GLC of α-muricholic acid (17). Cholesterol in plasma and liver, and sterols and bile acids in feces were determined by procedures described previously (4,13,16). Biliary phospholipid levels were determined by the Gomori method (18).

Statistical analyses (19,20) were performed as follows: Bartlett's test was applied to test for heterogeneity of variance between treatment groups. If no significant heterogeneity was detected, a (one-way) analysis of variance was carried out, and then the pooled standard error was calculated. If significant heterogeneity was found, typical transformations of data, such as log- and root-transformation, were applied. When no transformation satisfied the homogeneity of variance, the Kruskal-Wallis analysis of ranks was used. Analysis of variance was followed by Tukey's multiple comparison test. The Kruskal-Wallis analysis was followed by the non-parametric equivalents of Tukey's test.

RESULTS

The effects of ursocholic acid and ursodeoxycholic acid on the incidence of cholesterol crystals are shown in Table 1. After feeding the lithogenic diet for five weeks, the incidence was 80% in the control mice, but decreased after treatment with ursocholic and ursodeoxycholic acid.

The body weight, liver weight, and plasma and liver cholesterol levels are given in Table 2. The body weights were almost identical for all the groups; however, the liver weight was higher after feeding the lithogenic diet as compared with the ordinary diet. The liver weight was not changed by ursocholic acid treatment, but was decreased by the ursodeoxycholic acid treatment. Plasma and liver cholesterol levels were increased by the lithogenic diet and were decreased by ursodeoxycholic acid; ursocholic acid, however, had no effect.

The gallbladder lipid levels of the mice are shown in Table 3. The cholesterol level markedly increased. Since the amount of bile in one gallbladder was small, we analyzed the gallbladder, including the cholesterol crystals. Therefore, the cholesterol level measured did not correspond to the cholesterol concentration in the bile, but rather to the grade of the gallstones.

The biliary phospholipid level almost doubled after feeding the lithogenic diet compared to the ordinary diet; the levels after the treatment with both bile acids were intermediate. The biliary bile acid level was high in mice fed the lithogenic diet. The level was lower with ursocholic acid treatment, but not with ursodeoxycholic acid treatment.

The biliary bile acid composition is given in Table 4. Mouse bile contained a greater variety of bile acids than is shown in Table 4, *e.g.*, lithocholic, hyodeoxycholic, and some oxo-bile acids; however, their levels were small (less than 1-2%) and they are not listed. Since the lithogenic diet contained cholic acid, the mice fed this diet had more cholic and deoxycholic acids, and less β- and ω-muricholic and ursodeoxycholic acids than mice fed the ordinary diet.

After feeding ursocholic acid, the proportion of ursocholic acid was high, while cholic and β- and ω-muricholic acid levels were lower than in mice fed the lithogenic diet. Treatment with ursodeoxycholic acid increased α-muricholic, chenodeoxycholic and ursodeoxycholic acids and decreased cholic and β- and ω-muricholic acids.

Dietary intake, feces dry weight, and fecal sterol and bile acid levels are listed in Table 5. No significant change was found in dietary intake and feces dry weight among these mice. Fecal sterol excretion increased about four-fold in mice fed the lithogenic diet which contained 0.5% cholesterol. When ursocholic acid was given, the fecal cholesterol level did not change but the amount of coprostanol increased. When mice were fed ursodeoxycholic acid, fecal sterol excretion was higher than that of control mice fed the lithogenic diet. Fecal bile acid excretion also increased about five-fold in mice fed the lithogenic diet, and further increased, almost two-fold, after treatment with ursocholic acid and ursodeoxycholic acid.

Fecal bile acid composition is shown in Table 6. After feeding the lithogenic diet, the proportions of deoxycholic, cholic and 3α,12α,7=O bile acids were higher, while those of α- and ω-muricholic acids were lower, than when the ordinary diet was fed. Treatment with ursocholic acid increased the level of this bile acid, but decreased cholic, chenodeoxycholic, ω-muricholic, and 3α,12α,7=O bile acid levels. Treatment with ursodeoxycholic acid increased the proportions of ursodeoxycholic, hyodeoxycholic, and lithocholic acids, but decreased those of deoxycholic, cholic, ω-muricholic, and 3α,12α,7=O bile acids.

TABLE 1

Effects of Ursocholic Acid (UCA) and Ursodeoxycholic Acid (UDCA) on the Occurrence of Gallstones in Male Mice

Treatment	Diet	Gallstone grade[a]						Test
		0	I	II	III	IV	V	
Control	Lithogenic diet[b]	2		3	2	3		
UCA[c]	Lithogenic diet	6	1	2	1			*d*
UDCA[c]	Lithogenic diet	9		1				*d*
Normal	Ordinary diet	10						*d*

[a]Akiyoshi *et al.* (4).

[b]Lithogenic diet was given for five weeks *ad libitum*.

[c]Compounds were supplemented at the ratio of 0.25% to the lithogenic diet.

[d]Significant difference from control was detected at 0.05 by Wilcoxon's test.

DISCUSSION

The experiments demonstrate that ursocholic acid, like ursodeoxycholic acid, reduced the formation of biliary cholesterol crystals in mice fed a lithogenic diet. Plasma and liver cholesterol levels were decreased by treatment with ursodeoxycholic acid, but not by ursocholic acid treatment. These cholesterol levels were not related to biliary cholesterol crystal development. It is possible that the plasma and liver cholesterol levels reflect cholesterol absorption in the experimental mice (5). Therefore, we conclude that ursodeoxycholic acid reduced cholesterol absorption while ursocholic acid did not.

TABLE 2

Effects of Ursocholic Acid (UCA) and Ursodeoxycholic Acid (UDCA) on Plasma and Liver Cholesterol Levels (Mean and S.E.) in Male Mice[a]

		Lithogenic diet			Ordinary diet	
	Analysis	Control	UCA	UDCA	Normal	PSE
No. of mice		10	10	10	10	
Body weight (g)	P	37.7	39.4	36.2[b]	38.4	0.70
Liver weight (g/100 g BW)	PIT	6.4 ± 0.49	6.4 ± 0.25	5.1 ± 0.16[b,c]	4.9 ± 0.13[b,c]	
Plasma cholesterol (mg/100 mL)	P	169	175	136[b,c]	118[b,c]	8.1
Liver cholesterol (mg/g)	PLT	28.8 ± 3.07	27.6 ± 2.83	7.6 ± 0.48[b,c]	5.7 ± 0.36[b,c]	

[a]PSE, pooled S.E.; P, parametric analysis; PIT, parametric analysis based on inverse-transformed data; and PLT, parametric analysis based on log-transformed data.

[b]Significant difference from ursocholic acid group (at 0.05) by Tukey's multiple comparison tests.

[c]Significant difference from control group (at 0.05) by Tukey's multiple comparison tests.

TABLE 3

Effects of Ursocholic Acid (UCA) and Ursodeoxycholic Acid (UDCA) on Gallbladder Lipid Levels (Mean and S.E.) in Male Mice[a]

		Lithogenic diet			Ordinary diet	
	Analysis	Control	UCA	UDCA	Normal	PSE
No. of mice		10	10	10	10	
Gallbladder bile (μL)	PLT	41 ± 6.9	29 ± 3.2	35 ± 5.2	27 ± 2.8	
Cholesterol (mg/mL bile)	NP	35.3 ± 8.85	8.1 ± 1.90	3.7 ± 0.27[b]	1.4 ± 0.14[b,c]	
Phospholipids (mg/mL bile)	P	24.1	16.9[b]	17.2[b]	12.9[b]	1.24
Bile acids (mg/mL bile)	P	58.4	36.0[b]	50.2	40.7[b]	4.02

[a]PSE, pooled S.E.; PLT, parametric analysis based on log-transformed data; NP, nonparametric analysis; and P, parametric analysis.

[b]Significant difference from control group (at 0.05) by Tukey's multiple comparison tests.

[c]Significant difference from ursocholic acid group by Tukey's multiple comparison tests.

TABLE 4

Effects of Ursocholic Acid (UCA) and Ursodeoxycholic Acid (UDCA) on Biliary Bile Acid Composition (Mean and S.E.) in Male Mice[a]

		Lithogenic diet			Ordinary diet	
	Analysis	Control	UCA	UDCA	Normal	PSE
No. of mice		10	10	10	10	
Cholic acid (%)	NP	74 ± 0.7	53 ± 2.5	33 ± 1.5[b,c]	37 ± 2.3[b]	
Deoxycholic acid (%)	NP	3 ± 0.5	11 ± 4.1	6 ± 0.6	1 ± 0.2[c,d]	
Ursocholic acid (%)	NP	<1	23 ± 1.9[b]	<1[c]	<1[c]	
Chenodeoxycholic acid (%)	P	<1	<1	2[b,c]	<1[d]	0.1
Ursodeoxycholic acid (%)	PRT	<1	<1	48 ± 1.0[b,c]	2 ± 0.1[b,c,d]	
α-Muricholic acid (%)	P	1	<1	2[b,c]	2[b,c]	0.1
β-Muricholic acid (%)	PLT	12 ± 0.4	6 ± 0.4[b]	5 ± 0.5[b]	43 ± 2.5[b,c,d]	
ω-Muricholic acid (%)	NP	3 ± 0.2	1 ± 0.1	1 ± 0.1[b]	10 ± 0.7[c,d]	
Others (%)	1	8	6	7	7	

[a]PSE, pooled S.E.; NP, nonparametric analysis; P, parametric analysis; PRT, parametric analysis based on root-transformed data; and PLT, parametric analysis based on log-transformed data.

[b]Significant difference from control group (at 0.05) by Tukey's multiple comparison tests.

[c]Significant difference from ursocholic acid group by Tukey's multiple comparison tests.

[d]Significant difference from ursodeoxycholic acid group by Tukey's multiple comparison tests.

TABLE 5

Effects of Ursocholic Acid (UCA) and Ursodeoxycholic Acid (UDCA) on Fecal Excretion of Sterols and Bile Acids (Mean and S.E.) in Male Mice[a]

		Lithogenic diet			Ordinary diet	
	Analysis	Control	UCA	UDCA	Normal	PSE
No. of mice		5	5	5	5	
Diet intake (g/day)	P	5.1	5.4	5.9	5.5	0.22
Feces dry weight (g/day)	P	1.27	1.35	1.45	1.34	0.048
Total sterols (mg/day)	P	20.58	22.92	26.66	4.99	1.403
Cholesterol (mg/day)	P	19.80	20.79	23.07[b]	4.52[b,c,d]	1.269
Coprostanol (mg/day)	PLT	0.78 ± 0.092	2.13 ± 0.522[b]	3.59 ± 0.409[b]	0.47 ± 0.154[c,d]	
Total bile acids (mg/day)	PLT	5.04 ± 0.400	11.75 ± 0.786[b]	12.73 ± 0.718[b]	0.93 ± 0.093[b,c,d]	

[a]PSE, pooled S.E.; P, parametric analysis; and PLT, parametric analysis based on log-transformed data.

[b]Significant difference from control group (at 0.05) by Tukey's multiple comparison tests.

[c]Significant difference from ursocholic acid group by Tukey's multiple comparison tests.

[d]Significant difference from ursodeoxycholic acid group by Tukey's multiple comparison tests.

TABLE 6

Effects of Ursocholic Acid (UCA) and Ursodeoxycholic Acid (UDCA) on Fecal Bile Acid Composition (Mean and S.E.) in Male Mice[a]

		Lithogenic diet			Ordinary diet	
	Analysis	Control	UCA	UDCA	Normal	PSE
No. of mice		5	5	5	5	
Cholic acid (%)	PLT	22 ± 1.7	14 ± 1.2[b]	13 ± 1.4[b]	1 ± 0.2[b,c,d]	
Deoxycholic acid (%)	PLT	46 ± 3.9	36 ± 0.8[b]	26 ± 1.5[b,c]	27 ± 0.9[b,c]	
Ursocholic acid (%)	NP	<1	33 ± 0.8[b]	<1[c]	2 ± 0.6	
3α12α7=O (%)	P	6	4[b]	4[b]	4[b]	0.6
Chenodeoxycholic acid (%)	PRT	2 ± 0.2	<1[b]	b<1[b]	2 ± 0.5[c,d]	
Lithocholic acid (%)	PLT	<1	<1	8 ± 1.7[b,c]	2 ± 0.4[c,d]	
Hyodeoxycholic acid (%)	NP	<1	<1	2 ± 0.3[b,c]	<1[d]	
Ursodeoxycholic acid (%)	PLT	3 ± 0.2	3 ± 0.2	33 ± 1.3[b,c]	4 ± 0.4[b,d]	
α-Muricholic acid (%)	PLT	<1	<1	2 ± 0.2[b,c]	5 ± 1.0[b,c,d]	
β-Muricholic acid (%)	P	4	2	3	8[c]	1.5
ω-Muricholic acid (%)	PLT	7 ± 0.5	3 ± 0.2[b]	4 ± 0.3[b]	12 ± 1.9[b,c,d]	
Others (%)	PLT	8 ± 0.7	5 ± 0.4[b]	3 ± 0.2[b]	22 ± 3.4[b,c,d]	

[a]PSE, pooled S.E.; PLT, parametric analysis based on log-transformed data; NP, nonparametric analysis; P, parametric analysis; and PRT, parametric analysis for root-transformed data.

[b]Significant difference from control group (at 0.05) by Tukey's multiple comparison tests.

[c]Significant difference from ursocholic acid group by Tukey's multiple comparison tests.

[d]Significant difference from ursodeoxycholic acid group by Tukey's multiple comparison tests.

Development of biliary cholesterol crystals was reduced by treatment with these bile acids. The biliary phospholipid levels were low in the mice treated with both bile acids. These results are consistent with previous observations that ursocholic acid decreases biliary cholesterol and phospholipid levels (8–11).

After feeding ursocholic acid, this bile acid was well absorbed and constituted about 23% of the biliary bile acids; it was less than 1% in the control mice. These results agree with the observation of Loria *et al.* (11) who examined changes in biliary bile acids in patients after feeding them ursocholic acid. The proportions of cholic acid and β-muricholic acid in the bile decreased in our mice, while deoxycholic acid increased, although not significantly. In humans, deoxycholic acid in the bile markedly increased after oral administration of ursocholic acid (11). This difference between mice and humans might be due to the presence of deoxycholic acid 7α-hydroxylation activity in mouse liver. Ursocholic acid was dehydroxylated by the intestinal microflora forming deoxycholic acid, and deoxycholic acid was absorbed and transformed into cholic acid in mouse liver. Actually, the fecal excretion of deoxycholic acid increased from 2.3 mg/day/mouse in the control to 4.2 mg/day/mouse in the control to 4.2 mg/day/mouse after feeding ursocholic acid. The fecal excretion of cholic acid also increased after feeding ursocholic acid (1.1 mg/day *vs.* 1.6 mg/day). These results suggest that ursocholic acid is dehydroxylated to deoxycholic acid, and that ursocholic acid may compete with cholic acid absorption.

After feeding ursodeoxycholic acid, biliary ursodeoxycholic acid increased without resulting in any increase in muricholic acid, which suggests but does not prove that

ursodeoxycholic acid was not biotransformed to muricholic acid. Biliary cholic acid was decreased by ursodeoxycholic acid, which suggests that ursodeoxycholic acid may also compete with the absorption of cholic acid. Supporting these results are the findings that fecal excretion of ursodeoxycholic and lithocholic acids and of cholic and deoxycholic acids increased in mice fed ursodeoxycholic acid.

Our data suggest that ursocholic acid and ursodeoxycholic acid reduce crystal formation in mice fed the lithogenic diet in this animal model. Ursodeoxycholic acid decreases cholesterol absorption which results in a reduction of plasma and liver cholesterol levels compared with mice fed the lithogenic diet. Ursocholic acid, however, does not decrease these cholesterol levels. This suggests that the mechanism by which ursocholic acid reduces crystal gallstone formation differs from that of ursodeoxycholic acid. Crystal formation in this animal model does not seem to be related to plasma and liver cholesterol levels, but to bile acid composition. The relevance of the animal model to the human situation remains obscure.

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Phospholipid Requirement of Progesterone 5α-Reductase from Gastric Mucosa Microsomes of Guinea Pig

Kosuke Ichihara* and **Chikage Tanaka**
Department of Biochemistry, Kawasaki Medical School, Kurashiki, Okayama 701-01, Japan

Progesterone 5α-reductase partially purified from gastric mucosa microsomes was stimulated by short-chain synthetic phosphatidylcholines (PC), such as dilauroyl PC, but not by various PC from biological sources. Phosphatidylserine (PS) activated the gastric 5α-reductase to a limited extent compared to the liver 5α-reductase described previously [Ichihara, K., and Tanaka, C. (1987) *Biochem. Biophys. Res. Commun. 149,* 482–487]. In search of more effective phospholipid activators, we tested the effects of various lysophospholipids on 5α-reductase activity. Strongly stimulatory effects were observed when lysophosphatidylcholine (lysoPC) and lysophosphatidylethanolamine (lysoPE) were used instead of PC and phosphatidylethanolamine. Examination of synthetic lysoPC and lysoPE differing in acyl chain lengths showed that fatty chains of 12 to 16 carbons were effective in stimulating the 5α-reductase. By contrast, other lysophospholipids such as lysophosphatidic acid, lysophosphatidylglycerol or lysophosphatidylserine (lysoPS) greatly inhibited 5α-reductase activity. These findings suggest that gastric 5α-reductase may be under dual regulation; lysoPC and lysoPE may play important roles as positive effectors, whereas lysophosphatidic acid, lysophosphatidylglycerol and lysoPS act as negative effectors in progesterone 5α-reductase regulation.
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In recent years, the modulation of enzyme activities by phospholipids has been extensively investigated in biological membranes (1). Phospholipid activation has been observed for some membrane-bound enzymes when they are solubilized and purified by the use of detergents. Phospholipids also have been shown to influence the activity of soluble enzymes, such as protein kinase C (2,3).

Phospholipid stimulation of steroid 5α-reductase in microsomal membranes, first recognized by Golf and Graef (4) in rat liver, was subsequently observed by Cooke and Robaire (5) in rat epididymis. The phospholipid requirement was specific for phosphatidylcholine (PC) in both liver and epididymis (4,5). In conflict with these reports, we have recently shown that endogenous phosphatidylserine (PS) isolated from rat liver microsomes specifically stimulated progesterone 5α-reductase that had been solubilized from liver microsomes (6).

The most recent report from our laboratory (7) showed that gastric progesterone metabolism was initiated by reduction at C-5 of the steroid, which was catalyzed by steroid 5α-reductase. Furthermore, we demonstrated that 5α-reductase activity of stomach mucosa was the highest among gastrointestinal tissues of guinea pig. For further characterization of gastric 5α-reductase, we have examined the effect of phospholipids on reductase solubilized from gastric microsomes. The present study shows that some lysophospholipids, such as lysoPC and lysophosphatidylethanolamine (lysoPE), are effective stimulators of steroid 5α-reductase that had been partially purified from gastric mucosa microsomes of guinea pigs.

*To whom correspondence should be addressed.

Abbreviations: PA, phosphatidic acid; PAF, platelet activating factor, 1-*O*-alkyl-2-acetyl-*sn*-glycero-3-phosphocholine; PC, phosphatidylcholine; PE, phosphatidylethanolamine; PG, phosphatidylglycerol; PI, phosphatidylinositol; PS, phosphatidylserine.

MATERIALS AND METHODS

Materials. [4-^{14}C]Progesterone was purchased from Amersham International (Buckinghamshire, England). Natural PC (bovine liver, heart, brain and egg yolk), natural PE (bovine brain and liver), phosphatidic acid (PA) (egg yolk), phosphatidylglycerol (PG) (egg yolk), phosphatidylinositol (PI) (soybean), cardiolipin (heart), natural lysoPC (egg yolk), synthetic lysoPC (C_8, C_{10}, C_{12}, C_{14}, C_{16}, C_{18} and $C_{18:1}$), natural lysoPE (bovine brain and liver), synthetic lysoPE (C_{16}), lysoPI (soybean), lysoPG (soybean), lysoPS (bovine brain) and lysoPA ($C_{18:1}$) were obtained from Sigma Chemical Co. (St. Louis, MO). Synthetic PC (C_8, C_{10}, C_{12}, C_{14}, C_{16} and $C_{18:1}$) and PI (soybean) were purchased from Pharmacia Fine Chemicals (Uppsala, Sweden) and P-L Biochemicals (Milwaukee, WI), respectively. PS ($C_{18:1}$), lysoPE (C_{12}, C_{14}, C_{18} and $C_{18:1}$) and PAF were from Avanti Polar Lipids Inc. (Pelham, AL). PS (bovine brain) and precoated Silica Gel F_{254} plate were from Serdary Research Laboratories Inc. (London, Ontario, Canada) and Merck (Darmstadt, Germany), respectively. Dye reagent for protein determination was purchased from Bio-Rad Laboratories (Richmond, VA) and NADPH was from Oriental Yeast Co. (Tokyo, Japan). Digitonin and cholic acid were obtained from Wako Pure Chemical Co. (Osaka, Japan) and Nakarai Chemical Co. (Kyoto, Japan), respectively.

Endogenous phospholipids were separated from rat liver microsomes and guinea pig gastric microsomes exactly as described in our preceding paper (7).

Preparation of progesterone 5α-reductase. Solubilization and partial purification of 5α-reductase from gastric mucosa microsomes of guinea pig were carried out as described previously (7,8) with some modifications. Preparation of gastric mucosa microsomes and solubilization of 5α-reductase activity were performed as described previously (7). A cold 50% polyethylene glycol solution was added dropwise to the solubilized supernatant to arrive at a final concentration of 6.5% (w/v). After stirring for 10 min, the mixture was centrifuged at 10,000 × *g* for 15 min. Cold 50% polyethylene glycol was then added to the resulting supernatant to a final concentration of 13% (w/v). After centrifuging at 10000 × *g* for 20 min, the resulting precipitate was dissolved in 30 mM potassium phosphate buffer (pH 7.4)/0.4% (w/v) cholate/0.2 mM NADPH/20% (v/v) glycerol (designated 30 mM buffer), and then centrifuged at 100000 × *g* for 40 min. The supernatant was directly applied to a column (1.5 × 2.5

cm) of hydroxylapatite mixed with cellulose (1:1, w/w), which had been equilibrated with 30 mM buffer. After successive washes with 30 mM, 100 mM and 160 mM buffers, the column was eluted with 300 mM buffer. Cold 50% polyethylene glycol solution was added dropwise to the eluate to a final concentration of 20% (w/v). After being stirred for 20 min, the mixture was centrifuged at 100000 × *g* for 20 min. The resulting precipitate was dissolved in 30 mM potassium phosphate buffer (pH 7.4)/0.2 mM NADPH/20% (v/v) glycerol to yield a partially purified 5α-reductase preparation.

Assay methods. Progesterone 5α-reductase activity was determined as previously described (8) with minor modification. The standard reaction mixture contained potassium phosphate buffer (pH 7.0, 10 μmol), [4^{14}C]progesterone (2.4×10^4 dpm, 0.4 nmol) dissolved in 2 μL of methanol, NADPH (40 nmol) and enzyme preparation (2–3 μg of protein) in a final volume of 0.1 mL. After the substrate dissolved in methanol was evaporated to dryness, other reaction components were added. The various phospholipids tested were dispersed in 20 mM Tris-acetate buffer (pH 8.0) and 1 mM EDTA by sonicating for 1.0–1.5 min until no further decrease in turbidity of the sample was observed. The incubation was usually carried out at 37°C for 20 min. The enzyme assay was performed in duplicate and average values were calculated.

Protein was measured by using the Bio-Rad protein assay with bovine serum albumin as a standard.

RESULTS

Previous studies using endogenous phospholipids isolated from rat liver microsomes indicated that a lipid requirement for the steroid 5α-reduction in liver microsomes was specific for PS (6). These results were in conflict with the reports of Golf and Graef (4) and Cooke and Robaire (5), who had demonstrated an activation by PC in liver and epididymis, respectively. To clarify the possible role of phospholipids in gastric progesterone 5α-reduction, we examined the effects of commercially available PC (synthetic and naturally-occurring) on 5α-reductase activity. Although synthetic dilauroyl PC showed a significant stimulatory effect, most natural PC (from heart, egg yolk and liver) did not show any activation over a wide range of concentrations (Fig. 1). Upon examining a series of PC differing in the length of their saturated fatty acid chains (C_8 to C_{16}), and a PC with two unsaturated fatty acid chains ($C_{18:1}$), maximum stimulation was noted with dilauroyl PC (Fig. 2). It is of interest that dioleoylPC, like natural PC, did not exhibit any appreciable effect of 5α-reductase activity.

Among other phospholipids tested, synthetic PS which contained unsaturated fatty acid chains (dioleoyl) and a natural PS from bovine brain activated 5α-reductase (Fig. 3) to about the same extent as did dilauroyl PC (Figs. 1 and 2). Other phospholipids, such as PE (bovine liver and brain), PI (soybean), PA (egg yolk), PG (egg yolk) and cardiolipin (heart), were neither inhibitory nor stimulatory.

These results agree with our previous observations that endogenous PS specifically stimulated hepatic 5α-reductase activity (6), while natural PS from bovine brain activated gastric 5α-reductase only to a limited extent (130%) (Fig. 3). In search of more effective phospholipids

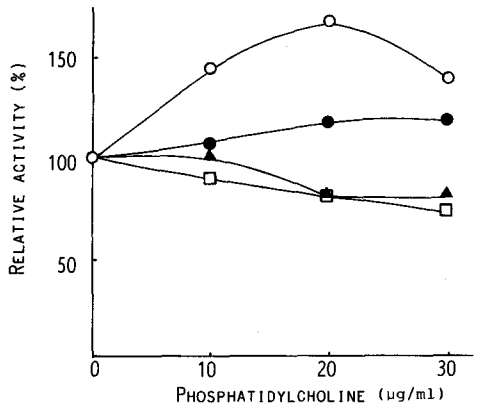


FIG. 1. Effect of various phosphatidylcholines on gastric progesterone 5α-reductase. Incubations were carried out under standard condition with 2 μg of 5α-reductase partially purified from gastric mucosa microsomes as described in Materials and Methods. PC at the concentration indicated was added to the incubation mixtures; synthetic dilauroyl PC (○) and natural PC, from the following sources: bovine liver (●), bovine heart (▲) and egg yolk (□). The enzyme activity was 0.42 nmol of products formed/min/mg, when PC was omitted from the reaction mixture.

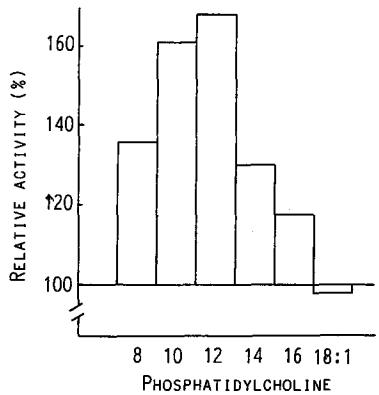


FIG. 2. Effect of synthetic phosphatidylcholines, differing in acyl chain lengths, on gastric progesterone 5α-reductase. Gastric 5α-reductase (2 μg) was incubated with 2–3 μg of PC, where maximum stimulation was observed. The numbers 8, 10, 12, 14 and 16 designate dicapryloyl, didecanoyl, dilauroyl, dimyristoyl and dipalmitoyl PC, respectively. 18:1 represents dioleoyl PC. The enzyme activity was 0.42 nmol of products formed/min/mg, when PC was omitted from the reaction mixture.

other than natural PS, we tested the effects of various lysophospholipids on 5α-reductase activity. A dramatic stimulatory effect was observed with oleoyl lysoPC and natural lysoPC from egg yolk (Fig. 4). PAF, which resembles lysoPC in molecular shape, activated reductase activity to a slightly lesser extent than did lysoPC (data not shown). It was of interest, therefore, to examine the effects of a series of lysoPC differing in the length of their fatty acid chains (C_8 to C_{18}), and to compare the results with those of the series of PC containing diacyl chains with 8 to 18 carbons (Fig. 2). Figure 5(A) shows the effects of various synthetic lysoPC compounds; maximum

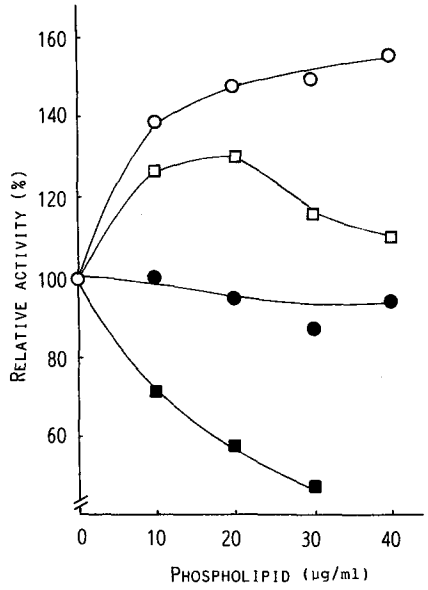


FIG. 3. Comparison between phosphatidylserines and phosphatidylcholines in stimulating gastric progesterone 5α-reductase. 5α-Reductase preparation (2 μg) was incubated with various phospholipids at the indicated concentrations: ○, dioleoyl PS; □, natural PS from bovine brain; ●, dioleoyl PC; ■, natural PC from bovine brain. The enzyme activity was 0.45 nmol of products formed/min/mg, when PS or PC was omitted from the reaction mixture.

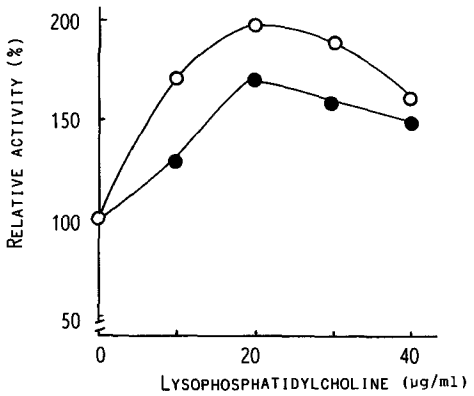


FIG. 4. Effect of lysophosphatidylcholines on gastric progesterone 5α-reductase. 5α-Reductase preparation (2 μg) was incubated with the indicated concentration of oleoyl lysoPC (○) or natural lysoPC from egg yolk (●). The enzyme activity was 0.45 nmol of products formed/min/mg, when lysoPC was omitted from the reaction mixture.

stimulation was observed with the C_{12}, C_{14} and C_{16} derivatives. While lysoPC with a saturated C_{18} fatty acid (stearoyl) brought about only limited enzyme activation, the presence of a single double bond in the acyl chain (oleoyl) greatly enhanced the stimulatory effect [Fig. 5(B)].

Similar results were obtained with lysoPE. While natural PE from bovine liver and brain did not exhibit any effect on 5α-reductase, the corresponding lysoPE compounds had marked stimulatory effects on enzyme activity [Fig. 6 (A) and (B)]. Like lysoPC, synthetic lysoPE with different fatty acyl chains activated 5α-reductase to various extents (Fig. 7). Of interest is that the presence of a single double bond in the acyl chain of lysoPE (Fig. 7) caused an enhancement of enzyme activation, as was observed for lysoPC (Fig. 5).

The effects of other lysophospholipids (lysoPI, lysoPS, lysoPG and lysoPA) on reductase activity are given in Figure 8. LysoPA (oleoyl), lysoPS (bovine brain) and lysoPG (soybean) were strongly inhibitory over a wide range of concentrations, whereas lysoPI (soybean) was weakly stimulatory in a narrow range of concentrations (10–20 μg/mL). In contrast to the stimulatory effect of PS, the corresponding lysoPS strongly inhibited 5α-reductase activity.

DISCUSSION

In our previous study (6) we demonstrated that endogenous PS had a specific stimulatory effect on progesterone 5α-reductase activity in rat liver microsomes. In contrast to these results, however,the observations of Golf and Graef (4) and Cooke and Robaire (5) indicated that the phospholipid requirement was specific for PC. It was, therefore, important to examine in detail the effect of phospholipids on gastric 5α-reductase, which we recently solubilized from gastric mucosa microsomes of guinea pig (7). After studying the effects of commercially available phospholipids on progesterone 5α-reductase activity, we found that natural PC did not exhibit any activation, whereas some shorter chain PC, such as dilauroyl PC, could partially activate the reductase. Among other phospholipids tested, both dioleoyl PS and naturally occurring PS (brain) activated the gastric enzyme to a limited extent as compared to the rat liver enzyme previously mentioned (6). Furthermore, in our preliminary experiments, endogenous PS prepared from gastric or hepatic microsomes was observed to be weakly stimulatory to gastric enzyme activity, but not to liver enzyme activity (6). On the basis of these results, it appeared conceivable that stimulatory effector(s) other than natural PS might play a role in regulating progesterone metabolism in gastric mucosa. Therefore, we examined the effects of various lysophospholipids on 5α-reductase activity. Our results demonstrate that both lysoPC and lysoPE from biological sources were most effective in their ability to activate the reductase; neither natural PC nor natural PE increased enzyme activity. Enzyme activation was observed to various extents with synthetic lysoPC and lysoPE compounds differing in acyl chain lengths; maximum stimulation was noted with C_{12}, C_{14} and C_{16} derivatives. With C_{18} derivatives, the presence of a single double bond in the acyl chain markedly enhanced enzyme stimulation. Among the synthetic phospholipids tested with various acyl chains, maximum stimulation was observed with the rather long-chain derivatives of both the lysophospholipids, unlike synthetic PC. Based on these observations, it is proposed that lysophospholipids, such as lysoPC and lysoPE, may play a physiological role in the activation of steroid 5α-reductase in gastric mucosa. It is conceivable that lysophospholipids may be generated from the corresponding phospholipids by the action of a phospholipase A_2, detected recently in the stomach mucosa associated with plasma and microsomal membranes (9–11). However, the generation of lysoPC and/or lysoPE under physiological conditions, which results in

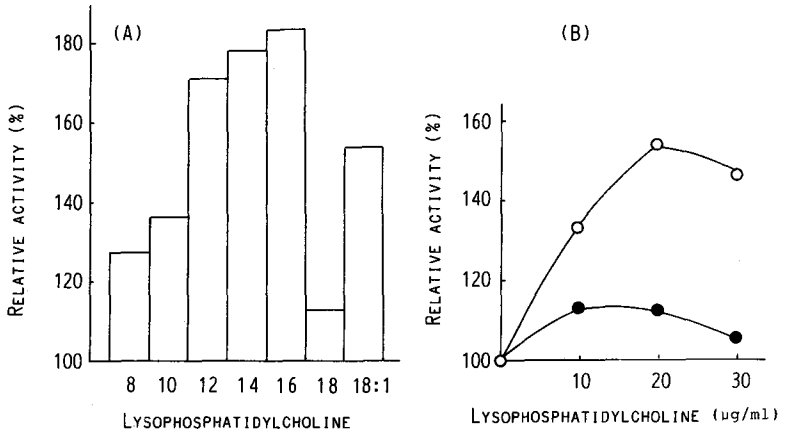


FIG. 5. Effect of synthetic lysophosphatidylcholines differing in the acyl chains on gastric progesterone 5α-reductase. (A) Effect of lysoPC with varying acyl chain lengths on 5α-reductase activity. 5α-Reductase preparation (1 μg) was incubataed with 2∼3 μg of lysoPC, where the maximum stimulation was observed. The numbers 8, 10, 12, 14, 16 and 18 represent capryloyl, decanoyl, lauroyl, myristoyl, palmitoyl and stearoyl lysoPC. 18:1 represents oleoyl lysoPC. (B) Comparison between oleoyl lysoPC (○) and stearoyl lysoPC (●) in stimulating the 5α-reductase (1 μg). The enzyme activity was 0.22 nmol of products formed/min/mg, when lysoPC was omitted from the reaction mixture.

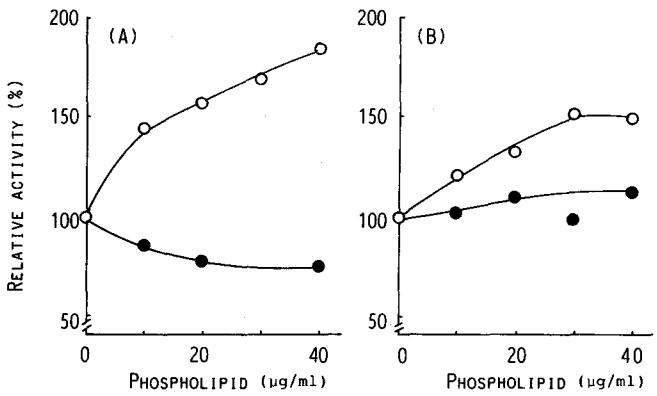


FIG. 6. Comparison between natural lysophosphatidylethanolamines and natural phosphatidylethanolamines in stimulating gastric progesterone 5α-reductase. 5α-Reductase preparation (1 μg) was incubated with the indicated concentration of lysoPE (○) or PE (●): (A) bovine brain; (B) bovine liver. The enzyme activity was 0.23 nmol of products formed/min/mg, when lysoPE or PE was omitted from the reaction mixture.

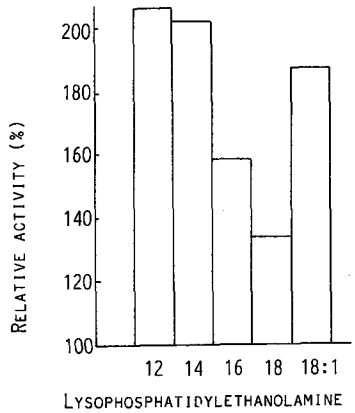


FIG. 7. Effect of synthetic lysophosphatidylethanolamines with various acyl chain lengths on gastric progesterone 5α-reductase. 5α-Reductase preparation (1.5 μg) was incubated with 2–3 μg of lysoPE, where the maximum stimulation was observed. The numbers 12, 14, 16 and 18 represent lauroyl, myristoyl, palmitoyl and stearoyl lysoPE, respectively; 18:1 represents oleoyl lysoPE. The enzyme activity was 0.32 nmol of products formed/min/mg, when lysoPE was omittd from the reaction mixture.

activation of gastric 5α-reductase, deserves further investigation.

Recently, certain lysophospholipids, lysoPC in particular, have been shown to regulate protein kinase C in a biphasic manner; stimulation at low concentration and inhibition at higher concentration (12). Likewise, the gastric 5α-reductase appears to be under dual regulation, that is, stimulation by lysoPC and lysoPE and inhibition by lysoPA, lysoPS and lysoPG. Indeed, further studies are required to determine whether the stimulation or inhibition of 5α-reductase afforded by these lysophospholipids could be functionally important in the regulation of gastric progesterone metabolism. Recently, similar results were obtained with heart acyl-CoA hydrolase (13) and erythrocyte Na^+, K^+-ATPase (14), where certain lysophospholipids exerted profound effects as inhibitors.

At present time, little is known about the mechanism by which phospholipids activate gastric 5α-reductase. Although the effect may be due to detergent action, this seems unlikely since detergents such as Triton X-100 produced a pronounced inhibition (data not shown). Also, the phospholipid requirement was rather specific for lysoPC and lysoPE in the case of gastric 5α-reductase. On the other hand, for neutral triacylglycerol hydrolase from rat heart (15), a detergent effect cannot be ruled out since

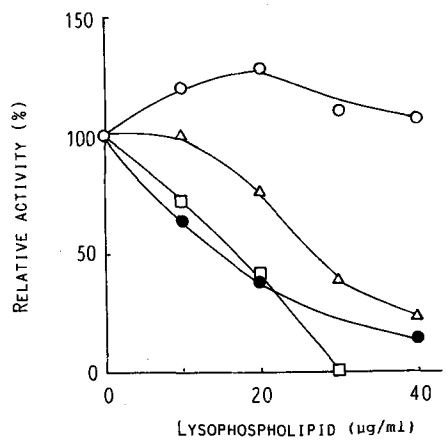


FIG. 8. Effect of various lysophospholipids on gastric progesterone 5α-reductase. 5α-Reductase preparation (1.5 μg) was incubated with the indicated concentrations of lysophospholipids: ○, lysoPI from soybean; △, lysoPG from soybean; ●, lysoPS from bovine brain; □, oleoyl lysoPA. The enzyme activity was 0.32 nmol of products formed/min/mg, when lysophospholipid was omitted from the reaction mixture.

besides lysoPE and PE, a neutral detergent stimulated hydrolase activity.

Whether lysophospholipids activate gastric 5α-reductase in cells remains to be established. It is not known whether the concentration of lysoPC or lysoPE found in gastric mucosa membrane is stimulatory. Recently, it has also been shown that a membrane-associated protein kinase in plants is stimulated by PAF, and also by both lysoPC and lysoPE (16). These results agree with our present findings, although PAF, which resembles lysoPC in molecular shape, stimulated gastric 5α-reductase to a lesser extent than did lysoPC and lysoPE. In the case of the plant protein kinase (16), a natural activator was proposed to be displaced by PAF and the lysophospholipids. Therefore, we cannot rule out the possibility that an endogenous activator structurally similar to PAF and lysoPC may exist in gastric mucosa. Much works remains to be done to pin-point the physiological activator(s) of the gastric progesterone 5α-reductase.

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Steryl Esters in a Cell Suspension Culture of Celery (*Apium graveolens*)

Lynne Dyas, Mark C. Prescott, Richard P. Evershed and L. John Goad*
Department of Biochemistry, University of Liverpool, Liverpool L69 3BX, United Kingdom

A range of analytical techniques was used to investigate the composition of the steryl fatty acyl esters in a cell suspension culture of celery *(Apium graveolens)*. Gas chromatography (GC) and GC-mass spectrometry (GC-MS), using electron ionization (EI) and negative ion chemical ionization (NICI), were employed to characterize the intact steryl esters. Assignments were supported by analysis of the sterol and fatty acid moieties released from the intact molecular species by alkaline hydrolysis. A selectivity for sterol esterification was noted, with the major free sterol, stigmasterol, occurring only in a very small amount in the esterified form. Instead, the precursors to Δ^5-phytosterols, particularly cycloartenol, predominated in the ester fraction. The pentacyclic triterpene, β-amyrin, was also found as the palmitate and linoleate esters. Changes in composition and abundance of the steryl esters during the different growth phases of a celery cell suspension culture were investigated. The total amount of esterified sterols exceeded that of free sterols throughout the growth cycle. The changes observed during growth highlighted differences between the esters of precursor sterols and those of the 4-desmethylsterols, and it is postulated that the various steryl esters perform different functions in cell metabolism.
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Steryl esters have been studied in numerous plant species (1–9) as well as in individual tissues and organelles (10–14). Despite this, their precise role in plants remains unclear. Suggested functions include a storage form of sterol (15,16) or a transport form, either intracellularly (17) or by translocation between tissues (15,18). Atallah and Nicholas (19) proposed that it is the liquid crystalline properties of steryl esters which determine their function, probably in association with membrane permeability. Steryl esters have also been implicated in a regulatory role in sterol biosynthesis (20,21).

Esterification of sterols has been extensively studied in animals due to the important part cholesteryl esters play, as constituents of plasma lipoproteins, and their involvement in clinical conditions such as atherosclerosis (22,23). As changes have been observed in the abundance of steryl esters at all vital stages of the plant life cycle, it is likely that they have an equally important role to play in plant metabolism. During seed germination of *Calendula officinalis* (16) and barley (24), a decrease in abundance of steryl esters occurs, accompanied by a concomitant increase in free sterols. Steryl esters accumulate again during flowering of *Calendula*, with the esterified form becoming more abundant than the free sterols in the inflorescence. The ripening of tomatoes (25) is characterized by an increase in total sterol content of the fruit, with the most marked increase being in the ester fraction (twelvefold). These changes were shown to be associated with the ripening process rather than aging, as a mutant non-ripening variety did not show similar increases at the same age. However, an increase in steryl ester abundance during senescence of tissues (15,16,26) has also been observed, with the implication that they are involved in the removal of sterols from the membranes (15).

In order to increase our understanding of the biosynthesis and metabolism of steryl esters in plants, we are investigating the changes in abundance and composition of steryl esters over the growth cycle of a single-cell suspension culture of celery *(Apium graveolens)*. This system has a growth curve exhibiting lag, logarithmic and stationary phases of growth.

Most studies concerning steryl esters have proceeded by alkaline hydrolysis of the intact molecules followed by separate analyses of the constituent sterol and acyl moieties. This method, however, loses potentially useful information on the nature of the intact esters. The use of negative ion chemical ionization gas chromatography-mass spectrometry (NICI GC-MS) has been demonstrated for the analysis of a range of intact steryl palmitates (27) and for the examination of relatively simple mixtures of steryl esters from animal and plant tissues (28). We have now used this technique to identify the steryl esters of celery, providing more precise information about individual esters, which may aid in the elucidation of their function.

EXPERIMENTAL

Growth conditions for celery. A celery cell suspension culture was provided by Dr. Hamish Collin, University of Liverpool, (Liverpool, U.K.). Cultures (60 mL) were grown in Murashige and Skoog medium (Flow Laboratories, Irvine, Scotland) supplemented with sucrose, kinetin and 2,4-dichlorophenoxyacetic acid. The cells were cultured in an orbital incubator at 20°C with constant shaking at 100 rpm and a 12-hr light/12-hr dark cycle. Cell growth was determined by cell counts and cell dry weight measurements.

Celery seedlings (White Pascal variety, Johnsons Seeds, Boston, U.K.) were grown in a greenhouse under natural night/day lighting, and harvested at 6 wk.

Extraction and fractionation of lipids. Cells were filtered from the medium through nylon, frozen in liquid nitrogen and freeze-dried. Lyophilized material was then extracted by stirring in chloroform/methanol (2:1, v/v; 30 mL per 50 mg tissue) followed by filtration. The cell residue was extracted two more times with $CHCl_3$/MeOH and the three extracts combined. Internal standards for quantification were added with the first volume of solvent, as detailed below. Combined filtrates were evaporated *in vacuo* to yield total lipids.

Total lipid extracts were applied to silica gel columns (70–230 mesh; 5 g; 5% deactivated) and eluted with

*To whom correspondence should be addressed.

Abbreviations: EI, electron impact ionization; FS, free sterols; GC, gas chromatography; M^+, molecular ion; MS, mass spectrometry; NICI, negative ion chemical ionization; SAM, S-adenosyl-L-methionine; SE, steryl esters.

hexane (50 mL) with increasing proportions of diethyl ether. Steryl esters were eluted in 2–4% Et_2O/hexane, and free sterols were eluted in 16–32% Et_2O/hexane fractions.

Hydrolysis of steryl esters. Steryl ester mixtures (*ca.* 5 mg) were heated at 70°C for 2 hr in 2 mL 8% KOH in 80% EtOH. Free sterols were extracted into 3 vol of hexane. The aqueous fraction was acidified with aqueous 20% (v/v) H_2SO_4, and the fatty acids were extracted into $CHCl_3$. The sterols were analyzed as their trimethylsilyl ethers, and the fatty acids were converted into methyl esters by heating at 70°C in methanolic 2.5% H_2SO_4 and then extracted into hexane (29).

Analyses of lipid fractions. Gas chromatographic analyses were performed on a Hewlett-Packard 5890 GC operated in on-column mode with helium (2 mL/min) as carrier gas. A 12 m × 0.22 mm i.d. BP1-coated (0.1 μm film thickness) capillary column (S.G.E., Milton Keynes, U.K.) was employed for intact steryl ester analyses, with the oven programmed from ambient to 340°C at 4°C/min. Sterols were determined as their trimethylsilyl derivatives on a 25 m × 0.22 mm BP5-coated (0.25 μm film thickness) column, and the oven was programmed from ambient to 290°C at 6°C/min. Fatty acids (as their methyl esters) were analyzed on a 25 m × 0.32 mm i.d. Carbowax (0.3 μm film thickness) capillary column (Hewlett-Packard), with the oven programmed from ambient to 230°C at 10°C/min.

GC-MS analyses were performed on a Pye Unicam 204 GC interfaced to a VG (Altrincham, U.K.) 7070H double-focussing magnetic sector mass spectrometer, coupled to a Finnigan (Hemel Hempstead, U.K.) INCOS#2300 data system. GC columns and operating conditions were as above. Electron impact spectra were recorded at 20 eV and 3 kV accelerating voltage. GC-MS with NICI was performed with ammonia as reagent gas, as reported previously (30).

Quantification of lipids. 5α-Cholestanol (100 μg) and cholesteryl myristate (100 μg) were added to the lyophilized cells prior to extraction to act as internal standards for quantification from GC peak areas. Quantification of the co-eluting compounds β-amyrin and sitosteryl linoleates, was performed by reference to the relative intensities of the mass spectral fragment ions at *m/z* 218, arising from β-amyrin and *m/z* 396 from sitosteryl linoleate. Isofucosterol and β-amyrin, from the free sterol fraction, were quantified in a similar manner, on the basis of their characteristic fragment ion abundances at *m/z* 386 and 218, respectively.

The mean value and variance for the absolute quantities of each compound were calculated from 5 replicate analyses, and Students' t-tests (employing Bessel correction for low sample number) were performed to establish significant changes between the differently aged cultures. All changes reported in the Results section were significant below $p = 0.05$.

Nomenclature. Trivial names of sterols have been used as follows: campesterol [(24*R*)-24-methylcholest-5-en-3β-ol]; stigmasterol [(22*E*)-(24*S*)-24-ethylcholesta-5,22-dien-3β-ol]; sitosterol [(24*R*)-24-ethylcholest-5-en-3β-ol]; isofucosterol [(24*Z*)-24-ethylcholesta-5,24(24^1)-dien-3β-ol]; cycloartenol [4,4,14α-trimethyl-9,19-cyclo-5α,19β-cholest-24-en-3β-ol]; obtusifoliol [4α,14α,24-trimethyl-5α-cholesta-8,24(24^1)-dien-3β-ol]; 24-methylenecycloartanol [4,4,14α,24-tetramethyl-9,19-cyclo-5α,19β-cholest-24(24^1)-en-3β-ol]; β-amyrin [olean-12-en-3β-ol]; episterol [24-methyl-5α-cholesta-7,24(24^1)-dien-3β-ol]; 24-methylenelophenol [4α,24-dimethyl-5α-cholesta-7,24(24^1)-dien-3β-ol]; Δ7-avenasterol [(24*Z*)-24-ethyl-5α-cholesta-7,24(24^1)-dien-3β-ol]; and 24-ethylidenelophenol [(24*Z*)-4α-methyl-24-ethylcholesta-7,24(24^1)-dien-3β-ol].

Trivial names of fatty acids have been used as follows: myristic acid, 14:0 (tetradecanoic acid); palmitic acid, 16:0 (hexadecanoic acid); stearic acid, 18:0 (octadecanoic acid); oleic acid, 18:1 (*cis*-9-octadecenoic acid); linoleic acid, 18:2 (*c*9,*c*12-octadecadienoic acid); linolenic acid, 18:3 (*c*9,*c*12, *c*15-octadecatrienoic acid).

RESULTS AND DISCUSSION

Identification of the steryl esters present in a celery cell culture. A typical GC profile of the intact steryl esters isolated from a celery cell suspension culture is shown in Figure 1. Table 1 lists those compounds identified by means of electron ionization (EI) and NICI mass spectrometry and GC elution order, together with the diagnostic mass spectral ions used for compound identification. As observed previously, molecular ions (M^+) were absent from the EI spectra of fatty acyl esters of Δ^5-sterols (27,31,32); hence, NICI mass spectrometry was employed to derive information regarding the nature of the fatty acyl moieties. Under the conditions employed in this study, molecular ions (M^+) of low abundance were detected for 4α,14α-dimethyl- and 4,4,14-trimethylsteryl esters. However, in order to assign all compounds with confidence, the determination of both the EI and NICI mass spectra and also the GC elution order was required. For example, isobaric ions occurring in the EI and NICI mass spectra of obtusifoliyl and cycloartenyl linoleates introduce an ambiguity which was resolved by consideration of the GC elution order of the compounds.

Evershed *et al.* (27) noted a characteristic $[RCO_2-19]^-$ ion in the NICI mass spectra of the steryl palmitates, and again in cholesteryl stearate (30). This ion was also observed in the present study. However, the fragment ion was not apparent in the spectra of the linoleate esters,

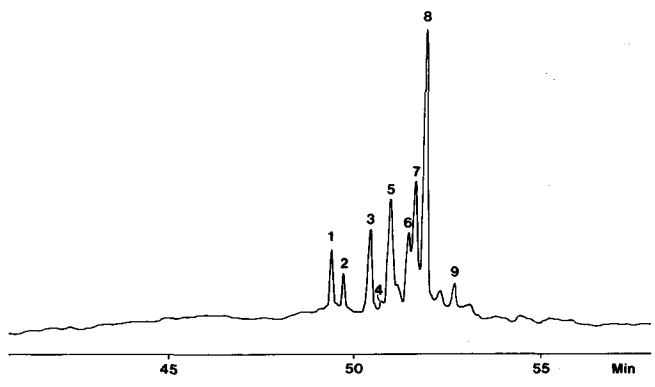


FIG. 1. A GC profile of the intact steryl esters extracted from a celery cell suspension culture. The identifications of the numbered peaks are listed by elution order in Table 1. For GC column and oven programming conditions, see Experimental.

TABLE 1

Identifications of the Intact Steryl Esters Isolated from a Cell Suspension Culture of Celery[a]

Compound	*m/z* (Relative abundance)	
	EI	NICI
β-Amyrin palmitate	189(28),203(50),218(100),393(1),408(1), 649(<1),664(<1)	237(100),236(22),255(17),407(18)
Cycloartenyl palmitate	241(26),286(5),297(11),339(7),365(9), 393(100),408(8),649(11),664(3)	237(100),236(18),255(25),407(11)
Campesteryl linoleate	213(23),255(42),367(35),382(99)	261(100),279(55),381(14)
Stigmasteryl linoleate	83(100),394(56)	261(100),279(15),393(1)
Obtusifoliyl linoleate	215(48),241(26),269(23),309(22),393(100), 409(16),673(27),688(5)	261(100),279(15),407(2)
Sitosteryl linoleate	213(14),255(35),275(36),288(44),381(30), 396(96)	261(100),279(24),395(8)
β-Amyrin linoleate	189(21),203(39),218(100),393(1),408(2)	261(100),279(52),407(4)
Cycloartenyl linoleate	241(11),286(12),297(16),339(26),365(23), 393(62),408(22),673(3),688(1)	261(100),279(15),407(6)
24-Methylenecycloartanyl linoleate	203(74),379(35),407(66),422(30)	261(100),279(9),421(11)

[a]Compounds are listed in the order of GC elution. Diagnostic ions from EI and NICI mass spectra are listed.

which suggests its occurrence is possibly confined to the esters of saturated fatty acids.

Alkaline hydrolysis of the steryl ester mixture revealed other sterol constituents (episterol, 24-methylenelophenol, Δ^7-avenasterol and 24-ethylidenelophenol) which were not apparent from the gas chromatogram (Fig. 1) or the mass spectra of the intact esters, due to their co-elution with, and similar MS behavior to, other compounds. Although the contribution of these four sterols to the total ester pool was relatively small, their presence again highlights the need for a variety of complementary analytical techniques in the analysis of the steryl esters to provide maximum compositional information.

The identification of the intact steryl esters revealed a selectivity of esterification of the various sterols. The free sterols of celery cultures consist almost exclusively of campesterol, stigmasterol, sitosterol and isofucosterol (33), whereas the steryl esters include a large proportion (*ca.* 70%) of esterified precursor sterols, particularly cycloartenyl linoleate. Nagai *et al.* (34) investigated the steryl esters of yeast (in which ergosterol is the major free sterol), and found that they were predominantly of ergosterol precursors, which were not methylated at C-24. In the same study it was shown that, in contrast to the free sterols, the steryl esters were poor substrates for S-adenosyl-L-methionine (SAM)-Δ^{24}-sterol methyltransferase. Hence, it appears that once esterified, the sterols are effectively removed from participating in biosynthetic reactions. The esterification of 4,4-dimethyl sterols at the C-3 position, as observed in the celery steryl esters, renders them unavailable for C-4 demethylation by inhibiting formation of the 3-oxosteroid intermediate. Esterification may therefore operate as a control step in phytosterol biosynthesis (21).

Also notable was the very small amount of esterified stigmasterol (<2%) even though it is the major component of the free sterol pool. Since stigmasterol is the major constituent (up to 44%) throughout the growth of the culture, its virtual absence from the steryl ester fraction implies some significant role for this sterol in cell metabolism. The need for a 24α-ethyl sterol for normal growth and cell division in celery cultures has been established, and stigmasterol was shown to be particularly active in this respect (33,35). It has also been demonstrated (36), using yeast sterol auxotrophs, that structural modifications in the sterol have an effect on membrane permeability and growth, and it was proposed that selective esterification of sterols by the cell would enable it to control the type of sterol available. Although it may appear that the results from the celery cultures agree with this hypothesis, insofar as there is an apparent discrimination against stigmasterol becoming esterified. stigmasterol does, nevertheless, occur as both the steryl glycoside and acylated steryl glycoside in this tissue (Dyas, L., unpublished data).

We have also determined the composition of free and esterified sterols of the roots and shoots of 6-week-old celery seedlings (Table 2). The roots and shoots of seedlings contained less cycloartenyl linoleate in relation to the amounts of campesteryl, stigmasteryl and, especially, sitosteryl linoleates. Although stigmasterol was esterified to both linoleic and palmitic acids in seedlings, it contributes only 4 and 16% to the esters of the shoots and roots, respectively, compared to 41 and 65% in the free sterol fractions, thus still exhibiting a degree of discrimination in the esterification process.

The NICI mass spectra of intact steryl esters from celery cultures, along with the analysis of the fatty acids resulting from their saponification, showed the presence of smaller amounts of other C_{18} fatty acids (18:0, 18:1 and 18:3) in addition to the dominant linoleate (18:2). This fatty acid complement reflected the relative proportions of total fatty acids found in celery cultures. However, a degree of selectivity was again noted; only two compounds, cycloartenol and the pentacyclic triterpene, β-amyrin, were found esterified to palmitic acid (16:0). A similar trend was observed in the celery seedling roots. The major palmitates were those of cycloartenol, obtusifoliol and β-amyrin, in contrast to the domination of

TABLE 2

Amounts (ng/mg dry weight) of Free and Esterified Sterols Isolated from 6-Week-Old Celery Seedling Roots and Shoots[a]

Compound	Root	Shoot
Free sterols		
Campesterol	200 (6.6)	60 (4.2)
Stigmasterol	1980 (65.3)	540 (41.4)
Sitosterol	710 (23.6)	550 (42.2)
Other[b]	140 (4.6)	150 (12.1)
Steryl esters		
Campesteryl palmitate	2 (0.9)	1 (2.8)
Stigmasteryl palmitate	6 (2.9)	nd[c]
Obtusifoliyl palmitate	9 (3.9)	2 (4.7)
β-Amyrin palmitate	4 (1.9)	nd
Sitosteryl palmitate	5 (2.0)	2 (4.3)
Cycloartenyl palmitate	24 (10.8)	1 (3.0)
Campesteryl linoleate	22 (9.8)	4 (7.7)
Stigmasteryl linoleate	28 (12.6)	2 (4.2)
Obtusifoliyl linoleate	11 (4.8)	1 (2.9)
β-Amyrin linoleate	9 (4.0)	nd
Sitosteryl linoleate	58 (25.7)	21 (41.2)
Cycloartenyl linoleate	15 (6.8)	1 (2.9)
Isofucosteryl linoleate	25 (11.0)	11 (22.1)
24-Methylenecycloartanyl linoleate	4 (1.9)	2 (4.3)

[a]Values in parentheses represent the % composition.

[b]Mainly cycloartenol.

[c]nd, not detected.

the linoleates by the typical Δ^5-phytosterols. Cycloartenol and β-amyrin are the initial products of 2,3-oxidosqualene cyclization, and their selective esterification to palmitic acid may prove significant when considering certain features of steryl ester biochemistry, such as the interrelationship of the sites of 2,3-oxidosqualene cyclization and sterol esterification, and the nature of the acyl donor.

Changes in abundance and composition of the steryl esters during growth. Celery cell suspension cultures exhibit a typical sigmoidal growth curve (33). Hence, cultures harvested at days 2, 7, 12 and 16 represent lag, log and early and late stationary phases of growth, respectively. Tables 3 and 4 list the amounts of free and esterified sterols extracted from each of these cultures.

At all growth stages of the cell culture, the amount of steryl esters considerably exceeded that of free sterols. In the majority of studies on plants, steryl esters rarely constitute the major sterol form, even when they appear to be metabolically significant, *e.g.* exhibiting a twelve-fold increase during the ripening of tomatoes (25). In this respect, the predominance of steryl esters in celery cultures is unusual. Whether this phenomenon is a feature of plant cell suspension cultures, or is restricted to that of celery, is unknown. However, in the celery seedlings the ratio of free-to-esterified sterols (FS/SE) is 27:1 in shoots and 14:1 in roots. Parks and co-workers (36–38) have extensively studied the esterification of sterols in yeast, and although they found that the steryl esters constituted up to 90% of total sterols during stationary phase, during logarithmic growth this figure was less than 10%. They have suggested that the conversion of free sterols to steryl esters is a result of the cessation of cell division, and excess sterols become esterified. The results obtained for the celery cultures do not, however, reveal sterol esterification following a similar pattern.

The FS/SE ratio in the celery cultures increased slightly from day 2 to day 7 (0.35:1 and 0.44:1, respectively), and more markedly to day 12 (0.60:1). The initial rise in the FS/SE ratio was due to an increase in concentration of total sterols at day 7 rather than to a decrease in the total steryl esters, which remained constant. It has been suggested that the steryl esters may serve as a storage form of sterol, which may be hydrolyzed to provide the free form in times of need, for example, prior to full development of sterol biosynthetic enzyme activity in germinating seeds (15,16,24), or when sterol biosynthesis is inhibited (39,40). This function is not apparent in this study during the growth cycle.

The amount of steryl esters in the cultures remained constant during the initial growth period, but by the

TABLE 3

Amounts (μg/mg dry weight) of Individual Free Sterols Extracted from Cell Suspension Cultures of Celery Harvested at Different Ages[a]

Compound	Days 2	7	12	16
Campesterol	0.33 ± 0.08 (17.6)[b]	0.51 ± 0.07 (20.7)	0.38 ± 0.08 (16.0)	0.41 ± 0.08 (15.5)
Stigmasterol	0.68 ± 0.08 (36.2)	0.90 ± 0.07 (36.6)	0.77 ± 0.13 (32.5)	1.17 ± 0.14 (44.2)
Sitosterol	0.41 ± 0.08 (21.8)	0.55 ± 0.08 (22.4)	0.53 ± 0.08 (22.4)	0.75 ± 0.12 (28.3)
Isofucosterol[c]	0.30 ± 0.07 (16.0)	0.39 ± 0.19 (15.8)	0.43 } ± 0.50 (18.1)	0.17 } ± 0.07 (6.4)
β-Amyrin[c]	nd[d]	nd	0.20 } (8.4)	0.12 } (4.5)
Others[e]	0.16 ± 0.06 (8.5)	0.11 ± 0.02 (4.5)	0.06 ± 0.05 (2.5)	0.03 ± 0.01 (1.1)
Total	1.88 ± 0.10	2.46 ± 0.14	2.37 ± 0.37	2.65 ± 0.16

[a]The data represent the mean values for five replicates.

[b]Values in parentheses represent the % composition.

[c]Standard deviation calculations were performed on replicate analyses of the mixed isofucosterol/β-amyrin GC peak area. Values given for the individual compounds are extrapolated from a single GC-MS analysis of each group.

[d]nd, not detected.

[e]Mainly cycloartenol.

TABLE 4

Amounts (μg/mg dry weight) of Individual Steryl Esters Extracted from Cell Suspension Cultures of Celery Harvested at Different Ages[a]

Compound	Days			
	2	7	12	16
β-Amyrin palmitate	0.09 ± 0.05 (1.7)[b]	0.10 ± 0.03 (1.8)	0.12 ± 0.09 (3.0)	0.35 ± 0.08 (5.2)
Cycloartenyl palmitate	0.21 ± 0.04 (4.0)	0.22 ± 0.04 (4.0)	0.11 ± 0.04 (2.8)	0.18 ± 0.04 (2.7)
Campesteryl linoleate[c]	0.35 ± 0.08 (6.7)	0.58 ± 0.13 (10.5)	0.49 ± 0.07 (12.4)	0.84 ± 0.22 (12.4)
Stigmasteryl linoleate	nd[d]	nd	0.07 ± 0.01 (1.8)	0.10 ± 0.05 (1.5)
Obtusifoliyl linoleate	0.66 ± 0.19 (12.6)	0.85 ± 0.19 (15.4)	0.69 ± 0.17 (17.5)	1.25 ± 0.53 (18.4)
β-Amyrin linoleate[e]	0.24 } ± 0.26 (4.6)	0.31 } ± 0.20 (5.6)	0.47 } ± 0.17 (11.9)	1.04 } ± 0.59 (15.3)
Sitosteryl linoleate[e]	0.41 } ± 0.26 (7.8)	0.51 } ± 0.20 (9.2)	0.56 } ± 0.17 (14.21)	0.70 } ± 0.59 (10.3)
Cycloartenyl linoleate	2.99 ± 0.85 (57.1)	2.70 ± 1.09 (48.8)	1.29 ± 0.39 (32.7)	2.06 ± 0.88 (30.4)
24-Methylenecycloartanyl linoleate	0.29 ± 0.10 (5.5)	0.26 ± 0.06 (4.7)	0.15 ± 0.02 (3.8)	0.26 ± 0.05 (3.8)
Total	5.24 ± 1.34	5.53 ± 1.41	3.95 ± 0.72	6.78 ± 2.10

[a]The data represent the mean values for five replicates.

[b]Values in parentheses represent the % composition.

[c]"Linoleate" includes small amounts of other C_{18} fatty acids; see Results.

[d]nd, not detected.

[e]Standard deviation calculations were performed on replicate analyses of the mixed β-amyrin/sitosteryl linoleates GC peak area. Values given for individual compounds are extrapolated from a single GC-MS analysis for each group.

beginning of the stationary phase (12 days) they showed a marked decrease. This change in the amount of total steryl esters was due almost entirely to a decline in the amounts of the esterified precursor sterols cycloartenol (as palmitate and linoleate) and 24-methylenecycloartanol (as linoleate). It seems unlikely that the cell is hydrolyzing the cyclopropylsteryl esters to provide their free counterpart for conversion to Δ^5-sterols, because if the cell requires the latter it would apparently be more efficient to hydrolyze the appropriate Δ^5-steryl esters than undertake several biosynthetic steps from the precursors. Moreover, the culture is approaching stationary phase where growth and division is slowing down and the demand for sterols is presumably diminished.

At late stationary phase (16 days), there was an equally marked increase in the amount of total steryl esters, accounted for by both a recovery of those precursor sterols which had declined at day 12, plus augmented amounts of the other steryl esters. The amounts of campesteryl and sitosteryl linoleates remained constant throughout the growth period and increased only at this late stage, whereas stigmasteryl linoleate was not detected during logarithmic growth but became apparent at stationary phase. The elevated amounts of esters of the pentacyclic triterpene β-amyrin were particularly noticeable in the 16-day-old cultures. β-Amyrin, like cycloartenol, is formed by the cyclization of 2,3-oxidosqualene, and it has been observed (41) that in pea seeds, the cyclization into cycloartenol or β-amyrin occurs at different times during the germination and seedling development, possibly depending on the relative rates of synthesis and degradation of the respective cyclase enzymes. The relative increase of β-amyrin in the free fraction at stationary phase accompanied by a decrease in precursor sterols (cycloartenol, 24-methylenelophenol, isofucosterol) implies that the rate of sterol biosynthesis has declined at this point. The pattern of esterification at this time is thus similar to that seen in yeast (36). It seems likely therefore, that the esterified 4-desmethylsterols and precursor sterols have different roles to play in plant metabolism.

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METHODS

Stereospecific Analysis of Triacyl-*sn*-glycerols by Chiral High-Performance Liquid Chromatography

Toru Takagi* and **Yasuhiro Ando**
Department of Chemistry, Faculty of Fisheries, Hokkaido University, Minato-cho, Hakodate, Japan 041

A method for the stereospecific analysis of triacyl-*sn*-glycerols by high-performance liquid chromatography (HPLC) on a chiral column is described. Triacyl-*sn*-glycerols were partially degraded with ethyl magnesium bromide, and the monoacylglycerols produced were separated as 1- and 2-monoacylglycerol fractions by thin-layer chromatography on boric acid-impregnated silica gel plates. The 1-monoacylglycerols were resolved into *sn*-1 and *sn*-3 monoacylglycerol fractions by HPLC on a chiral column (Sumichiral OA-4100) after derivatization with 3,5-dinitrophenyl isocyanate. Fatty acid methyl esters obtained from the original triacyl-*sn*-glycerols, 1- and 2-monoacylglycerol fractions, and *sn*-1 and *sn*-3 monoacylglycerol fractions were analyzed by gas chromatography on open-tubular columns. Stereospecific acyl distributions in triacyl-*sn*-glycerols were calculated from the data. The acyl distributions of several oils were obtained. The method is rapid, simple and gave reproducible results. *Lipids 26,* **542–547 (1991).**

The first stereospecific analysis of triacyl-*sn*-glycerols was reported by Brockerhoff in 1965 (1). The method involved the stereospecific cleavage with phospholipase A_2 to distinguish between the *sn*-1 and *sn*-3 positions in a triacyl-*sn*-glycerol molecule. Later, Lands and co-workers (2), Brockerhoff (3), Myher and Kuksis (4) and Christie and Moore (5) presented improved procedures for the stereospecific analysis of triacyl-*sn*-glycerols. The methods were enzymatic and involved the stereospecific lipolysis or esterification of diacylglycerols or their derivatives to discriminate between the chiralities (6,7).

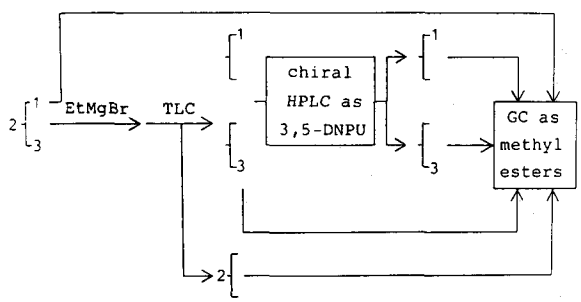
The present paper presents a novel method for the stereospecific analysis of acyl distributions in triacyl-*sn*-glycerols. In this analytical approach, high-performance liquid chromatography (HPLC) of monoacylglycerol enantiomers on a chiral stationary phase is employed instead of stereospecific enzyme reactions. We previously introduced part of the methodology (8). Triacyl-*sn*-glycerols were partially hydrolyzed by ethyl magnesium bromide, and the 1-monoacylglycerol products were resolved into *sn*-1 and *sn*-3 enantiomers by HPLC as 3,5-dinitrophenylurethanes (3,5-DNPU) on a chiral column (Sumipax OA-2100, Sumitomo Chemical Co., Osaka, Japan). Stereospecific distributions of acyl groups were calculated from area percentages of the enantiomer peak. On the other hand, Laakso and Christie (9) reported the chromatographic resolution of diastereomeric 1,2-diacylglycerol derivatives prepared with a chiral reagent on a column of silica gel for the stereospecific analysis of triacyl-*sn*-glycerols. Fatty acid residues of the *sn*-1,2- and the *sn*-2,3-diacylglycerol derivatives were converted to methyl esters, and then analyzed by gas chromatography (GC). The acyl compositions at *sn*-1, *sn*-2, and *sn*-3 positions were then calculated.

We recently improved the resolution of the monoacylglycerol enantiomers by modification of the HPLC method (10). The method permits the clear separations of a 1-monoacylglycerol mixture into *sn*-1 and *sn*-3 monoacylglycerols. In the present study, this modified HPLC method was applied to the stereospecific analysis of triacyl-*sn*-glycerols. The 1-monoacylglycerol products from the partial hydrolysis of natural triacyl-*sn*-glycerols were resolved into *sn*-1 and *sn*-3 monoacylglycerol fractions by a modified HPLC method, and the methyl esters prepared from the *sn*-1 and *sn*-3 fractions were analyzed by GC. The distribution of acyl groups between the *sn*-1, *sn*-2 and *sn*-3 positions was calculated based on the results of GC analyses.

MATERIALS AND METHODS

*Triacyl-*sn*-glycerols.* Yew *(Taxus cuspidata)* seeds were collected at Hakodate, Japan. High erucic acid rapeseeds were obtained from Yonezawa Seiyu Co. (Saitama-ken, Japan). Hawaiian macadamia nuts and soybean oil were purchased at Hakodate. Rapeseed and macadamia nut oils were prepared from seeds or nuts by crushing and diethyl ether extraction. Yew seeds and flesh meats of herrings were extracted by the method of Bligh and Dyer (11). Triacyl-*sn*-glycerol samples were purified by TLC on Kiesel Gel 60G plates (Merck, Darmstadt, Germany) with n-hexane/diethyl ether (4:1, v/v) for development. 1,3-Dipalmitoyl-2-linolenoylglycerol was prepared from 1,3-dipalmitoylglycerol by esterification of the secondary hydroxy group with linolenoyl chloride. 1,3-Dioleoyl-2-stearoylglycerol was obtained from Sigma Chemical Co. (St. Louis, MO).

*Partial hydrolysis of triacyl-*sn*-glycerols.* The procedures for the analysis of triacyl-*sn*-glycerols are outlined in Scheme 1. The first step is the partial hydrolysis of triacylglycerol with ethyl magnesium bromide by a modified Brockerhoff procedures (5,6). Triacyl-*sn*-glycerols (100 mg) were dissolved in 3 mL of dry diethyl ether, and ethyl magnesium bromide in dry diethyl ether (0.33 mL of 3 M solution) was added. The mixture was shaken for 1 min, and then 0.1 mL glacial acetic acid followed by



SCHEME 1. Procedures for stereospecific analysis of acyl distribution in triacyl-*sn*-glycerols by chiral HPLC.

*To whom correspondence should be addressed.

Abbreviations: 3,5-DNPU, 3,5-dinitrophenylurethane; GC, gas chromatography; HPLC, high-performance liquid chromatography; TLC, thin-layer chromatography.

water (3.3 mL) was added to stop the reaction. The products were extracted with diethyl ether. The ether extract was washed five times with 2% aqueous sodium bicarbonate, then washed with water, and dried over anhydrous sodium sulfate. After removal of the solvent at ambient temperature, the 1- and 2-monoacylglycerol fractions were separated by preparative TLC on boric acid-impregnated silica gel plates (20 × 20 cm, 0.5 mm thickness, boric acid 10% to Kiesel Gel 60G) developed in chloroform/methanol (98:2, v/v) under nitrogen.

Enantiomer separation of 1-monoacylglycerols by HPLC. The 1-monoacylglycerol fractions (1–3 mg) were dissolved in dry toluene (500 μL) and were reacted overnight at ambient temperature with 3,5-dinitrophenyl isocyanate (10–20 mg) in the presence of dry pyridine (50 μL). The chloroform solution of the products was spotted on a Kiesel Gel 60G plate and developed with n-hexane/1,2-dichloroethane/ethanol (40:15:6, v/v/v) under nitrogen. HPLC separations of the 1-monoacylglycerol 3,5-DNPU fractions were carried out using a Shimadzu LC-6A isocratic pump (Kyoto, Japan) with a SPD-1 ultraviolet spectrophotometric detector and a C-R3A integrator from the same company. For optimum resolution, a chiral column (25 or 50 cm × 4 mm i.d.) packed with 5 μm particles of N-(*R*)-1-(α-naphthyl)ethylaminocarbonyl-(*S*)-valine chemically-bonded to γ-aminopropyl silanized silica, Sumichiral OA-4100 (Sumitomo Chemical Co.) was used. The HPLC separation was done isocratically using a mixture of n-hexane/1,2-dichloroethane/ethanol (40:12:3, v/v/v) as mobile phase at a constant flow rate. The 1-monoacylglycerol 3,5-DNPU sample was dissolved in the minimum volume of chloroform for injection. Detection was at 254 nm.

GC analysis of methyl esters. Total triacyl-*sn*-glycerols, 1- and 2-monoacylglycerols (1–2 mg), and the 3,5-DNPU derivatives of *sn*-1 and *sn*-3 monoacylglycerols (0.1–0.3 mg) were placed into 10 mL centrifuge tubes. Two mL of diethyl ether and 1 M sodium methoxide/methanol solution (25 μL) were added. The content was gently shaken for 5 min before glacial acetic acid (6 μL) was added to stop the reaction. After removing the precipitate by centrifuging for 2 min at 2000 rpm, and evaporating the solvent, the products were extracted with n-hexane and injected onto a WCOT column (50 m × 0.25 mm i.d., 0.2 μm film thickness) coated with Silar 5CP in a Shimadzu GC-6AM instrument with a FID detector and a Shimadzu C-R6A integrator. The column temperature was 190°C, and the injector and detector temperatures were 230°C. Hydrogen was the carrier gas.

RESULTS AND DISCUSSION

HPLC resolution of 1-monoacylglycerols. Enantiomer separations of 1-monoacylglycerol homologue mixtures of 3,5-DNPU derivatives by HPLC on a chiral stationary phase are accompanied by peak separations according to the number of carbon atoms and olefinic bonds of the components because of the polar nature of the chiral stationary phase (8,10). In our previous paper (10), conditions for the resolution of enantiomers were reported that allow the separation of *sn*-1 and *sn*-3 groups of peaks. This was achieved mainly by using Sumichiral OA-4100 columns instead of the OA-2100, and by using less 1,2-dichloroethane in the eluent, *i.e.*, a n-hexane/1,2-dichloroethane/ethanol mixture (10). In the present study, 1-monoacylglycerol mixtures were obtained from triacyl-*sn*-glycerols of natural fats by partial hydrolysis with ethyl magnesium bromide. The 1-monoacylglycerol mixtures were separated into *sn*-1 and *sn*-3 enantiomer groups by HPLC on the OA-4100 column. The 1-monoacylglycerols derived from vegetable oils containing palmitic, stearic, oleic, linoleic and linolenic acids were separated into *sn*-1 and *sn*-3 enantiomer groups by HPLC using a 25 cm column and n-hexane/1,2-dichloroethane/ethanol (40:12:3, v/v/v) as mobile phase at ambient temperature. Figure 1 shows the HPLC resolution of the 1-monoacylglycerols prepared from soybean oil triacyl-*sn*-glycerols. Complete resolution of racemates into enantiomer groups was achieved for the 1-monoacylglycerol derivatives from soybean oil, macadamia nut oil, and yew seed oil triacyl-*sn*-glycerols even when using micro-preparative HPLC. The time required for the separation was less than 30 min. The sample amounts were about 2 and 200 μg per injection in analytical and micro-preparative HPLC, respectively. The 1-monoacylglycerols from high erucic acid-rapeseed triacylglycerols required the 50 cm column for the clear separation of the enantiomers under similar conditions.

HPLC of fish oil 1-monoacylglycerols. In HPLC of 1-monoacylglycerols on an OA-4100 chiral column, the components eluted in the order of decreasing acyl chain-length, while a double bond in the acyl chain lengthened the retention time by the equivalent of about 2 carbon atoms (10). Thus, the retention time of an *sn*-1 18:3 monoacylglycerol derivative was similar to that of an *sn*-1 12:0 monoacylglycerol. These were the last components to emerge in HPLC of the 1-monoacylglycerol derivatives from plant oils reprsenting the *sn*-1 group. By the modified HPLC method (10), it was possible to separate these components from the first eluting components representing the *sn*-3 group, namely the *sn*-3 20:0 and 22:1 monoacylglycerol derivatives. However, when fish oils were used, complete separation of enantiomeric groups was more difficult. The first peak in the *sn*-3 group, *i.e.*, 22:1 or 24:1 monoacylglycerol derivatives, and the last peak in the *sn*-1 group, *i.e.*, 16:4 or 22:6 monoacylglycerol derivatives, overlapped on the OA-4100 column at ambient

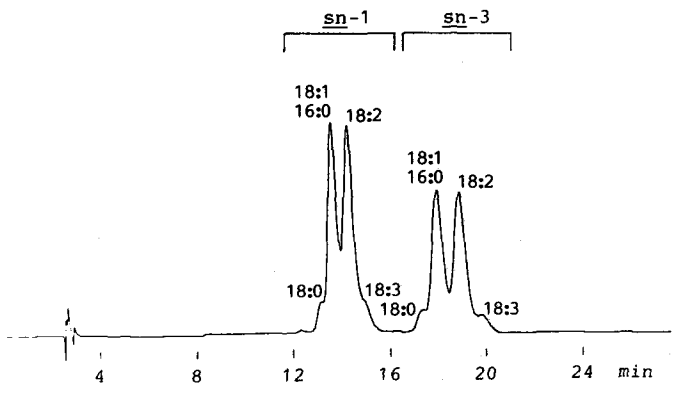


FIG. 1. Separation of 1-monoacylglycerols, formed by partial hydrolysis of soybean oil triacyl-*sn*-glycerols with ethyl magnesium bromide, as their 3,5-DNPU derivatives on a chiral column, OA-4100 (25 cm × 4 mm i.d.). Mobile phase, n-hexane/1,2-dichloroethane/ethanol (40:12:3, v/v/v). Flow rate, 1.0 mL/min; temperature, ambient; detection, 254 nm UV.

temperature. We observed that lowering the column temperature brought about a considerable improvement in enantiomer separation. Figure 2 shows a chromatogram obtained at −9°C using 50 cm column. The separation factor for the enantiomer resolution increased from about 1.4 to about 1.8 by lowering the column temperature from 20°C to −9°C. The time required for the separation of *sn*-1 and *sn*-3 monoacylglycerols from fish oils was about 200 min.

Differential losses in the conversions. Selective loss of monoacylglycerols in the preparation of 3,5-DNPU derivatives and methyl esters causes errors. To determine selective losses in the conversions, unsaturated 1-monoacylglycerols (A) were converted to 3,5-DNPU derivatives (B), and then to methyl esters (C) by the method described previously. In the present study, the following three samples were used (as A) in mixture with 1-stearoylglycerol: 1-oleoyl-, 1-linoleoyl- and 1-linolenoylglycerols (I); 1-arachidonoyl- and 1-eicosapentaenoylglycerols (II); and 1-docosahexaenoylglycerol (III). In each case, the results of HPLC of B and of GC of C were compared with the result of GC of the methyl esters obtained from A (Table 1). HPLC analysis of the 1-monoacylglycerol 3,5-DNPU was done as described in the previous paper (10). The results showed, in general, increasing losses as the unsaturation of the acyl groups increased. The apparent losses of methyl esters of unsaturated fatty acids could have been due to the short methylation time. 3,5-DNPU derivatives prepared from sample I were therefore converted to fatty acid methyl esters by transesterification in 1% sulfuric acid/methanol at 50°C overnight in a stoppered centrifuge tube. However, the fatty acid composition obtained by the latter method was close to that obtained by base-catalyzed transesterification as employed here.

Acyl compositions in sn-*1,* sn-*2 and* sn-*3 positions.* The composition (mole %) of the acyl group in each of the three positions was obtained by the following calculations using the procedure outlined in Scheme 1. The mean composition of each acyl group in the *sn*-1 and *sn*-3 positions [*sn*-1(3) position] was calculated from the GC analysis of

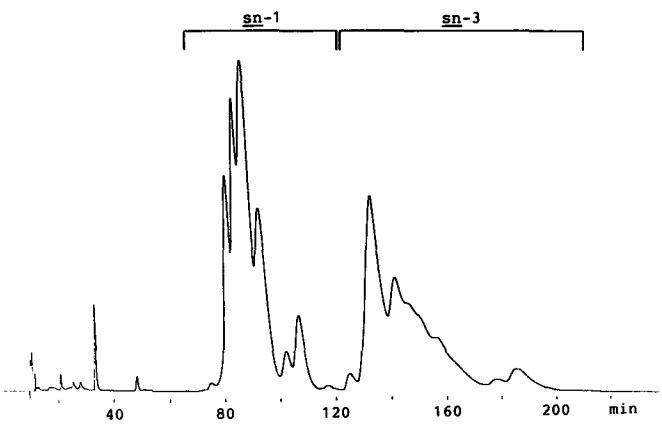


FIG. 2. Separation of 1-monoacylglycerols, formed by partial hydrolysis of herring oil triacyl-*sn*-glycerols with ethyl magnesium bromide, as their 3,5-DNPU derivatives on a chiral column, OA-4100 (50 cm × 4 mm i.d.). Mobile phase, n-hexane/1,2-dichloroethane/ethanol (40:12:3, v/v/v). Flow rate, 0.5 mL/min; temperature, −9°C; detection, 254 nm UV.

TABLE 1

Conversion of Unsaturated 1-Monoacylglycerols to 3,5-DNPU Derivatives and Methyl Esters

Acyl group	Conversion to[a]	
	3,5-DNPU[b]	Methyl ester[c]
18:0	1.000	1.000
18:1	1.014	0.998
18:2	0.972	0.913
18:3	1.041	0.900
20:4	0.905	0.815
20:5	0.865	0.765
22:6	0.885	0.667

[a] Change of ratios of unsaturated acyl groups to 18:0 acyl group in the conversion of 1-monoacylglycerols to 3,5-DNPU derivatives and methyl esters.

[b] Analyzed by HPLC using the procedure described previously (10).

[c] Analyzed by GC using the present procedure.

the original triacylglycerols and those of the 1- and 2-monoacylglycerols isolated by boric acid-impregnated silica gel TLC, *i.e.,* for each component:

$$sn\text{-}1(3)\ \text{position (mole \%)} = 3 \times \text{TG} \times [\text{1-MG}/(2 \times \text{1-MG} + \text{2-MG})]$$

where TG, 1-MG and 2-MG represent the proportions (mole %) of each acyl group in the original triacylglycerols, 1-monoacylglycerols and 2-monoacylglycerols, respectively. The composition of each acyl group in the *sn*-2 position was calculated in a similar manner as above, *i.e.*:

$$sn\text{-}2\ \text{position (mole \%)} = 3 \times \text{TG} \times [\text{2-MG}/(2 \times \text{1-MG} + \text{2-MG})]$$

The amount of each acyl group in the *sn*-1 and *sn*-3 positions was calculated from the concentration of each component in the *sn*-1(3) position and GC analyses of *sn*-1 and *sn*-3 monoacylglycerols resolved by HPLC on the chiral column, *i.e.*:

$$sn\text{-}1\ \text{position (mole \%)} = 2 \times sn\text{-}1(3)\ \text{position} \times [sn\text{-}1/(sn\text{-}1 + sn\text{-}3)]$$

$$sn\text{-}3\ \text{position (mole \%)} = 2 \times sn\text{-}1(3)\ \text{position} \times [sn\text{-}3/(sn\text{-}1 + sn\text{-}3)]$$

where *sn*-1 and *sn*-3 represent the proportions (mole %) of each acyl group in *sn*-1 and *sn*-3 monoacylglycerols analyzed by GC as methyl esters, respectively. These calculations avoid cumulative errors in the result for a particular position, and give reproducible and reliable results. In contrast, direct analysis of methyl esters from *sn*-1, *sn*-2 and *sn*-3 monoacylglycerols can involve considerable experimental errors. The composition of the acyl group in each of the three positions could also be obtained based on the following relationships:

$$sn\text{-}1\ \text{position} = 3 \times \text{TG} - \text{2-MG} - sn\text{-}3$$

$$sn\text{-}2\ \text{position} = 3 \times \text{TG} - sn\text{-}1 - sn\text{-}3$$

$$sn\text{-}3\ \text{position} = 3 \times \text{TG} - sn\text{-}1 - \text{2-MG}$$

However, this approach is not recommended, since the result for each position is subject to the cumulative experimental error of the other analyses.

TABLE 2

Reproducibility of Positional Distributions of Acyl Groups in Triacyl-*sn*-Glycerols from Soybean Oil (mole %)

No.	Acyl group	Triacylglycerol	Acyl group mole percent and standard deviation (n=4)			
			sn-1(3)[a]	*sn*-1	*sn*-2	*sn*-3
1	14:0	0.1	0.1[b] ± 0.0[c]	0.1 ± 0.0	0.2 ± 0.0	0.1 ± 0.0
2	16:0	11.6	16.0 ± 0.3	16.6 ± 0.2	3.0 ± 0.6	15.5 ± 0.6
3	16:1n-7	0.1	0.1 ± 0.0	0.1 ± 0.1	0.1 ± 0.0	0.1 ± 0.1
4	17:0	0.1	0.1 ± 0.0	0.1 ± 0.0	0.1 ± 0.0	0.1 ± 0.0
5	18:0	3.4	4.8 ± 0.1	4.9 ± 0.3	0.6 ± 0.2	4.7 ± 0.1
6	18:1n-9	22.7	22.6 ± 0.1	22.4 ± 0.1	23.0 ± 0.2	22.7 ± 0.2
7	18:1n-7	1.4	1.9 ± 0.0	1.9 ± 0.1	0.4 ± 0.1	1.9 ± 0.1
8	18:2n-6	53.9	47.2 ± 0.3	46.8 ± 0.3	67.0 ± 0.7	47.7 ± 0.5
9	18:3n-6	6.4	6.8 ± 0.1	6.7 ± 0.2	5.7 ± 0.1	6.9 ± 0.2
10	20:0	0.3	0.5 ± 0.0	0.5 ± 0.1	ND[d]	0.4 ± 0.0

[a] Primary position in triacyl-*sn*-glycerol.
[b] Mean value of quadruplicate analyses.
[c] Standard deviation.
[d] ND, not detected.

TABLE 3

Comparison of Stereospecific Distributions of Acyl Groups in Soybean Oil Triacyl-*sn*-Glycerols Obtained by HPLC and Enzymatic Methods

No.	Acyl group	Composition (mole %)					
		HPLC method[a]			Enzyme method[b]		
		sn-1	*sn*-2	*sn*-3	*sn*-1	*sn*-2	*sn*-3
1	14:0	0.1	0.1	0.1	0.3	0.3	−0.2
2	16:0	16.7	2.2	16.1	16.9	1.4	16.6
3	16:1n-7	0.1	0.1	0.1	0.1	0.1	0.0
4	17:0	0.1	0.0	0.1	0.1	0.0	0.0
5	18:0	5.4	0.3	4.6	5.2	0.3	4.7
6	18:1n-9	22.2	23.0	22.9	20.3	22.6	25.1
7	18:1n-7	2.1	0.4	1.7	2.0	0.4	1.8
8	18:2n-6	46.4	68.1	47.0	47.4	69.2	45.2
9	18:3n-6	6.4	5.7	7.0	7.5	5.7	5.9
10	20:0	0.5	ND[c]	0.4	0.2	ND	0.7

[a] Method presented here.
[b] Modified Brockerhoff method (6).
[c] ND, not detected.

Reproducibility of the stereospecific analysis. The reproducibility of the analytical method was checked for soybean oil triacyl-*sn*-glycerols. Table 2 shows the results. The stereospecific analyses carried out by four different persons showed good agreement. Another soybean oil triacyl-*sn*-glycerol sample was analyzed by the present method and by the Brockerhoff method as modified by Christie (6). The results are compared in Table 3. The positional distributions obtained differed little from each other.

Acyl migration in the course of analysis. Monoacylglycerols generally isomerize more rapidly than do diacylglycerols due to acyl migration. Isomerization affects the accuracy of a method. The accuracy of our method was determined by the stereospecific analysis of racemic synthetic triacyl-*sn*-glycerols (Table 4). The results showed that the *sn*-1 and *sn*-3 positions can be contaminated with 2.5–3.0% of acyl groups which have migrated from the *sn*-2 position. This would indicate that the error in the relative proportions at the *sn*-1 and *sn*-3 positions could be about 1%, when the acyl group in *sn*-2 position is 30% of the total. On the other hand, the *sn*-2 position contained 4–6% of acyl groups that migrated from the *sn*-1 and *sn*-3 positions (Table 4). However, the total acyl compositions suggest that the 1,3-dioleoyl-2-stearoylglycerol and 1,3-dipalmitoyl-2-linolenoylglycerol samples contained 1.5% 18:1 acyl and 5.1% 16:0 acyl in the *sn*-2 position, respectively. Thus, the *sn*-2 position can be contaminated with up to 3.0% of acyl groups which have migrated from the primary positions. These results demonstrate the relatively low rate of isomerization of monoacylglycerols, as compared to what has been reported for diacylglycerols

TABLE 4

Stereospecific Analysis of Synthetic Triacyl-*sn*-Glycerols to Examine Isomerization in the Procedure

Structure of sample	Acyl group	Composition (mole %)			
		Total	*sn*-1(3)[a]	*sn*-1	*sn*-2	*sn*-3
18:1-18:0-18:1	18:0	32.8	2.8	2.6	95.8	3.0
	18:1n-9	67.2	97.2	97.4	4.2	97.0
16:0-18:3-16:0	16:0	68.4	97.5	97.6	6.1	97.5
	18:3n-3	31.6	2.5	2.4	93.9	2.5

[a]Primary position in triacyl-*sn*-glycerol.

TABLE 5

Stereospecific Analysis of Macadamia Nut Triacyl-*sn*-Glycerols

No.	Acyl group	Composition (mole %)			
		Total	*sn*-1	*sn*-2	*sn*-3
1	12:0	0.4	ND[a]	0.4	0.8
2	14:0	1.4	1.3	1.0	1.9
3	16:0	9.0	12.5	3.1	10.8
4	16:1n-7	20.2	23.4	17.2	18.2
5	16:1n-5	0.3	4.3	3.0	2.0
6	18:0	3.1	3.2	0.6	5.3
7	18:1n-9	56.3	47.9	71.0	45.1
8	18:1n-7	3.4	4.5	1.3	4.0
9	18:2n-6	1.5	1.7	2.0	0.5
10	18:3n-3	0.1	ND	0.4	ND
11	20:0	2.2	0.3	0.0	6.1
12	20:1n-9	2.1	1.0	ND	5.2

[a]ND, not detected.

(5,12). For example, 1,3-diacylglycerols prepared by reaction with ethyl magnesium bromide contained 7–10% (or more) of acyl groups that had migrated from the *sn*-2 position (5,12). The *sn*-2 position of 1,2-diacylglycerols was contaminated with 4% of acyl groups that had migrated from the *sn*-1 and *sn*-3 positions (5,12).

The stereospecific analysis of the natural triacyl-*sn*-glycerols also supports the conclusion that acyl migration is minimal in the present method. Triacyl-*sn*-glycerols from macadaqmia nut oil, yew seed oil, high erucic acid rapeseed oil and herring oil were subjected to stereospecific analysis (Tables 5–8). Fatty acid compositions of macadamia nut and yew seed oils have been reported previously (13,14). Macadamia nut oil triacyl-*sn*-glycerols showed 20:1n-9 acyl groups at an abundance of 5.2% in the *sn*-3 position and of 1.0% in the *sn*-1 position, but these groups were not detected in the *sn*-2 position (Table 5). Yew seed oil triacyl-*sn*-glycerols showed all-*cis*-5,9,12-18:3 and 5,11,14-20:3 acyl groups at concentrations of 8.1 and 4.7%, respectively, in the *sn*-3 position, but these acyl groups were essentially absent in the other positions (Table 6). The rapeseed oil triacyl-*sn*-glycerols showed the 22:1n-9 acyl group at concentrations of 61.3% and 50.2% in the *sn*-3 and the *sn*-1 positions, respectively, but 22:1n-9 was only 0.8% abundant in *sn*-2 position (Table 7). Herring oil triacyl-*sn*-glycerols showed the 22:6n-3 acyl group at concentrations of 14.9% in the *sn*-2 position and of

TABLE 6

Stereospecific Analysis of Yew Seed Triacyl-*sn*-Glycerols

No.	Acyl group	Composition (mole %)			
		Total	*sn*-1	*sn*-2	*sn*-3
1	16:0	2.7	5.7	1.7	0.8
2	16:1n-9	0.1	0.0	0.1	0.0
3	18:0	1.4	3.7	0.4	0.2
4	18:1n-9	36.7	50.0	52.9	9.0
5	18:1n-7	0.6	0.2	0.3	1.2
6	18:2(5,9)	18.5	1.7	1.7	50.0
7	18:2n-6	31.6	30.8	41.5	23.3
8	18:3(5,9,12)[a]	2.9	0.2	ND[b]	8.1
9	18:3n-3	1.3	1.5	1.3	1.2
10	18:4(5,9,12,15)	0.2	ND	1.2	0.2
11	20:1n-9	1.5	4.2	ND	0.4
12	20:2n-6	1.0	2.0	ND	0.9
13	20:3(5,11,14)	1.6	ND	ND	4.7

[a]All-*cis*-5,9,12-octadecatrienoyl group. Other 5-olefinic acyl groups also have the *cis* configuration.

[b]ND, not detected.

TABLE 7

Stereospecific Analysis of Rapeseed Triacyl-*sn*-Glycerols

No.	Acyl group	Composition (mole %)			
		Total	*sn*-1	*sn*-2	*sn*-3
1	14:0	0.1	0.1	0.2	0.1
2	16:0	3.9	5.6	1.6	4.5
3	16:1n-9	0.1	ND[a]	0.1	0.6
4	16:1n-7	0.3	0.3	0.3	0.2
5	17:1n-8	0.1	ND	0.3	ND
6	18:0	1.3	1.9	0.4	1.7
7	18:1n-9	17.2	13.7	32.4	5.1
8	18:1n-7	1.4	2.8	1.0	0.5
9	18:2n-6	16.1	1.2	39.3	7.4
10	18:3n-3	8.7	3.3	21.3	1.3
11	20:0	0.8	0.6	0.3	1.5
12	20:1n-9	8.9	15.5	1.5	10.0
13	20:1n-7	1.5	1.9	0.4	2.0
14	20:2n-6	0.5	1.2	0.1	0.2
15	22:0	0.7	ND	ND	2.1
16	22:1n-9	37.4	50.2	0.8	61.3
17	22:1n-7	0.6	0.9	ND	1.0
18	22:2n-6	0.5	0.9	ND	0.7

[a]ND, not detected.

2.19% in the *sn*-1 position, but 22:6n-3 was not detected in the *sn*-3 position (Table 8). Such specificity in measured positional distribution indicates the low degree of isomerization of monoacylglycerols in the procedures of stereospecific analysis employed here.

Selectivity of acyl distributions. As a general rule, seed oils containing common fatty acids show a selective placement of unsaturated fatty acids at the *sn*-2 position, while long-chain or anomalous fatty acids are selectively enriched in the *sn*-3 position (7). Table 5 shows that the 18:1n-9 group was enriched (*ca.* 71%) at the *sn*-2 position, but that 18:1n-7 was more abundant at the *sn*-1 and *sn*-3 positions. This shows that the position of the olefinic bond

TABLE 8

Stereospecific Analysis of Herring Triacyl-*sn*-Glycerols

No.	Acyl group	Composition (mole %)			
		Total	*sn*-1	*sn*-2	*sn*-3
1	14:0	9.7	11.9	6.7	9.7
2	4,8,12-TMTD[a]	0.3	0.1	0.4	0.5
3	iso-15:0	0.1	0.1	0.2	0.1
4	15:0	0.6	0.4	0.9	0.5
5	16:0	15.7	15.2	17.9	12.5
6	16:1n-11	0.3	0.5	0.3	ND[b]
7	16:1n-9	0.2	0.1	0.5	0.1
8	16:1n-7	5.0	6.4	3.8	4.3
9	iso-17:0	0.5	0.6	0.4	0.4
10	17:0	0.6	1.4	0.3	ND
11	16:2n-4	0.4	0.5	0.6	0.1
12	16:3n-4	0.3	0.1	0.6	0.2
13	16:4n-1	0.4	0.9	0.4	ND
14	18:0	0.9	0.9	0.9	0.8
15	18:1n-9	9.9	11.3	8.7	9.0
16	18:1n-7	1.5	2.4	0.8	1.1
17	18:1n-5	0.5	0.6	0.5	0.3
18	18:2n-6	1.2	1.2	1.4	1.0
19	19:0	0.2	0.5	0.1	ND
20	18:3n-3	1.4	1.2	1.6	1.2
21	18:4n-3	3.8	4.6	3.9	2.4
22	20:0	0.1	0.1	0.1	0.2
23	20:1n-11	1.2	1.4	0.6	1.6
24	20:1n-9	11.8	16.3	4.2	14.4
25	20:2n-6	0.2	0.1	0.1	0.4
26	20:4n-6	0.2	0.0	0.2	0.2
27	20:4n-3	0.5	1.0	0.5	ND
28	20:5n-3	5.9	3.0	10.5	3.3
29	22:1n-11,n-13	16.0	12.3	5.2	31.6
30	22:1n-9	0.8	0.2	1.5	0.5
31	21:5n-3	0.3	0.3	0.5	ND
32	22:5n-3	0.5	0.2	1.2	ND
33	22:6n-3	6.1	2.2	14.9	ND
34	24:1n-9	0.7	0.5	0.3	1.4

[a]4,8,12-TMTD, 4,8,12-trimethyltetradecanoyl group.

[b]ND, not detected.

is an important factor in governing acyl distributions as is the number of carbon atoms and the number of double bonds. Table 6 shows that anomalous 5-olefinic acyl groups were selectively concentrated in the *sn*-3 position as predicted (7). Their accumulation at the *sn*-3 position, in turn, resulted in a lower relative proportion of 18:1n-9 at this position. Table 7 shows the positional distributions of 18 different acyl groups in rapeseed oil triacyl-*sn*-glycerols. The characteristic distribution patterns of seven components of acyl groups in triacyl-*sn*-glycerols from seeds of nine Cruciferae, as determined by Brockerhoff's method, were reported previously (15). The data presented in Table 7 are in generally good agreement with these in as much as 18:1n-9 was enriched at the *sn*-2 position, whereas 18:1n-7 was more abundant in the *sn*-1 position. Table 8 shows the distributions of 34 acyl groups of herring oil triacyl-*sn*-glycerols. The distributions of 11 acyl groups in herring oil had previously been reported by Brockerhoff *et al.* (16). The 11 most abundant components (16) amount to about 80% of acyl groups listed in Table 8. The method presented here is especially useful for the analysis of the complex acyl group compositions of the triacyl-*sn*-glycerols of marine lipids.

Comparison with the Brockerhoff method. In the Brockerhoff method the composition at the *sn*-3 position is not determined directly. So errors can occur particularly for minor components at the *sn*-3 position, and sometimes small negative values may be obtained. Another drawback can be the preferential hyrdolysis of certain acyl groups by phospholipase A_2, which affects the data for the *sn*-2 position. The most common source of phospholipase A_2 for structural studies are snake venoms. King cobra, *Ophiophagus hannah,* venom is preferred because it had been shown not to preferentially hydrolyze 16:0, 18:0, 18:1, 18:2 and 20:0 from lecithins (17). It has therefore been used for the stereochemical analysis of triacylglycerols.

However, triacyl-*sn*-glycerols from natural sources contain a great variety of acyl groups, and there is at present insufficient evidence for the nonpreferential hydrolysis of all types of acyl groups.

The method presented here permits the stereospecific analysis of triacyl-*sn*-glycerols. The procedures used are simple and rapid, and permit measurement of distributions even of minor components when high resolution capillary GC is employed.

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Evidence of Extensive Phospholipid Fatty Acid Methylation During the Assumed Selective Methylation of Plasma Free Fatty Acids by Diazomethane

Chu Lin, Edward W. Blank, Roberto L. Ceriani and Nome Baker*
John Muir Cancer and Aging Research Institute, Walnut Creek, California 94596

We compared a conventional method (Method I) for measuring plasma free fatty acid (FFA) concentrations with two more rapid procedures (Method II and Method III). Method I required total lipid extraction, separation of FFA by thin-layer chromatography, methylation, and gas-liquid chromatographic analysis of the fatty acid (FA) methyl esters. Method II was a colorimetric procedure. Method III relied upon diazomethane's presumed ability to selectively methylate FFA even in the presence of FA esters. The three methods were compared using plasma from fasted and from fed nude mice, tumor-bearing mice (MX-1 and ZR-75-1 human mammary carcinomas), and controls. Method II, was less reliable than Method I, but both gave similar mean values for plasma FFA levels in fasted mice. Both Methods I and II also showed similar lowering of plasma FFA levels after feeding previously fasted mice. Method III consistently gave values that were far greater than those obtained using Methods I and II. Moreover, highly significant differences between fasted and fed mice were obscured by direct methylation of plasma FFA with diazomethane (Method III). The excess FA methyl esters formed in Method III were derived from plasma phospholipids, but not from plasma triacylglycerols. After feeding fasted mice, plasma free palmitic acid and oleic acid levels fell (Method I); by contrast, the excess "FFA" formed by methylation of plasma phospholipid FA increased two-fold and fourteen-fold, respectively. Caution is therefore advised in the use of direct methylating agents when measuring total and individual plasma FFA levels.
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Before initiating a study of essential free fatty acid (FFA) transport to tumor cells in relation to tumor growth rates *in vivo*, we evaluated several methods for measuring plasma FFA concentrations. One was a colorimetric method (1) which we used earlier (2,3). The other two methods were based upon the analysis of fatty acid methyl esters (FAME) by gas-liquid chromatography (GLC). One of these methods is simple and rapid; it is based on the direct and supposedly selective methylation of FFA with diazomethane, which is added directly to the plasma (4). The other method, taken as the primary standard method, requires prior extraction of total plasma lipids, followed by separation of the FFA fraction by thin-layer chromatography (TLC) and methylation of FFA to form free fatty acid methyl esters (FFAME). Each method was tested under conditions that can be expected to perturb the FFA pool size, namely, fasting and feeding of control and tumor-bearing (TB) mice.

Using the colorimetric method (1) we could demonstrate a rapid decrease in plasma FFA levels induced by a fat-free meal in both control and TB mice, as reported for other strains of TB and control mice (5). However, the colorimetric method was less reliable than the standard reference (TLC/GLC) method, especially at low plasma FFA levels. We were unable to corroborate the earlier observation (4) that plasma FFA can be specifically methylated by diazomethane in the presence of other plasma lipids. We found that such direct methylation consistently resulted in artificially high plasma FFA levels. The artifacts in some cases were an order of magnitude greater than the results obtained by the "standard" technique. The artificial increment was shown to be due to the methylation of several classes of phospholipid fatty acids (PLFA), but not of triacylglycerol fatty acids (TGFA). The composition of the artificially formed FAME was highly dependent upon the nutritional state of the animals and was qualitatively different from the changes in plasma FFA composition following fasting and feeding.

METHODS

Animals. Female BALB/c (nu/nu) mice (7–8 wk old) was obtained from Simonson (Gilroy, CA). They were housed in autoclaved cages with filter tops and bedding in a temperature-controlled room with a 12-hr light (daytime) and 12-hr dark cycle.

Tumor. The estrogen receptor negative transplantable human breast carcinoma, MX-1, derived from a primary breast tumor, was obtained originally from the EG & G Mason Research Institute (Worchester, MA) and, since 1983, was transplanted serially in nude mice in our laboratory. Under sterile conditions, nude mice were injected subcutaneously with 35 mm^3 pieces of MX-1 tumor suspended in cell culture medium, proximal to the right #4 mammary gland using a 16-gauge needle. Human breast carcinoma cell line ZR-75-1 was grown in Dulbecco Modified Eagle's (DME) plus RPMI 1640 media (1:1, v/v; Gibco, Grand Island, NY), 5% fetal bovine serum, and 200 ng/mL of bovine insulin. ZR-75-1 was obtained from the ascites effusion of a 63-yr-old woman with an infiltrating ductal carcinoma. The ZR-75-1 tumor line was initiated by injecting one-half of the confluent cells grown

*To whom correspondence should be addressed at John Muir Cancer and Aging Research Institute, 2055 North Broadway, Walnut Creek, CA 94596.

Abbreviations: BHT, 3,5-di-*t*-butyl-4-hydroxytoluene; DME, Dulbecco modified Eagle's medium; EDTA, ethylenediaminetetraacetic acid; FAME, fatty acid methyl esters; FFA, free fatty acids; FFAME, free fatty acid methyl esters; GLC, gas-liquid chromatography; PC, phosphatidylcholine; PE, phosphatidylethanolamine; PG, phosphatidylglycerol; PL, phospholipid(s); PLFA, phospholipid fatty acids; PS, phosphatidylserine; TB, tumor-bearing; TG, triacylglycerol; TLC, thin-layer chromatography; TGFA, triacylglycerol fatty acids.

in a T-175 flask into the mid flank of an estrogen-primed nude mouse. Once the ZR-75-1 tumor was established it was serially passaged.

Diets. The mice were fed sterile, Purina laboratory chow (5068) from weaning until they were fasted for 24 hr. Animals that were fed for 20 hr following the fasting period were given fat-free pellets that contained (by weight) 64.75% sucrose, 24.0% vitamin-free casein, 3.75% salt mixture (AIN #76), 2.2% vitamin fortification mixture (ICN Nutritional Biochemicals, Cleveland, OH), 0.3% methionine, 5.0% cellulose, and 0.01% BHT (3,5-di-*t*-butyl-4-hydroxytoluene).

Experimental protocol. Six chow-fed animals (2 control, 2 bearing MX-1 tumors, and 2 bearing ZR-75-1 tumors) were fasted for 24 hr and were then fed the special fat-free diet for 20 hr. The mice were sacrificed along with another six 24-hr fasted animals (2 each of the corresponding C and TB groups). Retroorbital blood or blood following decapitation, both under ether anesthesia, was collected in tubes containing solid ethylenediaminetetraacetic acid (EDTA).

FFA analyses/Method I. Method I was taken to be the standard reference (TLC/GLC) method. Five μg of heptadecanoic acid in 0.05 mL of methanol were added to 0.1 mL of plasma. Total lipids were extracted according to Bligh and Dyer (6). The chloroform phase was concentrated and spotted on channels of a Whatman LK6D plate (Hillsboro, OR) and developed with the solvent system petroleum hydrocarbon/diethyl ether/acetic acid (80:20:1, v/v/v). A spray of 0.1% 2,7-dichlorofluorescein in ethanol was used to locate the FFA standard. A razor blade was used to scrape off the corresponding unsprayed FFA zone from the TLC plate. The scraped silica gel was stored in a 13 × 100 mm disposable glass tube with a Teflon cap. One mL ethereal diazomethane solution (4) was added to the tube, and methylation at room temperature was continued for about 10 min. A small desk-top centrifuge was used to centrifuge the reaction mixture. After removal of the supernatant, one mL of diethyl ether was added to wash the silica gel residue. The tubes were centrifuged again, and the ethereal supernatants were removed and added to the first extract. The ether extracts were evaporated to dryness under nitrogen. One-half mL each of hexane, water and methanol was added immediately to each tube. After vortexing and centrifuging, 0.4 mL of the hexane phase was transferred into a Wheaton (Millville, NJ) 2-mL glass sampling vial with cap. The hexane phase was then evaporated to dryness under nitrogen. Fifteen μL of hexane was added to the vial to dissolve the methyl esters. GLC analyses were carried out using 5 μL of this hexane solution.

FFA analyses/Method II. Method II was the colorimetric procedure of Hron and Menahan (1). Various concentrations of heptadecanoic acid were used to establish a standard curve. Chloroform/methanol (2:1, v/v) was used to wash all glass tubes prior to analyses in order to minimize blank values.

FFA analyses/Method III. Method III was the method of Pace-Asciak (4). Plasma FFA methylation was carried out with diazomethane in diethyl ether, which was added directly to plasma as described (4), except that heptadecanoic acid was used as internal standard. Diazide purchased from Aldrich (Milwaukee, WI) was used to prepare diazomethane by a method similar to that of Pace-Asciak (4), according to the instructions supplied by Aldrich. The following minor modifications were made in our later experiments in order to facilitate concentration of the FA methyl esters into convenient volumes and to insure dryness of the sample used for GLC analysis. After 800 μL of the ethereal reaction solution was evaporated to dryneses under nitrogen, two hundred μL each of methanol, water, and hexane were added to the tube. Samples were vortexed and quickly centrifuged, and the hexane phase was carefully transferred to a sampling vial. The hexane fraction was concentrated to dryness with nitrogen gas and reconstituted into a small volume for GLC injection.

Methylation of phospholipid. Phospholipid standards were purchased from Sigma Chemical Company (St. Louis, MO) and were purified by TLC using Whatman LK6D plates developed with choloroform/methanol/triethylamine/isopropanol/0.25 M KCl (30:9:18:25:6, by vol). Free fatty acids migrated almost to the solvent front. Phospholipids were detected by spraying the developed, air-dried plates with rhodamine B in ethanol. Methanol was used to elute the purified phospholipids from the silica gel. Methanol/acetyl chloride (50:1, v/v) (7), as well as diazomethane (4), were then used to determine the extent to which each phospholipid class would be transmethylated.

Instrumentation. Fatty acid analyses were performed on a Perkin-Elmer 8420 capillary gas chromatograph (Norwalk, CT) equipped with a flame-ionization detector (260°) and a 30-m Stabil wax column with a 0.32-mm internal diameter (Restek Corporation, Bellefonte, PA). The injector temperature was 260°. The initial temperature was set at 170° C and programmed at a rate of 3° C per min to a final temperature of 240° C and then held for 3 min. The total run time was 26.3 min. An electronic pressure readout of 14 psi was used for controlling the flow rate of helium as carrier gas. A split ratio of 10:1 was used for analysis. FAME retention times were determined by using a 68A methyl ester standard (Nu-Chek Prep, Elysian, MN). The weight percent response factor was the same for all 19 FAME standards analyzed under the conditions employed. A Perkin-Elmer data handling system set in the internal standard mode was used to calculate the results.

RESULTS

In Table 1 we compare the plasma FFA levels in 24-hr fasted control and TB mice measured by three different methods: Method I) the standard reference method (TLC/GLC of FFA methyl esters); Method II) The Hron and Menahan colorimetric method (1); and Method III) direct addition of diazomethane to plasma followed by GLC analysis of FA methyl esters (4). Using either the standard reference method (Method I) or the colorimetric method (Method II), we observed no differences between the mean plasma FFA concentrations of control and TB mice studied in the fasted state. This is consistent with earlier findings in fasted control and TB mice (with different tumors) (2,3,5). Therefore, the data obtained using each method for fasted mice (control and TB, combined) were averaged. As shown in Table 1, Methods I and II gave similar results, but Method III yielded values on average 2.7 times greater than those obtained using the standardized method. In each of the individual mouse

TABLE 1

Comparison of Plasma FFA Levels in 24-hr Fasted Control and TB Mice Using Three Different Methods[a]

Group[b]	Mouse no.	Plasma FFA (μM)		
		TLC/GLC	Colorimetric	Diazomethane
Nude controls	1	670	600	1,630
	2	590	760	2,800
ZR-75-1 mammary CA	3	690	560	1,720
	4	790	950	2,310
MX-1 mammary CA	5	740	360	1,290
	6	670	660	1,300
Grand mean (± S.E.)		690 (± 28)	650 (± 81)	1,840 (± 246)

[a] Methods used: Method I) standard reference method (TLC/GLC of FFAME); Method II) Hron and Menahan colorimetric method, and Method III) direct methylation of plasma with diazomethane and GLC of FAME. Method I, the standard reference method, is described in the present paper; Methods II and III are described in references 1 and 4, respectively. Statistical analysis: Method I *vs* Method II, n.s.; Method I *vs* Method III, $p \leq 0.001$.

[b] All mice were BALB/c (nu/nu) nude mice, bearing either the estrogen-sensitive (ZR-75-1) or the estrogen-insensitive (MX-1) human mammary carcinomas or no tumors (controls). (See footnote a.)

TABLE 2

Comparison of Plasma FFA Levels in Fed, Control and TB Mice Using Three Different Methods[a]

Group	Mouse no.	Plasma FFA (μM)		
		TLC/GLC	Colorimetric	Diazomethane
Nude controls	1	150	440	2,300
	2	180	250	2,100
ZR-75-1	3	130	250	1,600
	4	100	240	820
MX-1 mammary CA	5	200	120	500
	6	200	260	610
Grand mean (± S.E.)		160 (± 16)	260 (± 42)	1,300 (± 320)

[a] Methods used: Method I) standard reference method (TLC/GLC of FFAME); Method II) Hron and Menahan colorimetric method (1); Method III) Direct methylation of plasma with diazomethane and GLC of FAME (4). Statistical analysis: Method I *vs* Method II, n.s.; Method I *vs* Method III, $p \leq 0.01$. Mice were fasted 24 hr and then fed for 20 hr. See Table 1 legend for further experimental details.

samples, substantial artifactual formation of FAME was observed.

Evidence that Method III caused the artifactual formation of FAME from sources other than plasma FFA was obtained using plasma from another 6 mice that had been fasted for 24 hr and then fed a fat-free, sucrose-rich diet for 20 hr (Table 2). As expected, the plasma FFA levels were reduced significantly ($p \leq 0.001$) from 690 to 160 μM (Method I, Tables 1 and 2). Method II yielded far more inconsistent results, reflected in the S.E. values, than did Method I; however, the colorimetric method sufficed to demonstrate the significant effects of feeding on plasma FFA concentrations. Once again, with Method III we observed large artifactual elevations in the apparent plasma FFA levels in all 6 mice. The variation (Method III) in the fed mice was much greater than that seen in the fasted mice. Still, there was a consistent marked formation of FAME, which were not derived from FFA, in each of the 6 refed mice, just as had been found in the 6 fasted mice. Moreover, the formation of excess FAME obscured to a large extent the effects of feeding on the plasma FFA levels.

Changes in concentrations of the individual FFA moieties induced by feeding the previously fasted control mice are shown in Figure 1. These changes did not bear a consistent relationship to the formation of artifactually formed, "excess" FA moieties. This is additional evidence indicating that the "excess" FAME seen with Method III were derived from plasma lipids other than FFA. Under conditions in which each of the major peaks, except C_{20} and C_{22} polyunsaturated FFA, showed a significant fall in concentration in response to refeeding,

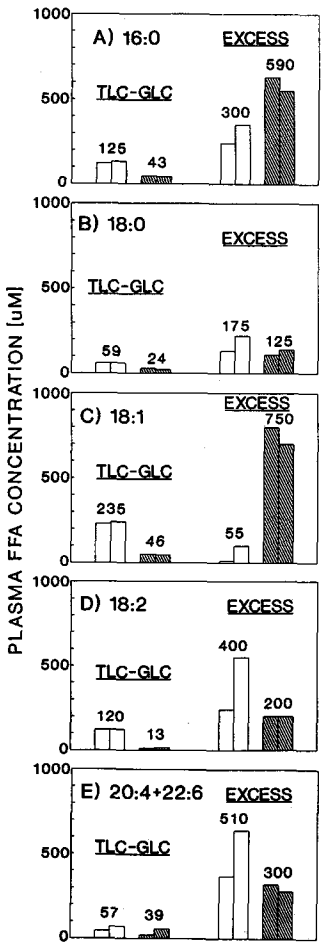


FIG. 1. Comparison of the major individual plasma FFA levels in 24-hr fasted and fasted-fed, control nude mice as measured by TLC/GLC (Method I) with the excess FA obtained by direct methylation of plasma FFA using diazomethane in diethyl ether. The plasma FFA concentration of each mouse, estimated using Method I, was substracted from the apparent FFA concentration obtained by Method III (diazomethane and GLC) to determine the excess FA formed by methylation of PLFA (see text). Data shown are those from two mice per dietary condition, each represented by a single bar, with the mean of the 2 values above them. Fasted, open bars; fasted-fed, hatched bars. Chain length and number of double bonds, separated by a colon, are indicated for each type or pair of FA.

the individual excess FA (Method I minus Method III) did not show a uniform response. Thus, the excesses of 16:0 and 18:1 both increased rather than decreased following refeeding, while excesses of 18:0, 18:2 and those of the more highly polyunsaturated FA either decreased or remained constant following refeeding.

Additional experiments were carried out to determine which of the major fatty acid ester fractions could be transmethylated under the conditions of Method III. Triglycerides (TG) did not serve as acyl donors under the latter conditions; they did not give rise to any detectable FAME. In contrast to TG, 4 classes of phospholipids (PL) were found to be acyl donors under the conditions described originally (4). When the standard phospholipids, which had been obtained commercially and then purified by TLC just before use, were treated according to Method III, the following results were obtained (values are % of total PLFA recovered as FAME): phosphatidylcholine (PC), 27%; phosphatidylethanolamine (PC), 27%; phosphatidylethanolamine (PE), 13%; phosphatidylglycerol (PG), 17%; phosphatidylserine (PS), 14%. We obtained similar results for PC, PE and PG using another supposedly specific FFA-methylating method in which acetyl chloride/methanol (1:50, v/v) was the methylating agent added directly to plasma under carefully controlled temperature and temporal conditions (7). Since PLFA is typically present in plasma lipoproteins in much higher concentrations than FFA, it seems logical to conclude that these were the sources of the excess apparent FFA found using Method III (compared to Method I).

DISCUSSION

We have compared a colorimetric method (Method II) for measuring plasma FFA levels with a standard TLC/GLC reference method (Method I). Both methods yielded the same mean plasma FFA concentrations for fasted mice and showed similar decreases in plasma FFA concentrations after carbohydrate feeding; however, the colorimetric method (1) was less reliable than the standard reference method.

We also tested two other GLC methods in which plasma FFA were methylated directly without prior extraction of total lipids and without separation of FFA by TLC. One of these (4) used diazomethane as a methylating agent (Method III). This method was selected for more detailed evaluation because it appeared to be faster and, theoretically, more specific than the other (7), which used acetyl chloride/methanol under carefully controlled conditions. Both of the latter methods (4,7) yielded plasma FFA levels far greater than those obtained using Methods I and II. The excess FAME that formed masked the effects of feeding on plasma FFA levels.

Further studies confirmed that extensive methylation of fatty acid (FA) esters (or of FFA derived from them after hydrolysis) by diazomethane had occurred; the degree of methylation was unreliable. Furthermore, the source of the FA moiety was phospholipids, not triacylglycerols. All four classes of PL tested served as effective FA donors. Whereas 16:0 and 18:1 plasma FFA concentrations fell markedly after feeding the fat-free diet to previously fasted mice, the excess 16:0 and 18:1 FAME formed by direct methylation with diazomethane (4) increased greatly. This finding indicates that one or more PL fraction(s), rich in 16:0 and 18:1, may have accumulated in the plasma during the 20-hr feeding period.

Clearly, direct methylation of plasma FFA is a risky procedure. It should only be used with great caution and validated by another more specific method. Perhaps we have inadvertently failed in some way to follow the authors' exact procedures (4,7). Nevertheless, we have reservations about the use of techniques in which total lipid extracts are injected onto the chromatographic column, which, in our experience, can ruin a column. In addition we have concerns about the potentially serious errors that may occur due to methylation of plasma esterified lipids when plasma FFA levels are low, as in fed animals, especially in the presence of hypertriglyceridemia, such as that seen in fed TB rodents. The acetyl chloride/methanol method for specific, direct methylation of plasma FFA (7) seems so sensitive to the analytical

conditions that it could conceivably give rise to a much greater degree of transesterification than that reported by the authors (3–5% of PLFA and TGFA). However, acetyl chloride is a convenient methylating reagent when used, as described by LePage and Roy (8), to measure plasma total lipid fatty acid composition, and we use that method routinely in our laboratory.

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COMMUNICATIONS

Maternal Protein Deficiency in Rat: Effects on Central Nervous System Gangliosides and Their Catabolizing Enzymes in the Offspring

V.V.T.S. Prasad[1]
Department of Biochemistry, Faculty of Science, MS University, Baroda-390002, India

Maternal protein deficiency imposed on rats a month prior to conception, and during gestation and lactation, resulted in a significant cell loss in cerebrum, cerebellum, brain stem and spinal cord of pups at weaning. The cerebellum was the most affected central nervous system (CNS) region; it contained only 25% of the normal cell number. Undernourished pups were also found to have a lower concentration of total gangliosides in cerebrum as compared to that of controls. However, the total ganglioside concentration was unaffected in the cerebellum, brain stem and spinal cord by maternal undernutrition. In all regions, undernutrition caused significant changes in the proportions of individual gangliosides; these alterations were region-specific. Sialidase, β-galactosidase, β-glucosidase, and β-hexosaminidase, which are involved in the catabolism of gangliosides, showed higher activities in all the regions of undernourished pups, suggesting that these enzymes may play a role in maintaining the porportions of various ganglioside fractions. *Lipids 26*, 553–556 (1991).

During the period of organogenesis, an organism is less equipped to cope with adverse environmental factors than it is at maturity (1). The brain is particularly vulnerable to genetic or epigenetic factors such as hormonal imbalance or nutritional deprivation during the period of cell division and during the most rapid phase of development (2). Though the development of rat central nervous system (CNS) begins around the end of the first week of gestation, growth spurt in brain development, which includes glial multiplication, axonal growth, dendritic arborization and synaptogenesis, is a postnatal event (3).

Several studies have shown that maternal protein deficiency impairs development of CNS of the offspring. Neonatal undernutrition is known to lower the concentration of CNS lipids such as cholesterol, phospholipids and galactolipids (4,5). However, reports on the effects of pre- and postnatal undernutrition on the CNS gangliosides are few and are not unequivocal (6–8). Further, there are no studies on the effects of maternal undernutrition imposed during gestation and lactation on the enzymes involved in ganglioside metabolism. The impact of maternal undernutrition imposed *in utero* and during lactation is particularly important as the gangliosides which are increasingly being implicated in differentiation, development and functions of the nervous system, accumulate at a rapid rate between 10 and 21 days of postnatal age. Hence the present experiment was designed to examine the effects of maternal undernutrition (imposed a month before conception, and during gestation and lactation) on cell number, levels of gangliosides and activities of sialidase, β-galactosidase, β-glucosidase and β-hexosaminidase in the cerebrum, cerebellum, brain stem and spinal cord of the pups at weaning.

MATERIALS AND METHODS

Experimental design and animals. Virgin female rats of the Charles Foster strain weighing 180–200 g were divided into two groups. The control group was fed a diet containing 20% casein and the undernourished group was fed a 6.5% casein diet. The 20% casein diet contained 20% casein, 20% sucrose, 7% peanut oil, 49% sago (*manihot utilissima*), 0.3% DL-methionine, 0.2% choline bitartrate, 3.5% mineral mixture and 1% vitamin mixture (9). The low protein diet contained 6.5% casein, 61.5% sago and other components similar to that of the 20% casein diet. The low protein diet of 6.5% casein was chosen because it is nutritionally similar to the diet of poor people in the area of Baroda, India. Both groups were maintained on the respective dietary regimen a month prior to conception, and during gestation and lactation. After birth, pups from different litters were culled randomly and nursed in litters of eight. Pups at 21 days of postnatal age were sacrificed by decapitation after the body weights were recorded. Intact whole brains and spinal cords were quickly dissected out on an ice-cold glass plate and the weights were recorded. Whole brains were separated into cerebrum, cerebellum and brain stem on ice-cold glass plates. Tissue samples for DNA, protein and enzyme assays were processed immediately, and samples for lipid analysis were stored until use in chloroform/methanol (1:1, v/v) at −20°C.

Biochemical analysis. DNA was extracted by the method of Schneider (10) and estimated according to the method of Burton (11). Cell numbers were calculated as described elsewhere (12). Protein was estimated according to Lowry *et al.* (13) using bovine serum albumin as the standard. Total gangliosides were isolated and purified by the method of Seyfried *et al.* (14) and ganglioside N-acetyl neuraminic acid (NANA) was assayed by the method of Warren (15) as modified by Skoza and Mohos (16). The details of these methods are described elsewhere (12). For the separation of individual gangliosides (GQ_1, GD_{1b}, DG_{1a}, GT_{1b}, and GM), 20 × 20-cm glass plates coated with silica gel G were developed by one ascending run with chloroform/methanol/water (58:38:9, v/v/v) that contained 0.02% $CaCl_2 \cdot 2H_2O$. The bands marked after exposure to iodine vapors were scraped from the plates and the ganglioside fractions were estimated as mentioned above. Since the concentrations of monosialo gangliosides such as GM_2 and GM_3 were extremely low, the bands corresponding to all monosialo gangliosides were pooled and the fraction was designated as monosialo ganglioside (GM). The bands were confirmed as gangliosides by using the resorcinol/HCl reagent on a duplicate set. The identity of the individual anglioside bands was confirmed by

[1]Present address: CINN Research Laboratory, Columbus Hospital, 2520 N. Lake View Avenue, Chicago, IL 60614

Abbreviations: CNS, central nervous system; NANA, *N*-acetylneuraminic acid.

TABLE 1

Effect of Maternal Undernutrition on DNA and Protein Levels in Rat CNS at Weaning[a]

Tissue	Group	Tissue weight (g)	DNA		Protein		Cell number (10^6)
			mg/g tissue	mg/tissue	mg/tissue	mg/tissue	
C	CT	1.098	1.85	2.03	123	135	327
	(n=6)	±0.006	±0.007	±0.02	±0.33	±0.99	±4.0
	UN	0.843[b]	1.92	1.60[b]	123	104[b]	261[b]
	(n=6)	±0.003	±0.015	±0.015	±0.46	±0.58	±2.4
CB	CT	0.186	9.2	1.7	104	19.3	244
	(n=6)	±0.002	±0.04	±0.016	±0.4	±0.19	±2.4
	UN	0.104[b]	5.0[b]	0.52[b]	103[b]	10.7[b]	83.6[b]
	(n=6)	±0.001	±0.07	±0.004	±0.52	±0.13	±0.7
BS	CT	0.153	2.14	0.326	104	15.8	52.6
	(n=6)	±0.012	±0.043	±0.005	±1.0	±0.12	±0.84
	UN	0.097[b]	1.57[b]	0.152[b]	101	10.1[b]	24.4[b]
	(n=6)	±0.002	±0.037	±0.003	±0.47	±0.4	±0.4
SC	CT	0.182	2.52	0.458	100	18.3	73.9
	(n=6)	±0.03	±0.04	±0.008	±0.67	±0.23	±1.31
	UN	0.145[b]	2.4	0.349[b]	101	14.5[b]	56.4[b]
	(n=6)	±0.002	±0.018	±0.006	±0.4	±0.21	±1.0

[a]CT, control pups born of mothers fed 20% casein diet; UN, undernourished pups born of mothers fed 6% casein diet; C, cerebrum; CB, cerebellum; BS, brain stem; SC, spinal cord; n, number of observations; values are mean ± SE.

[b]Values significantly different from the CT group, if $p<0.05$.

simultaneously running a reference bovine brain ganglioside mixture from Sigma Chemical Company (St. Louis, MO). A typical thin-layer chromatogram of the gangliosides is shown elsewhere (12).

Enzyme assays. A 10% (w/v) homogenate was prepared by homogenizing the tissue with ice-cold glass distilled water for three min at 2000–3000 rpm, upon ice, using a Potter-Elvehjem homogenizer. The assays were carried out as described earlier (12). Statistical comparison between group means was by Student's *t*-test (17). Differences between values were considered significant for $p<0.05$.

RESULTS

Maternal undernutrition resulted in significantly lowered body, cerebrum, cerebellum, brain stem and spinal cord weights of the rat pups at 21 days of age as compared to the controls. The body weight (data not shown) of the undernourished pups was 13.5 ± 0.24 g (mean ± SE) while the control pups weighed 43.8 ± 0.28 g. The tissue weights of cerebrum, cerebellum, brain stem and spinal cord of the undernourished pups were 23, 45, 37 and 16% of the control values, respectively (Table 1). Significant reductions in DNA content (mg DNA/tissue) amounting to 16, 12, 17, and 34% respectively, were observed in the cerebrum, cerebellum, brain stem and spinal cord of undernourished pups, compared to controls. This decrease in DNA content in the CNS tissues of the undernourished pups was reflected in the lowered cell number when compared to controls. Protein concentration (mg protein/g tissue) in the undernourished pups did not show any significant changes in CNS compared to the controls.

Undernutrition also lowered the concentration (μg NANA/g tissue) of total gangliosides in cerebrum but not in cerebellum, brain stem and spinal cord (Table 2). However, total ganglioside content (μg NANA/tissue) was lowered in cerebrum, cerebellum, brain stem and spinal cord of the undernourished pups compared to controls. In addition, maternal undernutrition was also found to alter the proportions of the individual gangliosides. Polysialoganglioside, GQ_1 increased in cerebrum, cerebellum and brain stem and decreased in spinal cord of the undernourished pups compared to controls. Trisialo ganglioside, GT_{1b}, was not altered in the cerebrum, but decreased in cerebellum and brain stem, and increased in the spinal cord. By comparison, disialo ganglioside, GD_{1b}, decreased in cerebrum, increased in cerebellum and brain stem, and was unaffected in the spinal cord. In the spinal cord and brain stem, the proportion of GD_{1a} increased while declining in the cerebrum. However, the monosialo ganglioside (GM) fraction increased in cerebrum and cerebellum, and decreased in brain stem and spinal cord. Sialidase activity was higher than that found in controls in cerebrum, cerebellum, brain stem and spinal cord of the undernourished pups (Table 3). β-Glucosidase and β-galactosidase showed a higher activity, in general, in all the CNS regions studied. β-Hexosaminidase showed no significant change in the cerebrum but was higher in the cerebellum, brain stem and spinal cord of pups born of mothers fed the 6.5% casein diet.

DISCUSSION

As a low protein diet and low tissue reserves of the mother can affect the quantity of milk (18,19), pre- and postnatal undernutrition in rat pups was produced by feeding mothers a 6.5% casein diet one month prior to conception, and during gestation and lactation. Maternal protein deprivation during the period of brain development caused reductions in the cell number of cerebrum, cerebellum, brain stem and spinal cord of the pups at weaning. Cerebellum was the most affected region, containing only 25% of the normal cell number. However, the concentration of total gangliosides was lowered only in cerebrum

TABLE 2

Effect of Maternal Undernutrition on CNS Gangliosides at Weaning[a]

		Total gangliosides		Ganglioside species (% of total ganglioside NANA)				
Tissue	Group	μg NANA/ g tissue	μg NANA/ tissue	GQ_1	GT_{1b}	GD_{1b}	GD_{1a}	GM
C	CT	706	791	9.9	21.1	14.1	43.8	11.1
	(n=8)	±4.6	±11.8	±0.4	±0.32	+0.37	±0.49	±0.49
	UN	491[b]	409[b]	12.1[b]	21.2	9.4[b]	41.3[b]	16.1[b]
	(n=8)	±9.8	±7	±0.33	±0.3	±0.32	±0.44	±0.14
CB	CT	398	71.1	6.7	44.3	10.3	26.5	12.5
	(n=8)	±6.5	±1.5	±0.5	±0.2	±0.3	±0.39	±0.38
	UN	370	36[b]	16.5[b]	28.3[b]	12.1[b]	26.3	16.7[b]
	(n=8)	±13	±1.0	±0.6	±0.4	±0.31	±0.49	±1.5
BS	CT	395	59.6	3.3	34.7	22.1	22	17.8
	(n=8)	±13	±2	±0.3	±0.3	±0.7	±1	±0.7
	UN	420	38.5[b]	7.8[b]	15.1[b]	28.1[b]	36.3[b]	13.3[b]
	(n=8)	±16	±2	±0.3	±0.2	±0.4	±0.5	±0.4
SC	CT	291	56	18.6	31.6	13.3	22	14.6
	(n=8)	±16	±1	±0.2	±0.8	±0.6	±0.4	±0.5
	UN	318	44.5[b]	13.2[b]	38[b]	13.5	27[b]	8.4[b]
	(n=8)	±6	±0.8	±0.4	±0.8	±0.3	±0.6	±0.6

[a,b]Same as in Table 1.

TABLE 3

Effect of Undernutrition on Rat CNS Ganglioside Catabolizing Enzymes at Weaning[a]

		β-D-Galactosidase				
Tissue	Group	Activity at pH 3.1	Activity at pH 4.5	β-D-Glucosidase	β-D-Hexosaminidase	Sialidase
		(Enzyme units/g tissue)				
C	CT	222	128	210	2930	13.1
	(n=10)	±0.9	±0.5	±4.5	±32	±0.41
	UN	233[b]	144[b]	229[b]	2936	16.0[b]
	(n=10)	±1.8	±1.8	±3.4	±50	±0.39
CB	CT	422	219	137	2897	7.9
	(n=10)	±4.4	±1.7	±2	±39	±0.78
	UN	435	288[b]	162[b]	3186	13.9[b]
	(n=10)	±4.3	±7.1	±2	±43	±0.35
BS	CT	388	225	171	2695	8.9
	(n=10)	±5.7	±2.5	±3.1	±60	±0.14
	UN	434[b]	248	197[b]	2978[b]	12.5[b]
	(n=10)	±8.8	±13.3	±5.3	±43	±0.24
SC	CT	330	155	184	1985	8.1
	(n=10)	±7.3	±2.9	±3.2	±39	±0.76
	UN	399[b]	277[b]	237[b]	2277[b]	10.8[b]
	(n=10)	±10.3	±8.9	±6.4	±43	±0.4

[a,b]Same as in Table 1.

of the undernourished pups. The reduced concentration of total gangliosides may be related to the reported impairments in dendritic development (20) and retarded synaptogenesis (21) following undernutrition. The observed decrease in cerebral ganglioside concentration in the present study agrees well with reports of decreased concentration of forebrain gangliosides in undernourished rat pups (6) and brain gangliosides in malnourished children (22). Karlsson and Svennerholm (8) found a lowered concentration of total gangliosides in the forebrains of undernourished pups at 10 and 15 days of postnatal age but not at 21 days. This may be due to the higher amounts of protein, duration of treatment and the smaller litter size employed in their study when compared to the present study. Even reports on effects of postnatal undernutrition on rat brain gangliosides were not consistent

(5,21,23–27). The inconsistency in the results may be due to differences in the strains of rats used, variations in the methods employed to produce undernutrition, and length of treatment.

In the present study, the concentration of total gangliosides was significantly lowered only in the cerebrum due to undernutrition. Although prenatal undernutrition was found to reduce the concentration of spinal cord gangliosides in neonatal pups (12), pre- and postnatal undernutrition did not alter the concentration of the spinal cord gangliosides in the pups at weaning. However, pre- and postnatal undernutrition induced significant alterations in the proportions of individual gangliosides in all the CNS regions. The effects on the different fractions were tissue specific. Lowered proportion of GD_{1b} in cerebrum of the undernourished pups is in agreement with the reported reduction in the GD_{1a} in the forebrain of malnourished children (22). As GD_{1a} is mostly located in the dendrites (28) and as undernutrition impairs the dendritic arborization in rat pups (20), the decrease in the GD_{1a} may be attributed to deficient dentritic growth following the pre- and postnatal undernutrition. Interestingly, the proportion of monosialo ganglioside (GM) increased in cerebrum and cerebellum and decreased in myelin-rich brain stem and spinal cord of the undernourished pups at weaning. This decrease in monosialo gangliosides, which are predominant in myelin, correlates well with reported deficits in myelin due to undernutrition (29).

In agreement with the lowered concentration of cerebral gangliosides, the activities of sialidase, β-galactosidase and β-glucosidase were higher in the cerebrum of the undernourished pups than in the controls. In contrast, a decrease in brain sialidase activity was reported in 21-day-old rat pups born of mothers fed a 8% casein diet (7). Adlard and Dobbing (30) did not find any change in brain β-galactosidase activity in rat pups born of mothers fed restricted amounts of food during gestation and lactation. However, in the present study, β-galactosidase activity increased in CNS of the undernourished pups. In cerebellum, brain stem and spinal cord, although the catabolizing enzyme activities were higher, alterations were observed only in the proportions of the individual gangliosides, but not in the concentration of total gangliosides following undernutrition. However, the degree of increase in the activities varied with the enzyme and the region, suggesting a role for these enzymes in altering the proportions of the individual gangliosides.

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Supplementation with Evening Primrose Oil in Atopic Dermatitis: Effect on Fatty Acids in Neutrophils and Epidermis

Liselotte Schäfer[a,*] and **Knud Kragballe**[b]

[a]Aarhus Oliefabrik A/S, Research and Development Laboratories, DK-8100 Aarhus C and [b]Marselisborg Hospital, Department of Dermatology, DK-8000 Aarhus C, Denmark

We investigated the effect of oral supplementation with evening primrose oil, containing 72% linoleic acid (18:2n-6) and 10% γ-linolenic acid (18:3n-6), on the epidermal and neutrophil phospholipid fatty acid composition in 15 patients with atopic dermatitis (AD). Three different dose levels, 4, 8 and 12 capsules per day containing 0.5 g oil, were given to three groups of patients. The only n-6 fatty acid showing a significant ($p < 0.05$) dose-related increase was dihomo-γ-linolenic acid (20:3n-6) in neutrophil phospholipids. The highest dose increased dihomo-γ-linolenic acid by 45% in neutrophil phospholipids, by 46% in lesion-free epidermal phosphatidylcholine, and by 15% in lesion-free epidermal phosphatidylethanolamine. In both lesional and lesion-free epidermis, supplementation resulted in a rise in the ratio between n-6 and monounsaturated fatty acids, reaching significance ($p < 0.05$) in lesional epidermis. This study shows that moderate and favorable fatty acid changes can be obtained in the epidermis of AD patients, when given 6 g per day of oil rich in n-6 fatty acids. The abnormal lipid and fatty acid pattern of the atopic epidermis may be involved in the pathogenesis of the disease, and should therefore be the target for future therapeutic approaches with fatty acid supplements.
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The importance of using fatty acid supplements as a treatment of atopic dermatitis (AD) has been discussed for more than half a century (1). In the last ten years, oil from the seeds of evening primrose *(Oenothera biennis)* has attracted special attention due to its unusually high content (10%) of γ-linolenic acid (GLA). In a recently published meta-analysis of nine double blind trials with evening primrose oil, Morse *et al.* (2) found that the overall disease severity of AD patients, especially itching, was significantly reduced in a dose-dependent manner by oral administration of this oil. The functioning of the Δ6 desaturase, an enzyme which converts linoleic acid (LA) into GLA, was thought to be defective in AD patients (3). If this were true, a direct supply of GLA would mean a bypass of this purported metabolic blockade. In previous fatty acid supplementation trials concerning patients with skin disease, only the plasma fatty acid composition was analyzed (4-6). Supplementation trials with oils containing GLA in humans have resulted in the elevation of plasma phospholipid levels of dihomo-γ-linolenic acid (DGLA), which is the precursor of the putative anti-inflammatory substances 15-hydroxy-eicosatrienoic acid and prostaglandin E_1 (7).

*To whom correspondence should be addressed at King Gustav V:s Research Institute, P.O. Box 60004, S-10401 Stockholm, Sweden.

Abbreviations: AA, arachidonic acid (20:4n-6); AD, atopic dermatitis; DGLA, dihomo-γ-linolenic acid (20:3n-6); FAME, fatty acid methyl ester; GLA, γ-linolenic acid (18:3n-6); LA, linoleic acid (18:2n-6); MUFA, monounsaturated fatty acids: PC, phosphatidylcholine; PE, phosphatidylethanolamine.

In two previous communications (8,9), we reported that our AD patients apparently did not have any abnormalities in fatty acid desaturation; neutrophil phospholipids and adipose tissue triglycerides showed a normal fatty acid pattern. However, the phospholipid content of the epidermis of these patients was abnormally high. Furthermore, the level of n-6 polyunsaturated fatty acids was reduced and that of monounsaturated fatty acids (MUFA) increased in the epidermal phospholipids, resulting in a highly abnormal ratio between these two fractions. The disease severity was directly related to the content of MUFA in the epidermis, and inversely related to the content of n-6 fatty acids. We interpreted these findings as being the consequence of incomplete lipid transformation combined with increased phospholipase A_2 activity in the epidermis of these AD patients.

The objective of the present study was to measure the magnitude of fatty acid changes in neutrophil and epidermal phospholipids attained by supplementation with evening primrose oil in doses commonly employed.

MATERIALS AND METHODS

Subjects and design. Fifteen adult patients with AD, 8 premenopausal women and 7 men, attending the outpatient clinic of the Department of Dermatology at Marselisborg Hospital (Aarhus, Denmark) agreed to participate in the study. The group of patients was the same as that described previously (8,9). Their age ranged from 19 to 51; the mean age was 32.3 ± 10.1 years. In 11 of the patients, eczema had commenced before the second year of life. In one patient it started at 15, and in three during adulthood. Five were smokers and four female patients used oral contraceptives. The affected body surface ranged from 3 to 70%. In 10 of the patients it ranged from 10 to 50%. None of the patients received topical or systemic treatment for 2 wk prior to the study. Subjects which were over- or underweight (more than 20% from the ideal weight), or which had diabetes or hyperlipidemia were excluded from the study. The patients were randomly assigned to three dose levels of evening primrose oil (Quest Vitamins, London, U.K.), namely 4, 8 or 12 capsules, each containing 0.5 g, per day. The fatty acid composition of the oil given as % by weight of total fatty acids was: 16:0 6.3%, 18:0 1.6%, 18:1 10.1%, 18:2n-6 71.5%, and 18:3n-6 9.9%. All patients were instructed to use only their usual emollient during the trial. Blood samples were obtained from fasted patients at week 0 and 10. Neutrophils were isolated by the Hypaque-Ficoll technique, according to Boyum (10). Skin biopsies, weighing 50-250 mg, were taken with a keratome set at 0.2 mm, and frozen immediately in liquid nitrogen, as previously described (11). Approximately two-thirds of the epidermis was removed.

Analytical procedures. Lipid extraction, separation, and analysis were carried out as previously described (8,9). Briefly, lipids were extracted from neutrophils and epidermal specimens with chloroform/methanol (2:1, v/v). Total

TABLE 1

Fatty Acid Composition of Epidermal Phosphatidylcholine from Lesion-Free and Lesional Areas Before and After Supplementation with Three Doses of Evening Primrose Oil for 10 Wk

		Lesion-free epidermal PC			Lesional epidermal PC		
Fatty acid	Dose[a]	Week 0[b]	Week 10[b]	Δ %[c]	Week 0[b]	Week 10[b]	Δ %[c]
18:2n-6	4	28.8 ± 2.8	27.3 ± 4.2	−3.4	25.2 ± 3.4	27.0 ± 3.4	8.8
	8	29.8 ± 1.9	31.2 ± 4.6	4.3	28.4 ± 3.4	28.6 ± 5.8	1.8
	12	31.9 ± 4.2	31.0 ± 1.4	−1.2	28.0 ± 4.5	27.6 ± 3.1	−0.7
20:3n-6	4	1.40 ± 0.43	1.53 ± 0.60	0.2	1.16 ± 0.37	1.58 ± 0.46	40.3
	8	1.27 ± 0.42	1.41 ± 0.43	15.0	1.24 ± 0.31	1.37 ± 0.64	6.6
	12	1.10 ± 0.26	1.60 ± 0.49[d]	46.3	0.94 ± 0.22	1.51 ± 0.26[d]	62.9
20:4n-6	4	4.12 ± 0.40	5.90 ± 1.93	37.3	3.37 ± 0.31	5.61 ± 1.76	68.2
	8	5.12 ± 1.66	5.84 ± 2.56	10.7	4.19 ± 1.53	4.40 ± 2.86	5.7
	12	3.67 ± 0.82	5.53 ± 1.41[d]	55.7	2.98 ± 0.32	5.04 ± 0.80[d]	69.9
Total n-6[e]	4	35.1 ± 2.2	35.6 ± 2.3	2.2	30.6 ± 3.5	35.4 ± 1.9[d]	16.6
	8	37.0 ± 1.8	39.3 ± 3.8	6.4	34.9 ± 2.7	35.4 ± 2.6	3.5
	12	37.5 ± 4.5	39.2 ± 2.5	5.3	32.8 ± 4.5	35.2 ± 4.0	7.5
Total MUFA[f]	4	28.7 ± 1.6	28.2 ± 5.5	−0.2	33.0 ± 3.3	27.0 ± 2.6[d]	−18.0
	8	25.8 ± 0.8	23.7 ± 2.0	−7.9	27.9 ± 2.8	27.7 ± 3.3	−3.7
	12	26.0 ± 4.0	23.8 ± 2.0[d]	−7.3	30.4 ± 4.4	27.1 ± 4.4[d]	−10.7
Total SFA[g]	4	35.1 ± 1.9	35.0 ± 5.3	−1.7	35.4 ± 1.3	36.3 ± 2.3	2.6
	8	36.0 ± 1.4	35.5 ± 2.0	−1.4	35.8 ± 1.2	35.5 ± 2.8	−0.2
	12	35.5 ± 1.0	35.7 ± 1.3	0.6	35.6 ± 0.8	36.3 ± 1.0	1.9
n-6/MUFA	4	1.23	1.29		0.94	1.33[d]	
	8	1.44	1.68		1.27	1.29	
	12	1.49	1.66		1.12	1.34[d]	

[a] Number of capsules (0.5 g) per day; each dose was taken by five subjects.

[b] Values are mean ± S.D. and given as % weight of total fatty acids.

[c] Average of individual changes in % of initial value.

[d] Significantly ($p < 0.05$) different from week 0 by Wilcoxon's signed rank test.

[e] Include 18:2, 18:3, 20:3, 20:4, 22:4, and 22:5.

[f] Include 14:1, 15:1, 16:1, 17:1, 18:1, 20:1n-11, and 20:1n-9.

[g] Include 14:0, 15:0, 16:0, 17:0, 18:0, 20:0, and 22:0.

phospholipids were isolated from the neutrophil lipid extract by solid-phase extraction on prepacked silica cartridges. Epidermal phospholipids were isolated from the epidermal lipid extract on an aminopropyl phase, packed in a glass column. Epidermal phospholipids were further separated into classes by normal phase high-pressure liquid chromatography on a silica column in a gradient of acetonitrile/water, as previously described (9). Phospholipids were detected at 203 nm. Individual phospholipid classes were identified using external standards. Individual phospholipid fractions were collected manually and taken to dryness in a rotary evaporator at 50°C. Fatty acid methyl esters (FAME) were prepared by transesterification with sodium methoxide. FAME were analyzed on a 50 m × 0.25 mm capillary fused silica column coated with a 0.2 μm film of CP SIL88 (Chrompack, Delft, The Netherlands). The carrier gas was helium and the flow rate 2 mL/min. Injection was on-column at 90°C. The oven was programmed to rise to 210°C within 6 min and then run isothermally for 18 min. A flame-ionization detector was used, and peak areas were integrated automatically. FAME were identified by comparison with a standard mixture of known composition (68A and 68B, NuChek Prep, Elysian, MN). The relative contents of major peaks of these standards agreed within 0.2% with the values stated by the manufacturer.

Statistical analysis. All data are presented as mean ± S.D. Dose response relationships were analyzed by ANOVA. The fatty acid changes within each dosage group were analyzed by Wilcoxon's signed rank test.

RESULTS

Linoleic acid (LA) comprised 72% (by weight of total fatty acids) of the oil supplement. However, the level of LA did not show any systematic change in either neutrophil or epidermal phospholipids after 10 wk of supplementation (Tables 1 and 2). The level of GLA, comprising 10% of the oil, remained unchanged at 0.1–0.2% (by weight of total fatty acids) in epidermal and neutrophil phospholipids (data not shown). The changes in DGLA and AA in the epidermis were positive in all three dosage groups, but significant ($p < 0.05$ by Wilcoxon's signed rank test) only in subjects on the highest dose (Table 1). The total content of n-6 fatty acids tended to increase upon supplementation in all three dosage groups in both lesional and lesion-free epidermal phosphatidylcholine (PC), while that of total MUFA tended to decrease. The ratio of n-6/MUFA significantly increased at week 10 in lesional epidermis in the lowest and highest dosage group (Table 1). In phosphatidylethanolamine (PE) from lesion-free epidermis, the average changes relative to week 0 on the highest dose (12 capsules/day) were 8% in LA, 15% in DGLA, 17% in AA, 2% in total n-6 fatty acids, 4% in MUFA, and −2% in saturated fatty acids. None of these changes reached

TABLE 2

Fatty Acid Composition of Neutrophil Total Phospholipids Before and After Supplementation With Evening Primrose Oil for 10 Wk

Fatty acid	Dose[a]	Week 0[b]	Week 10[b]	Δ %[c]
18:2n-6	4	12.3 ± 1.5	12.2 ± 1.7	−0.3
	8	12.3 ± 1.1	12.2 ± 1.2	−0.2
	12	11.9 ± 1.3	11.7 ± 1.6	−1.3
20:3n-6	4	2.71 ± 0.81	2.83 ± 0.66	6.1[d]
	8	1.91 ± 0.48	2.65 ± 0.52[e]	42.9[d]
	12	2.06 ± 0.65	2.91 ± 0.55[e]	45.1[d]
20:4n-6	4	15.3 ± 2.0	15.9 ± 1.4	5.3
	8	15.7 ± 2.1	16.1 ± 1.5	4.8
	12	16.9 ± 2.8	16.1 ± 1.0	−2.9
Total n-6[f]	4	33.6 ± 1.7	34.8 ± 1.8	3.5
	8	32.5 ± 1.7	34.1 ± 1.7	5.3
	12	34.0 ± 2.6	34.2 ± 2.2	0.8
Total MUFA[g]	4	32.7 ± 2.3	32.4 ± 2.2	−1.1
	8	33.5 ± 0.8	32.9 ± 2.8	−1.7
	12	33.6 ± 2.7	32.8 ± 1.9	−2.3
Total SFA[h]	4	29.4 ± 1.2	28.9 ± 0.6	−1.6
	8	29.4 ± 1.8	28.7 ± 1.2	−2.1
	12	28.0 ± 2.4	28.8 ± 0.7	3.3

[a]Number of capsules (0.5 g) per day; each dose was taken by five subjects.

[b]Values are mean ± S.D. and given as % weight of total fatty acids.

[c]Average of individual changes in % of initial value.

[d]Significant ($p < 0.05$) dose-related rise by ANOVA.

[e]Significantly ($p < 0.05$) different from week 0 by Wilcoxon's signed rank test.

[f]n-6 fatty acids include 18:2, 18:3, 20:3, 20:4, 22:4, and 22:5.

[g]Monounsaturated fatty acids include 14:1, 15:1, 16:1, 17:1, 18:1, 20:1n-11, and 20:1n-9.

[h]Saturated fatty acids include 14:0, 15:0, 16:0, 17:0, 18:0, 20:0, and 22:0.

statistical significance. The change in neutrophil phospholipid fatty acids upon supplementation is shown in Table 2. Here a significant dose-related rise in DGLA was found ($p < 0.05$ by ANOVA).

DISCUSSION

In previous studies on the effect of fatty acid supplementation on patients with AD, emphasis was placed on the clinical effect and on the plasma phospholipid fatty acid pattern (5,12,13). The effect of supplementation with n-6 fatty acids on epidermal phospholipids and neutrophils has, to our knowledge, not been investigated before in humans. Our data show that supplementation with evening primrose oil resulted in the elevation of DGLA in neutrophils and epidermal phopholipids, as has been shown in plasma (4). Despite the high concentration of LA and GLA in evening primrose oil, the levels of these fatty acids remained virtually unchanged. This shows that LA and GLA are rapidly desaturated and elongated when taken orally.

The effect of supplementation with evening primrose oil on the tissue phospholipid fatty acid composition has been investigated in animals. In mice macrophages, the DGLA concentration was increased 5-fold by feeding mice 25% (% by weight of total fatty acids) of GLA in the form of borage oil for three weeks (14). In another study with guinea pigs (7), using the same dose for eight weeks, a 10-, 4- and 1.3-fold elevation of GLA, DGLA and arachidonic acid (AA), respectively, and a 2-fold reduction in LA were seen in the epidermal phospholipid fraction, compared to animals fed a safflower oil-enriched diet. An analysis of the lipoxygenase products formed in the epidermis of these animals revealed that metabolites of DGLA, such as 15-hydroxy-eicosatrienoic acid and prostaglandin E_1 were synthesized. These substances have been proposed to have anti-inflammatory properties (7). Dietary GLA may therefore be a source for endogenous generation of anti-inflammatory metabolites in the epidermis which may suppress local inflammatory reactions (15). However, the highest dose of evening primrose oil normally given to patients is approximately 0.6% (% by weight of total dietary fat), corresponding to 12 × 0.5 g capsules per day. Our data show that this dose only leads to a 50% increase in DGLA in the epidermis after 10 wk of supplementation, reaching levels far less than in experimental animals. It is questionable whether the level of DGLA in human epidermis, attained by oral supplementation with evening primrose oil, would be large enough to support the synthesis of local anti-inflammatory metabolites, even if supplementation were continued for longer periods. The beneficial effect of n-6 fatty acid supplementation in AD, as reported by others (2), probably relies on other mechanisms. In a previous publication (9) we reported that the epidermis of AD patients had a lower n-6/MUFA ratio than the epidermis of healthy subjects, and that the disease severity correlated directly with the total MUFA content of the epidermal phospholipids. In essential fatty acid deficiency, which also results in dry scaling skin, an imbalance is found in the ratio between LA and MUFA (16). Although AD patients are not deficient in essential fatty acids, the characteristic dry skin could be caused by a partial substitution of polyunsaturated fatty acids by MUFA, leading to a defective structure of the epidermal water barrier. The present results show that supplementation with n-6 fatty acids results in a rise in the n-6/MUFA ratio, particularly in lesional epidermis. Subjects on the highest dose actually increased their n-6/MUFA ratio to a value of 1.68 in lesion-free epidermis. Previously we reported a value of 1.64 for healthy epidermis (9). Because this study was not controlled, we cannot relate the clinical outcome of the patients to the observed changes in the fatty acid composition of epidermal phospholipids. If and how these changes are causally related to the disease remains to be established. Because linoleic acid is an essential component of acylceramides, which constitute the epidermal water barrier (17), it would be of considerable interest to study the effect of n-6 fatty acid supplementation on the metabolism of acylceramides in the atopic epidermis.

In conclusion, this study shows that supplementation with up to 600 mg/day of evening primrose oil increases the level of n-6 fatty acids, especially DGLA, by 15–60% in neutrophil and epidermal phospholipids. Furthermore, supplementation results in a beneficial shift in the ratio between n-6 and MUFA. However, no clear evidence has yet been presented in favor of GLA as being the active component in evening primrose oil. New studies should clarify if more conventional vegetable oils containing high levels of linoleic acid, such as safflower or sunflower oil, may mediate the desired lipid changes in the epidermis, and if the dose should be further increased.

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4-Desmethylsterols from the Marine Bivalve *Macoma balthica*

Andrzej Jarzebski*
Institute of Oceanology, Polish Academy of Sciences, 81-967 Sopot, Poland

4-Desmethylsterols from *Macoma balthica* L. were investigated by gas chromatography and gas chromatography/mass spectrometry after isolation by argentation and column chromatography. Twenty-three Δ^0- and Δ^5-sterols with saturated and unsaturated side chains were identified. It is suggested that sterols in *M. balthica* are derived from dietary sources. The great diversity of minor 4-desmethylsterols is likely to be due to their role as intermediates in the metabolism of dietary sterols.
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The sterol composition of marine mollusks has been investigated in more detail than that of any other class of marine invertebrates (1-6). Application of modern analytical techniques has revealed that Bivalvia are unique in containing a great diversity of various Δ^5- and $\Delta^{5,22}$-sterols. Little is known about the origin of this complex mixture of sterols in bivalves.

Mollusks are an important link in the food chain in the Baltic Sea. One of these mollusks, *Macoma balthica* (a facultative filter/surface film deposit bivalve), is most abundant in the benthic fauna in the Gulf of Gdańsk. The major sterols of *M. balthica* have previously been investigated (7,8). Cholest-5-en-3β-ol (cholesterol) was the most abundant sterol; the other major *M. balthica* sterols were common constituents of bivalves (1-8). Some sterols (*e.g.* 24-methylcholesta-5,22-dien-3β-ol, 24-methylcholesta-5,24(28)-dien-3β-ol, 24-methyl-cholest-5-en-3β-ol) were typical phytoplankton sterols (4,5).

The minor sterols of *M. balthica* have previously not been characterized. In the present study, we therefore reexamined the sterols of *M. balthica* with special focus on the minor components.

MATERIALS AND METHODS

Gas chromatography was performed with a Carlo Erba (Milan, Italy) Fractovap 4100 gas chromatograph equipped with a 30 m × 0.3 mm I.D. OV-1 glass capillary column at 260°C with nitrogen as a carrier gas. Gas chromatography/mass spectrometry measurements were carried out with a Finnigan (Bremen, Germany) MAT 44S system using a OV-1 glass capillary column. ^{1}H nuclear magnetic resonance (NMR) spectra were recorded in $CDCl_3$ on a Bruker (Karlsruhe, Germany) 300-MHz spectrometer.

Argentation column chromatography was performed on silica gel 60 (0.040-0.063 mm; Merck, Darmstadt, Germany) (100 g) impregnated with silver nitrate (25 g) and activated at 100°C. Argentation thin-layer chromatography was carried out on Merck 20 × 20 cm aluminum sheets coated with silica gel impregnated with silver nitrate according to the method described by Pill *et al.* (9). Chromatograms were developed twice in chloroform/petroleum hydrocarbons (b.p. 40-60°C) (1:1, v/v).

Macoma balthica specimens (1.5 kg of wet weight) were collected from the Gulf of Gdańsk (Southern Baltic), and their lipids were extracted by the Bligh-Dyer method (10). Total sterols (690 mg, 3% of lipids) were isolated from the unsaponifiable part of the lipids as described previously (11). Re-chromatography of the total sterol fraction on a silica gel column using petroleum hydrocarbons (b.p. 40-60°C)/ethyl acetate yielded a fraction of 4-desmethylsterols (676 mg, 98.0% of total sterols), as detailed previously (12). 4-Desmethylsterols were acetylated with pyridine/acetic anhydride (1:1, v/v) for 24 hr at room temperature. The 4-desmethylsteryl acetates were chromatographed on a $AgNO_3$/silica gel gravity column (35 × 2.8 cm I.D.) with 420 mL each of 0, 2, 5, 10, 15, 20, 27.5, 35, 50 and 70% benzene in petroleum hydrocarbons (b.p. 40-60°C). Fractions (12 mL) were collected, analyzed by gas chromatography (GC) and combined as indicated to give nine fractions which were analyzed by gas chromatography/mass spectrometry (GC/MS).

RESULTS AND DISCUSSION

Total sterols isolated from *Macoma balthica* were chromatographed on a silica gel column to give fractions containing 20-isosterols (12) and 4-desmethylsterols. 4,4-Dimethylsterols and 4-methylsterols were not detected. This is important because these sterols are intermediates in the biosynthesis of cholesterol (the main *M. balthica* sterol) from simple precursors such as acetyl-CoA and mevalonic acid (13-15). The absence of 4,4-dimethylsterols and 4-methylsterols may be taken as an indication for the absence of *de novo* sterol synthesis in the bivalve studied. Therefore, sterols in *M. balthica* appear to be derived from dietary sources.

The 4-desmethylsterol fraction was acetylated in the usual manner. The acetate mixture was fractionated by argentation column chromatography. GC and GC/MS analyses of the fractions obtained by argentation column chromatography permitted the identification of the sterols, including the minor components. Sterols were identified by comparison of their retention times relative to cholesteryl acetate based on literature data (16) and by mass spectral fragmentation. The results are summarized in Table 1; the structures of the *M. balthica* sterols identified are shown in Figure 1.

The major 4-desmethylsterols, including C-24 alkylated Δ^5- and $\Delta^{5,22}$-sterols, were those previously detected in *M. balthica* (7,8). The C-24 configuration of C_{28}- and C_{29}-sterols occurring in marine invertebrates has received considerable attention in recent years. It was our intention to assign the stereochemistry at C-24 for 24-methylcholesta-5,22-dien-3β-ol, one of the major *M. balthica* sterols. Its content in the clam investigated underwent seasonal variations (17,18), which may be a further indication of the 24-methylcholesta-5,22-dien-3β-ol's exogenous origin.

*To whom correspondence should be addressed at Institute of Oceanology, Polish Academy of Sciences, ul. Powstańców Warszawy 55, 81-967 Sopot, Poland.

Abbreviations: GC, gas chromatography; GC/MS, gas chromatography/mass spectrometry; NMR, nuclear magnetic resonance.

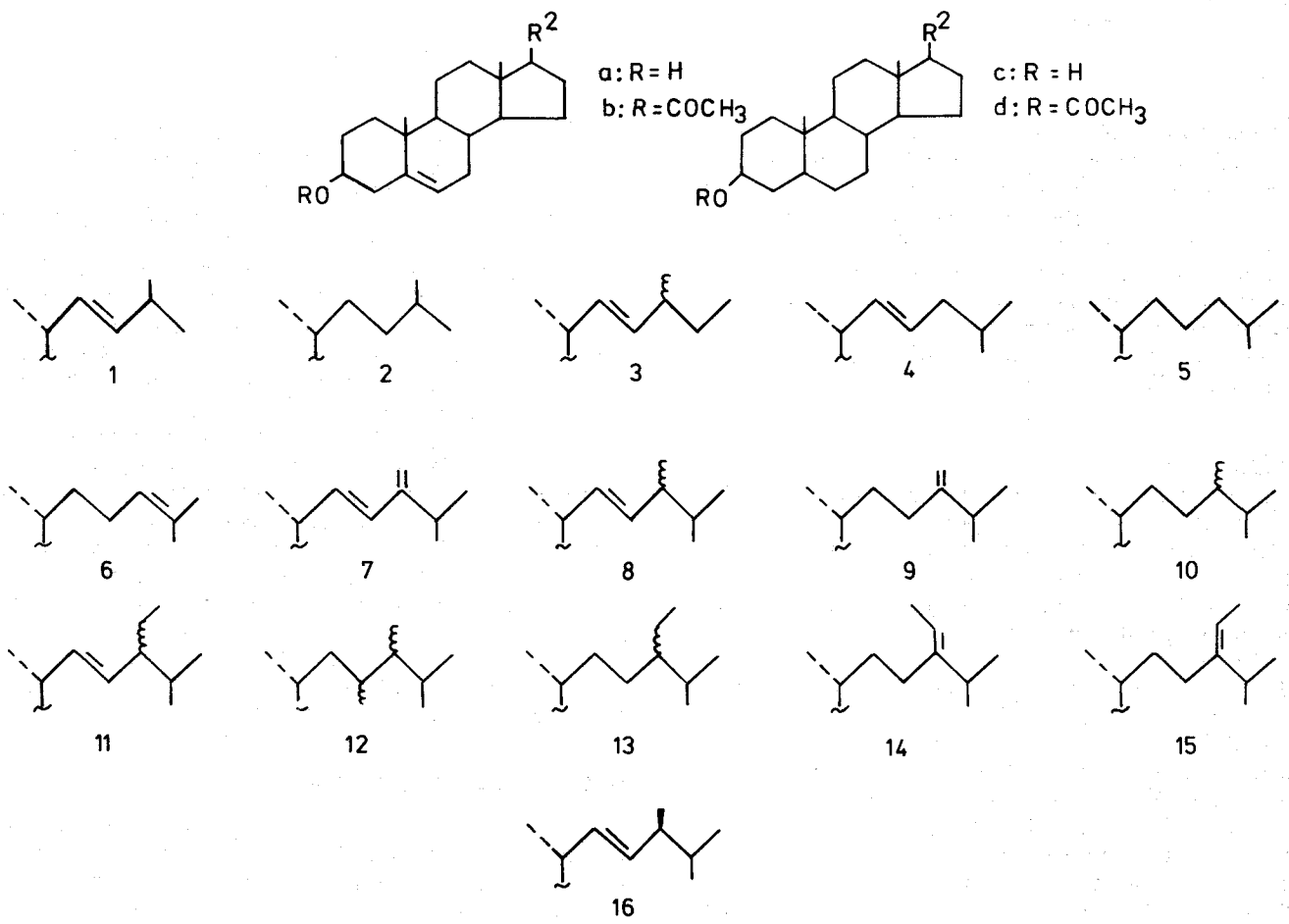


FIG. 1. 4-Desmethylsterols from *Macoma balthica*.

Fractions No. 150–160 from argentation chromatographic column contained 24-methylcholesta-5,22-dien-3β-yl acetate as the major component (identified by GC/MS) accompanied by a small quantity of acetates of: Δ^5-sterols (**5b, 10b, 13b**), other $\Delta^{5,22}$-sterols (**3b, 11b**) and some other minor sterols. Argentation thin-layer chromatography of the acetate mixture enabled us to isolate the major sterol of this group in satisfactory purity (about 95% by GC) to determine its C-24 stereochemistry. The ^{1}H NMR spectrum showed three methyl singlets at δ 0.692 (C-18), 1.018 (C-19) and 2.03 (CH_3CO); four doublets, corresponding to methyl groups, at δ 0.816 (J = 6.7 Hz, C-26 or C-27), 0.833 (J = 6.7 Hz, C-26 or C-27), 0.908 (J = 6.8 Hz, C-28) and 1.00 (J = 6.8 Hz, C-21); signals for olefinic protons at δ 5.16 (*m*, 2H, 22-H and 23-H) and 5.37 (*m*, 1H, 6-H); and a broad signal at δ 4.60 ppm (*m*, 1H, 3-H). These data are in good agreement with those reported in the literature for a (24S)-isomer (19). Therefore the structure of the sterol was assigned as (24S)-24-methylcholesta-5,22-dien-3β-ol. This sterol had first been isolated from the diatom *Phaeodactylum tricornutum* by Rubinstein and Goad (20). Later it was suggested that diatoms are the primary source of (24S)-24-methylcholesta-5,22-dien-3β-ol which is one of the prominent sterols in bivalves and other marine invertebrates (4,5). Its exclusive (no traces of 24R-isomer were observed in the ^{1}H NMR spectrum) occurrence in *M. balthica* is consistent with the hypothesis that diatoms are the main food source of this clam (21).

Among the minor components of the 4-desmethylsterol fraction from *M. balthica*, several C_{26}-C_{29} Δ^0- and Δ^5-sterols with saturated and unsaturated (Δ^{22} and/or Δ^{24}) side chains were found. In relation to other species of Bivalvia (3–6), *M. balthica* possesses relatively high content and diversity of stanols. The origin of stanols in the clam studied raises an interesting question. Stanols appear in many sediments as a result of microbiological transformation of Δ^5-sterols (22). Stanols were also found in large quantity in sediments from the Gulf of Gdańsk (Jarzebski, A., unpublished results). It is well-known that organic matter from surface sediments is an important component of the food of *M. balthica* (21). It could therefore be postulated that stanols occurring in *M. balthica* originate from sediments.

The most important source of food for *M. balthica* is phytoplankton (especially diatoms) and the organic matter derived from phytoplankton (21). The sterol composition of phytoplankton from the Gulf of Gdańsk is not

TABLE 1

4-Desmethylsterols from *Macoma balthica*

	Sterol	GC RRT[a]	%
1a	24-Norcholesta-5,22-dien-3β-ol	0.66	7.6
1c	24-Norcholest-22-en-3β-ol	0.68	0.2
2a	24-Norcholest-5-en-3β-ol	0.75	0.3
2c	24-Norcholestan-3β-ol	0.77	0.1
3a	27-Nor-24-methylcholesta-5,22-dien-3β-ol	0.88	1.2
4a	Cholesta-5,22-dien-3β-ol	0.91	6.4
4c	Cholest-22-en-3β-ol	0.93	0.2
5a	Cholest-5-en-3β-ol	1.00	41.4
5c	Cholestan-3β-ol	1.02	4.6
6a	Cholesta-5,24-dien-3β-ol	1.09	3.9
7a	24-Methylcholesta-5,22,24(28)-trien-3β-ol	1.10	0.1
16a	(24S)-24-Methylcholesta-5,22-dien-3β-ol	1.11	7.9
8c	24-Methylcholest-22-en-3β-ol	1.14	0.1
9a	24-Methylcholesta-5,24(28)-dien-3β-ol	1.27	9.5
10a	24-Methylcholest-5-en-3β-ol	1.29	7.3
10c	24-Methylcholestan-3β-ol	1.32	0.3
11a	24-Ethylcholesta-5,22-dien-3β-ol	1.37	1.5
11c	24-Ethylcholest-22-en-3β-ol	1.40	0.1
12c	23,24-Dimethylcholestan-3β-ol	1.55	0.1
13a	24-Ethylcholest-5-en-3β-ol	1.56	5.3
14a	24-Ethylcholesta-5,24(28)E-dien-3β-ol	1.57	0.2
13c	24-Ethylcholestan-3β-ol	1.59	0.3
15a	24-Ethylcholesta-5,24(28)Z-dien-3β-ol	1.63	0.8

[a]Retention times (RRT) of acetates relative to cholesteryl acetate (RRT, 1.00) on an OV-1 capillary column (for details, see Materials and Methods).

coincident with the sterol composition of *M. balthica*. The respective species of phytoplankton contain mainly C_{28} and C_{29} Δ^5-, $\Delta^{5,22}$- and $\Delta^{5,24(28)}$-sterols (Jarzebski, A., unpublished results). It can therefore be assumed that the sterol composition of *M. balthica* is the result of transformation of exogenous sterols. The dealkylation of 24-alkylphytosterols to produce C_{27}-sterols (*e.g.* cholesterol) has been recognized as one of the possible transformations of exogenous sterols in bivalve mollusks (5,23). Adaptation of sterols from dietary sources to satisfy the sterol requirements of the bivalve involves the formation of numerous intermediates, which may explain the great diversity of 4-desmethylsterols present in *M. balthica*.

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ARTICLE

Molecular Species Composition of the Major Diacyl Glycerophospholipids from Muscle, Liver, Retina and Brain of Cod (*Gadus morhua*)

M.V. Bell* and **J.R. Dick**
NERC Unit of Aquatic Biochemistry, School of Natural Sciences, University of Stirling, Stirling FK9 4LA, Scotland

The molecular species composition of phosphatidylcholine (PC), phosphatidylethanolamine (PE) and phosphatidylserine (PS) from white muscle, liver, retina and brain of cod (*Gadus morhua*) were determined by high-performance liquid chromatography of the respective 1,2-diacylglycerol 3,5-dinitrobenzoyl derivatives. A minimum of 69 diacyl species was identified. In muscle and liver saturated fatty acid/polyunsaturated fatty acid (PUFA) and monounsaturated fatty acid/PUFA molecular species were predominant, particularly 16:0/20:5 and 16:0/22:6 in PC, 16:0/22:6 and 18:1/22:6 in PE and 18:0/22:6 and 18:1/22:6 in PS. Didocosahexaenoyl species were major components of PC, PE and PS from retina, comprising 29.3, 71.8 and 59.7% of the respective totals. Didocosahexaenoyl species were also abundant in PE and PS from brain, accounting for 13.8 and 24.0% of the totals, respectively. DiPUFA species were important in muscle, totalling 21.2% in PC and 38.3% in PE. PC from all tissues had the largest amounts of species containing only saturated or monounsaturated fatty acids, accounting for 59.8% of PC from brain, including 12.8% of 18:1/24:1 plus 24:1/18:1.
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The main structural glycerophosholipids from vertebrate neural tissue contain large amounts of docosahexaenoic acid (DHA; 22:6n-3). Polyunsaturated fatty acids (PUFA) are very tenaciously retained by neural tissue (1), and two or three generations of dietary deprivation are required to reduce significantly the n-3 PUFA content of brain in rat (2) and brain and retina of rhesus monkeys (3). Depletion of n-3 PUFA in brain and retina results in reduced visual and cognitive abilities in rhesus monkeys (4). However, the precise role of 22:6n-3 in neural tissue is unknown.

Early attempts to separate phospholipids from bovine rod outer segments (ROS) according to the degree of unsaturation by adsorption thin-layer chromatography (TLC) (5), and later by argentation TLC (6), showed the presence of fractions containing more than six double bonds per molecule. Positional analyses of the fatty acids in PC and PE from frog ROS showed 22:6n-3 to be on both the *sn*-1 and *sn*-2 positions of the glyceride and it was deduced that diPUFA species were present (7). The development of methods for analyzing the molecular species composition of glycerophospholipids by high-performance liquid chromatography (HPLC) directly (8) or by preparing derivatives (9–12), or by gas-liquid chromatography (GLC) of volatile derivatives (13) has revealed the fully complexity of the lipid components of membrane structures, and these techniques have now been applied to many systems. The presence of diPUFA molecular species in frog retina was confirmed by direct analysis (14), while analyses of glycerophospholipids from the brain and retina of trout showed large amounts of di22:6n-3 species, especially in phosphatidylcholine (PC), phosphatidylethanolamine (PE) and phosphatidylserine (PS) from retina and in PE and PS from brain (15). Small amounts of diPUFA species also have been found in non-neural tissues including rat erythrocyte (11), rat testes (16), fish muscle (9) and cod roe (17). Marine fish are particularly rich in n-3 PUFA (18), and this paper presents molecular species analyses of PC, PE and PS of four tissues of cod (*Gadus morhua*). The results from two neural tissues, brain and retina, are compared with those from liver and muscle.

MATERIALS AND METHODS

Cod (*Gadus morhua*) (300–600 g weight) were obtained from the Marine Station (Millport on the Firth of Clyde, Scotland), maintained in a seawater aquarium at 10–14°C on a diet of chopped squid and used within two weeks of capture. Fish were killed by decapitation, and the eyes, liver and fillet of muscle removed and frozen at −70°C until required. Brains were removed and used immediately.

Phospholipase C from *Bacillus cereus* was obtained from Boehringer Corporation (London) Ltd. (Lewes, East Sussex, England). Butylated hydroxytoluene (BHT) was from Sigma Chemical Co. (Poole, Dorset, England). 3,5-Dinitrobenzoylchloride was from Aldrich Chemical Co. (Gillingham, Dorset, England) and was recrystallized from carbon tetrachloride before use. Standard lipids were obtained from Sigma and from Nu-Chek Prep (Elysian, MN), as detailed in (15). TLC and high-performance thin-layer chromatography (HPTLC) plates were coated with silica gel 60 (Merck, Darmstadt, Germany). Analar grade glacial acetic acid, carbon tetrachloride, propan-2-ol and pyridine were purchased from BDH Ltd. (Poole, Dorset, England). All other solvents of HPLC grade were from Rathburn Chemicals (Walkerburn, Peeblesshire, Scotland).

Ultrasphere ODS and Ultrasphere octyl HPLC columns (25 × 0.46 cm, 5 micron particle size) were obtained from Altex/Beckman (Beckman Instruments U.K. Ltd., High Wycombe, Bucks, England).

Extraction and purification of lipids. All solvents, apart from those used for TLC and HPLC, contained 0.01% (w/v) BHT. Samples were stored at −20°C under nitrogen between preparative procedures.

The brains were taken from 20 cod, the meninges re-

*To whom correspondence should be addressed.

Abbreviations BHT, butylated hydroxytoluene; GLC, gas-liquid chromatography; HPLC, high-performance liquid chromatography; HPTLC, high-performance thin-layer chromatography; MUFA, monounsaturated fatty acid; PC, phosphatidylcholine; PE, phosphatidylethanolamine; PI, phosphatidylinositol; PS, phosphatidylserine; PUFA, polyunsaturated fatty acid; ROS, rod outer segment; SFA, saturated fatty acid; TLC, thin-layer chromatography; VLCPUFA, very long chain PUFA. Molecular species are abbreviated as follows: *e.g.*, 16:0/22:6 PC is 1-palmitoyl-2-docosahexaenoyl-*sn*-glycero-3-phosphocholine.

moved and the remaining tissue (8.3 g) homogenized in 10 volumes of chloroform/methanol (2:1, v/v) using a Polytron tissue disrupter. The retinas from 40 frozen cod eyes (5.7 g tissue) and 20 chopped frozen livers (89 g) were each homogenized in 10 volumes of chloroform/methanol (2:1, v/v), and finely shipped frozen muscle fillets (211 g) from 20 fish were homogenized in 5 volumes of solvent. After homogenization total lipid was extracted by the method of Folch *et al.* (19).

Neutral lipid was first removed from the brain and liver extracts by preparative TLC in hexane/diethyl ether/acetic acid (70:30:1, v/v/v). Phospholipids were eluted from the origin with chloroform/methanol/water (5:5:1, v/v/v), dried by rotary evaporation under vacuum at 35°C and finally under a stream of nitrogen. Phospholipid classes were separated by TLC in chloroform/methanol/water/triethylamine (30:35:6:35, by vol) (20) and the lipids detected under UV light after spraying with 0.1% (w/v) 2′,7′-dichlorofluorescein in methanol containing 0.01% (w/v) BHT. Phospholipids were eluted from the silica gel with three 40-mL washes of chloroform/methanol/water (5:5:1, v/v/v) and dried as before. 2′,7′-Dichlorofluorescein was removed from lipids by extraction with a solution of 2% (w/v) $KHCO_3$. PC and PS were further purified by TLC in chloroform/acetone/methanol/acetic acid/water (10:4:2:2:1, by vol) (21), and detected and extracted as before. All phospholipids were finally checked for purity by HPTLC in methyl acetate/propan-2-ol/chloroform/methanol/0.25% (w/v) KCl (25:25:25:10:9, by vol) (22). Lipids were detected by spraying with 3% (w/v) copper acetate in 8% (v/v) phosphoric acid and charring at 160°C for 15 min.

Fatty acid methyl esters were prepared by esterification in 2 mL of 1% (v/v) concentrated sulfuric acid in methanol at 50°C for 16 hr under nitrogen. The fatty acid composition of the phospholipids was determined by GLC of fatty acid methyl esters in a Packard 436 chromatograph fitted with a CP Wax 52CB fused silica capillary column (50 m × 0.32 mm i.d.) (Chrompack U.K. Ltd., London, England) (23), and with a CP Sil5CB fused silica capillary column (50 × 0.34 mm i.d.) (Chrompack) (24), using hydrogen as carrier gas.

Preparation of 3,5-dinitrobenzoyl derivatives. One-mg portions of phospholipid were hydrolyzed with phospholipase C using a two phase system of 1 mL diethyl ether and 1 mL 0.1M sodium borate buffer, pH 7.5, at room temperature under nitrogen for 5 hr (25). At the end of the incubations 1,2-diacylglycerols were extracted and purified by TLC in hexane/diethyl ether/acetic acid (50:50:1, v/v/v) (17). 1-Alkenyl-2-acylglycerols and 1-alkyl-2-acylglycerols were separated at this stage. Amounts of phospholipid remaining after phospholipase C digestion were less than 2% of the starting material. Diacylglycerols were derivatized in dry pyridine with 3,5-dinitrobenzoyl chloride at 60°C for 45 min under nitrogen, extracted and washed as described by Takamura *et al.* (12). The purity of the product was checked by HPTLC in hexane/diethyl ether/acetic acid (70:30:1, v/v/v).

Separation of molecular species. The 1,2-diacyl-3-dinitrobenzoyl-*sn*-glycerols were separatd by HPLC at 19–21°C on reverse phase columns using a Pye Unicam 4010 pump (Pye Unicam Ltd., Cambridge, England) and three isocratic solvent systems. An ODS column was used with methanol/propan-2-ol (95:5, v/v), flow rate 1.0 mL/min; and acetonitrile/propan-2-ol (80:20, v/v), flow rate 1.0 mL/min (12); a C8 column was used with methanol/water/acetonitrile (93:5:2, v/v/v), flow rate 1.2 mL/min (17). Peaks were detected at 254 nm with a Pye Unicam 4020 detector and quantified using a Shimadzu C-R6A recording integrator (Anachem, Luton, England).

Peaks were identified from plots of $\log_{10}$ (relative retention time, RRT) *vs.* the effective carbon number on the *sn*-1 position of the glyceride as described by Patton *et al.* (8) and Takamura *et al.* (12) using 16:0/22:6 as a reference peak. Didocosahexaenoylglycerol (Nu-Chek Prep) was also available for direct comparison of retention times. The main n-3 PUFA-containing molecular species were identified directly by GLC of the fatty acid methyl esters (17), while 18:1/20:1 plus 20:1/18:1 and 18:1/24:1 plus 24:1/18:1 had previously been synthesized (15). Each sample was chromatographed three times in each of the solvent systems and the standard deviations calculated. Where final peak areas were calculated by subtraction, the standard deviations of the contributing peaks were added to give the final error. Results are given to one decimal place for clarity. The $\log_{10}$ (RRT × 10) of all the molecular species in each solvent system are given in the appendix.

RESULTS

In muscle SFA/PUFA species were the most abundant class, comprising over 50% of PC and PS with almost equal amounts of 16:0/20:5 and 16:0/22:6 in PC and mainly 18:0/22:6 in PS (Table 1). The most abundant MUFA/PUFA species in each class and a major component of PE and PS was 18:1/22:6. DiPUFA species were also abundant in PC (21.2%) and PE (38.3%), predominantly 22:6/20:5 plus 20:5/22:6 in PC and 22:6/22:6 in PE. Another nine diPUFA species were found. Only small amounts of species containing only saturated and monounsaturated fatty acids were present of which 16:0/18:1 was the most abundant comprising 6.4% of PC.

The molecular species composition of PC, PE and PS from liver was broadly similar to that of muscle (Table 2). SFA/PUFA were the most abundant species in PC and PS, and MUFA/PUFA species in PE. In all three glycerophospholipids 16:0/22:6 was a major species comprising a quarter of the PC, and 18:1/22:6 was abundant in PE and PS. In PS, 18:0/22:6 was a major species but was much less abundant than in PS from muscle; 18:1/22:6 and 18:0/22:6 were equally abundant in PS from liver. In PS from liver 20:1/22:6 was also a major species (Table 2). In addition, liver contained up to nine diPUFA species totaling 13.1% in PE with 22:6-containing species dominant. Species containing only saturated and monounsaturated fatty acids were again of minor importance with 16:0/18:1 comprising 6.4% of PC.

Di22:6 species were most abundant in PC, PE and PS from retina accounting for 29.3, 71.8 and 59.7%, respectively (Table 3). Other diPUFA species were of minor importance. In PC, 16:0/22:6, 16:0/18:1 and 18:0/22:6 were the only other species present at greater than 5.1%. Two unknown species totaling 9.2% were also present in PC and were probably very long chain PUFA-containing species (26). In PE, 16:0/22:6, 18:1/22:6 and 18:0/22:6 at 5–6% were the only other species present at more than 2%, while in PS 18:0/22:6 was again abundant (Table 3). Species com-

TABLE 1

Molecular Species Compositions of Phosphatidylcholine, Phosphatidylethanolamine and Phosphatidylserine from Cod Muscle[a]

Species	PC(mol%)	PE(mol%)	PS(mol%)
diPUFA			
20:5/20:5; (18:4/22:6)	2.4 ± 0.2	1.4 ± 0.0	—[b]
20:5/22:6	8.3 ± 0.0	9.5 ± 0.3	1.1 ± 0.1
22:6/22:6	6.4 ± 0.5	19.8 ± 1.1	5.0 ± 0.6
20:5/22:5; 20:4/22:6	0.9 ± 0.1	3.2 ± 0.1	tr[c]
22:5/22:6	1.8 ± 0.0	3.6 ± 0.1	1.8 ± 0.0
22:5/22:5; 22:4/22:6	1.1 ± 0.1	0.8 ± 0.0	2.0 ± 0.8
Total	21.2	38.3	10.2
SFA/PUFA			
14:0/22:6	1.6 ± 0.0	0.7 ± 0.0	—
16:0/20:5	18.2 ± 0.8	4.4 ± 0.1	0.9 ± 0.0
16:0/22:6	21.7 ± 0.2	13.0 ± 0.1	5.2 ± 0.1
16:0/20:4	4.7 ± 0.1	1.9 ± 0.0	1.0 ± 0.0
16:0/22:5	1.5 ± 0.1	1.1 ± 0.1	—
16:0/18:2	1.3 ± 0.0	0.5 ± 0.0	—
18:0/20:5	1.2 ± 0.1	2.5 ± 0.0	5.0 ± 0.1
18:0/22:6	0.8 ± 0.1	6.7 ± 0.1	35.7 ± 2.3
18:0/20:4	0.6 ± 0.0	0.7 ± 0.3	2.2 ± 0.0
18:0/22:5	tr	0.8 ± 0.0	4.9 ± 0.1
Total	54.3	33.5	57.5
MUFA/PUFA			
18:1/20:5	3.5 ± 0.1	5.4 ± 0.1	0.8 ± 0.0
18:1/22:6	4.0 ± 0.1	13.4 ± 0.3	10.9 ± 1.0
18:1/20:4	1.6 ± 0.2	1.8 ± 0.7	2.8 ± 0.1
18:1/22:5	1.5 ± 0.2	1.9 ± 0.4	3.4 ± 0.1
18:1/22:4	—	1.1 ± 0.0	1.5 ± 0.2
20:1/22:6	0.2 ± 0.1	1.9 ± 0.2	3.9 ± 0.1
Total	14.1	26.9	25.3
SFA/MUFA			
16:0/18:1	6.4 ± 0.1	0.4 ± 0.1	0.7 ± 0.1
18:0/18:1; (16:0/20:1)	0.5 ± 0.1	0.1 ± 0.1	1.7 ± 0.1
Total	7.8	0.7	3.3
MUFA/MUFA			
16:1/16:1	1.1 ± 0.1	—	—
18:1/18:1; (16:1/20:1)	0.6 ± 0.2	0.5 ± 0.0	2.2 ± 0.0
Total	2.3	0.6	3.1
Very long chain			
Total	0.4	—	0.7

[a]Species in italics have defined *sn*-1 and *sn*-2 fatty acid distributions, otherwise it was assumed that the most saturated fatty acid was on the *sn*-1 position. Only one fatty acid combination is given for those species with two equally likely positional isomers. Bracketed species are minor components based on fatty acid composition data. The errors are given as ± 1 standard deviation rounded to the nearest decimal place (see text). The following molecular species were also detected at <0.9% in at least one of the glycerophospholipids: 18:4/20:5, 22:4/20:5, *14:0/20:5, 14:0/22:5, 16:0/18:4, 16:0/22:4, 18:0/18:2, 18:0/22:4, 16:1/20:5, 16:1/22:6, 16:1/22:5, 20:1/20:5, 20:1/20:4, 20:1/22:5, 20:1/22:4,* 16:0/14:0, 14:0/16:1, 18:0/20:1, 16:1/18:1, 18:1/20:1, *24:1/20:5, 24:1/22:6,* 18:1/24:1.

[b]—, Not detected.

[c]tr, <0.1%.

prising only SFA and MUFA totalled just 2.1% and 5.9%, respectively, in PE and PS from retina.

PC from cod brain showed the most complex composition of any of the glycerophospholipids examined; a minimum of 54 species were found, including seven unknowns which totalled 6.6% (Table 4). At least 10 species containing 24:0, 24:1 and 26:1 fatty acids totalling 16.9% were found, the most important of which was 24:1/18:1 plus 18:1/24:1 (12.8%). These species containing very long chain fatty acids were found only as trace components of PE and as less than 1% of PS. PC from brain also contained 42.9% of species containing SFA or MUFA of which 16:0/18:1 (16.9%) and 18:0/18:1 (9.5%) were the most important. The only other major species present was

TABLE 2

Molecular Species Compositions of Phosphatidylcholine, Phosphatidylethanolamine and Phosphatidylserine from Cod Liver[a]

Species	PC(mol%)	PE(mol%)	PS(mol%)
diPUFA			
20:5/22:6	2.2 ± 0.1	3.7 ± 0.1	1.2 ± 0.1
22:6/22:6	1.9 ± 0.2	4.0 ± 0.5	1.8 ± 0.1
20:4/22:6; 20:5/22:5	2.0 ± 0.0	2.1 ± 0.1	0.1 ± 0.0
22:5/22:5; 22:4/22:6	—[b]	1.5 ± 0.1	0.6 ± 0.1
Total	6.6	13.5	4.0
SFA/PUFA			
14:0/22:6	2.0 ± 0.0	0.6 ± 0.0	0.4 ± 0.0
14:0/22:5	1.0 ± 0.1	0.4 ± 0.0	—
16:0/20:5	15.8 ± 1.0	7.5 ± 0.4	0.9 ± 0.1
16:0/22:6	25.2 ± 0.3	13.6 ± 0.6	13.5 ± 0.9
16:0/20:4	3.8 ± 0.0	0.1 ± 0.0	1.5 ± 0.1
16:0/22:5	2.1 ± 0.0	3.0 ± 0.0	1.8 ± 0.1
16:0/18:2	1.2 ± 0.0	1.4 ± 0.1	0.4 ± 0.1
18:0/20:5	1.5 ± 0.0	4.7 ± 0.3	1.0 ± 0.1
18:0/22:6	1.5 ± 0.3	4.6 ± 0.1	21.7 ± 0.1
18:0/20:4	1.0 ± 0.1	1.5 ± 0.1	2.1 ± 0.1
18:0/22:5	tr[b]	0.3 ± 0.1	2.0 ± 0.1
18:0/22:4	0.1 ± 0.0	0.4 ± 0.0	1.3 ± 0.5
Total	57.7	39.3	47.2
MUFA/PUFA			
16:1/20:5	1.8 ± 0.0	0.6 ± 0.1	tr
16:1/22:6	3.3 ± 0.0	0.7 ± 0.2	—
16:1/20:4	1.5 ± 0.1	0.6 ± 0.0	—
16:1/22:5	1.8 ± 0.0	0.1 ± 0.0	—
18:1/20:5	5.5 ± 0.1	8.7 ± 1.8	0.9 ± 0.0
18:1/22:6	5.9 ± 0.1	24.5 ± 0.1	22.0 ± 1.6
18:1/20:4	2.5 ± 0.1	0.7 ± 0.1	1.9 ± 0.1
18:1/22:5	0.7 ± 0.1	4.8 ± 0.1	3.8 ± 0.1
20:1/20:5	0.9 ± 0.1	0.9 ± 0.1	1.8 ± 0.1
20:1/22:6	0.9 ± 0.0	2.7 ± 0.2	10.8 ± 0.5
20:1/20:4	tr	0.5 ± 0.1	1.3 ± 0.1
Total	25.1	45.5	44.4
SFA/SFA			
Total	0.3	0.0	0.4
SFA/MUFA			
16:0/18:1	6.4 ± 0.1	0.2 ± 0.0	—
Total	7.4	0.7	1.4
MUFA/MUFA			
16:1/18:1	0.6 ± 0.1	0.3 ± 0.0	2.5 ± 0.4
18:1/18:1; (16:1/20:1)	1.3 ± 0.0	0.2 ± 0.0	—
Total	2.3	0.8	2.7
Very long chain			
Total	—	0.2	0.3

[a]Species in italics have defined *sn*-1 and *sn*-2 fatty acid distributions, otherwise it was assumed that the most saturated fatty acid was on the *sn*-1 position. Only one fatty acid combination is given for those species with two equally likely positional isomers. Bracketed species are minor components based on fatty acid composition data. The errors are given as ± 1 standard deviation rounded to the nearest decimal place (see text). The following molecular species were also detected at <0.9% in at least one of the glycerophospholipids: *20:5/20:5,* 22:5/22:6, 22:4/20:5, *14:0/20:5, 14:0/22:6, 16:0/18:4, 16:0/22:4, 18:0/18:2, 18:1/18:2, 18:1/22:4, 20:1/22:5, 20:1/22:4,* 14:0/16:0, *16:0/16:0,* 14:0/16:1, 18:0/18:1 + (16:0/20:1), *16:1/16:1,* 18:1/20:1, *20:1/20:1, 24:1/22:6,* 18:1/24:1.

[b]—, Not detected.

[c]tr, <0.1%.

TABLE 3

Molecular Species Compositions of Phosphatidylcholine, Phosphatidylethanolamine and Phosphatidylserine from Cod Retina[a]

Species	PC(mol%)	PE(mol%)	PS(mol%)
diPUFA			
20:5/22:6	0.7 ± 0.1	1.7 ± 0.1	0.4 ± 0.0
22:6/22:6	29.3 ± 0.9	71.8 ± 1.7	59.7 ± 1.4
22:5/22:6	0.2 ± 0.0	1.0 ± 0.1	0.4 ± 0.0
Total	30.2	74.6	60.5
SFA/PUFA			
16:0/20:5	2.6 ± 0.5	0.7 ± 0.1	0.1 ± 0.0
16:0/22:6	23.2 ± 0.1	5.9 ± 0.2	1.7 ± 0.1
16:0/20:4	1.7 ± 0.3	0.6 ± 0.0	0.4 ± 0.0
18:0/20:5	0.5 ± 0.1	0.8 ± 0.0	1.9 ± 0.5
18:0/22:6	9.1 ± 1.1	5.0 ± 0.1	16.2 ± 0.1
18:0/20:4	0.5 ± 0.0	1.0 ± 0.0	1.6 ± 0.5
Total	38.5	14.4	22.2
MUFA/PUFA			
18:1/20:5	0.6 ± 0.1	1.2 ± 0.2	0.4 ± 0.1
18:1/22:6	1.7 ± 0.5	5.9 ± 0.0	5.9 ± 0.4
20:1/20:4	0.2 ± 0.0	—[b]	1.7 ± 0.7
Total	4.1	9.0	11.3
SFA/SFA			
Total	0.7	tr[c]	0.5
SFA/MUFA			
16:0/18:1	13.4 ± 0.1	0.3 ± 0.0	0.3 ± 0.0
18:0/18:1; (16:0/20:1)	1.9 ± 0.1	0.5 ± 0.0	1.6 ± 0.2
Total	15.3	0.8	2.3
MUFA/MUFA			
18:1/18:1; (16:1/20:1)	1.3 ± 0.1	0.6 ± 0.0	2.5 ± 0.1
Total	2.3	1.3	3.1
Unknowns			
U1	5.1 ± 0.2	—	—
U2	4.1 ± 0.1	—	—

[a]Species in italics have defined *sn*-1 and *sn*-2 fatty acid distributions, otherwise it was assumed that the most saturated fatty acid was on the *sn*-1 position. Only one fatty acid combination is given for those species with two equally likely positional isomers. Bracketed species are minor components based on fatty acid composition data. The errors are given as ± 1 standard deviation rounded to the nearest decimal place (see text). The following molecular species were also detected as <0.9% in at least one of the glycerophospholipids: *20:5/20:5,* 22:5/22:6, *22:5/22:5, 14:0/22:6, 16:0/22:5, 18:0/22:5, 18:1/20:4, 18:1/22:5, 20:1/22:6, 20:1/22:5,* 14:0/16:0, 16:0/18:0, 18:0/16:1, 16:1/18:1, 18:1/20:1, *20:1/20:1,* 18:1/24:1.

[b]—, Not detected.

[c]tr, <0.1%.

16:0/22:6. PE and PS from brain were broadly similar in composition to those from muscle, liver and retina. Di22:6 was abundant in both classes, especially PS (24.0%), while 16:0/22:6, 18:1/22:6 and 18:0/22:6 were the other major species in PE, and 18:0/22:6 and 18:1/22:6 in PS.

DISCUSSION

The method used here is capable of resolving most of the molecular species present in glycerophospholipids from fish tissues. However, species containing fatty acids which are present in very small amounts (<0.5%) were not always detected, *e.g.,* 16:4n-3 and 18:3n-3. Isomers of unsaturated fatty acids are not separated, *e.g.,* n-7 and n-9 MUFA or n-3 and n-6 PUFA. Analysis by GLC showed that 22:5 was the n-3 isomer, 22:4 the n-6 isomer and 20:4 predominantly the n-6 isomer; 20:4n-3 was a very minor component in most samples. The chromatographic systems could not separate diPUFA species containing different fatty acids but the same total carbon number and double bonds. Very long chain fatty acid-containing species were also difficult to resolve, and the column and chromatography conditions

TABLE 4

Molecular Species Compositions of Phosphatidylcholine, Phosphatidylethanolamine and Phosphatidylserine from Cod Brain[a]

Species	PC(mol%)	PE(mol%)	PS(mol%)
diPUFA			
20:5/22:6	0.2 ± 0.0	1.4 ± 0.2	1.0 ± 0.0
22:6/22:6	0.6 ± 0.1	13.8 ± 1.1	24.0 ± 0.9
22:5/22:6	—[b]	0.2 ± 0.1	1.0 ± 0.0
Total	1.0	15.5	26.1
SFA/PUFA			
16:0/20:5	2.3 ± 0.0	1.9 ± 0.1	0.1 ± 0.0
16:0/22:6	17.5 ± 0.1	19.0 ± 0.2	1.5 ± 0.1
16:0/20:4	0.7 ± 0.0	1.3 ± 0.0	—
16:0/22:5	0.5 ± 0.0	1.4 ± 0.1	1.0 ± 0.1
18:0/20:5	1.3 ± 0.1	4.7 ± 1.0	2.6 ± 0.0
18:0/22:6	2.2 ± 0.1	14.7 ± 1.0	34.5 ± 0.4
18:0/20:4	0.8 ± 0.0	1.0 ± 0.1	0.4 ± 0.1
18:0/22:5	0.9 ± 0.1	0.7 ± 0.0	1.4 ± 0.0
Total	27.0	46.0	41.5
MUFA/PUFA			
18:1/20:5	0.4 ± 0.0	2.7 ± 0.1	0.8 ± 0.3
18:1/22:6	2.1 ± 0.4	14.6 ± 1.2	19.0 ± 0.2
18:1/20:4	0.1 ± 0.0	1.2 ± 0.1	0.1 ± 0.1
18:1/22:5	0.6 ± 0.0	1.7 ± 0.1	1.4 ± 1.0
20:1/22:6	0.8 ± 0.1	1.8 ± 0.3	0.6 ± 0.3
Total	5.2	22.2	22.1
SFA/SFA			
16:0/16:0	1.3 ± 0.1	tr[c]	—
16:0/18:0	1.2 ± 0.2	0.1 ± 0.0	—
Total	3.5	0.1	0.1
SFA/MUFA			
16:0/16:1	1.2 ± 0.1	0.2 ± 0.1	—
16:0/18:1	16.9 ± 0.5	3.1 ± 0.0	0.4 ± 0.0
18:0/18:1; (16:0/20:1)	9.5 ± 0.6	4.9 ± 0.1	3.7 ± 0.1
Total	28.9	8.2	4.5
MUFA/MUFA			
18:1/18:1; (16:1/20:1)	3.2 ± 0.0	4.8 ± 0.1	1.7 ± 0.0
18:1/20:1; 16:1/22:1	3.2 ± 0.0	1.8 ± 0.2	2.7 ± 0.1
20:1/20:1; 18:1/22:1	3.3 ± 0.3	0.4 ± 0.1	0.4 ± 0.0
Total	10.5	7.6	4.8
Very long chain			
18:1/24:1, (20:1/22:1)	12.8 ± 0.2	—	0.8 ± 0.1
16:0/24:0; 16:0/26:1	1.2 ± 0.1	—	—
Total	16.9	tr	0.9
Unknowns (7)			
Total	6.6	—	—

[a]Species in italics have defined *sn*-1 and *sn*-2 fatty acid distributions, otherwise it was assumed that the most saturated fatty acid was on the *sn*-1 position. Only one fatty acid combination is given for those species with two equally likely positional isomers. Bracketed species are minor components based on fatty acid composition data. The errors are given as ± 1 standard deviation rounded to the nearest decimal place (see text). The following molecular species were also detected at <0.9% in at least one of the glycerophospholipids: *20:5/20:5, 22:5/22:5, 14:0/22:6, 14:0/20:4, 14:0/22:5, 16:0/18:2, 20:1/20:5, 20:1/20:4, 20:1/22:5,* 14:0/16:0, *18:0/18:0,* 14:0/16:1, 18:0/20:1, *16:1/16:1,* 16:1/18:1, *24:1/20:5, 24:1/22:6,* 16:1/24:1, 16:0/24:1, 18:0/24:1 + (16:0/26:1), 20:1/24:1 + 18:1/26:1 + *(22:1/22:1), 24:0/20:5 + 26:1/20:5, 24:0/22:6 + 26:1/22:6.*

[b]—, Not detected.

[c]tr, <0.1%.

used could not separate the 24:0/PUFA and 26:1/PUFA pairs found in PC from brain (Table 4). The two solvent systems used with the C18 column can separate extreme positional isomers, *e.g.*, 20:5/16:0 from 16:0/20:5 but not 16:0/18:1 from 18:1/16:0. The presumption that most saturated fatty acids are located at the *sn*-1 position of the glycerol moiety is, therefore, supported by the results in all cases where the method can distinguish positional isomers.

Marine fish are particularly rich in n-3 PUFA, especially docosahexaenoic acid (18). The present results show the very unsaturated nature of the molecular species of glycerophospholipids from cod. PE was the most unsaturated glycerophospholipids in all tissues except brain, while PC was the most saturated and contained the shortest average chain length, both due to a lower proportion of 22:6n-3 containing species. PC from fish brain contains a much larger amount of SFA- and MUFA-containing species than glycerophospholipids from other fish tissues. Of particular note is the large amount of 18:1/24:1 plus 24:1/18:1, which was found in trout (9) and is even more abundant in cod. Since cod brain contains 1.0% of 22:1 fatty acid, 20:1/22:1 may be a minor component of this peak. Small amounts of other species containing very long chain fatty acids (24:0, 26:1) were also found in cod brain PC, raising the question of the special role of these molecular species in the structure and function of biomembranes in this tissue.

The increased sophistication of analytical techniques in recent years has led to the discovery of diPUFA species in an unexpectedly large number of tissues such as erythrocytes (11) and testes (16) from rat. Earlier molecular species analyses of fish muscle lecithins found appreciable amounts of diPUFA species (mainly 22:6/22:6, 20:5/22:6 and 22:5/22:6) in chum salmon *Oncorhynchus keta*, big-eyed tuna *Parathunnus obesus* and Alaska pollack *Theragra chalcogramma*, but not in carp *Cyprinus carpio* (9); the present results confirm these findings. The present study also found small amounts of diPUFA species in PC, PE and PS from cod liver and PS from cod muscle, while the large amount of di22:6 in PE from cod muscle is the greatest proportion of this molecular species yet reported in a non-neural tissue.

In bovine retina two unsaturated fatty acids were found in at least 43% of PS, 24% of PE and 24% of PC (6), and PE from frog retina contained about 50% diPUFA species, mainly 22:6n-3/22:6n-3 (14). Tissues from marine fish contain predominantly n-3 PUFA with very little n-6 PUFA, and therefore diPUFA species in fish retina are almost entirely 22:6/22:6. The amounts of di22:6 glycerophospholipids found in cod retina are the highest found in the retina of any species and are substantially higher than those found in trout retina (15). However, we found that PI from the retina of trout (15) and cod (27) does not contain diPUFA species. The molecular species of PI are usually dominated by $18:0/C_{20}$ PUFA species with 18:0/20:4n-6 a major species in the retina of fish (15,27) as in tissues from terrestrial animals (8,11,28), but 18:0/20:5n-3 is predominant in fish brain (15,27). PI is therefore much more saturated than other glycerophospholipids in retina.

Cod are visual feeders with the liver being a major depot for storage of neutral lipid, but other strategies exist for foraging and storing lipid in other species of fish. The patterns of molecular speciation of phospholipids in the tissues of cod described here therefore may not be representative of all species. Many laboratory studies with fish and other animals have shown that dietary lipid directly influences the fatty acid composition of tissue lipids (1). However, it has proved very difficult to deplete neural tissues of essential polyunsaturated fatty acids in mammals (1–3). The presence of large amounts of di22:6 glycerophospholipids in the retinas of a wide range of animal species suggests a special role for these molecules. It has been proposed that these unique lipids play a structural role by interacting with rhodopsin in the ROS membrane while also maintaining a very fluid membrane in which conformational changes can occur during the excitation cycle (29). The pigment epithelium of the eye of many teleost fish has a reflecting layer which consists of lipid spherules containing almost exclusively tridocosahexaenoylglycerol (30). This unique molecule could most easily be synthesized from a di22:6 glycerophospholipids.

Recently very long chain PUFA (VLCPUFA), with up to 36 carbons and six double bonds, of both the n-6 and n-3 series have been found in testes (31,32) and particularly in PC from retina (26). A survey of very long chain PUFA in retina from a variety of animal species found them to be present in all systems examined, including cod (26). The latter study also found extremely high levels of 22:6n-3 in cod retina and suggested a preponderance of di22:6 molecular species in this tissue (26), as was confirmed in the present study. The very long chain PUFA are esterified to the *sn*-1 position of PC with 22:6n-3 at the *sn*-2 position. Up to a third of PC from bovine ROS was found to consist of such VLCPUFA/22:6 species (29). The two unknowns found here in PC from cod retina behaved in a manner consistent with molecules containing VLCPUFA. However, the unknowns in PC from cod brain were different and did not appear to fit this pattern.

The roles of particular fatty acids in biomembrane structure has been the subject of much experimental work and debate (33,34). Molecular speciation of phospholipid classes from different tissues has shown surprising specificity indicating defined roles for some of these phospholipid species in certain membranes. However, the factors that make individual molecular species suited to particular biological roles, and the different tissue specificities of the enzymes involved in their synthesis and maintenance in the biomembrane remain to be elucidated.

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APPENDICES

APPENDIX 1

Log_{10} (RRT × 10 with Respect to 16:0/22:6) of Molecular Species in Methanol/Propan-2-ol, 95:5 (v/v)[a]

18:4/20:5	0.685	*16:0/22:6*	1.000	*20:1/22:5*	1.195
18:4/22:6	0.730	*18:1/22:6*	1.000	*18:0/18:2*	1.218
20:5/20:5	0.737	*16:0/20:4*	1.034	*20:1/18:2*	1.218
20:5/22:6	0.786	*18:1/20:4*	1.034	*18:0/22:4*	1.268
14:0/20:5	0.806	16:0/16:1	1.034	*20:1/22:4*	1.268
16:1/20:5	0.806	16:1/18:1	1.034	16:0/20:1	1.322

(continued on next page)

20:4/20:5	0.825	14:0/16:0	1.036	18:0/18:1	1.322
20:5/22:5	0.837	14:0/18:1	1.036	18:1/20:1	1.322
22:6/22:6	0.838	*16:0/22:5*	1.052	16:0/18:0	1.322
14:0/22:6	0.852	*18:1/22:5*	1.052	*24:1/20:5*	1.382
16:1/22:6	0.852	*16:0/18:2*	1.072	*24:1/22:6*	1.430
20:4/22:6	0.874	*18:1/18:2*	1.072	*18:0/18:0*	1.460
14:0/20:4	0.887	*18:0/20:5*	1.092	18:0/20:1	1.460
16:1/20:4	0.887	*20:1/20:5*	1.092	*20:1/20:1*	1.460
14:0/14:0	0.887	*16:0/22:4*	1.122	16:1/24:1	1.472
14:0/16:1	0.887	*18:1/22:4*	1.122	*24:0/20:5*	1.525
16:1/16:1	0.887	*18:0/22:6*	1.141	*26:1/20:5*	1.525
22:4/20:5	0.892	*20:1/22:6*	1.141	*24:0/22:6*	1.580
22:5/22:6	0.892	*18:0/20:4*	1.180	*26:1/22:6*	1.580
14:0/22:5	*0.905*	*20:1/20:4*	1.180	16:0/24:1	1.613
16:1/22:5	0.905	14:0/20:1	1.180	18:1/24:1	1.613
16:0/18:4	0.905	16:0/18:1	1.180	18:0/24:1	1.748
18:1/18:4	0.905	18:0/16:1	1.180	16:0/26:1	1.748
16:1/18:2	0.926	16:1/20:1	1.180	24:0/18:1	1.748
22:4/22:6	0.940	*18:1/18:1*	1.180	20:1/24:1	1.748
22:5/22:5	0.945	14:0/18:0	1.180	18:1/26:1	1.748
16:0/20:5	0.950	*16:0/16:0*	1.180	*22:1/22:1*	1.748
18:1/20:5	0.950	*18:0/22:5*	1.195		

[a]Species in italics have defined *sn*-1 and *sn*-2 fatty acid distributions. Only one fatty acid combination is given for those species with two equally probable positional isomers. In practice it is only possible to resolve molecular species with Log_{10} (RRT × 10) values differing by more than 0.02.

APPENDIX 2

Log_{10} (RRT × 10 with Respect to 16:0/22:6) of Molecular Species in Acetonitrile/Propan-2-ol, 80:20 (v/v)[a]

18:4/20:5	0.630	*14:0/14:0*	1.033	*18:0/18:2*	1.319
18:4/22:6	0.650	*18:1/22:5*	1.040	16:0/18:1	1.321
20:5/20:5	0.653	*16:0/22:5*	1.065	*20:1/22:4*	1.325
20:5/20:6	0.679	*18:1/20:4*	1.065	14:0/18:0	1.348
22:6/22:6	0.701	*16:0/20:4*	1.087	*16:0/16:0*	1.348
20:5/22:5	0.745	18:1/16:1	1.096	*18:0/22:4*	1.348
22:5/22:6	0.770	*20:1/20:5*	1.102	*24:1/20:5*	1.392
20:4/20:5	0.771	16:0/16:1	1.122	18:1/20:1	1.445
20:4/22:6	0.795	*18:0/20:5*	1.125	*24:1/22:6*	1.427
16:1/20:5	0.795	*20:1/22:6*	1.130	16:0/20:1	1.467
14:0/20:5	0.817	*18:1/18:2*	1.140	18:0/18:1	1.471
16:1/22:6	0.825	16:1/18:1	1.143	16:0/18:0	1.500
22:5/22:5	0.833	*18:0/22:6*	1.155		
14:0/22:6	0.848	*16:0/18:2*	1.165	*24:0/20:5*	1.583
22:4/20:5	0.878	14:0/18:1	1.167	*26:1/20:5*	1.583
16:1/22:5	0.888	*18:1/22:4*	1.170	*20:1/20:1*	1.598
22:4/22:6	0.897	14:0/16:0	1.190	16:1/24:1	1.613
14:0/22:5	0.912	*16:0/22:4*	1.195	*24:0/22:6*	1.613
16:1/20:4	0.913	*20:1/22:5*	1.195	*26:1/22:6*	1.613
18:1/18:4	0.920	*18:0/22:5*	1.220	18:0/20:1	1.622
14:0/20:4	0.936	*20:1/20:4*	1.220	*18:0/18:0*	1.650
16:0/18:4	0.945	*18:0/20:4*	1.245	18:1/24:1	1.765
18:1/20:5	0.946			16:0/24:1	1.790
16:1/16:1	0.948	18:0/16:1	1.275	*22:1/22:1*	1.916
14:0/16:1	0.970	16:1/20:1	1.290	20:1/24:1	1.916
16:0/20:5	0.974	*18:1/18:1*	1.294	18:1/26:1	1.916
18:1/22:6	0.975	20:1/18:2	1.295	18:0/24:1	1.944
16:1/18:2	0.988	14:0/20:1	1.313	16:0/26:1	1.944

[a]Species in italics have defined *sn*-1 and *sn*-2 fatty acid distributions. Only one fatty acid combination is given for those species with two equally probable positional isomers. In practice it is only possible to resolve molecular species with Log_{10} (RRT × 10) values differing by more than 0.02

APPENDIX 3

Log_{10} (RRT × 10 with Respect to 16:0/22:6) of Molecular Species in Methanol/Water/Acetonitrile, 93:5:2 (v/v/v)[a]

18:4/20:5	0.715	14:0/18:1	1.005	*20:1/18:2*	1.218
20:5/20:5	0.770	16:0/16:1	1.015	*18:0/22:5*	1.225
18:4/22:6	0.780	*16:0/20:4*	1.034	16:0/18:0	1.247
14:0/20:5	0.800	*18:1/22:6*	1.035	*20:1/22:5*	1.260
20:5/22:6	0.828	16:1/18:1	1.037	16:0/20:1	1.282
16:1/20:5	0.833	22:4/22:6	1.042	18:0/18:1	1.282
14:0/14:0	0.838	*16:0/18:2*	1.046	*18:0/22:4*	1.302
14:0/22:6	0.863	*22:5/22:5*	1.057	18:1/20:1	1.319
20:4/20:5	0.865	*18:1/20:4*	1.068	*20:1/22:4*	1.335
16:0/18:4	0.875	*18:0/20:5*	1.080	*18:0/18:0*	1.403
14:0/16:1	0.877	*18:1/18:2*	1.082	*24:1/20:5*	1.405
22:6/22:6	0.884	*16:0/22:5*	1.086	18:0/20:1	1.430
14:0/20:4	0.894	*20:1/20:5*	1.105	*20:1/20:1*	1.465
16:1/22:6	0.898	*16:0/16:0*	1.110	*24:1/22:6*	1.480
18:1/18:4	0.910	14:0/18:0	1.110	16:1/24:1	1.488
16:1/16:1	0.911	*18:1/22:5*	1.120	*24:0/20:5*	1.500
20:4/22:6	0.916	14:0/20:1	1.142	*26:1/20:5*	1.500
20:5/22:5	0.916	16:0/18:1	1.142	*24:0/22:6*	1.563
16:1/20:4	0.927	18:0/16:1	1.142	*26:1/22:6*	1.563
16:0/20:5	0.940	*18:0/22:6*	1.143	16:0/24:1	1.600
16:1/18:2	0.945	*20:1/22:6*	1.170	18:1/24:1	1.642
14:0/22:5	0.948	*18:0/20:4*	1.170	18:0/24:1	1.750
22:5/22:6	0.969	*16:0/22:4*	1.170	16:0/26:1	1.750
14:0/16:0	0.974	16:1/20:1	1.177	24:0/18:1	1.750
18:1/20:5	0.974	*18:1/18:1*	1.177	20:1/24:1	1.830
16:1/22:5	0.980	*18:0/18:2*	1.187	18:1/26:1	1.830
22:4/20:5	0.988	*20:1/20:4*	1.202	*22:1/22:1*	1.830
16:0/22:6	1.000	*18:1/22:4*	1.202		

[a]Species in italics have defined *sn*-1 and *sn*-2 fatty acid distributions. Only one fatty acid combination is given for those species with two equally probable positional isomers. In practice it is only possible to resolve molecular species with Log_{10} (RRT × 10) values differing by more than 0.02.

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Diacetone-D-glucosyl-3-*O*-(*N,N*-diethylamido)-thiophosphates of the Lipid-Soluble Vitamins D_2, D_3 and E

Stephan D. Stamatov[a,*] and **Salo Gronowitz**[b]

[a]Department of Organic Chemical Technology, University of Plovdiv, Bulgaria and [b]Division of Organic Chemistry 1, University of Lund, Chemical Center, Sweden

The acyclic triamides of phosphorous acid, after appropriate activation, offer a number of advantages over conventional reagents when biologically active lipids are to be phosphorylated. The hexaethyltriamide of phosphorous acid activated by addition of iodine at the optimum molar ratio of 1.05:0.05 was used as phosphorylating reagent to synthesize diacetone-D-glucosyl-3-*O*-(*N,N*-diethylamido)thiophosphates of ergocalciferol, cholecalciferol and DL-α-tocopherol in a "one-pot" procedure in overall yields of 83–87%. The high selectivity of the phosphorylation at the monoester formation stage permits the direct synthesis of asymmetrical diesters. Anisochronicity was detected in the ^{13}C and ^{31}P nuclear magnetic resonance spectra of the thiophosphates synthesized. The compounds represent new types of glycophospholipid structures which, in addition to carbohydrate and a phosphagen fragment, contain a lipid-soluble vitamin.
Lipids 26, **574–577 (1991).**

Although lipid-soluble D and E vitamins are considered essential for living organisms, their physiological functions have still not been conclusively determined. As a result, the synthesis of new phospholipids from steroids and tocopherols that can be used as models has received increasing attention in recent years (1–7). The synthesis of derivatives in which a carbohydrate fragment and a lipid-soluble vitamin are structurally linked has not yet been considered. Such compounds may be of interest for biochemical and pharmacological studies.

The present paper deals with the synthesis of diacetone-D-glucosyl-3-*O*-(*N,N*-diethylamido)thiophosphate derivatives of vitamins D_2, D_3 and E. These compounds represent a new glycophospholipid structure containing a phosphagen fragment and sulfur bonded to phosphorus (Fig. 1).

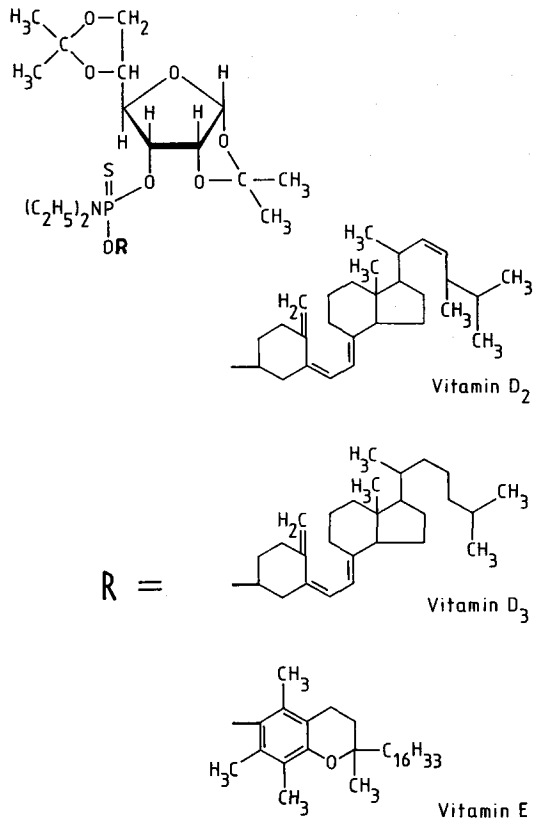


FIG. 1. Glucoamidothiophosphates of the vitamins D_2 (ergocalciferol), D_3 (cholecalciferol) and E (DL-α-tocopherol).

MATERIALS AND METHODS

Tris (*N,N*-diethyl)amide of phosphorous acid **1** (Schemes 1, 2 and 3) was prepared and freshly distilled as previously described (8). Ergocalciferol **a**, cholecalciferol **b**, DL-α-tocopherol **c** (Merck, Darmstadt, Germany), diacetone-D-glucose **d** (Fluka, Buchs, Switzerland) and all other reagents (Janssen, Stockholm, Sweden) were better than 98% pure. Benzene (Merck) was dried over sodium and freshly distilled. Reaction conditions were kept strictly anhydrous.

All processes were routinely monitored by analytical thin-layer chromatography (TLC) on pre-coated aluminum sheets of Silica Gel 60 F_{254} (Merck) using chloroform as the mobile phase. High-performance liquid chromatography (HPLC) was carried out (LDC Consta Metric III system, equipped with LKB 2142 Refractive Index Detector, Bromma, Sweden) using a Nucleosil silica gel column (500 × 10 mm) and n-heptane/ethyl acetate (8:2, v/v) as mobile phase.

^{13}C nuclear magnetic resonance (NMR) spectra were recorded on a Varian XL-300 spectrometer (Varian Assoc., Palo Alto, CA) at 75.43 MHz. ^{13}C chemical shifts are reported in ppm relative to tetramethylsilane (TMS). ^{31}P NMR spectra were recorded on the same instrument at 121.42 MHz. ^{31}P chemical shifts are reported in ppm relative to 85% phosphoric acid (external), where a positive sign is downfield from the standard. Infrared (IR) spectra were recorded on a Perkin-Elmer FT-IR 1750 (Perkin-Elmer, Norwalk, CI) spectrometer. Peak positions are reported in cm^{-1}. For **1aS–1cS** and **1adS–1cdS**, satisfactory microanalyses were obtained: C, ± 0.20; H, ± 0.11; P, ± 0.10; and S, ± 0.12.

*Ergocalciferyl-3-*0-bis(N,N-*diethylamido)thiophosphate* **(1aS)** *(representative procedure).* A mixture of iodine (0.025 g, 0.1 mmol) and the tris(*N,N*-diethyl)amide of

*To whom correspondence should be addressed at 31 Emily Street, Hurstville, NSW 2220, Australia.

Abbreviations: HPLC, high-performance liquid chromatography; NMR, nuclear magnetic resonance; TLC, thin-layer chromatography; TMS, tetramethylsilane.

phosphorous acid (1; 0.519 g, 2.1 mmol) in benzene (50 mL) was heated at 75°C in a stream of argon until the precipitate dissolved (about 15 min). The solution was cooled to room temperature (20–25°C), ergocalciferol (**a**; 0.793 g, 2.0 mmol) was added, and the reaction mixture was kept under these conditions for 4 hr to give **1a**. Sulfur (0.067 g, 2.1 mmol) was then added, and the mixture was stirred for 30 min. The solvent was removed under vacuum and the thiophosphate **1aS** was isolated by HPLC in analytically pure form. Yield of **1aS**: 1.17 g (97%); n_D^{40} = 1.5220; R_f($CHCl_3$) 0.80; $C_{36}H_{63}N_2OPS$ (603.1). ^{13}C NMR ($CDCl_3$) δ 12.4 ppm (*s*, C-18); 17.9 (*s*, C-28); 19.9 (*s*, C-26); 20.2 (*s*, C-27); 21.4 (*s*, C-21); 72.7 (*d*, C-3, J = 4 Hz); 108.2, 112.6 (each *s*, C-19); 116.9, 118.4 (each *s*, C-7); 121.5, 122.9 (each *s*, C-6); 132.3 (*s*, C-23); 135.3, 135.4 (each *s*, C-8); 136.2 (*s*, C-22); 141.8, 143.9 (each *s*, C-5); 145.8, 149.8 (each *s*, C-10): ergocalciferyl-3-*O*-fragment; 13.9 (*m*, CH_3CH_2N); 40.4 (*m*, CH_2N). ^{31}P NMR ($CDCl_3$) δ 77.2, 77.4 ppm (each *s*). IR (KBr, film) ν 3030 (CH=); 1645 (C=C); 1023, 816 (PO-C, P-OC); 732 (P-N); 695 cm^{-1} (P=S).

Cholecalciferyl-3-O-bis(N,N-diethylamido)thiophosphate (**1bS**). The derivative **1bS** was synthesized using cholecalciferol (**b**; 0.769 g, 2.0 mmol) and then purified in the same way as described for **1aS**. Yield of **1bS**: 1.13 g (96%); n_D^{40} =1.5189; R_f($CHCl_3$) 0.78; $C_{35}H_{63}N_2OPS$ (591.1). ^{13}C NMR ($CDCl_3$) δ 12.2, 12.3 ppm (each *s*, C-18); 19.1 (*s*, C-21);22.8 (*s*, C-26); 23.1 (*s*, C-27); 72.6, 73.5 (each *d*, C-3, J=4 Hz);108.2, 112.6 (each *s*, C-19); 116.9, 118.3 (each *s*, C-7); 121.5, 122.9 (each *s*, C-6); 135.2, 135.3 (each *s*, C-8); 141.8, 143.9 (each *s*, C-5); 145.7, 149.8 (each *s*, C-10): cholecalciferyl-3-*O*-fragment; 14.4 (*m*, CH_3CH_2N); 40.3 (*m*, CH_2N). ^{31}P NMR ($CDCl_3$) δ 75.6, 75.8 ppm (each *s*). IR (KBr, film) ν 3030 (CH=); 1642 (C=C); 1023, 813 (PO-C, P-OC); 729 (P-N); 690 cm^{-1} (P=6).

DL-α-Tocopheryl-6-O-bis(N,N-diethylamido)thiophosphate (**1cS**). The compound **1cS** was synthesized using DL-α-tocopherol (**c**; 0.861 g, 2.0 mmol), at 20–25°C for 10 hr, and then purified in the same way as described for **1aS**. Yield of **1cS**: 1.20 g (94%); n_D^{20} = 1.5140; R_f ($CHCl_3$) 0.89; $C_{37}H_{69}N_2O_2PS$ (637.2). ^{13}C NMR ($CDCl_3$) δ 12.8 ppm (*s*, CH_3-5); 15.4 (*s*, CH_3-7); 15.8 (*s*, CH_3-8); 21.7 (*s*, C-4); 21.9 (*s*, C-3); 23.3 (*s*, CH_3-2); 75.4 (*s*, C-2); 144.1 (*d*, C-6, J=9 Hz); 149.3 (*s*, C-9): nucleus; 20.4 (*m*, C-13, CH_3-12); 23.3 (*s*, CH_3-4, CH_3-8): chain, DL-α-tocopheryl-6-*O*-fragment; 14.5 (*d*, CH_3CH_2N, J=4 Hz); 41.5 (*d*, CH_2N, J=4 Hz). ^{31}P NMR ($CDCl_3$) δ 72.5 ppm (*s*). IR (KBr, film) ν 1249, 840 (PO-C, P-OC_{aryl}); 730 (P-N); 690 cm^{-1} (P=S).

Ergocalciferyl-3-O-(diacetone-D-glucosyl-3-O-)-(N,N-diethylamido)thiophosphate (**1adS**) *(representative procedure)*. The intermediate **1a** was prepared using ergocalciferol (**a**; 0.793 g, 2.0 mmol) as described for **1aS**. Diacetone-D-glucose (**d**; 0.521 g, 2.0 mmol) was then added and the mixture was heated at 70°C for 3.5 hr. Transformation to the thiophosphate **1adS** was accomplished by reaction with sulfur (0.067 g, 2.1 mmol) at 70°C for 5 min. The solvent was removed under vacuum, and the compound was isolated by HPLC in pure form. Yield of **1adS**: 1.37 g (87%); n_D^{40} = 1.5053; R_f ($CHCl_3$) 0.52; $C_{44}H_{72}NO_7PS$ (790.2). ^{13}C NMR ($CDCl_3$) δ 12.5 ppm (*m*, C-18); 18.0 (*s*, C-28); 19.9 (*s*, C-26); 20.2 (*s*, C-27); 74.9 (*m*, C-3); 116.5, 118.1 (each *d*, C-7, J=9 Hz); 121.6, 123.0 (each *d*, C-6, J=9 Hz): ergocalciferyl-3-*O*-fragment; 26.5 (*m*, C-11, C-12); 26.9 (*m*, C-8, C-9); 79.2, 80.9 (each *d*, C-3, J=9 Hz); 83.6, 83.9 (each *s*, C-2); 105.4, 105.6 (each *s*, C-1); 109.1, 109.4 (each *s*, C-7); 112.1 (*s*, C-10): glucosyl-3-*O*-fragment; 14.3 (*m*, CH_3CH_2N); 40.4 (*m*, CH_2N). ^{31}P NMR ($CDCl_3$) δ 73.2 ppm (*m*). IR (KBr, film) ν 1636 (C=C); 1024, 818 (PO-C, P-OC); 731 (P-N); 700 cm^{-1} (P=S).

Cholecalciferyl-3-O-(diacetone-D-glucosyl-3-O-)-(N,N-diethylamido)thiophosphate, (**1bdS**). The compound **1bdS** was prepared using cholecalciferol (**b**; 0.769 g, 2.0 mmol) and diacetone-D-glucose (**d**; 0.521 g, 2.0 mmol) as described for **1adS**. Yield of **1bdS**: 1.29 g (83%); n_D^{40} = 1.4975; R_f ($CHCl_3$) 0.50; $C_{43}H_{72}NO_7PS$ (778.2). ^{13}C NMR ($CDCl_3$) δ 12.2 ppm (*m*, C-18); 19.2 (*s*, C-21); 22.8 (*s*, C-26); 23.0 (*s*, C-27); 76.0 (*m*, C-3); 116.7, 118.0 (each *s*, C-7); 135.1, 135.3 (each *s*, C-8): cholecalciferyl-3-*O*-fragment; 26.3 (*m*, C-11, C-12); 26.7 (*m*, C-8, C-9); 79.0, 80.7 (each *d*, C-3, J=9 Hz); 83.7 (*m*, C-2); 105.0, 105.1 (each *s*, C-1); 109.0, 109.2 (each *s*, C-7); 112.0, 112.1 (each *s*, C-10): glucosyl-3-*O*-fragment; 14.0 (*m*, CH_3CH_2N); 40.0 (*m*, CH_2N). ^{31}P NMR ($CDCl_3$) δ 73.1 ppm (*m*). IR (KBr, film) ν 1637 (C=C); 1025, 817 (PO-C, P-OC); 730 (P-N); 705 cm^{-1} (P=S).

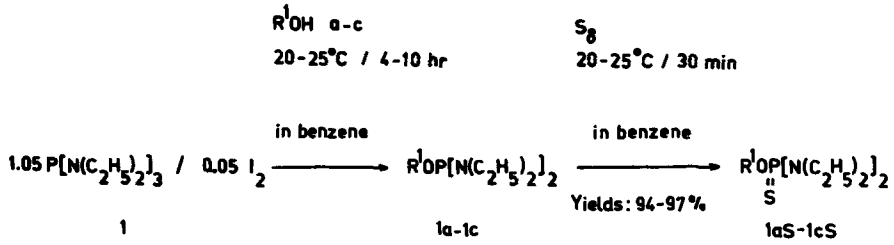
DL-α-Tocopheryl-6-O-(diacetone-D-glucosyl-3-O-)-(N,N-diethylamido)thiophosphate (**1cdS**). The thiophosphate **1cdS** was prepared by first using diacetone-D-glucose (**d**; 0.521 g, 2.0 mmol) (70°C; 15 min) and then DL-α-tocopherol (**c**; 0.861 g, 2.0 mmol) (second step: 70°C; 5 hr) in the same way as described for **1adS**. Yield of **1cdS**: 1.38 g (84%); n_D^{40} = 1.4923; R_f ($CHCl_3$) 0.44; $C_{45}H_{78}NO_8PS$ (824.3). ^{13}C NMR ($CDCl_3$) δ 11.9 ppm (*s*, CH_3-5); 14.8, 14.9 (each *s*, CH_3-7); 15.2 (*s*, CH_3-8); 74.8 (*s*, C-2); 141.7 (*d*, C-6, J=9 Hz); 148.5 (*d*, C-9, J=2 Hz): nucleus; 19.6 (*m*, C-13, CH_3-12); 22.5, 22.6 (each *s*, CH_3-4, CH_3-8): chain, DL-α-tocopheryl-6-*O*-fragment; 26.1 (*s*, C-11, C-12); 26.5 (*m*, C-8, C-9); 79.5 (*d*, C-3, J=5 Hz); 80.7 (*d*, C-4, J=8 Hz); 104.7 (*s*, C-1); 108.9 (*s*, C-7); 112.0 (*s*, C-10): glucosyl-3-*O*-fragment; 13.9 (*m*, CH_3CH_2N); 40.6 (*m*, CH_2N). ^{31}P NMR ($CDCl_3$) δ 73.1 ppm (*m*). IR (KBr, film) ν 1240, 840 (PO-C, P-OC_{aryl}); 750 (P-N); 635 cm^{-1} (P=S).

RESULTS AND DISCUSSION

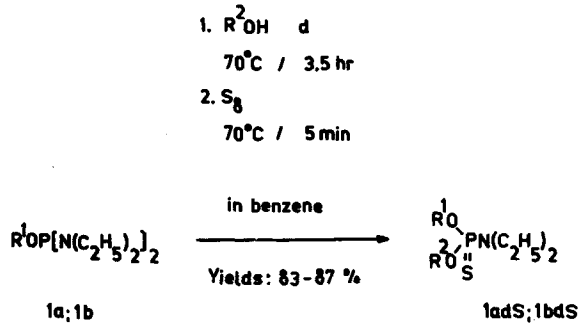
It is well known that the phosphorylation of lipids by phosphorus oxychloride, chlorophosphates and other conventional reagents can frequently be accompanied by side reactions, *e.g.*, nucleophilic substitution at the carbon with the phosphatoxyl group, molecular rearrangements, eliminations, etc. (5). Use of acyclic triamides of phosphorous acid after activation with iodine avoids these problems (5,7,8). In the present study, the tris(*N,N*-diethyl)-amide of phosphorous acid **1** (Schemes 1, 2 and 3) with iodine at an optimal molar ratio of 1.05:0.05 was used as reagent. Ergocalciferol **a**, cholecalciferol **b**, DL-α-tocopherol **c**, and diacetone-D-glucose **d** were selected as substrates (Schemes 1, 2 and 3).

The synthesis was carried out according to a method proposed recently (8). The activated phosphamide **1** was reacted with the vitamin **a-c** in stoichiometric amounts at room temperature (20–25°C) for 4 hr (10 hr for DL-α-tocopherol) to give the *bis*(*N,N*-diethylamido)phosphite **1a-1c** in close to quantitative yield. This was proven by transformation of **1a-1c** to the corresponding thiophosphate derivatives **1aS-1cS** (Scheme 1).

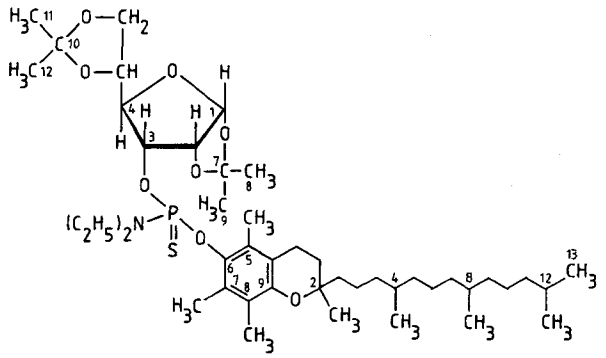
The high selectivity of the process at the monoester formation stage permits the next step, *viz.*, the synthesis of unsymmetrical diesters, to be performed as a "one-pot" procedure. In order to avoid extended reaction times at

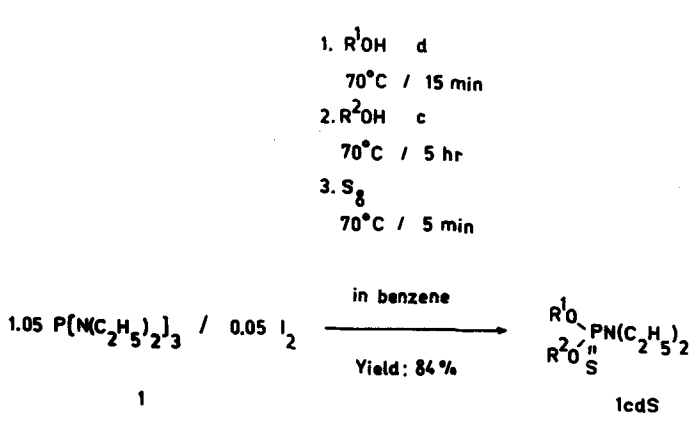


SCHEME 1. For a, 1a and 1aS: R^1 = ergocalciferyl; b, 1b and 1bS: R^1 = cholecalciferyl; c, 1c and 1cS: R^1 = DL-*α*-tocopheryl.



SCHEME 2. For 1a and 1adS: R^1 = ergocalciferyl; 1b and 1bdS: R^1 = cholecalciferyl; d, 1adS and 1bdS: R^2 = diacetone-D-glucosyl.





SCHEME 3. For d and 1cdS: R^1 = diacetone-D-glucosyl; c and 1cdS: R^2 = DL-*α*-tocopheryl.

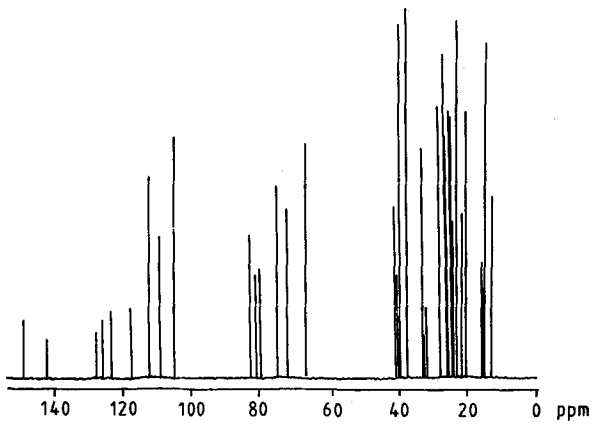


FIG. 2. Proton-decoupled 75.43 MHz ^{13}C NMR spectrum of DL-*α*-tocopheryl-6-*O*-(diacetone-D-glucosyl-3-*O*-), (*N,N*-diethylamido)thiophosphate.

room temperature, the second P-O bond was introduced by moderate heating. Thus, the consecutive treatment of the crude intermediate **1a**, **1b** with an equivalent quantity of diacetone-D-glucose **d** and sulfur at 70°C for 3.5 hr and 5 min, respectively, afforded the thiophosphate derivative **1adS**, **1bdS** in high yield (Scheme 2). An approach which used diacetone-D-glucosyl-3-*O*-*bis*-(*N,N*-diethylamido)phosphite as the initial key intermediate was not effective because transesterification occurred.

Treatment of the DL-*α*-tocopheryl-6-*O*-*bis*(*N,N*-diethylamido)phosphite **1c** with diacetone-D-glucose **d** at 70°C led to displacement of the tocopheryl radical. Thus, an alternative "one-pot" scheme with diacetone-D-glucosyl-3-*O*-*bis*(*N,N*-diethylamido)phosphite as initial key intermediate was used in the synthesis of the DL-*α*-tocopheryl-6-*O*-(diacetone-D-glucosyl-3-*O*-)-(*N,N*-diethylamido)thiophosphate **1cdS** (Scheme 3; Fig. 2).Conclusions in regard to the structures of the compounds synthesized were drawn based on spectral and microanalytical data.

The thiophosphates reported here were difficult to analyze by ^1H NMR. Individual signals were found to overlap in the extremely complex proton spectra. Thus, structure elucidation heavily relied on ^{13}C NMR data, where signal assignments were based mainly on broad band decoupling, off-resonance coherent decoupling and chemical shift comparison of the starting substrates **a-d** with their thiophosphate derivatives **1aS-1cS** and **1adS-1cdS**. Two-dimensional ^1H-^{13}C correlation NMR

techniques were also used to gather additional information on the tocopherol structures.

Multiplicities were observed for the phosphorus ester carbons C-3 and C-6. Other anisochronicities (9) detected in the ^{13}C NMR spectra (for example, **1aS**: two singlets, 108.2, 112.6 ppm, C-19; **1bS**: two singlets, 12.2, 12.3 ppm, C-18; etc.) were attributed to diastereoisomerism. The multiplicities observed in the ^{31}P NMR spectra (**1aS**: two singlets, 77.2, 77.4 ppm; **1adS**: multiplet, 73.2 ppm; **1cdS**: multiplet, 73.1 ppm; etc.) agree with the latter interpretation. The absence of anisochronicities in the ^{13}C NMR spectra of the starting substrates **a-d** is also consistent with this view. Our attempts to separate the indicated isomers by HPLC were not successful.

ACKNOWLEDGMENT

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METHOD

Ion-Pair High-Performance Liquid Chromatography of Bile Salt Conjugates: Application to Pig Bile

Véronique Legrand-Defretin[a,*], Catherine Juste[a], Robert Henry[b] and Tristan Corring[a]

[a]Laboratoire d'Ecologie et de Physiologie du Système Digestif, C.R.J.-I.N.R.A., 78350 Jouy-en-Josas, France, and [b]Laboratoire de Chimie Organique, I.N.S.A. Bâtiment 403, 69621 Villeurbanne, France

The high-performance liquid chromatographic separation and quantitation of conjugated bile salts from pig bile is reported. Synthetic standards and bile samples were chromatographed on a C_{18} reversed phase column using acetonitrile/water/tetrabutyl ammonium phosphate as an isocratic mobile phase at a flow rate of 1 mL/min. Detection of the ion-pairs was at 214 nm. The method permits efficient separation of all conjugated pig biliary bile salts without prior modification or treatment of the samples. Analysis of 12 pig biles showed that 85% of the bile salts are conjugated to glycine. The three main conjugated bile salts were glyco-3α,6α,7α-trihydroxy-5β-cholanoic acid (GHC), glyco-3α,7α-dihydroxy-5β-cholanoic acid (GCDC), and glyco-3α,6α-dihydroxy-5β-cholanoic acid (GHDC). Glyco-3α-hydroxy-6-oxo-5β-cholanoic acid (G3α6oxo), tauro-3α,7α-dihydroxy-5β-cholanoic acid (TCDC), tauro-3α,6α,7α-trihydroxy-5β-cholanoic acid (THC), and tauro-3α,6α-dihydroxy-5β-cholanoic acid (THDC) were found to contribute each for 4 to 5% ot the total. An excellent correlation was found between the sum of conjugated bile salts quantitated by high-performance liquid chromatography (HPLC) and values obtained by conventional enzymatic assay. Simplicity, efficiency and relative rapidity of the method render it suitable for routine analyses.

Lipids 26, 578–583 (1991).

In recent years, high-performance liquid chromatography (HPLC) has been applied to the analysis of bile acids in human (1–9), rat (1,10), dog (9), rabbit (9), and hamster (11) bile. Although the pig is considered an interesting model for biomedical research (12–20), the analysis of pig bile has not been previously reported. Among the species studied, pig bile is closest to man with respect to bile salt/cholesterol and phospholipid/cholesterol ratios (12,13).

The present paper describes a reliable method for the routine analysis of biliary bile salts from pig. Among various chromatographic systems available to-date, some (2–4) are rather time-consuming because they require prior hydrolysis and derivatization, or group separation of bile salts, and thus do not offer substantial improvements over earlier gas and thin-layer chromatographic techniques. Moreover, decomposition of conjugated bile salts can occur. We therefore retained only those steps of earlier techniques that do not cause these disadvantages. They include a reversed-phase separation either under strict pH control of the mobile phase (1,5,7,11) or addition of ion-pair chromatographic reagents (6,8,9,21). The latter approach seems more appropriate for routine analysis since the problem of strict pH control is circumvented. We applied this approach (6,9) to the analysis of pig bile, which contains the unusual hyocholic, hyodeoxycholic, and 3α-hydroxy-6-oxo-5β-cholanoic conjugates, and we optimized the isocratic high-performance liquid chromatographic separation of pig biliary bile salts. Using this method, it was possible for the first time to quantitatively determine all bile salt species in pig bile.

*To whom correspondence should be addressed.

Abbreviations:HPLC, high-performance liquid chromatography; C, GC, TC, 3α,7α,12α-trihydroxy-5β-cholanoic acid and its glyco- and tauroconjugates; CDC, GCDC, TCDC, 3α,7α-dihydroxy-5β-cholanoic acid and its glyco- and tauroconjugates; DC, GDC, TDC, 3α,12α-dihydroxy-5β-cholanoic acid and its glyco- and tauroconjugates; HC, GHC, THC, 3α,6α,7α-trihydroxy-5β-cholanoic acid and its glyco- and tauroconjugates; HDC, GHDC, THDC, 3α,6α-dihydroxy-5β-cholanoic acid and its glyco- and tauroconjugates; 3α6oxo, G3α6oxo, T3α6oxo, 3α-hydroxy-6-oxo-5β-cholanoic acid and its glyco- and tauroconjugates; MC, GMC, TMC, 3α,6β-dihydroxy-5β-cholanoic acid and its glyco- and tauroconjugates; TBAP, tetrabutyl ammonium phosphate; RRT, relative retention time; AUFS, absorbance units full scale.

MATERIALS AND METHODS

Equipment. The HPLC equipment used was a Beckman high-performance liquid chromatograph (Model 110-A Pump, Model SpH-125 FIX Autoinjector with a 20-μL sample loop, and a Model 160 Detector, Beckman Instruments, Palo Alto, CA) equipped with a Model CR-3A Integrator (Shimadzu, Tokyo, Japan).

Reagents. All reagents were of HPLC grade. Water was purchased from Farmitalia Carlo Erba (Milano, Italy). Acetonitrile, methanol, and ethanol were purchased from Merck (Darmstadt, Germany). Tetrabutyl ammonium phosphate 0.5 M (TBAP) was obtained from Regis Chemical (Morton Grove, IL). Sep-Pak C_{18} cartridges (Waters Associates, Milford, MA) were used to purify the bile samples.

Glycocholate (GC), taurocholate (TC), glycochenodeoxycholate (GCDC), taurochenodeoxycholate (TCDC), glycodeoxycholate (GDC), and taurodeoxycholate (TDC) were of the highest purity available from Sigma (St. Louis, MO). Taurohyocholate (THC) was kindly provided by Dr. J.Cl. Montet (INSERM U-31, Marseille, France).

Synthesis of bile salts. Glycohyocholate (GHC), glycohyodeoxycholate (GHDC), and taurohyodeoxycholate (THDC) were synthesized according to the method of Tserng *et al.* (22). Attempts to synthesize glyco-3α-hydroxy-6-oxo-5β-cholanoic acid (G3α6oxo) from 3α-hydroxy-6-oxo-5β-cholanoic acid led to a mixture of epimerization products in which the 5α-isomers predominated. Epimerization was avoided by oxidizing GHDC with Jones' reagent (23).

For this purpose, Jones' reagent (1.33 mmol in 1.23 mL) was added to a solution of GHDC (2 mmol) in acetone (50 mL) at 0°C under stirring. The mixture was left for 1 hr at 0°C, then 2 drops of isopropanol were added. The mixture was then poured into ice-water (200 mL). It was successively extracted with ethyl acetate (3 times, 150 mL each), washed with water (50 mL) and a saturated NaCl

solution (20 mL), and evaporated to dryness. A white solid residue (520 mg) was obtained which showed two main fractions (Rf 0.62 and 0.55) in thin-layer chromatography (Silicagel 60 F, Merck; eluent, chloroform/ethanol/ethyl acetate/acetic acid/water, 8:6:5:1:1, by vol, Solvent A; spray reagent, phosphomolybdic acid). The residue (520 mg) was subjected to column chromatography on silica gel 60 (100 g, 70-230 mesh, Merck). Elution with chloroform/ethanol/ethyl acetate/water (8:6:5:1, by vol) and evaporation to dryness gave G3α6oxo (57 mg) as a white and very hygroscopic solid (Rf 0.55, using Solvent A). Final purity (99%) was checked by gas chromatography after hydrolysis with cholylglycine hydrolase.

Operating conditions. An Ultrasphere IP column (particle size 5 μm, 250 × 4.6 mm i.d. Beckman Instruments) was used as a stationary phase. Loss of resolution of the column was observed with time, but the column could be regenerated by washing it successively with water, methanol, acetonitrile/tetrahydrofuran (50:50, v/v), methanol, and finally again with water. The mobile phase was prepared by mixing 65 mL water, 35 mL acetonitrile, and 1 mL TBAP 0.5 M. The flow rate was 1 mL/min. Detection was at 214 mm and 0.1 absorbance units full scale (AUFS).

Samples. Mixtures of synthetic bile salts were prepared from pure bile salts dissolved in the eluent.

Choledocal bile samples were obtained from 12 healthy castrated large white pigs (46.7 ± 1.9 kg at the beginning of the experiment, Experimental Stock Farm, Institut National de la Recherche Agronomique, La Minière, France), fed a standard diet (dry matter, 87.80%; crude protein, 14.87%; fat, 3.44% of fresh matter; energy value, 3.85 kcal/g of fresh matter). Pigs were fitted with biliary and upper duodenal fistulas to exteriorize the enterohepatic circulation according to a method previously described (24,25). A representative 24-hr sample for each of the 12 pigs was obtained by continuous 2% bile derivation over a day-night cycle. This method of bile collection does not affect the enterohepatic circulation of bile salts in pig (25). Following these collection periods, bile restitution to the duodenum was discontinued (13). Biliary collection was then followed up and 12 samples with low bile salt concentrations were collected. All bile samples were frozen at −80°C until analysis.

Bile purification was done as follows (11): 50 to 150 μL of bile were diluted with 2 mL of 0.5 M phosphate buffer (pH 7.0), and the solution was passed through a Sep-Pak C_{18} cartridge previously washed with 3 mL of methanol and 6 mL of water. The cartridge was then washed with 8 mL of water and 2 mL of 1.5% ethanol. Finally, bile salts were eluted with 5 mL of the mobile phase, and 20 μL of the solution was injected into the HPLC.

Enzymatic analysis. Total bile salt in bile was determined using 3α-hydroxysteroid dehydrogenase (Sigma) according to Turley and Dietschy (26).

Expression of results and statistical analysis. Data were expressed as means ± SEM. All relative retention times (RRT) were determined with respect to GCDC. All relations among the data (peak area *versus* amount of bile salts injected; HPLC determination *versus* enzymatic determination) were explored using correlation and linear regression analysis (least square method).

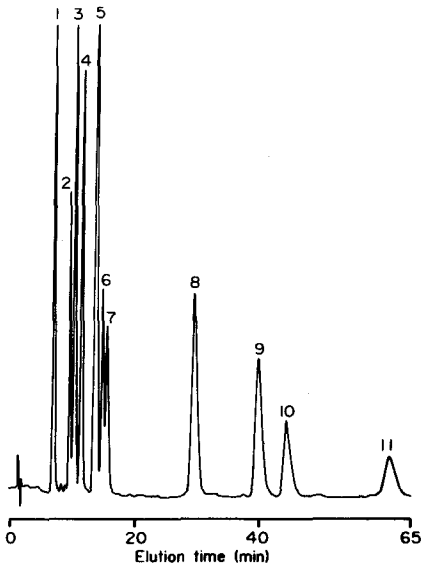


FIG. 1. Elution profile of a standard solution of pure bile salts. Chromatographic conditions: stationary phase, column Ultrasphere IP; solvent, water/acetonitrile/TBAP (65:35:1, v/v/v); flow-rate, 1 mL/min; detector wavelength, 214 nm, 0.1 AUFS. Peaks: 1 = GHC; 2 = THC; 3 = GHDC; 4 = GC; 5 = G3α6oxo; 6 = THDC; 7 = TC; 8 = GCDC; 9 = GDC; 10 = TCDC; 11 = TDC.

TABLE 1

Relative Retention Time of Bile Salt Conjugates[a]

Bile salt	Relative retention time[b]
Glycohyocholate	0.250 ± 0.003
Taurohyocholate	0.335 ± 0.004
Glycohyodeoxycholate	0.357 ± 0.005
Glycocholate	0.385 ± 0.003
G3α6oxo[c]	0.481 ± 0.007
Taurohyodeoxycholate	0.498 ± 0.003
Taurocholate	0.511 ± 0.001
Glycochenodeoxycholate	1.000
Glycodeoxycholate	1.301 ± 0.005
Taurochenodeoxycholate	1.468 ± 0.005
Taurodeoxycholate	1.887 ± 0.001

[a]Chromatographic conditions as in Figure 1.

[b]Relative to glycochenodeoxycholate = 1.000; n = 10.

[c]Glyco-3α-hydroxy-6-oxo-5β-cholanoic acid.

RESULTS

Separation of synthetic conjugated bile salts. A representative chromatographic tracing of purified standards to serve as reference is shown in Figure 1. Elution of the 11 bile salt conjugates tested in this study was completed in 1 hr and the separation achieved was very sharp for each. A close similarity in chromatographic behavior was observed between the glyco- and tauroconjugates. Indeed, for both glyco- and tauroconjugates the elution order was as follows: HC, HDC, C, CDC, DC. Glycine conjugates always emerged before their taurine analogues. Table 1 lists the RRT of the 11 conjugated bile salts studied.

Quantification of bile salts. The calibration curves for all bile salt conjugates are shown in Figure 2. At 214 nm, the molar absorbance of tauroconjugates on one hand, and

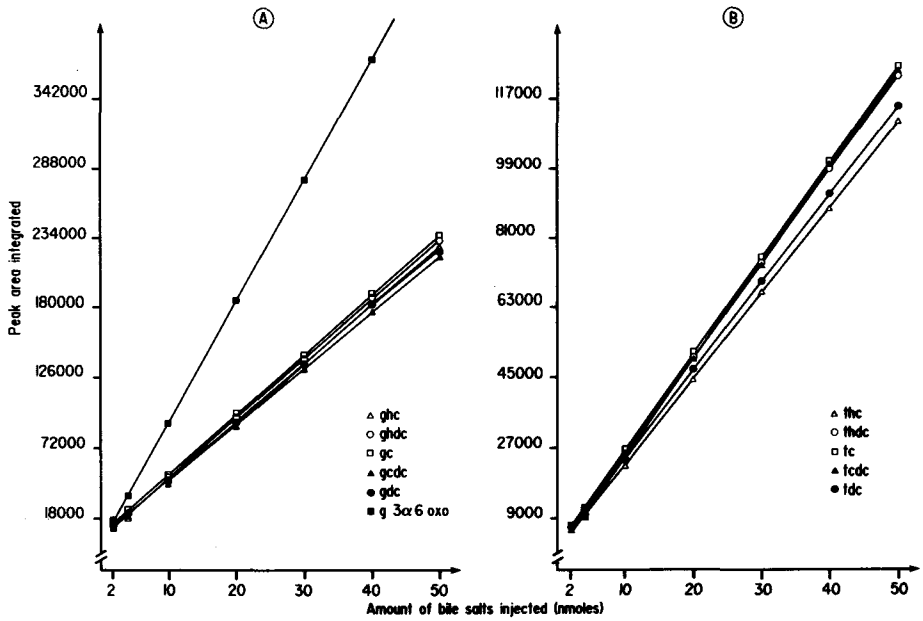


FIG. 2. Amounts of bile salt standards relative to the peak integrated areas for glycoconjugates (A) and tauroconjugates (B). Correlation coefficients of linear regression ranged from 0.963 to 0.999 for glycoconjugates and 0.995 to 0.999 for tauroconjugates. Chromatographic conditions as in Figure 1.

all glycoconjugates except G3α6oxo on the other were in the same range. Moreover, the molar extinction coefficient of a glycoconjugate was always higher than the corresponding tauroconjugate. As expected, the absorbance of the ketonic G3α6oxo was higher than of other glycoconjugates.

Within the range of concentrations tested (0.1 to 2.5 mM), all peak areas were proportional to the amount of bile salt injected into the HPLC. At 0.1 AUFS, the detection limit was 0.5 nmol for GHC, THC, GHDC, GC, GCDC, and GDC, and 1 nmol for THDC, TC, TCDC, and TDC. Correlation coefficients (r values) of linear regression ranged from 0.963 to 0.999 ($P<0.001$) and from 0.995 to 0.999 ($P<0.001$) for glycine and taurine conjugates, respectively.

To measure recoveries, standard samples of pure conjugated bile salts were added (10 nmol) to pig bile and subjected to HPLC after bile purification as described above. RRT were not affected by the conjugated bile salts present in the sample. Mean recoveries ranged from 89 to 102% (Table 2).

Biliary sample preparation. To test the recovery of bile salts after preparation of biliary samples with the Sep-Pak C_{18} cartridges, 10 bile samples were subjected to enzymatic assay of total bile salt concentration before and after the purification procedure. The mean recovery was 97 ± 1%. A special vacuum apparatus (Sep-Pak Cartridge Rak, Waters Associates) was employed to facilitate the extraction of a large number of bile samples. Ten cartridges were then run simultaneously for 20 min. This made the procedure feasible for routine analysis.

Analysis of pig bile. A representative separation of bile salts from pig bile is shown in Figure 3. Seven bile salt conjugates were identified in all chromatograms: GHC, THC, GHDC, G3α6oxo, THDC, GCDC, and TCDC. A minor unidentified peak (peak X, Fig. 3) occasionally appeared (only in chromatograms with high proportion of G3α6oxo) between TC and GCDC. Its RRT was 0.661 ± 0.001.

TABLE 2

Recovery of Bile Salts Added[a] to Pig Bile[b]

Bile salt (n)[c]	Recovery (%)
Glycohyocholate (15)	98
Taurohyocholate (10)	102
Glycohyodeoxycholate (15)	94
Glycocholate (15)	93
Taurohyodeoxycholate (10)	96
Taurocholate (10)	89
Glycochenodeoxycholate (15)	98
Glycodeoxycholate (15)	100
Taurochenodeoxycholate (10)	99
Taurodeoxycholate (10)	92

[a]Bile samples were supplemented with 10 nmol of each bile salt.
[b]Chromatographic conditions as in Figure 1.
[c]Number of assays.

Twelve choledocal bile samples collected from twelve pigs over a day-night cycle were analyzed according to the method developed. Table 3 shows that 85% of the bile salts found in porcine bile are conjugated to glycine. The primary bile salts, GHC and GCDC, are on average, the most abundant, comprising about 60% of the total. The dihydroxy-bile salt, GHDC, is only slightly less abundant (about 20% of the total). Respective proportions of these three main bile salts can, however, greatly differ between animals. G3α6oxo and the tauroconjugates, THC, THDC, and TCDC, each constitute about 4 to 5% of total bile salts measured (Table 3).

To check the chromatographic separation and quantita-

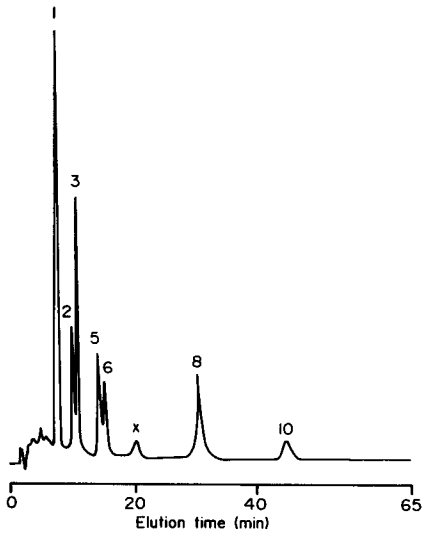


FIG. 3. Typical chromatogram of bile salts from normal pig bile. Peaks: 1 = GHC; 2 = THC; 3 = GHDC; 5 = G3α6oxo; 6 = THDC; 8 = GCDC; 10 = TCDC; X = unknown, see text. Chromatographic conditions as in Figure 1.

tion of bile salt conjugates in porcine bile, the summed value of individual bile salt concentrations obtained in HPLC was compared to enzymatically determined total bile salt concentration. This test was carried out on each of 24 samples which were collected in fistulated pigs with and without enterohepatic circulation in order to cover a wide range of bile salt concentrations. The slope and intercept of the regression line (Fig. 4) were 1.02 ± 0.01 and 0.77 ± 0.61, respectively. The correlation coefficient was 0.999 ($P<0.001$).

DISCUSSION

Addition of ion-pair chromatography reagent to the mobile phase allows better separation of ionic compounds and eliminates the problems of precise pH control in reversed phase HPLC (1,5,7,11). Thus in this study we developed a rapid, reproducible, and sensitive ion-pair technique for the simultaneous determination and quantitation of 11 bile salt conjugates. Particular attention was paid to the composition of the mobile phase (acetonitrile/water/counter-ion) in order to efficiently separate hyocholate and hyodeoxycholate conjugates. These bile salts had not been resolved before by HPLC in their conjugated forms (1,2,10).

Compared to reversed-phase chromatography (1,5,7,27), the addition of TBAP changes the elution sequence so that bile salts conjugated with glycine are eluted ahead of taurine conjugates. A reversed elution order for tauro- and glycoconjugates in reversed-phase and ion-pair HPLC systems has been observed previously (6,8,9,21), presumably due to differences in the interaction of bile salts and "ion-paired bile salts" with the stationary phase.

Disregarding the nature of conjugation, elution order of bile salt species, HDC, C, CDC, and DC, was identical with the order reported in reversed-phase chromatography (1,5,11,27). This has been discussed previously (28,29). The emergence of HC before C may be explained by the closer proximity of the 3α,6α,7α-hydroxyl groups than of the 3α,7α,12α-hydroxyl groups.

Linear regression between the amount injected and the peak area for each bile salt conjugate allows direct determination of concentrations. Due to our interest in measuring individual bile salt concentrations in pig bile (0.2 to 37.2 mM), no effort was made to extrapolate the standard curves to lower ranges of concentration. The detection limit was, however, 0.5 to 1 nmol which is within the range previously reported in ion-pair and reversed-phase HPLC (0.1 to 20 nmol, 1,5,7,11,27). To enhance detectability, more sophisticated techniques such as HPLC coupled with immobilized 3α-hydroxysteroid dehydrogenase column have been successful (10,30). Nevertheless, the expense involved limits the routine use of these techniques.

Knowledge about the profile of conjugated bile salts in pig bile remains fragmentary (14,20,31–37). The aim of the present work was to devise a rapid procedure that allows simultaneous determination and quantitation of glyco- and tauroconjugated bile salts present in pig bile. The use of a Sep-Pak cartridge allowed the direct and reproducible clean-up of bile samples without prior deproteinization. The elution profile of pig bile samples showed G3α6oxo and glyco- and tauroconjugates of the three bile salts, HC, HDC, and CDC. All these bile salts were separated in a single run while they had previously only been characterized separately by thin-layer and gas chromatography (14,31,33,34,36). Cholic and deoxycholic conjugates were not detected in pig bile, while one previous study on gallbladder porcine bile reported trace amounts of cholic acid (12). However, in the latter study, no 3α6oxo was detected. Surprisingly, pig hepatocytes in culture (20) were recently shown to synthesize small quantities of 3α,6β-dihydroxy-5β-cholanoic acid (murocholic acid, MC). With the present method, murocholic conjugates were shown to emerge with a relative retention time of 0.275 and 0.376 for glyco- and tauroconjugates, respectively, which is quite different from the relative retention times of all bile salt species identified in pig bile. 6-Epimerization of MC into HDC could prevent the secretion of MC into bile *in vivo*. One unknown bile salt, for which a reference standard was not available, appeared in very small amounts in some chromatograms. This compound may be tentatively assigned as the tauroconjugate of 3α6oxo as it fulfills the following criteria: (i) it appears only in chromatograms with high proportions of G3α6oxo; (ii) when the elution order of glyco- and tauroconjugates is juxtaposed, the unidentified peak emerges between TC and TCDC in the same manner as G3α6oxo emerges between GC and GCDC. Unfortunately, attempts to predict the RRT of T3α6oxo from a variance analysis of the RRT of the 11 standard bile salts (using the GENSTAT procedures, REGRE, FIT, and DROP) were unsuccessful. Confirmation of the identity of this compound as T3α6oxo is thus still necessary.

Quantitative determination of the bile salt spectrum for the 12 pigs clearly showed that, whatever the bile salt species, 83.7 to 86.0% of the material was conjugated with glycine. This is somewhat higher than reported in an earlier study (38). Especially high proportions of 6α-hydroxylated bile salts (GHC + THC = 38.6%, GHDC + THDC = 24.3%) were found in porcine bile. The pig is the only known species to exhibit this type of high 6α-hydroxylation activity although this pathway has been

TABLE 3

Bile Salt Composition in Pig Bile[a]

Pig	GHC (mM)	THC (mM)	GHDC (mM)	G3α6oxo (mM)	THDC (mM)	GCDC (mM)	TCDC (mM)
1	27.4	3.4	10.4	3.6	1.4	19.6	1.9
2	15.0	1.7	28.5	6.4	5.9	28.5	3.7
3	33.2	5.0	13.6	2.6	1.8	23.9	3.3
4	37.2	8.1	20.2	3.8	3.3	7.8	1.1
5	15.3	1.8	19.1	4.0	5.1	18.9	2.7
6	24.5	5.6	6.3	0.2	1.3	16.5	6.9
7	6.5	1.1	10.7	3.7	1.8	10.4	1.7
8	22.8	2.9	10.3	1.6	1.9	15.6	2.6
9	20.9	3.2	6.9	2.3	0.4	14.0	2.6
10	10.2	0.6	6.9	1.8	0.9	14.3	3.3
11	34.7	8.8	11.5	4.3	4.0	17.0	6.5
12	22.5	1.8	20.4	3.1	4.8	23.0	4.7
Mean ± SEM	22.5[b] 2.8	3.7[d] 0.8	13.7[c] 2.0	3.1[d] 0.5	2.7[d] 0.5	17.4[b,c] 1.7	3.4[d] 0.5
Range	6.5–37.2	0.6–8.8	6.3–28.5	0.2–6.4	0.4–5.9	7.8–28.5	1.1–6.9

[a]Chromatographic conditions as in Figure 1. Choledocal bile samples were collected from 12 pigs as described in Materials and Methods.
[b,c,d]Means with a common superscript in the same row are not significantly different (P>0.05).

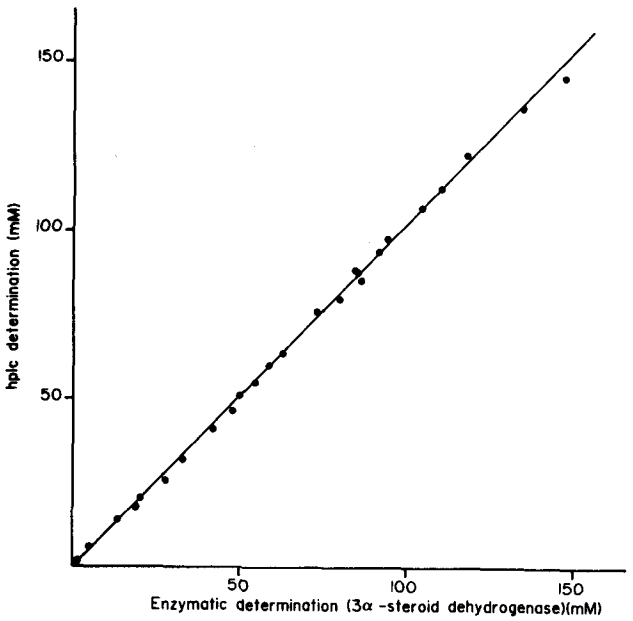


FIG. 4. Correlation between total bile salt concentrations in pig bile (n = 24) measured enzymatically and by summing the individual bile salt concentrations determined by HPLC. Slope (1.02 ± 0.01) not different from 1; intercept (0.77 ± 0.61) not different from 0; correlation coefficient r = 0.999 (P<0.001). Chromatographic conditions as in Figure 1.

shown to also exist in other species. Trace amounts of HC (<0.5%) and HDC (<1.5%) have been detected in rat bile (39). During the prenatal phase in man, 6α-hydroxylation of bile acid is a major metabolic pathway (40,41). Considerable repression of this activity then seems to occur, although certain mechanisms of derepression seem to become apparent in cases of cholestatic liver disease (42,43). The three main conjugated bile salts in porcine bile were GHC, GCDC, and GHDC. Although pigs were from the same breed and fed the same diet, appreciable differences were found in their respective proportions of GHC and GHDC. GCDC proportions were somewhat more constant. It is highly probable that the intestinal 7α-dehydroxylation of GHC in GHDC was more efficient in certain pigs. Another possible explanation might be hepatic regulation of the GHC/GHDC ratio. Although regulation through less active hepatic 7α-rehydroxylation of GHDC in GHC is not possible (34), other metabolic pathways may be involved. Indeed, GHDC may be generated by the liver, either through 6α-rehydroxylation of lithocholic acid, or through direct hepatic *de novo* synthesis, though the latter pathway still needs to be studied (37,44). The other bile salts (G3α6oxo and tauroconjugates) identified in this study each constitute less than 5.5% of total bile salts measured.

In summary, we showed that ion-pair HPLC is a simple and useful technique for the analysis of conjugated bile salts in pig bile. This method preserves the structural integrity of the conjugated bile salts since prior modification and treatment of the sample is avoided. The relative rapidity of the method renders it suitable for routine analysis. In our study it was possible to quantitatively determine the overall bile salt composition of pig bile within less than one hour using one mobile phase under isocratic conditions.

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REVIEW

Sterol Biosynthesis Inhibitors: Their Current Status and Modes of Action[1]

E.I. Mercer
Department of Biochemistry, University College of Wales, Aberystwyth, Dyfed SY23 3DD, United Kingdom

The mechanism of each of the reactions in the post-squalene segment of the fungal and higher plant sterol biosynthetic pathway is outlined. The inhibitors of the enzymes catalyzing the reactions are described and how inhibition is brought about is explained in the areas where it is known.
Lipids 26, 584–597 (1991).

During the past twenty years, the sterol biosynthetic pathway has become of interest to agrochemical and pharmaceutical companies. The reason for this is that the primary fungitoxic effect of several classes of commercially-important fungicides has proved to be the inhibition of one or more of the enzyme catalyzing steps in sterol biosynthesis. These fungicides, now known as sterol biosynthesis inhibitors (SBI), were discovered by routine screening procedures. However, the realization that sterol biosynthesis in fungi is a prime target for fungicides, and the fear of the widespread appearance of resistance to the existing classes of SBI, has led to attempts to rationally design compounds that will inhibit other enzymes in the pathway in the hope that from amongst them will emerge new, commercially exploitable SBI.

Although inhibitors of sterol biosynthesis that act in the pre-squalene segment of the pathway are known (1), the SBI fungicides presently used in agriculture and medicine inhibit enzymes in the post-squalene segment of the fungal sterol biosynthetic pathway. The *prima facie* reason for this is that the pre-squalene segment suffers from selectivity problems which stem from the fact that it is mechanistically the same in fungi, animals and higher plants and is common to the biosynthesis of other physiologically important polyisoprenoid derivatives (*e.g.*, ubiquinone, plastoquinone, carotenoids, chlorophyll). The post-squalene segment, however, is significantly different in fungi, animals and higher plants and is common only to the polycyclic triterpenoids, of which the sterols appear to be the only class to have profound physiological importance. This is almost certainly too simplistic an explanation because it takes no account of intracellular compartmentation and the fact that some SBI exhibit marked selectivity in spite of the fact that the reaction they block (*e.g.*, 14-demethylation) is common to sterol biosynthesis in fungi, animals and higher plants. However, despite the reasons for it, whether right or wrong, the post-squalene segment of the pathway has been regarded as the most likely one to yield new SBI fungicides and therefore has been the subject of most of the recent investigations into sterol biosynthesis inhibitors in fungi and higher plants. Therefore, this review will deal exclusively with inhibitors of the post-squalene segment of the sterol biosynthetic pathway in these organisms. Other recent review literature in this area can be found in references 2–4.

POST-SQUALENE STEROL BIOSYNTHESIS

In fungi. All fungi except the Pythiaceae are able to synthesize sterols, and most fungi synthesize ergosterol as their main sterol. The conversion of squalene into ergosterol includes the following steps:

(a) oxidation of squalene to 2,3-oxidosqualene
(b) cyclization of 2,3-oxidosqualene to lanosterol
(c) methylation at C-24 to yield a 24-methylene sterol
(d) demethylation at C-14 to yield a 4,4-dimethyl-$\Delta^{8,14}$-sterol
(e) saturation of the Δ^{14}-double bond
(f) successive removal of the 4α- and 4β-methyl groups to yield a 4-demethyl-Δ^{8}-sterol
(g) isomerization of the Δ^{8}-double bond to yield a 4-demethyl-Δ^{7}-sterol
(h) introduction of a Δ^{5}-double bond to yield a 4-demethyl-$\Delta^{5,7}$-sterol
(i) introduction of a Δ^{22}-double bond
(j) saturation of the $\Delta^{24(28)}$-double bond introduced in step (c)

The sequence in which these steps occur is not the same in all ergosterol-producing fungi. In general, it may be said that i) the demethylation reactions occur before the double bond transformations in ring B and in the side chain, with that at C-14 occurring before those at C-4, where the 4α-methyl is removed first; ii) in filamentous fungi methylation at C-24 occurs before the demethylation reactions whilst the reverse is true of yeast; iii) the double bond transformations in ring B occur in the sequence $\Delta^{8}\rightarrow\Delta^{7}\rightarrow\Delta^{5,7}$; and iv) the way in which the double bond

[1]Based on a paper presented at the Symposium on Plant and Fungal Sterols: Biosynthesis, Metabolism and Function, held at the AOCS Annual Meeting, Baltimore, MD, April 1990.

Nomenclature used: Campesterol, 24α-methylcholest-5-en-3β-ol; cholesterol, cholest-5-en-3β-ol; cycloartenol, 4,4,14α-trimethyl-9β,-19-cyclo-5α-cholest-24-en-3β-ol; cycloeucalenol, 4α,14α-dimethyl-9β,-19-cyclo-5α-ergost-24(28)-en-3β-ol; desmosterol, cholesta-5,24-dien-3β-ol; dihydrobrassicasterol, 24β-methylcholest-5-en-3β-ol; ergosterol, 24β-methylcholesta-5,7,22(Z)-trien-3β-ol; fecosterol, 5α-ergosta-8,24(28)-dien-3β-ol; fucosterol, stigmasta-5,24(28E)-dien-3β-ol; lanosterol, 4,4,14α-trimethyl-5α-cholesta-8,24-dien-3β-ol; 24-methylene cholesterol, ergosta-5,24(28)-dien-3β-ol; 24-methylene cycloartanol, 4,4,14α-trimethyl-9β,19-cyclo-5α-ergost-24(28)-en-3β-ol; 24-methylene-24,25-dihydrolanosterol, 4,4,14α-trimethyl-5α-ergosta-8,24(28)-dien-3β-ol; 24-methylene lophenol, 4α-methyl-5α-ergosta-7,24(28)-3β-ol; obtusifoliol, 4α,14α-dimethyl-5α-ergosta-8,-24(28)-dien-3β-dien-ol; sitosterol, 24α-ethylcholest-5-en-3β-ol; stigmasterol, 24α-ethylcholesta-5,22(E)-dien-3β-ol; zymosterol, 5α-cholesta-8,24-dien-3β-ol.

Abbreviations: COI, cycloeucalenol obtusifoliol isomerase; Δ^{8}SI, sterol $\Delta^{8}\rightarrow\Delta^{7}$ isomerase; Δ^{14}SR, sterol Δ^{14}-reductase; DMI, demethylation inhibitor(s); FAD, flavin adenine dinucleotide; HEI, high energy intermediate(s); I_{50}, the concentration of an inhibitor required to inhibit the activity of an enzyme by 50%; LDAO, *N*-lauryl-dimethylamino-*N*-oxide; SAM, *S*-adenosylmethionine; SBI, sterol biosynthesis inhibitor(s); SMT, sterol methyltransferase(s); TS, transition state.

transformations in the side chain intermesh with those in ring B accounts for most of the sequence variations found in different fungal species.

In list of reactions involved in the post-squalene segment of the biosynthetic pathway of those fungi that do not produce ergosterol as their main sterol differs slightly from that given above (a–j) in that some extra reactions may occur and/or others may be omitted. For instance; i) fungi of the classes Chytridiomycetes and Hypochytridiomycetes synthesize cholesterol and 24-methyl and 24-ethylcholesterols; therefore they must be able to carry out a second transmethylation, introduce a Δ^5-double bond and saturate a $\Delta^{24(25)}$-double bond, but be unable to form a Δ^{22}-double bond; ii) fungi of the orders Saprolegniales and Leptomitales synthesize cholesterol, desmosterol, 24-methylene cholesterol and fucosterol and therefore must be unable to saturate a $\Delta^{24(28)}$-double bond in addition to having the peculiarities of the fungi of i); and iii) fungi of the order Uredinales have 24-ethylidene- and 24-ethyl-Δ^7-sterols as their main sterols and therefore must be able to carry out a second transmethylation, but be unable to introduce Δ^5- or Δ^{22}-double bonds (5).

In higher plants. All higher plants are able to synthesize sterols, and the predominant ones are sitosterol, stigmasterol and campesterol or dihydrobrassicasterol (6). The conversion of squalene into these sterols includes the following steps (6):

(a) oxidation of squalene to 2,3-oxidosqualene
(b) cyclization of 2,3-oxidosqualene to cycloartenol
(c) methylation at C-24 to yield 24-methylene cycloartanol (first chain methylation)
(d) demethylation at C-4 to yield a 4α,14α-dimethyl sterol (cycloeucalenol)
(e) opening of the 9β,19-cyclopropane ring to yield a 4α,14α-dimethyl-Δ^8-sterol (obtusifoliol)
(f) demethylation at C-14 to yield a 4α-methyl-$\Delta^{8,14}$-sterol
(g) saturation of the Δ^{14}-double bond introduced in step (f) to yield a 4α-methyl-Δ^8-sterol
(h) isomerization of the Δ^8-double bond to yield a 4α-methyl-Δ^7-sterol
(i) demethylation at C-4 to yield a 4-demethyl-Δ^7-sterol
(j) introduction of a Δ^5-double bond to yield a 4-demethyl-$\Delta^{5,7}$-sterol
(k) saturation of the Δ^7-double bond to yield a 4-demethyl-Δ^5-sterol
(l) methylation at C-28 to yield a 24(Z)-ethylidene sterol (second side chain methylation)
(m) saturation of $\Delta^{24(28)}$-double bond in 24-methylene- and 24-ethylidene sterols giving 24-methyl and 24-ethyl sterols, respectively
(n) introduction of a Δ^{22}-double bond

Although the sequence in which these steps occur is not the same in all higher plants, there is nevertheless a preferred sequence in most of them (6). It begins with the sequence (a)→(b)→(c)→(d)→(e)→(f)→(g)→(h), which then bifurcates. One fork, namely (h)→(i)→(j)→(k)→(m), gives the 24-methyl sterols; whilst the other, namely (h)→(l)→(i)→(j)→(k)→(m), gives the 24-ethyl sterols. It is not clear when step (n) occurs in the reaction sequences, but it is generally believed to be a late reaction (6).

A comparison of the higher plant reactions with those of fungi reveals two fundamental differences. Firstly, cycloartenol, a 9β,19-cyclopropyl sterol, is produced by the cyclization reaction rather than lanosterol, a Δ^8-sterol. This means that higher plant sterol biosynthesis has to include a reaction that is not seen in the fungal or the higher animal pathway, namely the opening up of the 9β,19-cyclopropane ring to generate the angular methyl group on C-10 and coincidentally to introduce a Δ^8-double bond. This reaction is catalyzed by the enzyme cycloeucalenol-obtusifoliol isomerase (COI). Secondly, the demethylation reactions occur in the sequence 4α, 14α, 4β, whereas in fungi the sequence is 14α, 4α, 4β (4β here refers to the biosynthetic origin or the methyl group rather than its orientation when demethylation occurs, which is 4α because the removal of the first methyl group from C-4 causes inversion of configuration).

INHIBITION OF POST SQUALENE STEROL BIOSYNTHESIS IN FUNGI AND HIGHER PLANTS

Squalene epoxidase. Squalene epoxidase (EC 1.14.99.7) catalyzes the oxidation of squalene to 2,3-oxidosqualene. It is monooxygenase and thus requires O_2 and reduced pyridine nucleotide for this purpose. The enzyme from yeasts is a microsomal flavin adenine dinucleotide (FAD)-requiring flavoprotein; that from *Candida* spp. prefers NADH (7,8), whilst that from *Saccharomyces cerevisiae* prefers NADPH (9,10). In fungi, the catalytic activity of squalene epoxidase requires the presence of a reductase which occurs with it in the membrane. The function of the reductase is to supply the squalene epoxidase with reducing equivalents from NAD(P)H. The catalytic activity of squalene epoxidase is also enhanced by a soluble protein factor(s) whose function is not clear (7,8). The *C. albicans* enzyme has been shown to be inactivated by Cu^{2+}, partially inhibited by thiol reagents and unaffected by CN^- (7). Little is known about the requirements of the higher plant enzyme.

The allylamine compounds, naftifine and terbinafine (Fig. 1), are antimycotics, now used in medicine, which are potent inhibitors of fungal squalene epoxidase (8,11–16) but are only weakly inhibitory towards the mammalian enzyme (8). They are primarily fungicidal against dermatophytes, and filamentous and dimorphic fungi, and fungistatic against some *Candida* species (17). Naftifine is used as a topical antimycotic whilst terbinafine can be used both topically and systemically. Naftifine and terbinafine are reversible, non-competitive inhibitors of squalene epoxidase (13,18). However, it is not yet clear how they bring about this inhibition. There is no evidence that they interrupt electron flow from NAD(P)H to the epoxidase; the reversible, non-competitive kinetics of inhibition effectively rule out a mechanism-based process; and their lack of an obvious structural resemblance to squalene suggests that they do not act as substrate analogues. Ryder (18) has suggested two possible inhibitory mechanisms. In one (Fig. 2) the allylamine molecule binds fairly loosely to two separate sites on the epoxidase, the naphthalene ring to the squalene binding site (A) and the side chain to a nearby lipophilic binding site (B). The enzyme is known to interact with various lipids (19) implying that it has a lipophilic binding site; moreover, the fungal and mammalian enzymes have different affinities for lipids which could explain the selec-

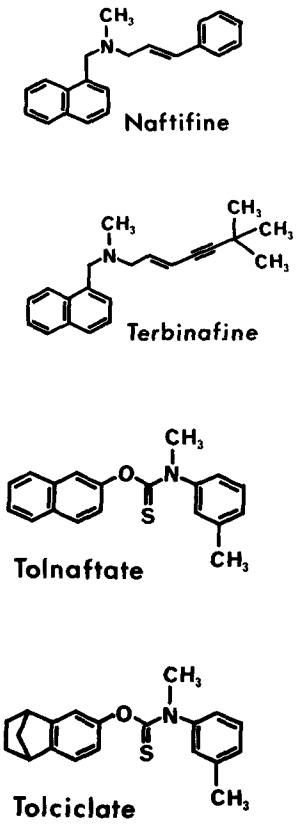


FIG. 1. Squalene epoxidase inhibitors.

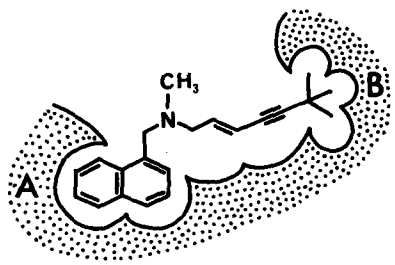


FIG. 2. Postulated mechanism of squalene epoxidase inhibition by allylamine fungicides, redrawn after Ryder (18). A is the squalene binding site and B is a lipophilic binding site on the enzyme.

tivity of the allylamines. Also in favor of this mechanism is the recent finding that naphthalene and 1-methylnaphthalene are weak inhibitors of the *Candida* squalene epoxidase (18).

The effect of the allylamines on higher plant epoxidase has not been tested but the fact that naftifine, applied to the roots of radish plants, caused a relatively weak inhibition of their growth (20) gives a *prima facie* indication that they may be only poor inhibitors of the higher plant enzyme. No fungicide of the allylamine type has been developed yet for use against fungi causing diseases of higher plants.

The thiocarbamate antifungals, tolnaftate and tolciclate (Fig. 1) have also been shown to inhibit squalene epoxidase in *Candida* spp. and *Trichophyton mentagrophytes* (21,22), but are less active than terbinafine (21).

The compounds 2,3-aza-2,3-dihydrosqualene and 1,1',2-tris-*nor*-3-trimethylammonium squalene iodide, designed as inhibitors of the 2,3-oxidosqualene cyclase (23,24), have been shown to inhibit squalene epoxidase in *Candida albicans* although they inhibited the 2,3-oxidosqualene lanosterol cyclase better (25). This fits in with the fact that, as analogues of the carbocationic high energy intermediates of the cyclization reaction, they should bind more tightly to the catalytic site of the cyclase than they would to that of the epoxidase, for which they are only substrate analogues.

2,3-Oxidosqualene cyclases. The fact that the product of 2,3-oxidosqualene cyclization is lanosterol in fungi and cycloartenol in higher plants would indicate that different enzymes catalyze the cyclization reaction in sterol biosynthesis in these organisms, namely 2,3-oxidosqualene lanosterol cyclase (EC5.4.99.7) and 2,3-oxidosqualene cycloartenol cyclase (EC 5.4.99.8), respectively. The substrate for both enzymes is the all-*trans*-stereoisomer of (3S)-2,3-oxidosqualene. For the cyclization reaction to proceed the substrate has to be folded, from its epoxy end, into a chair-boat-chair-boat sequence of potential cyclohexanoid rings. It is believed that this conformation is forced upon the substrate by the shape of the catalytic site of the cyclase during the formation of the enzyme-substrate complex. The C_8-unit at the non-epoxy end remains unfolded, and therefore does not take part in the cyclization reaction; it ultimately becomes the sterol side chain. Cyclization is apparently initiated by an electrophilic attack by a proton, probably derived from an appropriately located group at the catalytic site on the epoxy-O-C(2) bond. This leads to a wave of electron shifts from neighboring π-bonds running forward through the molecule, which ultimately results in the formation of four fused rings, three cyclohexanoid (sterol rings A, B and C) and one cyclopentanoid (sterol ring D). There is evidence (26,27) to suggest that the overall annulation process proceeds through a series of conformationally rigid carbocationic intermediates and terminates when the electron deficiency reaches C-19 (ultimately the sterol C-20), which is the start of the unfolded section of the molecule, yielding a carbocationic protosterol. This is the same for both cyclases, but has only a transient existence before undergoing a backward rearrangement by way of a series of Wagner-Meerwein shifts. There are four of these shifts for the lanosterol cyclase and five for the cycloartenol cyclase. These shifts involve the movement of H^- or H_3C^- from one carbon to an immediately adjacent carbon when the latter has an electron deficiency; they occur by way of a bridged carbocation and are permitted only when the moving and leaving groups are *trans* and coplanar.

Using the sterol numbering system, the sequence of these shifts for the lanosterol cyclase is i) 17β-H to C-20; ii) 13α-H to C-17; iii) 14β-methyl to C-13; and iv) 8α-methyl to C-14; the resulting C-8 carbocation is then stabilized by the removal of the 9β-H as proton, presumably by an appropriately placed nucleophilic group at the catalytic site.

The first four shifts in the sequence catalyzed by the cycloartenol cyclase are identical to those above, and then continue with v) 9β-H to C-8. The resulting C-9 carbocation is ultimately stabilized by the removal of a 19-H as a proton and the formation of a C-9 to C-19 bond resulting

in the 9β,19-cyclopropane ring. However, for steric reasons, the formation of this C-C bond cannot be concerted with the migration of the 9-H. This means that the final stages of the rearrangement/stabilization sequence should involve a discrete C-9 carbocationic intermediate, probably temporarily stabilized by a nucleophilic group of the cyclase, before 19-H elimination occurs.

This reaction has recently attracted a good deal of attention from designers of sterol biosynthesis inhibitors, probably because it is perceived as a potential target for fungicides, and its mechanism would allow for a rational approach to the design of such compounds. Cattel and Benveniste (*e.g.*, refs. 23,24) separately and in collaboration, have been the leaders in this field. Their aim has been to design high energy intermediate (HEI) or transition state (TS) analogues for three of the key postulated steps of the cyclization reaction sequence, namely: i) the initial step in which the attacking proton causes polarization of the epoxy-O-C(2) bond and thereby an electron deficiency, manifested as a fractional positive charge, on C-2 (A, Fig. 3); ii) the step leading to the formation of the C-20 carbocationic protosterol (B, Fig. 3); and iii) the final step in which the C-8 carbocation or C-(8,9) bridged carbocationic HEI is generated (C_1 and C_2; Fig. 3) (28).

The first compounds designed to inhibit step i) that were successful in the sterol fungal/higher plant context were structural variants of 2-aza-2,3-dihydrosqualene (Fig. 4), differing in the type (methyl, ethyl and isopropyl) and number (0–3) of alkyl substituents on the positively charged nitrogen (23,29). All except the isopropyl derivatives noncompetitively inhibited the cycloartenol cyclase of maize seedlings. It was then found that the *N*-oxide analogues of these compounds were even more effective (30,31), which was to be expected since they are nearer to the TS analogue ideal (28). 2-Aza-2,3-dihydrosqualene, its *N*-oxide, and *N*-laurel-dimethylamino-*N*-oxide (LDAO) inhibited the growth of bramble cells in suspension culture and caused them to accumulate 2,3-oxidosqualene, indicating that the cycloartenol cyclase had been inhibited (32). Squalene *N*-methyloxaziridine (33) and *N,N*-diethylazasqualene, its *N*-oxide and their bis-*N,N*-diethyl derivatives (34) inhibit the lanosterol cyclase of *S. cerevisiae*, presumably by blocking step i). Other compounds (Fig. 5) that probably inhibit the fungal/higher plant cyclases in this manner are 2,3-iminosqualene (35), AMO 1618 (36) and U-18666A (3β-[2-diethylaminoethoxy]-androst-5-en-17-one) (32,37).

To date, few inhibitors of the fungal/higher plant cyclase operating at step ii) have been discovered (Fig. 6). 20-Azadammarenol, which after protonation can be considered a HEI analogue of the C-20 protosterol, proved not to be an inhibitor of the plant cyclase (28). However, *N*-[2-(4,8-dimethyl-3(E),7-nonadienyl)phenylmethyl]-*N'*,*N'*-diethyl-1,2-ethanediamine has been found to inhibit the lanosterol cyclase of *C. albicans* (38).

In an attempt to produce a cyclase inhibitor operating at step iii), a number of *N*-substituted-8-aza-decalins have been synthesized and tested. Of these, *N*-[(1,5,9)-trimethyldecyl]-4α,10-dimethyl-8-aza-*trans*-decal-3β-ol and its 1,5,9-trimethyldeca-4,8-dienyl derivative (Fig. 7) proved to be good inhibitors of the cycloartenol cyclase of maize seedlings (28,39,40).

C-24 and C-28 S-adenosylmethionine (SAM):sterol methyltransferases (24- and 28-SMT). The methyl donor

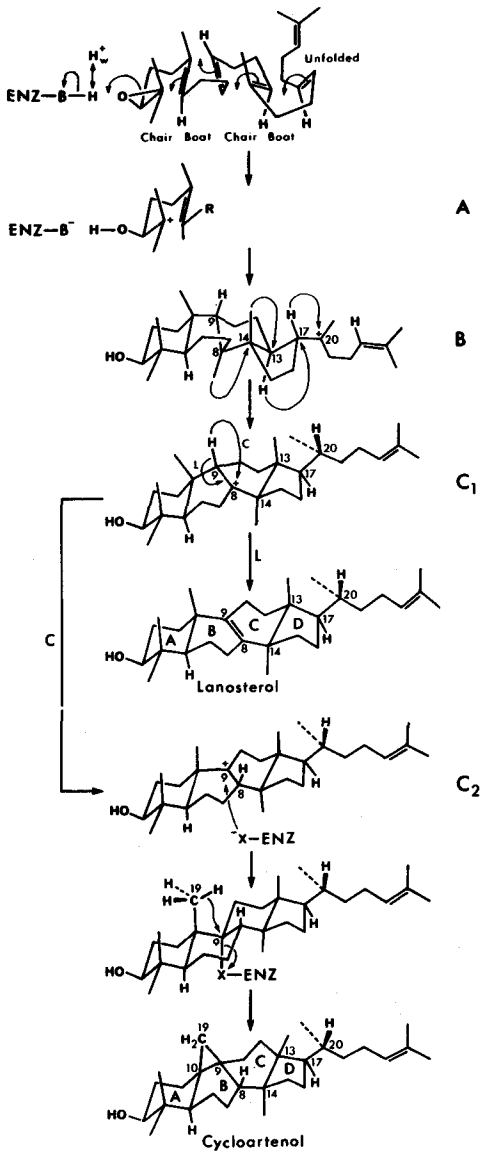


FIG. 3. Mechanism of the cyclization of 2,3-oxidosqualene to lanosterol (L) and cycloartenol (C). A, B, C_1 and C_2 are stages in the reaction sequence for which inhibitors have been designed.

for both methyltransferases is *S*-adenosylmethionine (SAM), and the acceptor sterol has to have an appropriately placed double bond, namely $\Delta^{24(25)}$ for the 24-SMT and $\Delta^{24(28)}$ for the 28-SMT. The acceptor sterols for the 24-SMT of filamentous fungi, yeast and higher plants are lanosterol, zymosterol and cycloartenol, respectively. The acceptor sterol for the 28-SMT is a 24-methylene sterol generated by the 24-SMT; this is 24-methylene lophenol in higher plants and may be 24-methylene-24,25-dihyrolanosterol in those fungi that are capable of synthesis of 24-ethylidene and 24-ethyl sterols. Although variations in the mechanism of the 24- and 28-transmethylation reactions have been observed (6,41), certain generalizations can be made, namely that: i) the 24-SMT generates a C-25 carbocationic intermediate; ii) in ergosterol-producing fungi and in 24-ethylidene/ethyl-sterol-producing organisms the 24-SMT also generates a C-24 carbocationic intermediate as a result of a 1,2-H^- shift from C-25 to

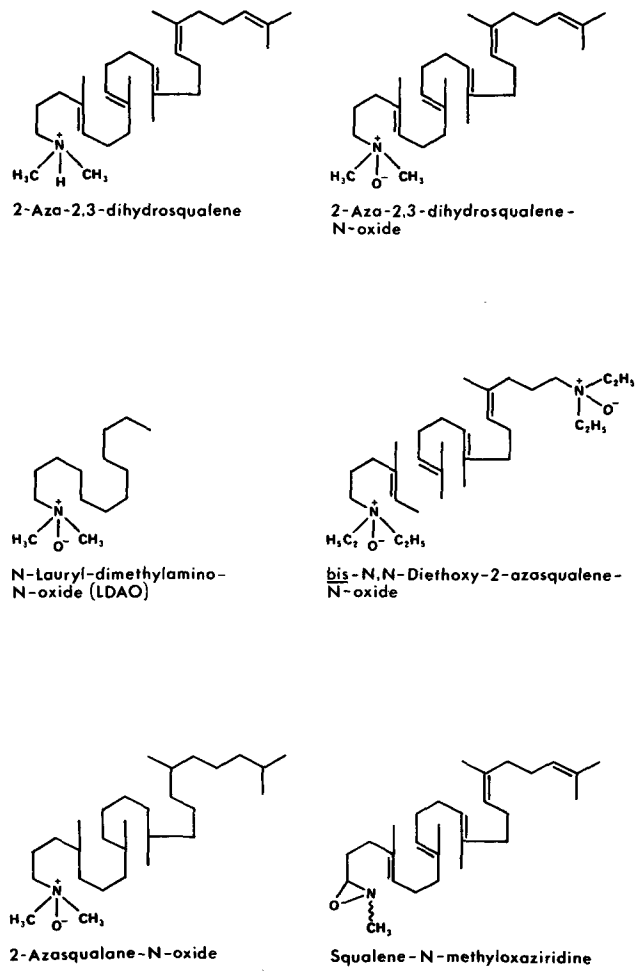


FIG. 4. Compounds designed to inhibit 2,3-oxidosqualene cyclases at stage A (see Fig. 3) of the cyclization reaction sequence.

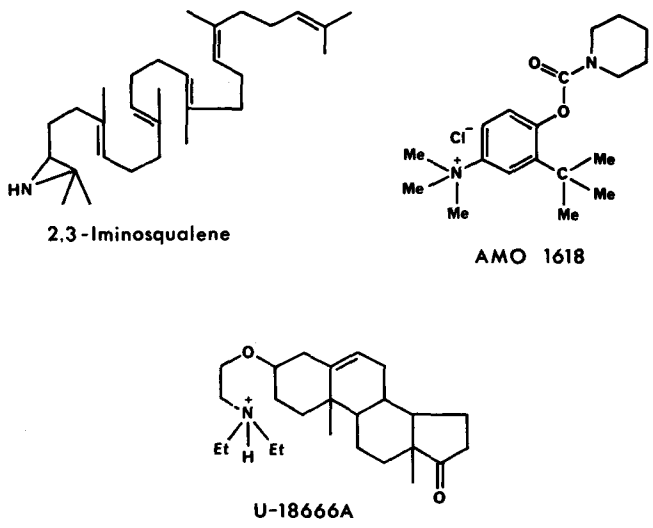


FIG. 5. Other compounds that inhibit 2,3-oxidosqualene cyclases at stage A (see Fig. 3) of the cyclization reaction sequence.

C-24; and iii) the 28-SMT generates a C-24 carbocationic intermediate. The participation of these two carbocationic intermediates in these reactions has stimulated the rational design of inhibitors of the 24- and 28-SMT. In general, these compounds are steroids with a positively charged heteroatom replacing either C-24 or C-25 in the hope that they would be satisfactory HEI analogues.

In aerobically-growing *S. cerevisiae*, 25-aza-24,25-dihydrozymosterol was shown to inhibit the 24-SMT (42), with the result that no 24-methylene- or 24-methyl-sterols were produced, and zymosterol accumulated. Tertiary amines such as this have a pK_a close to 10 and, consequently, mainly exist in the protonated state at physiological pH, thus placing a positive charge on the nitrogen atom. 23-Azacholesterol, with its N atom non-ideally placed to inhibit the 24-SMT, nevertheless did so (71% inhibition at 10 μM) in *S. cerevisiae* (43), although it was a far better inhibitor of the sterol $\Delta^{24(28)}$-reductase (100% inhibition at 1 μM). A 1 μM concentration of 23-azacholesterol in the growth medium of the *erg-2, erg-3* and *erg-5* mutants of *S. cerevisiae* also blocked the 24-SMT (44), causing zymosterol accumulation. Using a 24-SMT enzyme preparation from *S. cerevisiae*, the inhibitory power (expressed as μM I_{50} values) of a series of azasterols (Fig. 8) was found to be: 25-azacholesterol HCl (0.05) > 25-aza-24,25-dihydrozymosterol (0.08) > 25-azacholesterol = 25-azacholestanol (0.14) > (20*R*)- and (20*S*)-22,25-diazacholesterol (0.18) > 24-azacholesterol (0.22) > 25-aza-24,25-dihydrolanosterol (1.14) > 23-azacholesterol (4.8) (45). The 24-SMT of *Saprolegnia ferax* also has been shown to be inhibited by 25-azacholesterol (46), whilst that of *Gibberella fujikuroi* is inhibited by 24-epiiminolanosterol (47).

The 24- and 28-SMT present in microsomes prepared from maize embryos were shown to be inhibited by 25-azacycloartanol (48); moreover, a 1 μM concentration of this compound cause a marked decrease in 24-alkylated sterols in suspension cultures of bramble cells (48). The relative potencies of 25-azacycloartanol and an extensive range of related compounds as inhibitors (Fig. 9) of the 24-SMT of maize microsomes have been determined (49). The investigation showed that: i) the most potent inhibitors (K_i values 15–50 nM) had a sterol-like structure with a positively charged atom replacing C-25; ii) the sterol nucleus could be that of cycloartenol, lanosterol or cholesterol and the positively charged atom could be N, S or As; iii) the positive charge on the heteroatom could arise as a result of protonation at physiological pH of a neutral amine such as 25-azacycloartanol to give a tertiary ammonium group, polarization of the carbonyl group of an amide such as 24-oxo-25-azacycloartanol to give a species with a partial positive charge on the N atom, or the presence of an N-O bond which, in tertiary amines such as 24-methyl-25,26,27-trisnor-24-dimethylamino-*N*-oxide cycloartanol, has a strong permanent dipole moment (N^+-O^-); iv) compounds with a positively charged atom replacing C-24 or C-23 were also inhibitory (K_i values 90–180 nM) but were less so than those in which the positively charged atom replaced C-25; and v) compounds with a positively charged atom replacing C-20 were not significantly inhibitory. These effects have been discussed further in the literature (6,28,40).

The only other inhibitors of the 24- and 28-SMT of note are analogues of the immediate or more remote methyl

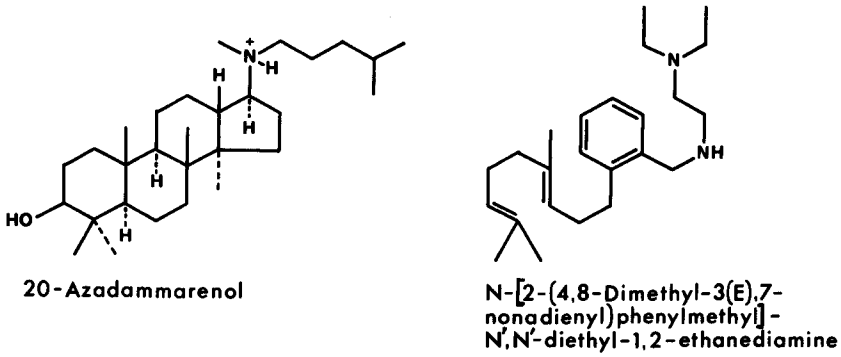


FIG. 6. Compounds designed to inhibit 2,3-oxidosqualene cyclases at stage B (see Fig. 3) of the cyclization reaction sequence.

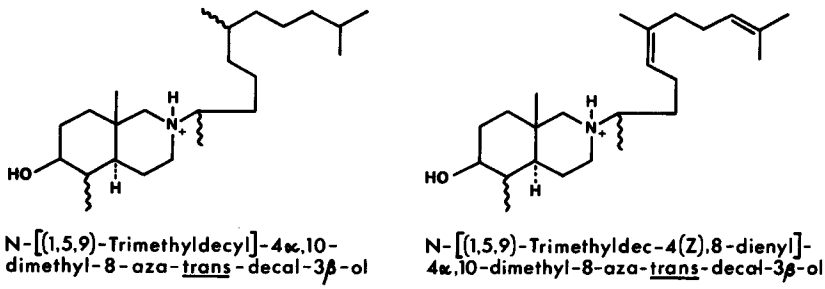


FIG. 7. Compounds designed to inhibit 2,3-oxidosqualene cyclases at stage C_2 (see Fig. 3) of the cyclization reaction sequence.

donors, SAM and *L*-methionine, respectively. Sinefungin, an antibiotic analogue of SAM isolated from *Streptomyces griseolus*, inhibits the 24-SMT of *S. cerevisiae* (50), as does *D,L*-ethionine (51).

Sterol 14-demethylase. Sterol 14-demethylation takes place in three O_2 and NADPH requiring steps, namely: i) oxidation of the 14α-methyl group to a 14α-hydroxymethyl group; ii) oxidation of the 14α-hydroxymethyl group to a 14α-formyl group; and iii) oxidative removal of the 14α-formyl group as formate along with the 15α-hydrogen atom to generate a Δ^{14}-double bond (52, and refs. therein). It is now known that a cytochrome P-450 catalyzes all three steps in *S. cerevisiae* (53,54) and rat liver (55–58). The substrate for this enzyme is lanosterol and the product is 4,4-dimethyl-5α-cholesta-8,14,24-trien-3β-ol. It has been given the abbreviated name cyt. P-450_{14DM} but, in view of the distinct possibility that a similar cytochrome P-450 exists in filamentous fungi whose substrate and product are 24-methylene-24,25-dihydrolanosterol and 4,4-dimethyl-5α-ergosta-8,14,24(28)-trien-3β-ol, respectively, a better name would be cyt. P-$450_{Lano.14DM}$, so as to differentiate it from the (as yet hypothetical) cyt. P-$450_{24Melano.14DM}$. This would also bring the nomenclature in line with the abbreviated name given to the higher plant 14-demethylase, cyt. P-$450_{Obt.14DM}$ (59), whose substrate and product are obtusifoliol and 4α-methyl-5α-ergosta-8,14,24(28)-trien-3β-ol, respectively. It would also allow the abbreviation cyt. P-450_{14DM} to be used for sterol 14-demethylases in general, regardless of their source or specificity; it is in this manner that the term cyt. P-450_{14DM} will be used in the rest of this review.

Cyt. P-450_{14DM} are located in microsomal membranes in close association with a flavoprotein enzyme whose function is to catalyze the transfer of two electrons, one at a time, from NADPH to the protoheme iron of this and other cytochrome P-450; it may thus be called NADPH:cyt. P-450 oxidoreductase. Since cytochrome P-450 are *b*-type cytochromes, their prosthetic group is protoheme, which is believed to be situated in a cleft within the apoenzyme (60). The protoheme iron has six ligands, four are the pyrrole N-atoms of the porphyrin ring, the fifth is the S-atom of an apoenzyme cysteine residue, and the sixth is oxygen during the catalytic cycle, but is probably water when the enzyme is inactive. The apoenzyme also has a lipophilic binding site to which the substrate that is to be oxidized (in this case a 14α-methyl sterol) binds non-covalently. The location of this binding site is not definitely known, but it is clear that it must be positioned sufficiently closely to the protoheme to allow the substrate, when bound to it, to react with oxygen bonded to the protoheme iron; it is therefore likely that the binding site is associated with the protoheme-containing cleft (6,61).

A cytochrome P-450 catalytic cycle begins with the binding of the substrate to the lipophilic binding site; for this to be possible the protoheme iron must be in the Fe^{3+} state. An electron, received from NADPH *via* the NADPH:cyt. P-450 oxidoreductase, then reduces the iron to the Fe^{2+} state, allowing a molecule of O_2 to become the

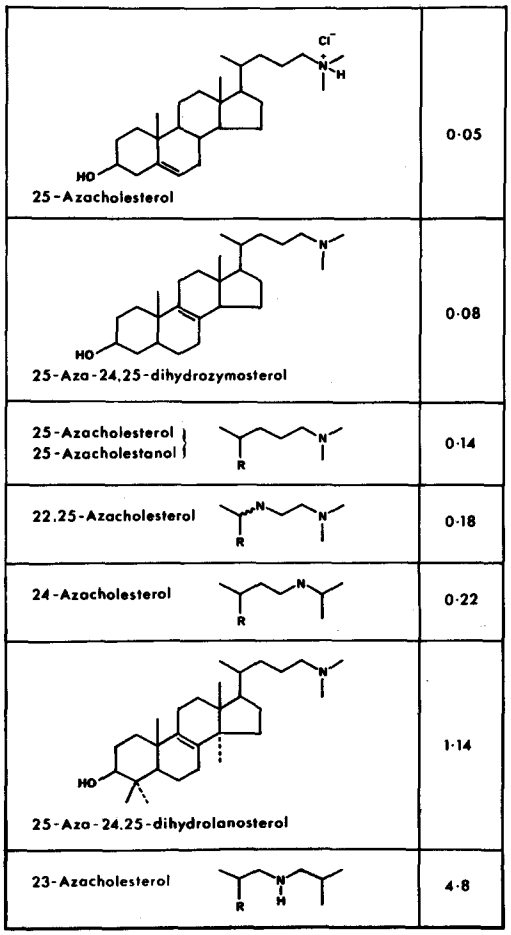


FIG. 8. Relative efficacy of various azasterols as inhibitors of the sterol 24-methyltransferase of *S. cerevisiae* (45).

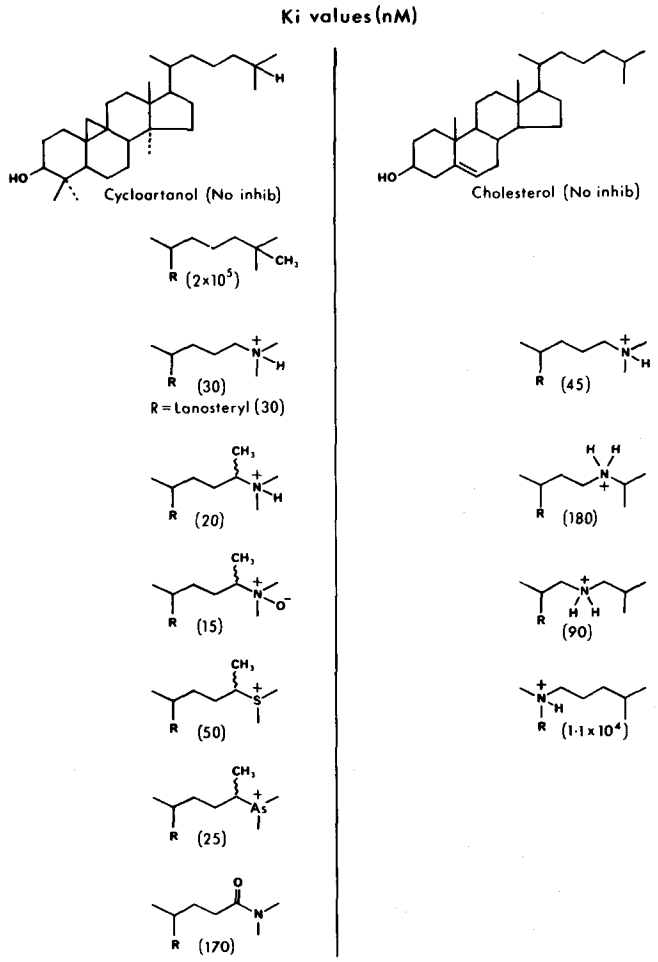


FIG. 9. Relative efficacy of various azasterols and related hetero compounds as inhibitors of the sterol 24- and 28-methyltransferases present in maize embryo microsomes (49). The compounds on the left are derivatives of cycloartanol (or, in one case, lanosterol), while those on the right are derivatives of cholesterol.

sixth ligand. There then follows a complex and poorly understood sequence of events which includes the receipt of a second electron from NADPH, cleavage of the O-O bond with loss of one atom of oxygen as water, reaction of the remaining oxygen atom (probably as an oxene) with the substrate carbon atom that is to be oxidized, and terminates with the formation of the oxidized product and reestablishment of the protoheme iron in the Fe^{3+} state. In the case of the cyt. P-450_{14DM} there must be three such cycles, with the product of the first, the 14-hydroxymethyl sterol, becoming the substrate of the second, and the product of the second, the 14α-formyl sterol, becoming the substrate of the third. In such a sequence it is presumed that only the product of the third catalytic cycle is released from the lipophilic binding site.

Inhibition of the cyt. P-450_{14DM} has been a source of great interest since it was discovered (62) that the pyrimidine fungicide triarimol (Fig. 10) inhibited the 14-demethylation step in sterol biosynthesis. Since then a considerable number of fungicides have been shown to exert their primary fungitoxic action by inhibiting the cyt. P-450_{14DM} and have consequently been called demethylation inhibiting fungicides or DMI; they have been the subject of a considerable number of reviews (63–72). Structurally, the DMI consist of an N-containing heterocyclic ring which may be 5- or 6-membered with 1, 2 or 3 N-atoms, and which is linked to a relatively large lipophilic moiety of quite varied structure—Fig. 10 shows some representative examples. It has been shown that both fungitoxicity (73) and 14-demethylase-inhibiting ability (74) reside in the *N*-heterocycle and that it is dependent upon the presence of an unsubstituted and unhindered N-atom *meta* with respect to the ring atom carrying the lipophilic moiety.

DMI inhibit the cyt. P-450_{14DM} by utilizing the non-bonded electron pair on the *meta*-N of the N-heterocycle to form a coordinate bond with the positively charged protoheme iron. The evidence for this is i) the appearance of a type 2 binding spectrum when a DMI is incubated with the cyt. P-450_{14DM} (59,61,72,75); type 2 binding spectra are produced when binding to the protoheme iron occurs (76); and ii) the ability of DMI to compete with CO for binding to the sixth coordination position of the protoheme when the iron is in the Fe^{2+} state (72).

Although the main inhibitory interaction of DMI with cyt. P-450_{14DM} is between the N-heterocycle and the pro-

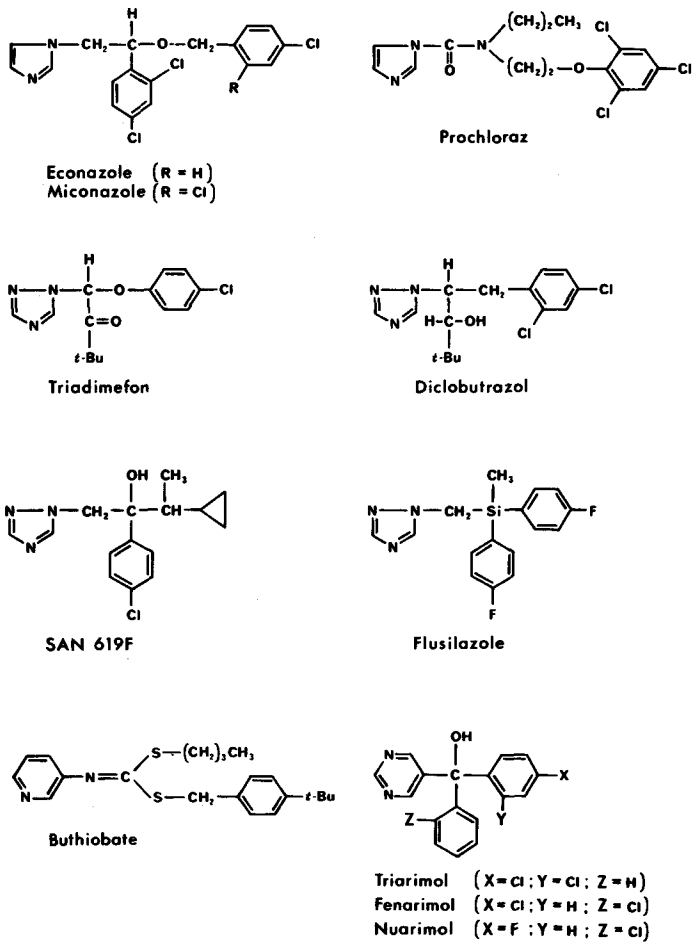


FIG. 10. Structures of some sterol 14-demethylase inhibiting (DMI) fungicides.

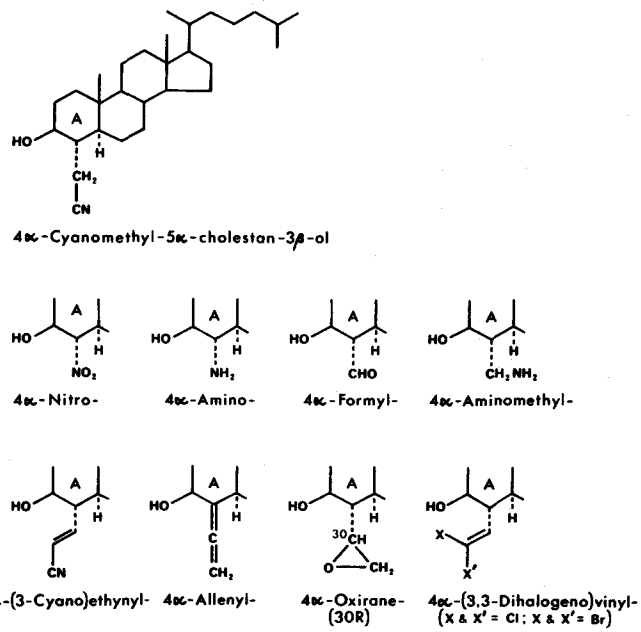


FIG. 11. Compounds designed to inhibit sterol 4-demethylation (82–84).

toheme iron, it has been assumed that some interaction also occurs between the lipophilic moiety and the lipophilic binding site and that this accounts for differences in the inhibitory power of different DMI towards the cyt. $P\text{-}450_{14DM}$ from a given organism and for differences in the inhibitory power of a given DMI towards cyt. $P\text{-}450_{14DM}$ from different organisms. However, a recent study (77) has cast some doubt upon this. It was found that the difference in the efficacy of flusilazole (Fig. 10) as an inhibitor of the cyt. $P\text{-}450_{14DM}$ of *S. cerevisiae* and rat liver diminished markedly as the two enzyme preparations were purified.

Sterol 4-demethylation. Demethylation at C-4 involves a complex of microsomal enzymes rather than a single sterol 4-demethylase. Thus, there are more opportunities for blocking the process since each of the component enzymes is available for inhibition. Despite this, inhibitors of 4-demethylation are few, and to date no fungicide has been developed which acts by inhibiting C-4 demethylation.

The enzyme complex from rat liver (78–80) and presumably that of fungi and higher plants consists of a monooxygenase, an NAD^+-dependent sterol 4-decarboxylase and an NADPH-dependent 3-ketosteroid reductase. The monooxygenase catalyzes the stepwise oxidation of the 4α-methyl group of a 4,4-dimethyl- or 4α-methyl sterol to a 4α-carboxyl group, *via* a 4α-hydroxymethyl- and a 4α-formyl group, each step requiring one molecule of O_2 and one of NADPH. It is not a cytochrome P-450 since it is not inhibited by CO. The resulting 4α-carboxy-4β-methyl- or 4α-carboxy sterol is then oxidatively decarboxylated by the NAD^+-dependent sterol 4-decarboxylase. In this reaction it is likely that the loss of the carboxyl group, as CO_2, is facilitated by the concerted removal of the 3α-H, as H^-, by NAD^+ to yield the enol of the corresponding 4-methyl-3-ketosteroid or 3-ketosteroid, respectively. This is then followed by spontaneous tautomerization of these enols to their more stable keto forms. When the enol undergoing this tautomerization has a residual methyl group at C-4, as is the case during the removal of the first of the two C-4 methyl groups, this methyl takes up the 4α position in the resulting 4-methyl-3-ketosteroid because, as such, it has the spatially more satisfactory equatorial orientation. Thus, the methyl that was 4β in the 4,4-dimethyl sterol becomes 4α in the 4-monomethyl sterol and is consequently positioned correctly for the monooxygenase at the start of the second 4-demethylation reaction sequence. In the final step of the process, the NADPH-dependent 3-ketosteroid reductase catalyzes the reduction of the 3-keto moiety to yield a 4α-methyl- or a 4-demethyl sterol.

The monooxygenase component of the rat liver 4-demethylase complex has been shown to be weakly inhibited (15%) by 1.5 mM cyanide ions (81). It is also inhibited by 4α-cyanomethyl-5α-cholestan-3β-ol (Fig. 11), a compound specifically designed for this purpose (82). This inhibition is irreversible and only occurs in the presence of NADPH. It has been suggested that inhibition is caused by the generation, as a result of catalytic action by the monooxygenase, of an electrophilic 4α-acyl cyanide which then reacts with a nucleophilic group at the active site of the enzyme. The 4α-nitro-, 4α-amino-, 4α-formyl-, 4α-

aminomethyl-, 4α-(3-cyano)ethynyl-, 4α-allenyl-, 4α-oxirane-, 4α-(3,3-dichloro)vinyl-, and 4α-(3,3-dibromo)vinyl-derivatives of 5α-cholestan-3β-ol (Fig. 11) recently have been synthesized as other possible inhibitors of the monooxygenase (83,84), but as yet no reports as to their efficacy have been published. It will be interesting to see whether any of these compounds are selectively inhibitory towards the animal, fungal or higher plant 4-demethylase complexes.

Sterol Δ^{14}-reductase, sterol $\Delta^{8}\rightarrow\Delta^{7}$-isomerase and cycloeucalenol obtusifoliol isomerase. These three enzymes are treated together because they are all inhibited, to a greater or lesser extent, by the morpholine and 3-phenylpropylamine group of fungicides (6,85) and by many of the compounds designed in recent years to inhibit one or the other (6).

The sterol Δ^{14}-reductase (Δ^{14}SR) catalyzes the reduction of the Δ^{14}-double bond that was introduced by 14-demethylation. Its preferred substrate in filamentous fungi is 4,4-dimethyl-5α-ergosta-8,14,24(28)-trien-3β-ol, whilst in yeast it is 4,4-dimethyl-5α-cholesta-8,14,24-trien-3β-ol, and in higher plants it is 4α-methyl-5α-ergosta-8,14,24(28)-trien-3β-ol; its preferred reductant in all cases is NADPH (52,86).

The sterol $\Delta^{8}\rightarrow\Delta^{7}$-isomerase ($\Delta^{8\rightarrow7}$SI) catalyzes the isomerization of the Δ^{8}-double bond that was originally present in the lanosterol in fungi and was generated by the action of COI in higher plants, to a Δ^{7}-double bond. Its preferred substrate in fungi is fecosterol, whilst that in higher plants is 4α-methyl-5α-ergosta-8,24(28)-3β-ol (6,52). There is no evidence to suggest that it needs a cofactor.

Cycloeucalenol obtusifoliol isomerase (COI), an enzyme found in higher plants but not fungi, catalyzes the opening of the 9β,19-cyclopropane ring of cycloeucalenol so as to generate the $\Delta^{8(9)}$-double bond and a β-orientated methyl group at C-10 that are found in the product of the reaction, obtusifoliol (6). Again, there is no evidence of a cofactor requirement.

The mechanism of the reactions catalyzed by these enzymes is outlined in Fig. 12 (also see refs. 6 and 85). They are all initiated by an electrophilic attack by a H^+, probably derived from an appropriately positioned group at the catalytic site of the relevant enzyme (and exchangeable with H^+ in the aqueous milieu), on a nucleophilic carbon of the sterol substrate which results in the generation of a carbocationic HEI intermediate. The formal location of the positive charge in the carbocationic HEI generated by the Δ^{14}SR, $\Delta^{8\rightarrow7}$SI and COI are relatively close to one another, being C-14, C-8 and C-9, respectively. This proximity of the positive charges and the probability that each is delocalized to some extent around the adjacent carbon atoms, thereby emphasizing the proximity, is believed to be the key to the observation that, in many cases, all three enzymes are subject to inhibition by the same compound, particularly when that compound, by accident or design, is a structural and electronic mimic of one of the HEI.

This first became apparent when the primary fungitoxic effect of the morpholine fungicides—tridemorph and fenpropimorph, and subsequently the 3-phenylpropylamine fungicide, fenpropidin (Fig. 13)—was eventually tracked down to inhibition of sterol biosynthesis at the steps catalyzed by Δ^{14}SR and $\Delta^{8\rightarrow7}$SI (87–93). During the same period, Benveniste and his co-workers (94–97) showed that tridemorph and fenpropimorph were potent inhibitors of COI in higher plants (94–97); they achieved an intellectual breakthrough in explaining the mode of action by pointing out that at physiological pH these compounds were protonated and, as such, were both structural and electronic mimics of the carbocationic HEI of all three enzymes. Consequently, they would bind to the catalytic sites of these enzymes much more tightly than the true substrates and would in this way inhibit the enzymes (6,96–100). This followed from the concept of transition state analogues as potent enzyme inhibitors (101–105). Figure 14 shows how a morpholine fungicide would bind to the catalytic sites of the three enzymes so as to mimic the relevant carbocationic HEI.

Based on this proposed mode of action, Benveniste's group synthesized a range of components derived from 8-aza-4α,10-dimethyl-*trans*-decal-3β-ol (Fig. 15, 1–7) which mimics the sterol rings A and B and has a positively charged N-atom at the equivalent of the sterol C-8, thereby mimicking the carbocationic HEI. All the derivatives have a relatively large group that can be folded or positioned to look somewhat like all or part of the rest of the sterol molecule which is attached to the N-atom. Based upon the predominant type of sterol, 9β,19-cyclopropyl-, Δ^{8}- or $\Delta^{8,14}$-, that accumulated when maize seedlings and bramble cells were grown in the presence of these compounds, the following conclusions were drawn: i) compounds **3** and **7** (the *N*-oxides) were powerful inhibitors of Δ^{14}SR; ii) compound **2** was a powerful inhibitor of $\Delta^{8\rightarrow7}$SI; and iii) compounds **4**, **5** and **6** are particularly powerful inhibitors of COI (106). An assay system for Δ^{14}SR using a microsomal preparation from maize embryos has shown that compounds **2**, **4**, **5** and **6** are particularly good inhibitors, and compound **1** is a fairly good inhibitor of this enzyme (86). Assay systems based on the maize embryo microsomal preparation have shown that compound **2** is a good inhibitor of $\Delta^{8\rightarrow7}$SI and that compounds **2**, **2A**, **2B**, **2C** and **5** are good inhibitors of COI (28,40,107). That the nature of the *N*-substituent of such compounds may play a part in determining which of the three enzymes is inhibited most strongly is shown by the finding that the 2*S*-2-methyl isomer of fenpropimorph causes a greater accumulation of 9β,19-cyclopropyl sterols than Δ^{8}-sterols in wheat caryopses, whilst the 2*R*-2-methyl isomer causes the reverse (108).

It would appear that 2-aza-2,3-dihydrosqualene, its *N*-oxide and LDAO (Fig. 4), as well as inhibiting cycloartenol cyclase, can also inhibit $\Delta^{8\rightarrow7}$SI in bramble cells (32), although it is doubtful whether they do this by mimicking the carbocationic HEI. COI has been shown to be inhibited reversibly and highly specifically by 7-oxo-24(28)-dihydroeucalenol (Fig. 16) in bramble cells (109). Again, this inhibition is probably not due to the mimicking of the carbocation HEI. It is suggested (109) that the carbonyl group at C-7 of the inhibitor could compete with the 9β,19-cyclopropyl ring of the substrate for the H^+ supplied by the enzyme. A compound that is a substrate analogue, rather than a HEI analogue, is 15-aza-24-methylene-*D*-homocholesta-8,14-dien-3β-ol (often called 15-azasterol; Fig. 16), an antimycotic (A25822B) which has been shown to inhibit Δ^{14}SR in *S. cerevisiae* (110,111) and bramble cells in suspension culture (112).

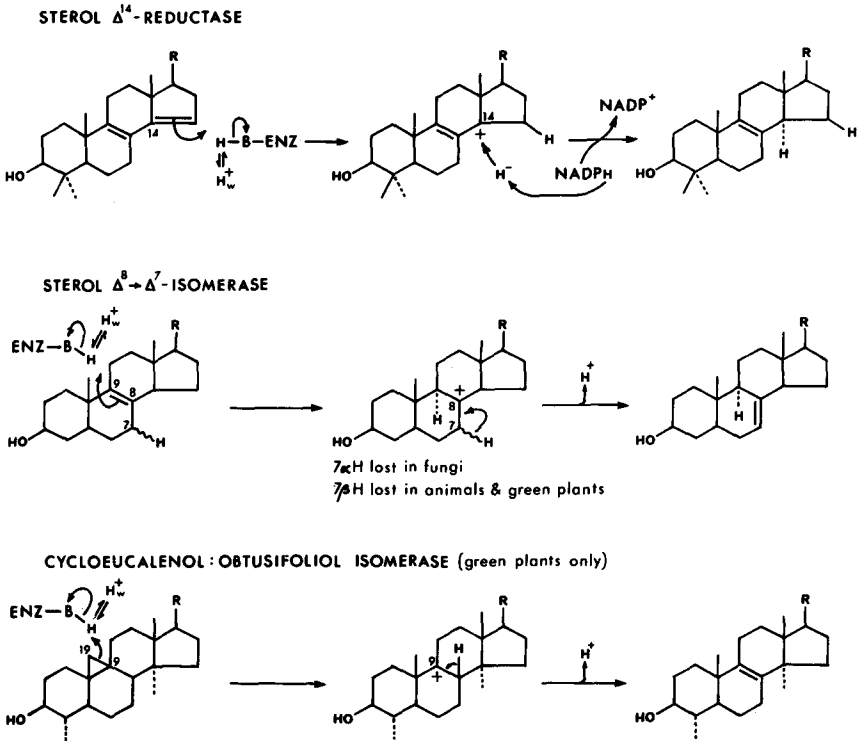


FIG. 12. Mechanism of the reactions catalyzed by the sterol Δ^{14}-reductase, the sterol $\Delta^{8}\rightarrow\Delta^{7}$-isomerase and cycloeucalenol obtusifoliol isomerase.

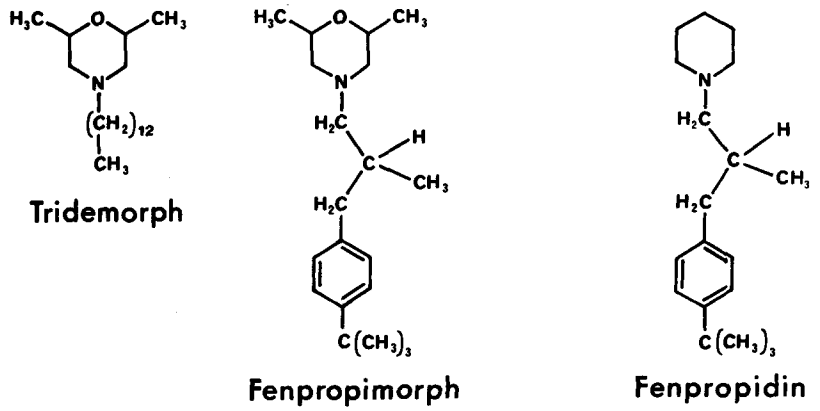


FIG. 13. Structure of some morpholine and 3-phenylpropylamine fungicides.

Other compounds which have been shown to inhibit Δ^{14}SR and $\Delta^{8\rightarrow7}$SI are AY-9944 and triparanol (Fig. 16). AY-9944 caused the accumulation of Δ^{8}-sterols in bramble suspension cultures (113), while triparanol did likewise in *S. cerevisiae* (114), indicating inhibition of $\Delta^{8\rightarrow7}$SI. The two compounds had a considerable effect on the sterol composition of three *Chlorella* species when present in their growth medium, which suggested inhibition of one or the other of these two enzymes, along with other possible enzymes. Their effect varied from species to species (115).

Δ^{5} *Desaturation.* Insertion of the Δ^{5}-double bond occurs at the Δ^{7}-sterol level, thereby generating a $\Delta^{5,7}$-sterol; it involves the loss of the 5α and 6α H-atoms (116–118). The overall reaction is obligatorily aerobic (119), and requires NAD(P)H (120–122), suggesting the participation of a monooxygenase. However, its mechanism is not clear and two different processes have been suggested. The first involves two steps, an initial aerobic hydroxylation at C-5 or C-6 which is followed by an anaerobic dehydration. The second involves a single step desaturation catalyzed by a sterol Δ^{5} desaturase, of the fatty acid desaturase type, which is an oxygenase sited at the terminus of an electron transport chain whose other components are a flavoprotein [NAD(P)H:cytochrome b_5 oxidoreductase], cytochrome b_5, and a cyanide-sensitive protein (52).

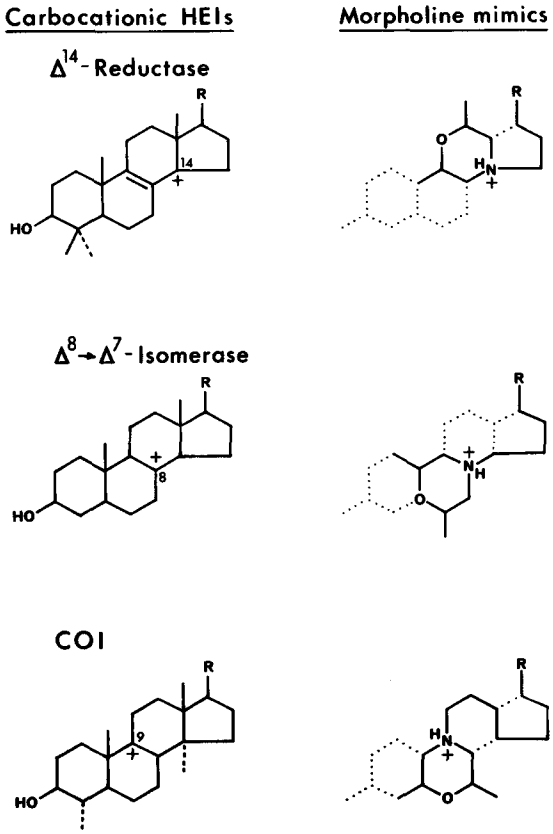
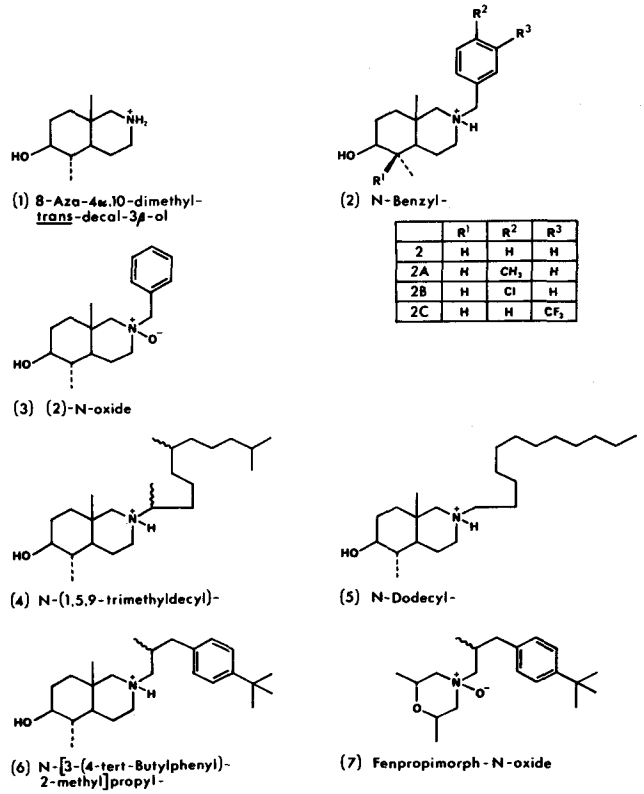


FIG. 14. Morpholine fungicides as structural and electronic mimics of the carbocationic HEI generated in the reactions catalyzed by the sterol Δ^{14}-reductase, the sterol $\Delta^{8}\rightarrow\Delta^{7}$-isomerase and cycloeucalenol obtusifoliol isomerase.



	R1	R2	R3
2	H	H	H
2A	H	CH_3	H
2B	H	Cl	H
2C	H	H	CF_3

FIG. 15. Compounds based on 8-aza-4α,10-dimethyl-*trans*-decal-3β-ol which have been designed as potential inhibitors of the sterol Δ^{14}-reductase, the sterol $\Delta^{8}\rightarrow\Delta^{7}$-isomerase and cycloeucalenol obtusifoliol isomerase (28,40,86,105,106).

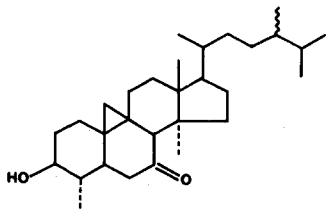
Specific inhibitors of Δ^5 desaturation have yet to be found. Cyanide but not CO has been shown to block the enzyme in *S. cerevisiae* (123). Moreover, there is an indication that 14-demethylase inhibitors may partially block it. The first hint of this was that *Ustilago maydis* accumulated 14α-methyl-5α-ergosta-8,24(28)-dien-3β,6α-diol when treated with etaconazole (124). This sterol has now been detected in other fungi treated with DMI (66,125) but, significantly, not in an *erg3* mutant (A2) of *S. cerevisiae* (125), which does not have a functional Δ^5 desaturation system. This suggests that DMI are inhibiting the Δ^5 desaturation system and, in doing so, are causing the accumulation of the 3α,6β-diol, which may or may not be a normal intermediate in the Δ^5 desaturation process.

Δ^7 *Saturation.* Little is known about the mechanism of Δ^7 saturation. It is a process that does not occur in many fungi but is a usual reaction in sterol biosynthesis in higher plants. By analogy with the animal system (126), the reaction ought to involve the *trans* addition of H-atoms at 7α and 8β, with the former arising from NADPH and the latter from the aqueous milieu (6).

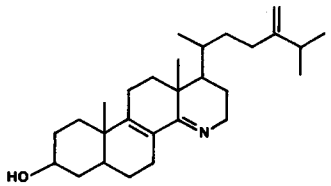
The classical inhibitor of this reaction in cholesterol biosynthesis in rat liver is AY-9944 (Fig. 16; 127,128). However, this compound has been shown to be much less specific in its inhibitory actions on sterol biosynthesis in the plant kingdom (115) and, to date, there is no good, specific inhibitor of this reaction in plants or in those fungi that can carry it out.

$\Delta^{24(28)}$ *Saturation.* Saturation of the $\Delta^{24(28)}$-double bond in fungi appears to be catalyzed by a microsomal, NADPH-dependent enzyme which adds the H^- from NADPH to C-24 and H^+ from the aqueous milieu to C-28 (52). It is inhibited by 23-azacholesterol (43,44).

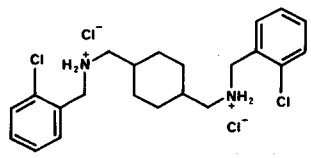
Δ^{22} *Desaturation.* The Δ^{22}-double bond in the fungal and higher plant sterols has the *trans* configuration and is introduced by the removal of the 22-*pro-S* and 23-*pro-S* H-atoms (116,117). Little is known about the mechanism of the reaction, but the introduction of the Δ^{22}-double bond into ergosta-5,7-dien-3β-ol in *S. cerevisiae* has been shown to be catalyzed by a cytochrome P-450 monooxygenase. This probably explains why the fungicide, triarimol (Fig. 10), an inhibitor of the cyt. $P\text{-}450_{14DM}$, was found to have an inhibitory effect on Δ^{22} desaturation in *U. maydis* (62). It is quite possible that many such DMI would be shown to inhibit Δ^{22} desaturation if they were assayed against the Δ^{22} desaturase itself. The presence of a marked inhibition of this enzyme would be missed if, as is usual, the inhibitory qualities of a DMI are assessed by its ability to: i) inhibit the cyt. $P\text{-}450_{14DM}$ in an enzyme assay procedure; or ii) alter the sterol profile of an organism grown in its presence. The reason why such a marked inhibition of the Δ^{22} desaturase would not be apparent with the latter procedure is because 14-demethylation precedes Δ^{22} desaturation in the biosynthetic pathway and so the better the compound is as a DMI, the less sterol would be available for Δ^{22} desaturation and, consequently, the less obvious the lack



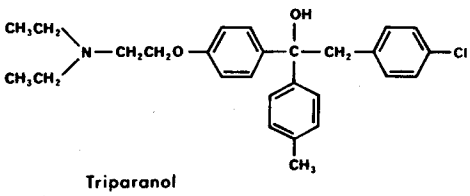
7-Oxo-24(28)-dihydrocycloeucalenol



15-Azasterol (A25822B)



AY 9944



Triparanol

FIG. 16. Other compounds capable of inhibiting one or more of the enzymes, sterol Δ^{14}-reductase, sterol $\Delta^{8}\rightarrow\Delta^{7}$-isomerase and cycloeucalenol obtusifoliol isomerase.

of Δ^{22}-sterols in the sterol profile would be. It is, however, clear that the Δ^{22} desaturase cytochrome P-450 is distinct from cyt. $P\text{-}450_{Lano,14DM}$, because fungal mutants deficient in the Δ^{22}-desaturase are able to carry out 14-demethylation (67). Thus, a DMI which also inhibited Δ^{22} desaturation would be exhibiting the lack of specificity towards cyt. $P\text{-}450_{14DM}$ also seen in those which inhibit the cytochrome P-450-dependent *ent*-kaurene to *ent*-kaurenoic acid step in the gibberellin biosynthetic pathway.

CONCLUSIONS

During the past decade, it has been gratifying to see the elucidation of the mechanism by which some of the known sterol biosynthesis inhibitors act, and the rational approach being taken in the development of new inhibitors. The rational approach is dependent upon a sound knowledge of the mechanism of the reaction whose enzyme is to be inhibited. In this respect, inhibitor designers are fortunate because such knowledge is available for many of the reactions of sterol biosynthesis. However successful though this approach has been, its limitations are becoming apparent in that compounds designed to inhibit a particular enzyme may work well with the enzyme from one organism but not with that from another. The reason for this must be in the nature of the enzymes themselves. Although the enzymes from the organisms in question catalyze the same reaction, there must be significant differences in their catalytic sites which affect the binding of the inhibitor and, therefore, its efficacy. The consequence of this is that inhibitor designers should perhaps switch their sights from the mechanism of the reaction to the enzyme catalyzing it. They should isolate the enzyme and, using the skills of the molecular biologist, produce sufficient amounts of it to submit the protein to X-ray crystallography for three-dimensional structural analysis. From there it is a relatively small step to the elucidation of the structure of the catalytic site and the design of compounds that would bind to it. By examining the enzyme derived from different organisms, differences in the structures of the catalytic sites would become apparent and the way would be open for design of organism-selective inhibitors.

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Involvement of Heme Components in Sterol Metabolism of *Saccharomyces cerevisiae*[1]

R. Todd Lorenz and L.W. Parks*
Department of Microbiology, North Carolina State University, Raleigh, North Carolina 27695-7615

There is an intimate association between sterol biosynthesis in yeast and aerobicity. Besides the requirement for molecular oxygen for the epoxidation of squalene, cytochrome hemoproteins are involved in demethylation and desaturation steps. Regulatory effects of hemes on sterol formation have been demonstrated using specifically defective mutants of yeast. Heme competency participates in a mechanism whereby wild-type cells are prevented from taking exogenous sterols from the growth media. The multiple interactions of hemes and sterols appear to be associated with the variously defined functions for sterols in the yeast cells.
***Lipids* 26, 598-603 (1991).**

Yeasts are the most primitive organisms that synthesize abundant amounts of sterols. *Saccharomyces cerevisiae* is especially suitable for studies on sterol function and synthesis because of its ease of culture, well defined genetic mechanism, amenability to molecular biological manipulations, and wealth of biochemical information. Its principal sterol is ergosterol, which in common with many plant sterols has an unsaturation and an alkylation in the side chain (see Fig. 1). In this report we shall review the interactions between hemes and sterols and how these affect the physiology of the organism.

STEROL ESTERIFICATION

Sterols exist in two forms in yeast, in the free hydroxyl form, and as esters of long-chain fatty acids. Free sterols are intercalated within each leaflet of the phospholipid bilayer. The hydroxyl group is orientated toward the aqueous environment, and the ring structure and side chain are surrounded by the hydrophobic fatty acids of the phospholipids. Free sterols are deposited within all phospholipid bilayers of yeast cells such as the endoplasmic reticulum, golgi apparatus, mitochondria and the nuclear and plasma membranes. Steryl esters are extramembranous lipids which are deposited in globules within the cytoplasm. The lipid globules are closely associated with the endoplasmic reticulum and contain predominately steryl esters, triacylglycerol, and phospholipids (1-3). The free sterol and the steryl ester pools undergo a culture-cycle dependent interconversion. The free sterol pool remains relatively constant, but the steryl ester pool is very low during exponential growth and increases dramatically as the culture enters stationary phase (4). When stationary phase cells are transferred to fresh medium, the steryl esters are rapidly hydrolyzed to free sterol and fatty acids, and utilized for new membrane biogenesis (5; see Fig. 2).

We have shown that the ratio of the amount of sterol in the free and esterified form is variable (6). Using a sterol auxotroph that cannot synthesize sterols, we found that by titrating the cellular response to increasing concentra-

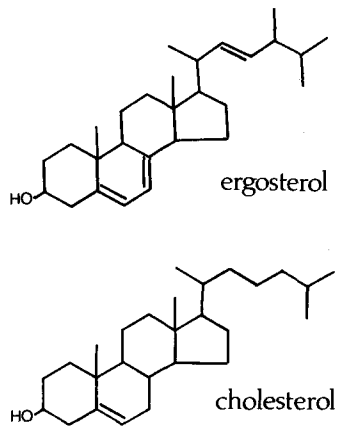


FIG. 1. Ergosterol and cholesterol.

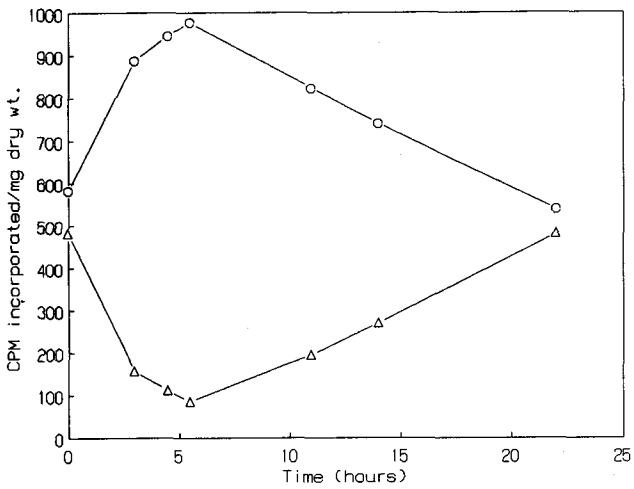


FIG. 2. Interconversion of free sterol and steryl ester during the growth cycle. A wild-type strain, X2180-1A, was pregrown to stationary phase in the presence of [^{14}C]acetate to label the lipids. Cells were then washed and inoculated into fresh media without [^{14}C]acetate. Samples were taken at the designated time points, harvested, and prepared for extraction. Free sterol (O) and steryl esters (△) were separated by thin-layer chromatography, and the accumulated radioactivity was determined by scintillation counting.

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*To whom correspondence should be addressed.

Abbreviations: ALA, δ-aminolevulinic acid; DOS, dioxidosqualene; HMG-CoA, 3-hydroxy-3-methylglutaryl coenzyme A.

tions of free sterol in the medium, there is a minimal cellular concentration of free sterol below which the culture cannot proliferate (6). We have defined this level of sterol as the domain function. As the amounts of exogenous sterol are increased, the cellular levels of free sterol increase until a saturating concentration of free sterol is eventually reached. This pool of free sterol of variable concentration of sterol we have defined as the bulk function. As the cellular concentration of the bulk pool increases toward saturation there is an increasingly greater portion of the accumulated sterol that can be converted to steryl ester. Above the bulk concentration, all additional sterol is deposited as ester.

The complex cycling of sterols into and out of the ester fraction provides little insight into the physiological role of sterols in cellular metabolism. We reasoned that if there were diverse functions for sterols in the organism, it was unlikely that the different functions would have the same precise requirements for the different features of the ergosterol molecule. Additionally, it seemed unlikely that there would be the same quantitative requirements. Using a sterol auxotrophic mutant of yeast, we have varied quantitatively and qualitatively the sterols that were provided to the organism to determine which features of the ergosterol molecule were required for growth.

STEROL AUXOTROPHY

By feeding different sterols and stanols to the sterol auxotroph RD5-R (*hem1, erg3, erg7*), it was found that growth could not occur when highly purified cholestanol was supplied as the sole sterol source. However, if ergosterol was supplied in trace amounts (10 ng/mL) in the presence of excess cholestanol (5 μg/mL), the growth rate was similar to that of cells grown on excess ergosterol. Cells were not able to grow on the cholestanol or microamounts of ergosterol separately (7). The role fulfilled by the microamount of ergosterol has been termed "the sparking function" (8). The cholestanol could be replaced by a wide range of sterols and stanols, but the sparking function could only be satisfied by sterols possessing C-5 unsaturation (9).

Other studies with yeast have also demonstrated growth differences from various sterols, depending on culture conditions. The sterol auxotroph GL7 (*hem3, erg7*) had higher growth rates and yields in media supplemented with cholesterol and growth-limiting amounts of ergosterol, when compared to the growth of the organism on either sterol alone (10). Sterol preferences for optimal activity of a protein kinase (11), a methyltransferase (12), and for polyphosphoinositide metabolism (13) have been reported. It has also been proposed that a sterol with a C-24 β-methyl group is required when the wild-type organism is grown under anaerobic conditions in the presence of the synthetic sterol inhibitor, 2,3-iminosqualene (14,15).

Apparently, anaerobically cultured cells have a different sterol requirement than heme mutants that are cultured aerobically. For example, GL7 does not grow on lanosterol (which lacks the C-5,6 unsaturation) aerobically, but does grow on lanosterol anaerobically (16,17). However, one factor which has been overlooked in many studies is the unsaturated fatty acid source used to supplement sterol auxotrophs. The ratios are critical, since the resulting phospholipid composition can alter the growth response to different sterols (18). This may account for discrepancies that have been seen between different systems that have been studied.

Correlating biochemical and biophysical activities with physiological functions involves a number of factors in addition to the accumulated sterol in the cell. Involved in this somewhat complicated process are the interrelationships between sterols, molecular oxygen and heme biosynthesis.

HEME INTERACTIONS

The biosynthesis of ergosterol, unsaturated fatty acids, hemes and methionine in *S. cerevisiae* is an aerobic process (19,20). Molecular oxygen is required early in the ergosterol pathway for the conversion of squalene to 2,3-oxidosqualene (21). Additionally, oxygen is necessary for coproporphyrinogen decarboxylation and protoporphyrinogen oxidation, in the yeast heme pathway (22,23). Therefore, in the absence of oxygen, hemes cannot be synthesized, and the ergosterol precursor, squalene, accumulates (24).

Cytochrome P-450 hemoproteins are required in the ergosterol pathway for the oxidative demethylation of lanosterol (25,26) and the desaturation at the C-22 position of the side chain (27). Cytochrome b_5 hemoproteins are necessary for the desaturation at the C-5 position of the sterol molecule (28,29), and for fatty acid desaturase which introduces a double bond at C-9 of palmitic and stearic acids (30,31). A siroheme is an integral component of the sulfite reductase enzyme which is necessary for methionine synthesis (32). Therefore, anaerobically cultured cells or heme mutants must be supplemented with an exogenous source of sterol, unsaturated fatty acids, and methionine for growth to occur. Hemes also play a regulatory role in sterol biosynthesis at 3-hydroxy-3-methylglutaryl coenzyme A (HMG-CoA) reductase (see below). Finally, hemes participate in a process, termed "aerobic sterol exclusion," which precludes the accumulation of exogenous sterols. Thus, a defect in heme biosynthesis has broad effects on the cells; the mutants cannot synthesize methionine, unsaturated fatty acids, ergosterol, or hemes which results in respiratory deficiency.

The rate-determining enzyme of the ergosterol pathway in yeast is HMG-CoA reductase (33,34). This pathway produces all isoprenoid-containing compounds, including sterols, ubiquinone, dolichol, and isopentenylated adenosine. Regulation of enzyme activity in response to varying growth conditions or mutations in the sterol biosynthetic pathway have been described (35–38). Other reports indicate a feedback inhibition on the activity of HMG-CoA reductase by ergosterol (39,40). Unsaturated fatty acids and glucose have been reported to be inducers of HMG-CoA reductase activity (41,42). Squalene epoxidase may be another highly regulated enzyme of the ergosterol biosynthetic pathway because of the requirement for unsaturated fatty acids in the conversion of squalene to 2,3-oxidosqualene (43).

A direct effect of hemes on sterol formation was observed using mutants with defects in the cyclization of squalene (*erg7*) and in the synthesis of δ-aminolevulinic acid (*hem1*). This defect in heme biosynthesis can be remedied by the addition of δ-aminolevulinic acid (ALA)

to the medium. We observed that the organism had a five-fold increase in HMG-CoA reductase activity when the cells attained heme competency by the addition of ALA. Appropriate controls showed that it was not ALA *per se*, but the acquisition of heme competency that elicited the response. The increase in HMG-CoA reductase was dependent also on the presence of glucose as the carbon and energy source.

Attendant to the stimulation of HMG-CoA reductase activity was a marked accumulation of 2,3-oxidosqualene and, later in the incubation, 2,3,22,23-dioxidosqualene (DOS; see Fig. 3). This demonstrated that heme competency caused an enhanced diversion of cellular carbon and energy to the ergosterol biosynthetic pathway.

HMG-CoA reductase activity is derived from two isozymes with separate structural genes (*HMG1* and *HMG2*) and different controlling mechanisms (44,45). The expression of *HMG1* is enhanced by heme competency *via* the *hap1* gene product, while *HMG2* responds in a contrary manner (46). However, under the conditions of these experiments, the depletion of hemes did not affect the total activity of HMG-CoA reductase *in vitro*. Although this appears to be in conflict with our results, other factors such as differences between *in vitro* and *in vivo* enzyme activities, and compartmentalization of the isozymes may account for the excessive accumulation of sterol precursors by heme competency that we see in our experiments.

Mevinolin is an unmethylated derivative of compactin, and both are competitive inhibitors of HMG-CoA reductase (47,48). We tested the effect of mevinolin on the growth of yeast cultures. Only a slight inhibition of growth was observed with up to 75 μg/mL of mevinolin. Above 150 μg/mL of mevinolin, growth was seriously impaired (unpublished experiment). An interesting observation was made when we analyzed the free sterol and the steryl ester concentrations of the cells. The ester content was decreased dramatically in the stationary phase with low mevinolin concentrations. Only when the bulk concentration of sterols was substantially reduced was growth reduction observed. Growth ceased when the domain level of free sterol was attained.

We were surprised that such dramatic changes in sterol content elicited such minor variations in growth rate. The apparently excessive concentrations of both bulk and ester components of sterols would seem to provide little physiological advantage to the cells. This apparent overproduction may be of significance in nature where the yeast would be exposed to varying aerobic and anaerobic conditions. As mentioned earlier, there is a dependency for molecular oxygen and hemes in ergosterol formation. Yet, yeast cells can grow efficiently under anaerobic conditions. Thus it appears that the variable bulk pool and ester fractions serve as reserve components that can be mobilized when endogenous sterol synthesis is precluded by the lack of oxygen. We have shown that the bulk pool can be maintained through hydrolysis of the steryl ester fraction. Esterification as a storage mechanism is a physiologically expensive process. A considerable saving would be realized by storing as much sterol as possible in the free form. What dictates the upper limit of the bulk pool of sterol remains an interesting point for discussion.

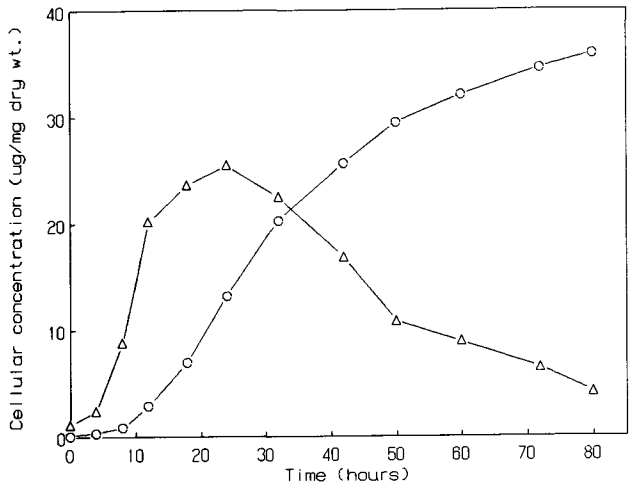


FIG. 3. Overproduction of sterol intermediates by heme competency. Strain FY3 (*hem1, erg7*) was pregrown on 5 μg/mL of cholesterol and transferred to fresh medium containing 100 μg of δ-aminolevulinic acid (ALA) per mL. Samples were taken at the designated time points, harvested, and prepared for extraction. Lipids were separated by thin-layer chromatography, eluted, and quantified by gas chromatography. When heme competency was attained by the addition of ALA to bypass the *hem1* mutation, there was a dramatic increase in the synthesis of 2,3-oxidosqualene; 2,3-oxidosqualene, (▽); DOS (○).

AEROBIC STEROL EXCLUSION

A peculiar situation exists with wild-type yeast in their inability to take up sterols from the environment under aerobic conditions. This is an interesting phenomenon, since it might be anticipated that the cells would prefer to use exogenous sterols and thus save the enormous metabolic costs of synthesizing the large amount of sterols that are found in the cell. Usually, when there is a cellular metabolite that is available in the medium, there is a preference to use the exogenous source to spare endogenous synthesis. Feed-back control and down-regulation of endogenous synthesis is a customary response to many external supplies. However, an exception to this situation exists with respect to the 28-carbon lipid, ergosterol. In the presence of exogenous sterol, aerobically grown wild-type yeast cells continue to produce large amounts of ergosterol and do not take up a significant quantity of the sterol supplied (49,50). This phenomenon has been termed aerobic sterol exclusion.

Under anaerobic conditions, heme and sterol synthesis are precluded, and sterol uptake occurs readily (14,15). Sterol auxotrophs blocked early in the heme pathway also accumulate exogenous sterol from the medium (18,51).

The mechanism and regulation of aerobic sterol exclusion was investigated using a strain of yeast, FY3, that has a defect in *hem1* (aminolevulinic acid synthetase), and a block at oxidosqualene cyclase (*erg7*). Cultures of FY3 were grown on differing concentrations of cholesterol, harvested at mid-exponential phase, and the sterols were extracted and quantified. As the exogenous concentration of cholesterol was decreased, there was a concomitant decrease in the free sterol concentration of the cell until a minimal level of 0.62 μg/mg dry wt was reached. These cells, grown on 1 μg of the cholesterol or less were termed

"sterol-depleted cells." Conversely, as the concentration of cholesterol in the medium was increased, there was an attendant increase in the cellular concentration of free sterol until a maximal level of 3.94 μg/mg dry wt was attained. Cells grown on 11 μg of cholesterol or ergosterol per mL or more did not have an increased free sterol concentration. These cells were termed "free sterol saturated" (see Fig. 4).

Parallel experiments revealed that the amount of sterol taken up from the medium was inversely proportional to the amount of cellular free sterol. This mechanism of sterol uptake regulation is thus termed "sterol-dependent."

Sterol uptake by these heme-incompetent cells was not an active uptake process for the following reasons: (i) no media components were required, (ii) sterol uptake was unaffected by inhibitors of cellular energy transformations known to affect uptake of actively transported nutrients, (iii) no pH optimum was observed, and (iv) cellular viability was not required.

Strain FY14 (*hem1, erg1::URA3, leu2, ade2, ura3*) was constructed to determine the effects of heme competency on the ability of cells to take up exogenous sterol. FY14 has an insertionally inactivated gene for squalene epoxidase, preventing the synthesis of any sterol. The *hem1* defect can be by-passed by the addition of ALA. A culture of FY14 was grown under sterol-depleting conditions (1μg/mL cholesterol) and exposed to 100 μg/mL ALA for 48 hr. Sterol uptake assays that were performed on these cells revealed that the cells were severely inhibited in regard to sterol uptake. Compared to a control culture not exposed to ALA, heme competency decreased sterol uptake by 71% (see Fig. 5). The *HAP* (heme-activating protein) gene family appears to comprise a global activation system for products involved in respiration (52). However, further experiments demonstrated that the *HAP1* and *HAP2* gene products are not likely to be involved in this exclusion process. Thus, sterol uptake is also controlled in some way by heme competency, and this mechanism of regulation is termed "sterol-independent." These two mechanisms, sterol-dependent and heme-dependent, control sterol uptake such that exogenous sterols can enter during heme incompetent situations (*e.g.*, anaerobic conditions) and are excluded during heme competency.

A mutant of the aerobic sterol exclusion system has been isolated. Strain upc20 (mutant of uptake control) is heme-competent, produces ergosterol as its endproduct sterol, and takes up about 3.2 μg of exogenous sterol per mg dry wt. Wild-type yeast only take up about 0.08 μg of exogenous sterol per mg dry wt. A most striking characteristic of upc20 is a five-fold overproduction of steryl esters, which appears to be a result of an abnormal esterification/hydrolysis partitioning cycle of sterols. Steryl esters produced in upc20 are not hydrolyzed to free steryl and fatty acids when cells are shifted to new culture medium. The sterol uptake phenotype of upc20 is genetically inseparable from the esterification phenotype. The pleiotropic mutation is known to be recessive and involves only one gene (50).

An interesting complication of aerobic sterol exclusion in the selection of sterol auxotrophs has caused considerable difficulty in preparing those mutants. Since heme competency elicits sterol-independent exclusion, mutants that are auxotrophic for sterols but prototrophic for hemes (*e.g., hem1, erg7*) have not been isolated. It

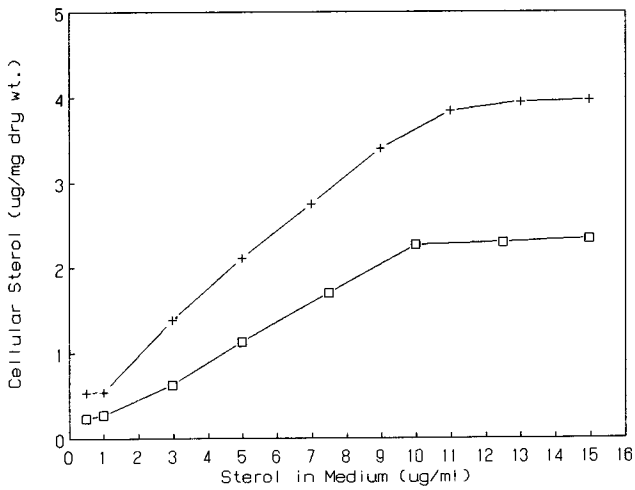


FIG. 4. Free sterol saturation curves of cholesterol and ergosterol. FY3 was grown on different concentrations of exogenous cholesterol or ergosterol and harvested at late log phase. Lipids were extracted, and the amount of sterol associated with the free sterol fraction was quantified by gas chromatography. Cells grown on 1 μg/mL or less sterol are termed "sterol-depleted" representing the domain level. Cholesterol, (+); ergosterol, (□).

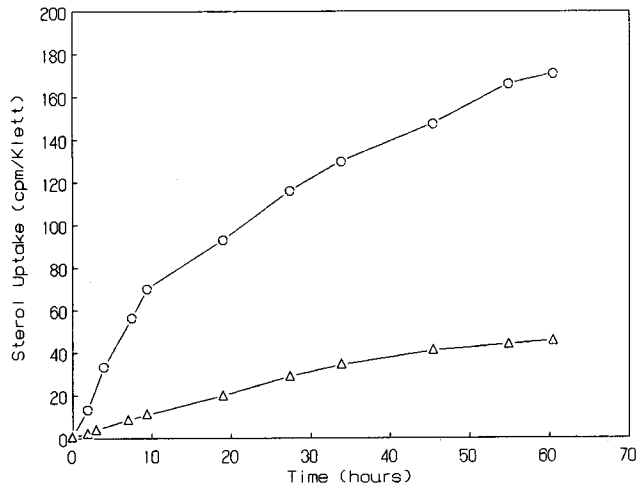


FIG. 5. Effect of heme competency on cholesterol uptake of a sterol auxotroph. Strain FY14 (*hem1, erg1::URA3*) was constructed to determine the influence of heme competency on the ability to take up exogenous sterol. FY14 was pregrown on 1 μg/mL of cholesterol, and transferred to fresh media containing ^{14}C-labelled cholesterol with and without 100 μg/mL ALA. Samples were taken at the designated time points, filtered, and washed, and the amount of radiolabelled cholesterol taken up was determined by scintillation counting. FY14 control, (○); FY14 + 100 μg/mL ALA, (△).

appears that the exclusion is so effective that sterol starvation prevents germination of the spores, even though an abundance of suitable sterol is provided in the medium. Interestingly, both sterol exclusion processes are vitiated in the upc20 strain.

The survival advantage of aerobic sterol exclusion to the yeast cell is not immediately apparent. The substantial physiological advantage to having no exclusion for a

variety of cellular components has been mentioned. It might be anticipated that aerobic sterol exclusion is indeed of survival advantage to the organism, or the process would have been eliminated by natural selection. Yeast appear to compete best in nature in hypertonic environments. Yeast flourish on fruit, in flower nectars, in salt-preserved meats and other high osmotic environments. These media also have an abundance of sterols from the plant or animal sources. We have shown that there are multiple functions for sterols in yeast and that ergosterol is indeed the preferred sterol. In sterol auxotrophs, cholesterol will allow abundant growth, but enormously higher concentrations of the animal sterol are needed for some functions in yeast (51). As the predominant sterol in yeast, ergosterol best fulfills the multiple roles. Preferences for different features of the sterol for optimal function in the different physiological roles have been demonstrated. Ergosterol must represent a consensus structure that is able to satisfy the distinctive and cumulative needs of the sterol functions for the survival of the cells. If the organism were free to take up a sterol different from ergosterol, and one that was less suitable for the specific requirements of the cell, that cell would be at a distinct disadvantage to one which was synthesizing and utilizing ergosterol. Despite the enormous metabolic expense of synthesizing sterol, fulfilling the precise requirements with ergosterol rather than an exogenous substitute sterol may offer such a physiological advantage that endogenous synthesis of ergosterol is preferred by the yeast.

Unlike most small-molecular-weight cellular constituents, like amino acids, purines, pyrimidines, vitamin cofactors, *etc.*, which have a single defined role, sterols appear to have multiple functions. This substantially complicates attempts to define the different roles. Interactions between sterols and hemes introduce another level of complexity in defining sterol functions. Nevertheless, the challenge is exciting and has practical ramifications. Most antifungal agents that are being used intercede in one fashion or another in sterol physiology. By understanding the precise physiological functions of the fungal sterols, it may be possible to design specific controlling agents for the fungi. Those antifungals, being targeted at a distinctive cellular activity, might be needed in only very low concentrations, potentially minimizing toxic effects. In addition, physiological manipulation of the fungi, such that the organism has enhanced sensitivity to antifungal agents, would lower the effective minimal inhibitory concentrations of the drug.

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Diversity of Sterol Biosynthetic Capacity in the Caryophyllidae[1]

Thomas A. Salt[a,*], Sihua Xu[b], Glenn W. Patterson[b] and John H. Adler[c]
[a]USDA/Plant Variety Protection Office, Beltsville, Maryland 20705, [b]Department of Botany, University of Maryland, College Park, Maryland 20742, and [c]Department of Biological Sciences, Michigan Technological University, Houghton, Michigan 49931

The order Caryophyllales, along with its two associated orders, the Polygonales and Plumbaginales, comprise the angiosperm subclass Caryophyllidae. We have now characterized the sterol compositon of 231 members of this subclass. This includes 210 species and 21 cultivars in 108 genera within the 14 families of these three orders. From these data, clear differences in biosynthetic capability and putative relationships between taxa have been established. Members of the two monofamilial orders (Polygonales and Plumbaginales) contain Δ^5-sterols in ratios typical of "main line" angiosperms. Members of families in the Caryophyllales contain Δ^5-sterols, or Δ^7-sterols or mixtures of Δ^5- and Δ^7-sterols. In the majority of species where Δ^7-sterols are the dominant sterols produced, trace amounts to almost equal amounts of Δ^5-sterols are also present. Replicate samples of many of these species have shown that the ratio of Δ^5-sterol to Δ^7-sterol in these species is stable over time and/or location. From these data, it appears that the conversion of Δ^7-sterols to Δ^5-sterols is highly regulated in the majority of species within this order. In these families, similarities in sterol composition correlate well with taxonomic relatedness. Relationships between these taxa with respect to biosynthetic capability can now be postulated.
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The order Caryophyllales, along with its two associated orders, the Polygonales and Plumbaginales, comprise the angiosperm subclass Caryophyllidae. The Polygonales and Plumbaginales are both monofamilial orders (Polygonaceae and Plumbaginaceae) customarily associated, but with an uncertain taxonomic relationship to the Caryophyllales (1). Together, these two orders contain approximately 10% of the species in the subclass.

The Caryophyllales consists of 12 families (Fig. 1) with approximately 10,000 species (1) and is generally agreed (1–3) to include the following families: Phytolaccaceae, Achatocarpaceae, Nyctaginaceae, Aizoaceae, Didiereaceae, Cactaceae, Chenopodiaceae, Amaranthaceae, Portulacaceae, Basellaceae, Molluginaceae and Caryophyllaceae. In this paper, the Stegnospermataceae (frequently included in the Phytolaccaceae) and the Gyrostemonaceae (moved to the order Dilleniidae) are also presented for the comparison of sterol composition.

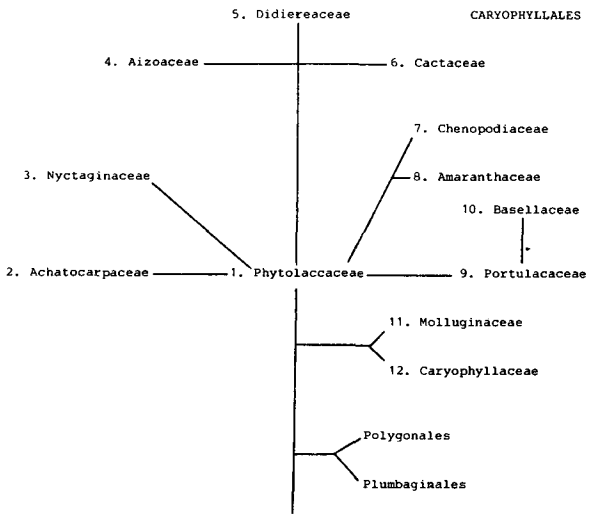


FIG. 1. Putative relationships among the families and orders of the Caryophyllidae (1).

Prior to 1980, the distribution of sterols in angiosperms was based on a sparse sampling of a few genera in many families. The majority of angiosperms are reported to produce 24-alkyl-Δ^5-sterols as the dominant 4-desmethylsterols (4–7). However, several species were reported which deviated from these "main line" angiosperms. Species in the Theaceae and Cucurbitaceae were reported to produce 24-alkyl-Δ^7-sterols as their dominant 4-desmethylsterols (4,6). While the Asteraceae and Fabaceae are considered "main line" families, at least one species in each produces exclusively 24-alkyl-Δ^7-sterols (8,9). Within the Caryophyllales, *Spinacia oleracea* (Chenopodiaceae) (9–11), *Lophocereus schottii* (Cactaceae) (12–13) and *Phytolacca americana* and *P. esculenta* (Phytolaccaceae) (14–16) were the only species reported and also contain exclusely 24-alkyl-Δ^7-sterols.

Plants exclusively producing 24-alkyl-Δ^7-sterols are widely scattered throughout the angiosperms and are considered to be "rare" except in the Cucurbitaceae and Theaceae. In 1980, realizing the relationship of the above four species, we began to characterize the sterol composition of families in the Caryophyllales to determine if other families existed which were similar to the Cucurbitaceae and Theaceae. The relationship of the Chenopodiaceae, Cactaceae and Phytolaccaceae presented the first opportunity to study sterol biosynthesis at a taxonomic level above the genus or family.

[1]Based on a paper presented at the Symposium on Plant and Fungal Sterols: Biosynthesis, Metabolism and Function, held at the AOCS Annual Meeting, Baltimore, Maryland, April 1990.

*To whom correspondence should be addressed at the USDA/Plant Variety Protection Office, 10301 Baltimore Blvd., Beltsville, MD 20705.

TABLE 1

Percent Desmethylsterol Composition of The Polygonales and Plumbaginales

Family	Sterols												
	A	B	C	D	E	F	G	H	I	J	K	L	(Ref.)
Polygonaceae													
Atraphaxis muschketowii	2	—	—	3	8	69	6	1	2	2	7	—	17
Muehlenbeckia axillaris	1	—	—	3	3	88	—	—	2	—	3	—	17
M. varians	3	—	2	3	8	77	—	1	6	—	—	—	17
Polygonella articulata	—	—	—	9	2	89	—	—	—	—	—	—	17
Polygonum amphibium	—	—	—	10	17	73	—	—	—	—	—	—	17
P. aviculare	—	—	—	7	3	90	—	—	—	—	—	—	17
P. cuspidatum	—	—	—	2	8	90	—	—	—	—	—	—	17
P. pensylvanicum	—	—	—	7	5	88	—	—	—	—	—	—	17
P. punctatum	—	—	—	7	7	86	—	—	—	—	—	—	17
Rumex acetosella	—	—	—	9	4	87	—	—	—	—	—	—	17
R. crispus	—	—	—	5	6	89	—	—	—	—	—	—	17
Rumex sp.	—	—	—	6	5	89	—	—	—	—	—	—	17
Plumbaginaceae													
Acantholimon glumaceum	1	4	4	10	4	70	—	—	—	—	—	7	17
Limonium europea	1	—	—	4	5	71	8	—	—	—	11	—	17
L. nashii	—	—	—	5	4	91	—	—	—	—	—	—	17
L. thouinii	6	—	7	5	17	54	2	8	1	—	—	—	17
Plumbago rosea	10	—	—	2	10	75	3	—	—	—	—	—	17
P. zeylanica	6	—	—	5	7	70	—	7	2	—	3	—	17

A, cholest-5-enol.
B, 24-methylcholesta-5,24(28)-dienol.
C, 24-methylcholesta-5,22-dienol.
D, 24-methylcholest-5-enol.
E, 24-ethycholesta-5,22-dienol.
F, 24-ethylcholest-5-enol.
G, 24-ethylcholesta-5,24(28)Z-dienol.
H, cholest-7-enol.
I, 24-ethylcholest-7-enol.
J, 24-ethylcholesta-7,24(28)Z-dienol.
K, 24-methylcholestanol.
L, 24-ethylcholestanol.

TAXONOMIC ARRANGEMENT

The taxonomic relationships between families and orders in the Caryophyllidae are presented in Figure 1. Within the Caryophyllales, the Phytolaccaceae is regarded as the basal family from which all other families are derived. The Achatocarpaceae and Nyctaginaceae are closely related and only marginally removed from the Phytolaccaceae (1). The Aizoaceae, Didiereaceae and Cactaceae are "linked" families and appear in the linear sequence. Similarly, the Chenopodiaceae and Amaranthaceae, the Portulacaceae and Basellaceae, and the Molluginaceae and Caryophyllaceae are "linked" pairs of families reflected by the linear arrangement of the order (1).

POLYGONALES AND PLUMBAGINALES

The species examined in the Polygonales and Plumbaginales all synthesize Δ^5-sterols from 87–100% of the desmethylsterols (Table 1). The 24-methylcholest-5-enol, 24-ethylcholest-5-enol and 24-ethylcholesta-5,22-dienol were isolated consistently from all species in ratios generally characteristic of "main line" angiosperms (4,6,7). Species in the Polygonales and Plumbaginales are distinctly different, with respect to their sterol composition, than the majority of species examined in the Caryophyllales below.

CARYOPHYLLALES

Phytolaccaceae and Stegnospermataceae. The Phytolaccaceae, including the Stegnospermataceae, is considered to be the most basal family in the Caryophyllales and all other families are believed to be derived from it either directly or indirectly (1). The family is presented in Table 2 in relation to the current taxonomic position of the species (20).

Species in the Phytolaccaceae, within the subfamilies Gisekioideae and Phytolaccoideae, produce predominantly Δ^7-sterols. However, these species also produce trace to considerable quantities of Δ^5-sterols. While these species are capable of producing Δ^5-sterols, Δ^7-sterols appear to be the preferred sterol species. This would suggest that a genetic block, as previously postulated for the Cucurbitaceae (6), does not exist within these species. It would appear that the conversion of Δ^7-sterols to Δ^5-sterols is, instead, regulated to produce the preferred Δ^7-sterol products. This would also amend the previous belief that if an organism is capable of producing Δ^5-sterols, then it is committed to do so (4,6).

Those species which are capable of producing Δ^5-sterols also produce Δ^0-sterols (stanols), presumably through a Δ^4-3-one intermediate, as demonstrated in a few other organisms (21,22). This may also represent a point of regulation within these species. Whether the stanols represent the final product in sterol metabolism in these species is unknown. Other organisms have been demonstrated to be capable of introducing a Δ^7-double bond into a saturated sterol nucleus (21–26). This would create a "stanol pathway" of Δ^7-sterol $\rightarrow$ Δ^5-sterol $\rightarrow$ Δ^0-sterol $\rightarrow$ Δ^7-sterol. While this has not been demonstrated in plants, it could present a mechanism by which the ratio of Δ^7 : Δ^5 : Δ^0-sterol might be maintained in these species.

TABLE 2

Percent Desmethylsterol Composition of The Phytolaccaceae and Stegnospermataceae

Family	Sterols														
	A	B	C	D	E	F	G	H	I	J	K	L	M	N	(Ref.)
Phytolaccaceae															
Subfamily: Gisekioideae															
Gisekia miltus	6	—	6	5	9	2	5	22	14	5	2	7	17	—	18
G. pharnaceoides	3	—	3	5	—	—	8	36	22	—	7	—	14	2	18
Subfamily: Phytolaccoideae															
Anisomeria coriacca	2	—	—	2	—	—	—	28	55	5	3	—	5	—	18
A. littoralis	2	1	5	4	—	—	2	34	34	5	3	4	5	1	18
Ercilla spicata	—	—	1	1	9	—	—	34	44	—	1	—	10	—	18
Phytolacca americana	—	—	t	t	—	—	—	86	13	—	—	—	—	—	18,19
P. dioica	—	—	—	4	5	—	7	26	32	5	2	—	19	—	18
Subfamily: Rivinoideae															
Tribe: Microteeae															
Microtea debilis	4	3	7	6	3	—	—	58	8	—	—	—	10	—	18
M. maypurensis	—	—	—	—	37	—	—	39	24	—	—	—	—	—	18
M. paniculata	—	2	8	2	27	—	—	37	21	—	—	—	3	—	18
Tribe: Seguierieae															
Gallesia integrifolia	1	—	3	15	23	2	—	2	5	4	1	3	32	9	18
Seguieria aculeata	3	—	—	1	7	—	—	57	28	3	—	—	1	—	18
S. americana	6	—	—	5	—	—	—	48	31	—	2	—	8	—	18
S. langsdorfii	2	—	1	1	—	—	—	21	1	—	1	1	15	57	18
Tribe: Rivineae															
Hilleria latifolia	2	—	1	4	4	—	—	15	12	—	—	—	46	16	18
H. secunda	4	—	—	—	7	—	—	18	7	—	—	—	45	19	18
Ledenbergia macrantha	3	1	1	1	—	—	—	36	9	2	—	2	26	19	18
L. peruviana	2	—	1	3	10	—	13	55	4	2	—	—	10	—	18
L. sequirerioides	5	—	2	2	4	—	—	54	28	2	—	2	1	—	18
Monococcus echinophorus	3	—	—	2	5	—	—	44	9	—	—	3	17	17	18
Petiveria alliaceae	3	—	1	4	2	—	—	15	4	—	—	1	34	36	18
Psammotropha myariantha	4	—	7	6	10	—	1	59	3	—	—	3	6	—	18
Schindleria racemosa	3	—	2	26	27	—	—	2	3	—	—	1	23	15	18
S. densiflora	1	—	7	29	39	—	—	9	4	—	1	—	10	—	18
Trichostigma octandium	2	—	2	1	2	—	—	67	3	1	—	3	4	15	18
T. polyandrum	5	—	4	27	46	—	—	2	1	—	—	1	9	5	18
Subfamily: Agdestidoideae															
Agdestis clematidea	5	—	7	18	56	—	—	—	9	1	—	—	3	1	18
Subfamily: Lophiocarpoideae															
Lophiocarpus polystachyus	5	1	13	11	56	3	6	3	—	—	—	—	2	—	18
Stegnospermataceae															
Stegnosperma cubense	6	—	3	11	14	—	2	42	18	—	—	2	—	2	18
S. halimifolia	4	—	—	—	3	—	2	39	50	2	—	—	—	—	18
S. watsonii	4	—	3	6	6	3	5	35	34	4	—	—	—	—	18

A, cholest-5-enol.
B, 24-methlcholesta-5,24(28)-dienol.
C, 24-methylcholest-5-enol.
D, 24-ethylcholesta-5,22-dienol.
E, 24-ethylcholest-5-enol.
F, 24-ethylcholesta-5,24(28)Z-dienol.
G, 24-methylcholest-7-enol.
H, 24-ethylcholesta-7,22-dienol.
I, 24-ethylcholest-7-enol.
J, 24-ethylcholesta-7,25(27)-dienol.
K, 24-ethylcholesta-7,24(28)Z-dienol.
L, 24-methylcholestanol.
M, 24-ethylcholestanol.
N, 24-ethylcholest-22-enol.
t, Trace amount (<1%).

Metabolism appears to shift away from the Δ^7-sterols toward Δ^5-sterols and Δ^0-sterols in species within the subfamily Rivinoideae. Within the tribes Seguierieae and Rivineae, metabolism is shifted to the extent that in many species Δ^0-sterols are among the dominant sterols produced. This is in contrast to the final two subfamilies (Agdestidoideae and Lophiocarpoideae), where the Δ^5-sterols are the preferred sterol species and ratios of sterol composition approximate those of "main line" plants.

Gyrostemonaceae, Achatocarpaceae and Nyctaginaceae. The Gyrostemonaceae is a small family of 5 genera and 17 species, originally included in the Caryophyllales and later excluded (1,20). The Achatocarpaceae is the smallest family of the order with only two genera and eight species, while the Nyctaginaceae consists of 30 genera with 300 species (1). The latter two families are closely allied to the Phytolaccaceae and only marginally removed within the taxonomic relationships of the order.

The species within these three families exhibit a strong preference toward the Δ^5-sterols (Table 3). Spe-

TABLE 3

Percent Desmethylsterol Composition of The Gyrostemonaceae, Achatocarpaceae, Nyctaginaceae, Aizoaceae and Didiereaceae

	Sterols														
Family	A	B	C	D	E	F	G	H	I	J	K	L	M	N	(Ref.)
Gyrostemonaceae															
Codonocarpus continifolius	6	—	3	6	71	—	—	—	—	—	—	1	13	—	27
C. australis	3	—	—	10	79	—	—	—	—	—	—	—	8	—	27
Didymotheca pleiococca	4	—	1	8	58	—	16	8	—	—	—	—	5	—	27
Gyrostemon australasicus	6	—	6	18	43	—	3	—	6	—	—	3	15	—	27
G. ramulosus	5	—	5	29	61	—	—	—	—	—	—	—	—	—	27
Tersonia brevipes	4	1	6	12	50	—	—	—	—	2	—	2	20	3	27
Achatocarpaceae															
Achatocarpus spinulosus	—	—	1	5	93	—	—	—	1	—	—	—	—	—	27
A. nigricans	1	—	1	6	90	—	—	—	1	—	—	—	1	—	27
A. pubescens	1	—	1	2	93	—	—	—	2	—	—	—	1	—	27
Phaulothamnus spinescens	4	—	—	8	74	—	—	7	7	—	—	—	—	—	27
Nyctaginaceae															
Allonia latifolia	3	2	4	22	42	—	1	21	2	—	—	3	—	—	27
Commicarpus chinensis	2	—	2	21	72	1	1	—	—	—	—	—	—	—	27
C. raynalii	2	—	3	19	75	—	1	—	—	—	—	—	—	—	27
Mirabilis jalapa	—	—	4	11	72	—	—	2	10	—	—	—	—	—	27
Oxybaphus viscosus	3	—	4	8	69	—	—	—	2	—	3	—	10	—	27
Aizoaceae															
Aptenia cordifolia	—	1	15	15	32	—	—	17	3	—	2	—	14	—	27
Carpobrotus aequiliaterus	7	—	12	24	49	—	—	6	2	—	—	—	—	—	27
C. edulis	—	—	9	2	59	—	—	16	1	—	1	—	12	—	27
Drosanthemum hispidium	—	—	—	10	27	—	—	—	3	3	—	—	51	6	27
Eberlanzia hospitalis	4	—	4	7	50	13	—	—	3	—	6	3	6	4	27
Mesembryanthemum crystallinum	—	1	17	1	68	—	—	5	2	—	1	—	5	—	27
M. nodiflorum	—	3	4	9	27	—	—	15	11	—	2	1	23	4	27
Psilocaulon absmile	6	4	5	7	35	2	1	2	3	—	—	3	28	4	27
P. trothai	4	2	3	6	30	3	—	—	17	—	1	2	30	2	27
Ruschia tumidula	6	1	8	3	57	3	—	3	3	—	3	1	10	2	27
Tetragonia tetragonioides	2	—	5	8	40	—	2	11	21	3	3	—	5	—	27,19
Didiereaceae															
Alluaudia adscendens	5	—	4	2	74	—	—	1	2	—	—	—	12	—	27
A. humbertii	4	—	—	5	68	—	—	1	5	—	4	—	13	—	27
A. procera	5	—	—	7	55	—	—	3	—	—	5	—	25	—	27
Didierea madagascariensis	3	—	4	2	87	—	—	—	—	—	2	—	2	—	27
D. trolii	5	—	9	3	78	—	—	1	2	—	2	—	—	—	27

A, cholest-5-enol.
B, 24-methlcholesta-5,24(28)-dienol.
C, 24-methylcholest-5-enol.
D, 24-ethylcholesta-5,22-dienol.
E, 24-ethylcholest-5-enol.
F, 24-ethylcholesta-5,24(28)Z-dienol.
G, 24-methylcholest-7-enol.
H, 24-ethylcholesta-7,22-dienol.
I, 24-ethylcholest-7-enol.
J, 24-ethylcholesta-7,25(27)-dienol.
K, 24-ethylcholesta-7,24(28)Z-dienol.
L, 24-methylcholestanol.
M, 24-ethylcholestanol.
N, 24-ethylcholest-22-enol.

TABLE 4

Percent Desmethylsterol Composition of the Cactaceae

	Sterols							
Family	A	B	C	D	E	F	G	(Ref.)
Cactaceae								
Echinopsis tubiflora	—	33	—	67	—	—	—	19,28
Epiphyllum sp.	—	9	2	76	8	t	4	19,28
Hylocereus undatus	t	18	8	73	—	—	—	19,28
Notocactus scopa	3	12	3	74	—	t	8	19,28
Opuntia sp.	—	4	8	88	—	—	—	
O. comonduensis	4	9	—	87	—	—	—	19,28
O. humifusa	5	8	—	87	—	—	—	19,28
Pereskia aculeata	3	19	6	72	—	—	—	19,28
Schlumbergera bridgesii	t	10	—	81	8	—	—	19,28
Stenocereus thurberi	10	20	—	70	—	—	—	29
S. gummosus	10	20	—	70	—	—	—	29

A, cholest-5-enol.
B, 24-methylcholest-5-enol.
C, 24-ethylcholesta-5,22-dienol.
D, 24-ethylcholest-5-enol.
E, 24-ethylcholesta-7,24(28)Z-dienol.
F, 24-methylcholestanol.
G, 24-ethylcholestanol.
t, Trace amount (<1%).

TABLE 5

Percent Desmethylsterol Composition of the Chenopodiaceae

Family	Sterols																
	A	B	C	D	E	F	G	H	I	J	K	L	M	N	O	P	(Ref.)
Chenopodiaceae																	
Subfamily: Chenopodioideae																	
Atriplex arenaria	—	—	3	25	53	—	—	—	—	—	16	—	3	—	—	—	19,30
A. canescens	8	—	2	10	40	8	—	—	3	4	21	—	4	—	—	—	32
A. hymenolytra	—	—	—	5	2	—	—	—	3	27	12	4	45	—	—	2	32
A. lentiformis	1	—	t	7	43	6	—	—	1	2	34	1	4	—	—	t	32
A. patula	2	—	—	11	46	6	—	—	—	7	19	—	9	—	—	—	32
A. polycarpa (female)	6	—	6	10	43	3	—	—	2	4	24	—	1	—	—	1	32
A. polycarpa (male)	7	—	8	19	44	4	—	—	—	4	14	—	—	—	—	—	32
A. semibaccata	—	—	t	5	2	—	—	—	1	44	34	1	11	—	t	1	32
A. watsonii (female)	1	—	1	7	22	25	—	—	1	2	12	2	27	—	—	—	32
A. watsonii (male)	2	—	2	13	33	20	—	—	t	1	12	—	16	—	—	—	32
Beta vulgaris	—	—	6	7	13	—	—	—	—	48	25	—	1	—	—	—	19,30,31
Bassia hirsuta	5	—	11	15	45	19	—	—	—	—	—	—	5	—	—	—	19,30
Kochia scoparia	—	—	2	10	88	—	—	—	—	—	—	—	—	—	—	—	19,30
Salicornia bigelovii	—	—	2	29	1	18	—	—	—	31	10	—	—	4	5	—	19,30
S. europaea	—	—	4	25	15	—	—	—	—	38	13	—	—	4	1	—	19,30
S. subterminalis	2	—	2	24	58	—	—	—	—	6	—	1	—	—	7	—	32
S. virginica	—	—	—	21	35	—	—	—	—	4	—	—	—	—	36	4	32
Spinacea oleracea	t	t	—	2	—	—	—	8	—	35	45	—	8	—	—	—	9
S. oleracea	—	—	—	—	—	—	—	—	2	76	22	—	—	—	—	—	11
Genus: Chenopodium																	
Section: Chenopodium																	
Subsection: Cellulata																	
C. berlandieri ssp. *berlandieri*	2	—	2	12	24	—	3	—	1	15	34	—	3	—	4	—	32
C. berlandieri ssp. *nuttalliae*	1	t	3	14	16	—	4	1	2	23	29	1	t	2	3	—	32
C. quinoa ssp. *quinoa*	1	—	2	8	14	—	7	1	3	19	39	3	3	—	—	—	32
Subsection: Leiosperma																	
C. petiolare	3	—	2	9	26	—	3	—	2	28	27	—	—	—	—	—	32
C. fremontii	8	1	3	8	42	—	2	t	2	4	19	1	1	1	7	—	32
C. standleyanum	5	—	4	11	21	—	6	—	3	12	29	2	—	—	7	—	32
Subsection: Chenopodium																	
C. album	5	—	3	10	20	—	12	1	3	18	21	1	3	—	3	—	32
C. album	3	5	2	10	24	—	—	—	—	16	38	—	—	1	1	—	19,30
C. oahuense	3	—	3	15	28	—	7	1	—	20	17	—	4	1	1	—	32
Subsection: Grossefoveata																	
C. gigantospermum	6	—	4	13	16	—	4	—	3	35	19	—	—	—	—	—	32
Section: Scottsbergia																	
C. sanctae-clare	4	—	—	8	13	—	1	—	—	24	42	—	8	—	—	—	32
Section: Ambrina																	
C. ambrosioides	2	—	—	3	10	—	3	2	—	55	11	—	10	1	3	—	32
C. ambrosioides	—	—	—	—	—	—	—	—	—	68	10	—	22	—	—	—	30
Section: Botryoides																	
Subsection: Botrys																	
C. graveolens	1	—	1	5	6	—	—	—	1	54	26	2	t	1	2	—	32
Subfamily: Salisoloideae																	
Ceratoides lanata	6	12	14	10	38	7	2	—	3	1	—	2	2	3	—	—	32
Salsola kali	—	—	21	3	67	—	—	—	—	—	—	—	3	—	6	—	19,30
Suaeda linearis	—	—	6	13	40	16	—	—	—	—	—	—	—	6	19	—	19,30
Chenopodium rubrum[a]	—	—	—	—	—	—	—	—	—	54	— 23	—	23	—	—	—	19,30
C. urbicum[a]	—	1	2	7	42	—	—	—	—	7	38	—	2	1	—	—	19,30
C. leptophyllum[a]	—	—	1	10	45	—	—	—	—	—	42	—	2	—	—	—	19,30

[a]Unknown taxomonic position.

A, cholest-5-enol.
B, 24-methylcholesta-5,22-dienol.
C, 24-methylcholest-5-enol.
D, 24-ethylcholesta-5,22-dienol.
E, 24-ethylcholest-5-enol.
F, 24-ethylcholesta-5,24(28)Z-dienol.
G, cholest-7-enol.
H, 24-methylcholesta-7,24(28)-dienol.
I, 24-methylcholest-7-enol.
J, 24-ethylcholesta-7,22-dienol.
K, 24-ethylcholest-7-enol.
L, 24-ethylcholesta-7,25(27)-dienol.
M, 24-ethylcholesta-7,24(28)Z-dienol.
N, 24-methylcholestanol.
O, 24-ethylcholestanol.
P, 24-ethylcholest-22-enol.
t, Trace amount (<1%).

TABLE 6

Percent Desmethylsterol Composition of the Amaranthaceae

Family	Sterols													
	A	B	C	D	E	F	G	H	I	J	K	L	M	(Ref.)
Amaranthaceae														
Subfamily: Deeringioideae														
Deeringia muricata	—	—	4	4	20	—	38	14	—	—	3	14	3	35
Subfamily: Amaranthoideae														
Achyranthes bidentata	—	1	5	9	20	4	17	35	—	1	1	3	4	35
Aerva javanica	—	—	11	21	20	7	13	16	3	—	2	3	4	35
A. persica	—	—	12	8	25	8	7	19	—	—	3	5	13	35
Alternanthera maritima	—	—	2	8	14	4	26	34	—	—	—	7	5	35
A. halimifolia	—	—	4	12	12	4	39	16	—	—	—	4	9	35
A. paronychioides	—	3	7	24	—	3	42	3	—	1	2	2	13	35
A. canescens	—	—	11	14	12	5	21	20	2	—	3	5	7	35
A. caracasana	—	—	5	11	24	4	24	20	1	—	—	6	5	35
A. flavescens	—	—	4	10	22	3	26	24	1	—	—	7	3	35
A brasiliana	—	—	4	14	18	3	29	26	—	1	—	2	3	35
Chionothrix latifolia	—	1	7	42	36	1	5	t	1	—	—	3	4	35
Cyphocarpa angustifolia	—	—	7	12	30	4	12	18	—	—	—	5	12	35
Nototrichium humile	—	—	6	23	32	3	10	11	—	—	—	7	8	35
N. sandwicense	—	—	6	32	21	1	13	16	—	2	—	3	6	35
Ptilotus parvifolius	—	—	2	6	32	3	12	34	—	1	—	5	5	35
P. obovatus	—	t	5	9	22	2	3	46	—	2	—	7	4	35
P. atriplicinifolius	—	t	9	9	33	2	5	18	9	3	—	7	4	35
Sericocomopsis pallida	—	—	10	28	47	2	—	4	1	—	—	5	3	35
Seriostachys tomentosa	—	—	3	27	57	—	—	8	1	—	—	3	1	35
Subfamily: Gomphrenoidaea														
Brayulinea densa	—	1	10	14	36	2	2	6	—	2	4	16	7	35
Froelichia arizonica	—	—	5	17	55	1	—	3	—	—	—	15	4	35
F. floridana	—	—	6	25	42	1	—	2	—	—	—	18	6	35
Gomphrena sonorae	—	—	12	11	60	1	—	4	—	—	1	6	5	35
G. dispersa	—	t	10	14	57	3	—	8	—	—	—	4	3	35
G. officinalis	—	—	18	9	55	3	—	6	4	—	—	4	1	35
Pfaffia grandiflora	—	—	2	7	32	3	14	17	5	—	4	11	5	35
P. stenophylla	—	—	4	12	52	1	5	17	—	—	—	6	3	35
P. hookeriana	—	—	4	15	32	3	10	22	—	2	1	7	4	35
P. elata	—	—	14	5	38	—	—	—	—	—	2	5	36	35

A, cholest-5-enol.
B, 24-methylcholesta-5,24(28)-dienol.
C, 24-methylcholest-5-enol.
D, 24-ethylcholesta-5,22-dienol.
E, 24-ethylcholest-5-enol.
F, 24-methylcholest-7-enol.
G, 24-ethylcholesta-7,22-dienol.
H, 24-ethylcholest-7-enol.
I, 24-ethylcholesta-7,24(28)Z-dienol.
J, 24-ethylcholesta-7,25(27)-dienol.
K, 24-methylcholestanol.
L, 24-ethylcholestanol.
M, 24-ethylcholest-22-enol.
t, Trace amount (<1%).

cies in the Achatocarpaceae and Nyctaginaceae contain a sterol composition which is typical of other "main line" angiosperms. Only species in the Gyrostemonaceae exhibit a continued metabolism to the Δ^0-sterols. Based on the sterol data, it would appear that the origin of the Achatocarpaceae and Nyctaginaceae would be in or near the subfamilies Agdestioideae and Lophiocarpoideae of the basal family Phytolaccaceae.

Aizoaceae, Didiereaceae and Cactaceae. The Aizoaceae, Didiereaceae and Cactaceae are the cactus and cactus-like succulent group within the Caryophyllales (1). With the exception of *Lophocereus schottii* in the Cactaceae (12,13), these three families are similar to the previous families in exhibiting a strong preference toward Δ^5-sterol production (Tables 3 and 4). While species in the Cactaceae produce predominantly Δ^5-sterols, species in the Aizoaceae and Didiereaceae also produce substantial quantities of Δ^0-sterols. In *Drosanthemum hispidium* (Aizoaceae), Δ^0-sterols were the preferred sterol species (57%). It appears that regulation of sterol metabolism in these families is similar to that previously stated for the subfamily Rivinoideae in the basal family Phytolaccaceae.

Chenopodiaceae and Amaranthaceae. The Chenopodiaceae and Amaranthaceae have been referred to as the archetypical core of the Caryophyllales (1). The Chenopodiaceae (Table 5) is organized by the subfamily arrangement of Blackwell (33). The treatment of the genus *Chenopodium* is that of Aellen and Just (34).

Species within the Chenopodiaceae are very diverse in their sterol biosynthetic capacity. Within the subfamily Chenopodioideae, sterol metabolism ranges from those species which produce in excess of 98% Δ^7-sterols to species which produce 1:1 ratios of Δ^7-:Δ^5-sterols to species which produce in excess of 98% Δ^5-sterols. In genera where many species have been examined, percent sterol composition does not appear to be consistent at the genus level. Most species within the

TABLE 7

Percent Desmethylsterol Composition of The Genera Amaranthus and Celosia (Amaranthaceae)

Family	Sterols						
	A	B	C	D	E	F	(Ref.)
Amaranthaceae							
Subfamily: Amaranthoideae							
Amaranthus tricolor	2	2	4	6	67	19	36
A. tricolor 'Hong Kong'	1	1	4	5	74	15	36
A. tricolor '9 Ind.'	—	t	4	5	78	13	36
A. tricolor 'Chin'	—	t	2	5	77	16	36
A. tricolor 'P.R.C.'	t	4	5	6	69	16	36
A. tricolor 'Coz'	t	1	t	5	76	18	36
A. tricolor 'Splendor'	2	t	4	7	70	17	36
A. gangeticus	1	2	2	6	71	18	36
A. hypochondriacus '79-R101'	—	2	2	7	72	17	36
A. hypochondriacus (seed)	1	t	2	23	49	25	36
A. hypochondriacus (callus)	—	t	10	2	76	12	36
A. cruentus '79-R123'	—	9	7	6	60	18	36
A. caudatus 'Kiss Me'	1	1	3	9	62	24	36
A. caudatus	10	3	6	5	59	17	36
A. retroflexus	—	1	6	4	69	20	36
A. leucocarpus	—	t	2	5	77	15	36
Subfamily: Deeringioideae							
Celosia plumosa 'Fancy Plumes'	—	3	5	11	69	12	36
C. plumosa 'Fire Dragon'	3	t	t	7	53	36	36
C. cristata 'Red Velvet'	—	—	—	2	68	30	36
C. cristata 'Semidwarf'	1	—	3	5	72	19	36
Seed							
Amaranthus tricolor '78S-113'	—	—	12	15	56	17	36
A. tricolor '79W-294'	—	—	14	16	52	18	36
A. retroflexus '78S-73'	—	—	10	16	52	17	36
A. hybridus '81S-394'	—	—	12	13	58	17	36
A. dubius '78S-223'	—	—	13	16	52	19	36
A. cruentus '78S-40'	—	—	11	16	56	17	36

A, cholest-5-enol.
B, 24-methylcholest-5-enol.
C, 24-ethylcholesta-5,22-dienol.
D, 24-methylcholest-7-enol.
E, 24-ethylcholesta-7,22-dienol.
F, 24-ethylcholest-7-enol.
t, Trace amount (<1%).

genus *Chenopodium* produce more Δ^7-sterols than Δ^5-sterols. In only three species was this trend reversed. However, repeated characterization of the sterol composition of a species over time and/or location has shown that the bulk sterol composition of an individual species is stable (19,30).

In the genus *Atriplex*, some species are monoecious while others are dioecious. In the two dioecious species examined (*A. watsonii* and *A. polycarpa*), male and female plants were analyzed separately (32). While both sexes produce mixtures of Δ^7- and Δ^5-sterols, male plants produced higher proportions of Δ^5-sterols. Whether all dioecious *Atriplex* species exhibit this pattern is not known.

The species examined in the subfamily Salisoloideae all produce predominantly Δ^5-sterols in ratios comparable to "main line" angiosperms. While all species in the Chenopodiaceae are capable of producing Δ^5-sterols, further metabolism to the Δ^0-sterols appears to be limited except in two species. This would suggest that the conversion of Δ^5-sterols to Δ^0-sterols is being regulated or that Δ^0-sterols are metabolized further to some other products.

The Amaranthaceae is one of the three largest families in the order (1). The family (Tables 6 and 7) is organized according to Engler and Prontl (38). The Amaranthaceae is similar to the Chenopodiaceae with respect to the sterol composition of its member species. The majority of species in the subfamilies Deeringioideae and Amaranthoideae produce mixtures of Δ^7- and Δ^5-sterols (Table 6). In several species in the subfamily Amaranthoideae and all species in the Gomphrenoideae, sterol metabolism is shifted to favor Δ^5-sterol production.

Unlike members of the related Chenopodiaceae, all members of the Amaranthaceae further metabolize Δ^5-sterols to Δ^0-sterols. In most species, Δ^0-sterols are present in excess of 10% of the total desmethylsterol. In this respect, species in the Amaranthaceae are more similar to species in the basal family Phytolaccaceae than to species in the Chenopodiaceae. This would lend support to the belief that the Chenopodiaceae and Amaranthaceae are derived from a common ancestor and not from each other (1).

Portulacaceae and Basellaceae. The Portulacaceae consists of approximately 20 genera while the Basellaceae consists of only four genera (1). The Basellaceae is derived from the closely related Portulacaceae and is generally considered to be more advanced (1).

Members of the Portulacaceae may be divided into

TABLE 8

Percent Desmethylsterol Composition of the Portulacaceae, Basellaceae and Molluginaceae

Family	Sterols														
	A	B	C	D	E	F	G	H	I	J	K	L	M	N	(Ref.)
Portulacaceae															
Calandrinia caulescens	6	—	13	—	8	—	2	32	18	—	6	2	10	3	18
C. gayana	5	4	2	1	5	—	3	58	7	—	—	4	5	6	18
Claytonia virginica	3	—	1	1	8	—	6	11	60	3	—	2	5	—	18,19
Grahamia bracteata	—	—	2	12	19	5	—	21	25	—	—	—	7	9	18
Lewisia rediviva	4	—	2	6	14	6	2	36	20	—	4	—	6	—	18
Montia parvifolia	5	—	8	3	37	13	—	—	—	—	2	1	—	31	18
Portulace elatior	7	1	13	30	47	—	—	—	2	—	—	—	—	—	18
P. grandiflora	—	—	20	11	69	—	—	—	—	—	—	—	—	—	18,19
P. halimoides	6	—	13	7	42	—	2	—	2	—	—	—	5	23	18
P. oleracea	—	—	14	14	72	—	—	—	—	—	—	—	—	—	18
Basellaceae															
Anredera cordifolia	1	—	4	8	5	—	9	35	29	—	—	5	4	—	18
A. vesicaria	3	2	1	14	15	2	2	23	33	—	1	2	—	2	18
A. ramosa	2	—	1	9	14	1	1	21	44	—	1	1	—	5	18
Basella alba	—	—	3	18	11	—	2	24	39	—	3	—	—	—	18
Molluginaceae															
Adenogramma galioides	5	—	2	6	9	2	5	46	15	—	1	—	9	—	18
A. glomerata	3	—	3	4	16	—	2	47	7	—	—	1	17	—	18
Glinus orygiodes	8	—	1	9	9	—	3	58	11	—	—	1	—	—	18
Limeum aethiopicum	2	—	7	15	71	—	—	—	5	—	—	—	—	—	18
L. dinteri	2	—	3	28	39	—	—	4	2	6	1	—	15	—	18
L. fenestrata	3	—	6	7	80	—	—	—	4	—	—	—	—	—	18
Macarthuria apetala	3	—	8	21	7	—	4	31	9	—	7	—	5	5	18
Mollugo nudicaulis	3	8	5	9	13	2	4	28	8	—	6	4	7	3	18
M. pentaphylla	4	7	7	12	8	7	4	32	6	—	5	4	1	3	18
M. pinosia	2	4	10	3	29	10	5	25	5	—	1	3	3	—	18
M. verticillata	1	—	14	37	39	—	1	—	4	—	—	1	3	—	18
Pharnaceum serpyllifolium	3	7	5	12	12	3	3	30	6	—	7	3	5	4	18

A, cholest-5-enol.
B, 24-methylcholesta-5,24(28)-dienol.
C, 24-methylcholest-5-enol.
D, 24-ethylcholesta-5,22-dienol.
E, 24-ethylcholest-5-enol.
F, 24-ethylcholesta-5,24(28)Z-dienol.
G, 24-methylcholest-7-enol.
H, 24-ethylcholesta-7,22-dienol.
I, 24-ethylcholest-7-enol.
J, 24-ethylcholesta-7,25(27)-dienol.
K, 24-ethylcholesta-7,24(28)Z-dienol.
L, 24-methylcholestanol.
M, 24-ethylcholestanol.
N, 24-ethylcholest-22-enol.

two groups (Table 8). Species in the genera *Calandrinia, Claytonia, Grahamia* and *Lewisia* produce Δ^7-sterols in excess of Δ^5-sterols. In these species, substantial quantities of Δ^0-sterols are also produced. In *Montia* and *Portulaca,* sterol metabolism is shifted to favor the production of Δ^5-sterols. In two species, Δ^5-sterols presumably are further metabolized to produce substantial quantities of Δ^0-sterols, while in the remaining species Δ^0-sterols appear to be absent.

Species in the Basellaceae are believed to be derived from species in the Portulacaceae (1). It would appear appropriate that these species would have an origin in or near the first group above. While the sterol composition of species in the Basellaceae is similar to the first group in the Portulacaceae, the production of Δ^0-sterols appears to be more limited. Whether this represents an "advancement" in biosynthetic regulation in the Basellaceae is equivocal.

Molluginaceae and Caryophyllaceae. The Molluginaceae and Caryophyllaceae may represent the group with the earliest dichotomy from the Phytolaccaceae. The Molluginaceae and Caryophyllaceae are unique within the Caryophyllales in that all other families of the order produce betalains (unique and characteristic of the order), whereas the Molluginaceae and Caryophyllaceae produce anthocyanins (1). These two pigments have not been demonstrated to coexist within the same species. While the production of betalains has received taxonomic significance within this order, other unique morphological characteristics clearly support the inclusion of the Molluginaceae and Caryophyllaceae in this order.

The Caryophyllaceae is the type family of the order. The tribal arrangement of the family (Table 9) is that of Thomson (39), the subtribal arrangement of the Alsineae is that of McNeill (40).

The sterol composition of species in the Molluginaceae (Table 8) is most similar to that of species in the tribe Rivineae in the basal family Phytolaccaceae. Species in the Molluginaceae produce mixtures of Δ^7- and Δ^5-sterols or Δ^5-sterols. In both groups, Δ^5-sterols are further metabolized to the Δ^0-sterols.

The species in the Caryophyllaceae exhibit a diversity of sterol biosynthetic capacities which is unique within the angiosperms (Table 9). Species in the tribes Sclerantheae, Paronychieae, Lychnideae and Diantheae

TABLE 9

Percent Desmethylsterol Composition of the Caryophyllaceae

Family	Sterols														
	A	B	C	D	E	F	G	H	I	J	K	L	M	N	(Ref.)
Caryophyllaceae															
Tribe: Scleranthea															
Scleranthus annuus	—	—	—	—	—	—	—	42	58	—	—	—	—	—	19,41
Tribe: Paronychieae															
Paronychia virginica	—	—	—	—	—	—	—	55	45	—	—	—	—	—	19,41
Tribe: Lychnideae															
Lychnis alba	—	—	—	—	—	—	6	62	32	—	—	—	—	—	19,41
Silene cucubalus	—	—	—	—	—	—		72	28	—	—	—	—	—	19,41
Tribe: Diantheae															
Dianthus armeria	—	—	—	—	—	—	6	73	21	—	—	—	—	—	19,41
D. chinensis	—	t	t	1	—	1	12	51	33	—	—	—	—	—	[a]
D. heddewigii	—	—	—	—	—	6	4	41	49	—	—	—	—	—	[a]
D. pubera	—	4	2	—	—	t	8	38	46	—	—	—	—	—	[a]
Gypsophila paniculata	—	—	—	—	—	—	—	63	37	—	—	—	—	—	19,41
Saponaria officinalis	—	—	—	—	—	—	—	80	20	—	—	—	—	—	19,41
Tribe: Alsineae															
Subtribe: Sabulininae															
Sagina decumbens	—	4	2	9	2	8	2	18	50	5	—	—	—	—	[a]
Spergula arvensis	—	—	—	—	—	—	—	3	17	11	69	—	—	—	19,41
Minuartia caroliniana	—	—	—	—	—	—	4	47	5	38	6	—	—	—	19,41
Subtribe: Stellariinae	—														
Stellaria media	—	4	5	—	—	5	10	56	20	—	—	—	—	—	[a]
Myosoton aquaticum	—	—	—	—	—	—	6	66	28	—	—	—	—	—	19,41
Cerastium arvense	—	2	—	18	—	—	8	8	57	—	—	1	6	—	19,41
C. vulgatum	—	1	—	22	—	—	—	10	42	—	—	2	23	—	19,41
Arenaria serpyllifolia	—	2	—	15	—	—	—	38	29	—	—	2	14	—	19,41
Tribe: Polycarpeae															
Pycnophyllum molle	4	2	3	5	—	4	5	37	18	14	—	—	5	3	[a]

[a]Previously unreported.

A, cholest-5-enol.
B, 24-methylcholest-5-enol.
C, 24-ethylcholesta-5,22-dienol.
D, 24-ethylcholest-5-enol.
E, 24-ethylcholesta-5,24(28)Z-dienol.
F, 24-methylcholest-7-enol.
G, 24-ethylcholesta-7,24(28)Z-dienol.
H, 24-ethylcholesta-7,22-dienol.
I, 24-ethylcholest-7-enol.
J, 24-ethylcholesta-7,25(27)-dienol.
K, 24-ethylcholesta-7,22,25(27)-trienol.
L, 24-methylcholestanol.
M, 24-ethylcholestanol.
N, 24-ethylcholest-22-enol.
t, Trace amount (<1%).

produce Δ^7-sterols exclusively (19,41). However, these analyses were performed on packed GLC columns (41). The analyses of two additional species of *Dianthus* with capillary GLC have indicated that minor quantities of Δ^5-sterols may be present in these four tribes.

Species in the tribe Alsineae, subtribes Sabulininae and Polycarpeae, produce Δ^7-sterols with both 24α-ethyl and 24β-ethyl side chain configurations. Conformational analyses of sterol species with 360 MH_z NMR (41) have demonstrated that the $\Delta^{25(27)}$-sterols in these species possess the 24β-alkyl orientation. In *Spergula arvensis,* 24β-ethylsterols are the preferred sterol species even though 24α-ethylsterols are also produced.

The majority of species in the subtribe Stellariinae (except *Myosoton aquaticum*) produce prominantly Δ^7-sterols with lesser amounts of Δ^5-sterols. As the ratio of Δ^5-sterols to Δ^7-sterols increases, Δ^0-sterols are also produced.

SUMMARY

The Caryophyllales possesses other unique chemical features (1) and sterol chemistry may now be listed among these. Species within the families of the Caryophyllales possess a diversity of sterol metabolism which is currently unique in angiosperms.

The fossil record indicates that the Caryophyllales originated approximately 70 million years ago (1). The Caryophyllales may represent an order where evolution and attempts at regulation of sterol biosynthesis are most prominant. Alternatively, most species in this order are xerotolerant or halotolerant and many species are highly adaptable weeds found in waste places. Many species can tolerate conditions across all of these environments. If functional requirements for sterols with altered structures or stereospecificity exists in plants, the diversity of sterols in these species may facilitate tolerance to widely differing and harsh condi-

tions if sterols of differing structure are readily available. If, however, this diversity reflects the evolution of regulation within the sterol pathway, the species in the Caryophyllales could serve as a useful tool for investigating these control mechanisms.

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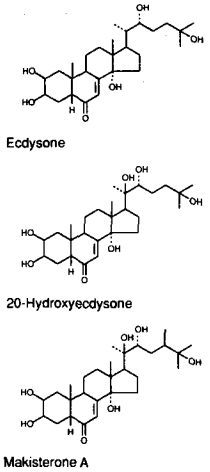
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Neutral Sterol Metabolism in Insects[1]

James A. Svoboda* and **Mark F. Feldlaufer**
Insect Hormone Laboratory, Agricultural Research Service, USDA, Beltsville, Maryland 20705

Since they are unable to biosynthesize sterols, many phytophagous and omnivorous insects satisfy their cholesterol requirement by side chain dealkylation of the C-24 alkyl group of dietary C_{28} and C_{29} phytosterols. However, not all insects that can dealkylate the phytosterol side chain produce cholesterol. In addition, certain insects, *e.g.*, some Hymenoptera, Hemiptera, and Diptera, are unable to dealkylate the sterol side chain. Although C_{27} ecdysteroids (molting hormones), which are biosynthesized from cholesterol, are the major ecdysteroids in most insects, many of those species that are unable to dealkylate phytosterols utilize campesterol as a precursor for the C_{28} ecdysteroid makisterone A. The considerable diversity of steroid utilization between certain insect species makes it difficult to generalize about insect steroid biochemistry. The ability to disrupt certain unique aspects of steroid utilization and metabolism in insects might be exploited for developing new insect control technology.
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SCHEME 1. Structures of ecdysteroids referred to in this review.

Insects are unable to biosynthesize the steroid nucleus (1) and require a dietary source of sterol to support normal development and reproduction (2). This presents an important area of biochemical difference between insects and many other species which might be exploited for developing new insect control technology by selectively disrupting steroid metabolism in insects. Cholesterol will generally (with few known exceptions) satisfy the dietary requirement of insects for sterol both as a membrane component and for physiological purposes such as ecdysteroid (molting hormone) biosynthesis (3). Ecdysone and 20-hydroxyecdysone (Scheme 1), the two most common ecdysteroids found in insects, are C_{27} steroids derived from cholesterol (4). Ecdysteroids are essential for every molt or morphological change that an insect undergoes. Obtaining a dietary source of cholesterol is no problem for insects that feed on animals or animal products which contain an abundance of cholesterol. However, for phytophagous insects that must subsist on a diet of plant or plant-derived material containing little or essentially no cholesterol, there would exist a major problem had these insects not evolved the ability to dealkylate C_{28} and C_{29} phytosterols to produce cholesterol (Scheme 2). Thus, it follows that a disruption of sterol metabolism at any metabolic step between the dietary phytosterol and cholesterol or between cholesterol and the molting hormone would result in mortality during early larval molts, which would be highly desirable with crop pests. It is necessary, first of all, to understand the variability of steroid metabolism among insects in order to be able to logically develop specific inhibitors of these essential biochemical pathways.

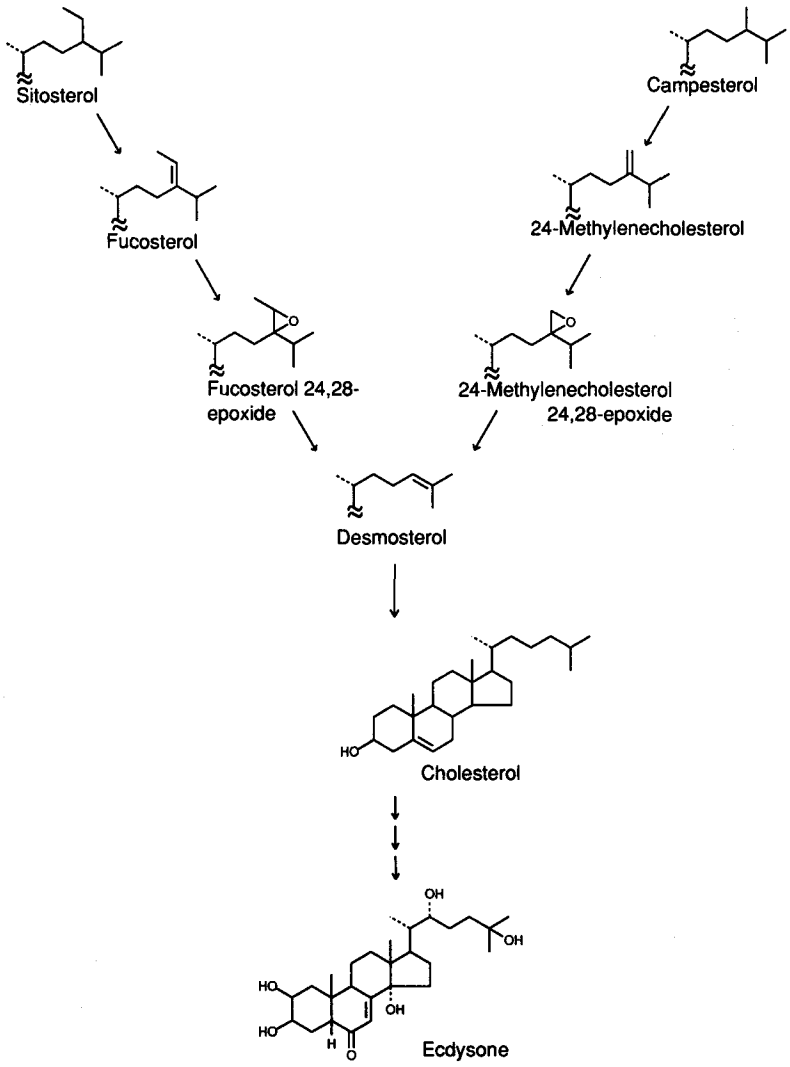
In this review, we will discuss recent research in several areas of neutral sterol metabolism in insects and the relationships of neutral sterols to ecdysteroid biosynthesis. We will not attempt to cover this area of research historically. The reader is referred to several recent reviews that treat this area of insect steroid metabolism in considerable depth (5–8). We will consider: i) certain aspects of dealkylation of C_{28} and C_{29} phytosterols; ii) sterol utilization in certain Hymenoptera; iii) some comparative studies with Hemiptera; and iv) comparative studies with Diptera.

SIDE CHAIN DEALKYLATION STUDIES

Much of the research on conversion of C_{28} and C_{29} phytosterols to cholesterol was done with sitosterol and campesterol (Scheme 2), but stigmasterol ($\Delta^{5,22E}$-24-ethyl) is also one of the most ubiquitous C-24 alkyl phytosterols. The metabolism of stigmasterol was examined in some depth recently in two separate studies. To date, the Mexican bean beetle, *Epilachna varivestis*, differs from any other insect that has been examined rigorously with respect to the metabolism of dietary C_{28} and C_{29} phytosterols. Regardless of the dietary sterol involved, whether sitosterol or stigmasterol, this insect reduces the Δ^5 bond before dealkylating the C-24 substituent, thus producing mainly cholestanol, some of which is subsequently converted to Δ^7-cholestenol (9). Three new intermediates were isolated and identified as metabolites of [^{3}H]stigmasterol and were found to accumulate in the insect tissues when azasteroid inhibitors were fed to the insects in combination with [^{3}H]stigmasterol (10). The three new insect

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*To whom correspondence should be addressed at USDA-ARS, Insect Hormone Laboratory, Building 467, BARC-East, Beltsville, MD 20705.



SCHEME 2. Generalized scheme for dealkylation and conversion of sitosterol and campesterol to cholesterol in insects.

sterols are 22-stigmastenol, 22,24-cholestadienol and 24-cholestenol. Thus, the pathway for dealkylation of stigmasterol in this species appears to be:

$$\Delta^{5,22}\text{-24-ethyl} \rightarrow \Delta^{22}\text{-24-ethyl} \rightarrow \Delta^{22,24} \rightarrow \Delta^{24} \rightarrow \Delta^{0} \rightarrow \Delta^{7}$$

The side chain metabolism proceeds in the same sequence as found in other insects that do not reduce the Δ^5 bond prior to dealkylation:

$$\Delta^{5,22}\text{-24-ethyl} \rightarrow \Delta^{5,22,24} \rightarrow \Delta^{5,24} \rightarrow \Delta^{5}$$

It is of interest to note, however, that stigmastanol is not dealkylated nearly as efficiently as stigmasterol, nor does Δ^7-stigmastenol appear to be metabolized by this insect. The Δ^5 bond must be present to obtain good metabolism of dietary sterols in the Mexican bean beetle.

The fate of [^{3}H]stigmasterol also was studied in a lepidopteran, *Spodoptera littoralis,* and stigmasterol-24,28-epoxide and stigmasta-5,22E,24(28E)-trien-3β-ol were identified for the first time from insects as intermediates in the dealkylation and conversion of stigmasterol to cholesterol (11). These two new sterols, plus cholesta-5,22E,24-trien-3β-ol, accumulated in the insect tissues when an azasteroid inhibitor was fed in combination with stigmasterol in the diet. Thus, it appears that intermediates in the conversion of stigmasterol to cholesterol in this lepidopteran occur that are analogous to fucosterol and fucosterol-24,28-epoxide in the conversion of sitosterol to cholesterol in other species (Scheme 2). The available evidence indicates that in *S. littoralis* the following pathway exists: stigmasterol → stigmasta-5,22E,24(28E)-trien-3β-ol → stigmasterol-24,28-epoxide → cholesta-5,22E,24-trien-3β-ol → desmosterol → cholesterol. Earlier indirect evidence from sitosterol metabolism studies with *Bombyx mori* is consistent with these results from *S. littoralis* (12). Later *in vitro* and *in vivo* metabolic studies with silkworms fed sitosterol provided more direct evidence for the presence of an epoxide as an intermediate in dealkylation (13,14). Additional information on the involvement of an

epoxide in dealkylation of phytosterols in several species was provided by a number of laboratories concerned with the mechanism involved (15–20). The review by Svoboda and Thompson (6) contains a detailed discussion of this mechanism.

The dealkylation of C_{28} phytosterols, such as campesterol, proceeds similarly to that of C_{29} sterols, with 24-methylene (21) and 24,28-epoxide (5) compounds serving as intermediates. However, *Heliothis zea*, which readily converts Δ^5-C_{28} and Δ^5-C_{29} phytosterols to cholesterol, utilizes $\Delta^{5,7}$-24-alkyl sterols differently (22). In metabolic studies with *H. zea* fed ergosterol ($\Delta^{5,7,22E}$-C_{28} sterol), the major insect sterol was 7-dehydrocholesterol, indicating that the side chain could be metabolized to a typical cholesterol side chain, but little saturation of the Δ^7 bond occurred in the B ring (22). In fact, less cholesterol was formed from ergosterol than when 7-dehydrocholesterol was the added dietary sterol. This is in contrast to the capability of *Tribolium confusum* to utilize 7-dehydrocholesterol, since this sterol and cholesterol occur in equilibrium in *T. confusum* regardless of the dietary sterol used (6).

STEROL UTILIZATION IN HYMENOPTERA

The honey bee, *Apis mellifera*, is one of a growing number of phytophagous insect species known to be incapable of dealkylating C_{28} and C_{29} phytosterols to produce cholesterol. Extensive studies with both C_{28} and C_{29} radiolabeled sterols, including campesterol, sitosterol, and 24-methylenecholesterol, using a well-defined diet, provided no proof of conversion of any of these 24-alkyl sterols to cholesterol (23). Regardless of the dietary sterol added, 24-methylenecholesterol was always the major sterol of the next generation. It was determined that the nurse bees have a very unusual ability to selectively transfer sterols from their own endogenous sterol pools in order to maintain a high level of 24-methylenecholesterol in the brood food and, thus, provide high levels of this sterol to the developing brood in the cells (23). Sitosterol and isofucosterol also seem to be favored by this selective transfer mechanism, although the significance of this selective sterol transfer remains unknown.

Of particular interest in recent research with the honey bee was the discovery that the C_{28} ecdysteroid, makisterone A (Scheme 1), is the major ecdysteroid identified in the pupa and ovaries of this species (24,25). This finding is consistent with what was found with several species of Hemiptera, all of which are also unable to dealkylate and convert C_{28} and C_{29} phytosterols to cholesterol, but utilize makisterone A as their molting hormone (26,27). In metabolic studies with labeled sterols, campesterol was identified as the precursor of makisterone A in the honey bee (28), although it was suspected prior to this that 24-methylenecholesterol, the major sterol of the honey bee, may have been the precursor of this ecdysteroid. The honey bee converts [^{3}H]campesterol, but not [^{3}H]24-methylenecholesterol, to makisterone A. It also converts [^{14}C]cholesterol to [^{14}C]20-hydroxyecdysone, although it does so less efficiently (28).

In comparative studies, another phytophagous hymenopteran, the alfalfa leafcutter bee, *Megachile rotundata*, was found to have 24-methylenecholesterol as a major component of its tissue sterols, with very little cholesterol occurring (0.5% of the total sterols), indicating that this solitary bee utilizes dietary sterols similarly to the honey bee (29). In this same study, the sterols of four omnivorous species of Hymenoptera, the baldfaced hornet, *Dolichovespula maculata*, the eastern yellowjacket, *Vespula maculifrons*, the Allegheny mound ant, *Formica exsectoides*, and the red imported fire ant, *Solenopsis invicta*, contained cholesterol as the major sterol, reflecting use of dietary sterols with no metabolic change, since their diet is usually high in cholesterol content (29). A very significant report of sterol utilization in a hymenopteran concerned the leaf-cutting ant, *Atta cephalotes isthmicola*. This paper described the first study in which no cholesterol could be detected in an insect (30). Apparently this phytophagous species cannot dealkylate the sterol side chain and the principal sterol of the brain and whole body was 24-methylcholesta-5,7,24(28)-trien-3β-ol. This provides the first evidence that a functional nervous system can exist in an animal without cholesterol.

STEROL UTILIZATION IN HEMIPTERA

The milkweed bug, *Oncopeltus fasciatus*, was the first phytophagous species found to be unable to dealkylate and convert 24-alkyl phytosterols to cholesterol (31). In addition, it was the first insect shown to produce makisterone A (32). Subsequent studies showed that other related Hemiptera also were able to produce makisterone A as their major ecdysteroids (26,27). This led to investigations of neutral sterol metabolism in a number of Hemiptera belonging to two different groups, the Pentatomomorpha and the Cimicomorpha. Three species of the first group, *O. fasciatus*, *Nezara viridula* and *Dysdercus cingulatus* are phytophagous and cannot dealkylate phytosterols; a fourth member of this group, *Podisus maculiventris*, is predacious and obtains adequate cholesterol from its diet (33). However, all of these species produce makisterone A as their major molting hormone (26). It is interesting to note that *P. maculiventris* is secondarily predacious but has maintained the ecdysteroid biosynthesizing capabilities of its ancestors. The three members of the Cimicomorpha group included in this study, *Rhodnius prolixus*, *Arilus cristatus*, and *Cimex lectularius*, are all blood feeders and cholesterol is their major tissue sterol, as it is normally in abundance in their diet (33). These blood feeders were found to produce only C_{27} ecdysteroids, such as ecdysone and 20-hydroxyecdysone (34). Another phytophagous true bug, *Megalotomus quinquespinosus*, although belonging to a different taxonomic group than the Hemiptera previously discussed, is likewise unable to dealkylate C_{28} and C_{29} phytosterols to form cholesterol (35). This species also produces makisterone A as its major ecdysteroid (35), thus fitting into the pattern of those phytophagous Hemiptera and other species that are unable to dealkylate but are able to utilize a dietary phytosterol unchanged, to produce makisterone A as their major ecdysteroid.

STEROL UTILIZATION IN DIPTERA

The house fly, *Musca domestica*, in studies using adequate biochemical methodology, was shown to be unable to convert [^{3}H]sitosterol to cholesterol over 25 years ago (36,37). For many years after, it was assumed that all

Diptera lacked this capability. However, eventually it was found that larvae of the yellow fever mosquito, *Aedes aegypti*, have the necessary enzymes to carry out such a conversion (38). In this study, dietary radiolabeled sitosterol, campesterol, and desmosterol were used to verify that this dealkylation capacity existed in *A. aegypti*. The 24-sterol reductase inhibitor, 25-azacholesterol, was also used in combination with [^{14}C]sitosterol to further demonstrate that a 24-reductase enzyme was present and could be inhibited, bringing about an accumulation of desmosterol with little cholesterol produced.

Until recently, the literature on phytosterol utilization in *Drosophila melanogaster* was unclear. Early growth studies with *D. melanogaster* led to the conclusion by the authors that this insect was able to dealkylate phytosterols to produce cholesterol (39). More recently, from another study, it was assumed that ergosterol was dealkylated by *D. melanogaster* (40). Neither of these studies used selective, valid biochemical or analytical techniques. Still other researchers used gas-liquid chromatography (GLC) to show metabolism of yeast sterols fed to *D. melanogaster*, but it is difficult to conclude that no cholesterol was present in the insects reared on the wild type yeast strain (41). However, in order to clarify this situation, and by using thorough metabolic studies, we were able to definitively demonstrate that neither dietary [^{14}C]sitosterol nor [^{14}C]desmosterol are converted to [^{14}C]cholesterol by *D. melanogaster* (42). It is noteworthy that *D. melanogaster* is classified among the higher Diptera as is the house fly, which it resembles with respect to sterol dealkylation.

From related research, it was concluded that several cactophilic *Drosophila* species could not dealkylate. They were fed cactus and the sterols were analyzed by GLC. From additional studies with axenic cultures, it was determined that these *Drosophila* were capable of some selective uptake of sterols (43), and demonstrated poor development when fed desmosterol as the sole added sterol (44). Since insects that dealkylate should thrive on a diet supplemented with desmosterol, this provides further proof of the inability of certain cactophilic species to dealkylate.

Recent investigations on the capability of *M. domestica* and *D. melanogaster* to utilize various dietary sterols and synthesize certain ecdysteroids were carried out in light of the fact that neither species is capable of dealkylating 24-alkyl sterols to produce cholesterol (45). These studies indicated that both species are capable of producing makisterone A from campesterol when this sterol is included at a fairly high concentration in the artificial medium. Through selective uptake, trace contaminants of cholesterol in the diet still allow both species to produce predominantly C_{27} ecdysteroids (ecdysone and 20-hydroxyecdysone). Whether the makisterone A has any physiological significance in these species is as yet unknown.

CONCLUSION

As we learn more about all aspects of steroid biochemistry in insects, the variety of ways that insects utilize and metabolize dietary neutral sterols becomes increasingly fascinating. This variability is a reflection of the ability of insects to adapt to living in virtually every conceivable ecological niche and to utilize whatever medium is available. Basic information on steroid metabolism provides important comparisons from a taxonomic standpoint, but also such indepth knowledge is necessary to enable discovery and development of specific inhibitors or other means of disrupting this important and unique area of insect biochemistry.

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Metabolism of Plant Sterols by Nematodes[1]

David J. Chitwood* and **William R. Lusby**
Nematology Laboratory and Insect Neurobiology and Hormone Laboratory, USDA-ARS, Building 467, BARC-East, Beltsville, Maryland 20705

Parasitic nematodes do not biosynthesize sterols *de novo* and therefore possess a nutritional requirement for sterol, which must be obtained from their hosts. Consequently, the metabolism of phytosterols by plant-parasitic nematodes is an important process with potential for selective exploitation. The sterol compositions of several species of plant-parasitic nematodes were determined by capillary gas chromatography-mass spectrometry and compared with the sterol compositions of their hosts. Saturation of the phytosterol nucleus was the major metabolic transformation performed by the root-knot nematodes *Meloidogyne arenaria* and *M. incognita* and the corn root lesion nematode, *Pratylenchus agilis*. In addition to saturation, the corn cyst nematode, *Heterodera zeae*, dealkylated its host sterols at C-24. Because free-living nematodes can be cultured in sterol-defined artificial medium, they have been successfully used as model organisms for investigation of sterol metabolism in plant-parasitic nematodes. Major pathways of phytosterol metabolism in *Caenorhabditis elegans*, *Turbatrix aceti* and *Panagrellus redivivus* included C-24 dealkylation and 4α-methylation (a pathway unique to nematodes). *C. elegans* and *T. aceti* introduced double bonds at C-7, and *T. aceti* and *P. redivivus* saturated the sterol nucleus similarly to the plant-parasitic species examined. Several azasteroids and long-chain dimethylalkylamines inhibited growth and development of *C. elegans* and also the Δ^{24}-sterol reductase enzyme system involved in the nematode C-24 dealkylation pathway.
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One of the few major biochemical differences between parasitic nematodes and their plant or vertebrate hosts is that nematodes possess a nutritional requirement for sterol because they are incapable of *de novo* sterol biosynthesis (1,2). Because higher plants synthesize 24-alkylsterols almost exclusively and because most animals predominantly contain cholesterol (3), one might expect plant-parasitic nematodes to remove the C-24 alkyl substituents of common phytosterols and thereby produce cholesterol. Not only is phytosterol dealkylation by nematodes of interest to the steroid biochemist, but this pathway offers the potential for being selectively exploited by novel nematode control agents. Therefore, we have examined various aspects of nematode sterol metabolism in depth in recent years, especially phytosterol metabolism and its disruption (4–16). The purpose of this review is to consolidate this research in which over 60 sterols have been identified in nematodes, with emphasis upon our recent work with *Pratylenchus agilis*.

Nematodes can be divided into at least three major groups according to their feeding habits: plant-parasitic nematodes, animal-parasitic nematodes, and the somewhat misnamed free-living nematodes, which in nature primarily feed upon microorganisms (17). The most significant obstacle in investigation of the biochemistry of parasitic nematodes is the present inability of biologists to readily culture parasitic nematodes throughout their entire life cycles in artificial medium. As a result, all of our research involving the metabolism of supplemented (usually radiolabeled) sterols by nematodes has been restricted to free-living nematodes. We have focused such studies upon *Caenorhabditis elegans* because it has received intensive study by molecular geneticists and developmental biologists (18). For comparative investigations of metabolic diversity in free-living nematodes, we examined *Panagrellus redivivus* and *Turbatrix aceti*. The plant-parasitic species investigated were representatives of the two most agronomically important groups of nematodes: the cyst nematodes *(Heterodera, Globodera)* and the root-knot nematodes *(Meloidogyne)*. These nematodes are sedentary endoparasites, *i.e.*, they establish themselves within plant roots and establish permanent feeding sites. We also investigated the lesion nematode *Pratylenchus agilis* because large quantities of it can be reared in sterile culture on excised corn root explants. This species is a migratory parasite that feeds both endoparasitically and ectoparasitically on corn roots (19).

STEROL IDENTIFICATION

The sterols we identified in nematodes were identical to authentic standards (when in our possession) by gas-liquid chromatography (GLC) relative retention times (RRT; Table 1) and gas-chromatography-mass spectrometry (GC-MS) (Table 2). In our previous investigations, the identification of compounds for which we lacked authentic material (Table 1, compounds 6, 7, 14, 15, 17, 18, 20, 26, 28, 30, 33, 45, 48, 50, 52–54, 56, 57, 59, 60, and 63) was accomplished by careful interpretation of data from GLC, GC-MS, ultraviolet (UV), and argentation column and thin-layer chromatography, with corroboration being obtained from MS and GLC data in the literature. Similarly, in our more recent investigations with *P. agilis*, we identified compounds 13, 34, 41, 44, 51, 55, 58, and 61 by comparison of GLC and GC-MS data with data associated with structurally related, previously identified compounds, as well as comparison with GLC and mass spectrometry (MS) data from the literature (20–28). In experiments with free-living nematodes, dietary sterols with known stereochemistry at C-24 were used: *e.g.*, sitosterol (24α-ethylcholesterol), campesterol (24α-methylcholesterol), or stigmasterol (24α-ethylcholesta-5,22(*E*)-

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*To whom correspondence should be addressed.

Abbreviations: GC-MS, gas chromatography-mass spectrometry; GLC, gas-liquid chromatography; NMR, nuclear magnetic resonance; RRT, relative retention time; UV, ultraviolet spectroscopy.

TABLE 1

Gas-Liquid Chromatographic Relative Retention Times (RRT) of Sterols from Nematodes or Their Diets or Host Plants, Expressed Relative to Cholesterol[a]

Sterol	DB-1	OV-17	Sterol	DB-1	OV-17
1. Cholesta-5,7,9(11)-trienol	0.98	1.06	42. 24-Ethylcholesta-5,7-dienol	1.75	1.87
2. Cholest-8(14)-enol	0.99	1.02	43. 24-Ethyllathosterol	1.81	1.96
3. Cholesterol	1.00	1.00	44. 24-Ethylcholest-24(25)-enol	1.81	2.04
4. Cholestanol	1.02	1.02	45. 24Z-Ethylidenelathosterol	1.89	2.20
5. Cholest-8(9)-enol	1.05	1.05	46. 4α-Methylcholest-8(14)-enol	1.18	1.15
6. Cholesta-7,9(11)-dienol	1.05	1.16		(1.15)[b]	(1.13)
7. Cholesta-5,7,9(11),24-tetraenol	1.07	1.28	47. 4α-Methylcholestanol	1.20	1.15
8. Desmosterol	1.08	1.20		(1.17)	(1.13)
9. 7-Dehydrocholesterol	1.10	1.16	48. 4α-Methylcholesta-8(14),24-dienol	1.29	1.43
10. 24-Methylcholesta-5,22(*E*)-dienol	1.11	1.14		(1.26)	(1.39)
11. Cholest-24-enol	1.12	1.26	49. 4α-Methylcholest-7-enol	1.31	1.36
12. Lathosterol	1.12	1.19		(1.27)	(1.33)
13. 24-Methylcholest-22(*E*)-enol	1.14	1.17	50. 4α-Methylcholesta-7,24-dienol	1.42	1.66
14. Cholesta-8(9),24-dienol	1.15	1.28		(1.38)	(1.61)
15. Cholesta-5,7,9(11),22(*E*),24-pentaenol	1.16	1.48	51. Obtusifoliol	1.52	1.56
16. Cholesta-5,22(*E*),24-trienol	1.17	1.43		(1.49)	(1.50)
17. Cholesta-5,7,24-trienol	1.21	1.42	52. 4α,24-Dimethylcholest-8(14)-enol	1.54	1.52
18. Cholesta-7,24-dienol	1.23	1.44		(1.49)	(1.48)
19. 24-Methylcholesta-5,23-dienol	1.25	1.38	53. 4α,24-Dimethylcholestanol	1.58	1.52
20. 24-Methylcholesta-5,7,9(11)-trienol	1.26	1.42		(1.53)	(1.48)
21. 24-Methylenecholesterol	1.26	1.37	54. 4α,24-Dimethylcholest-7-enol	1.69	1.80
22. Cholesta-5,7,22(*E*),24-tetraenol	1.29	1.68		(1.64)	(1.76)
23. 24-Methylenecholestanol	1.30	1.39	55. 4α-Methyl-24-ethylcholest-22(*E*)-enol	1.71	1.66
24. 24-Methylcholesterol	1.30	1.33		(1.67)	(1.61)
25. 24-Methylcholestanol	1.33	1.35	56. 4α-Methyl-24-methylenelathosterol	1.75	1.86
26. 24-Methylenecholesta-5,7-dienol	1.39	1.57		(1.68)	(1.80)
27. 24-Ethylcholesta-5,22(*E*)-dienol	1.40	1.45	57. 4α-Methyl-24-ethylcholestanol	2.10	1.87
28. 24-Methylcholesta-7,24(28)-dienol	1.41	1.63		(1.91)	(1.87)
29. 24-Methylcholesta-5,7-dienol	1.42	1.54	58. 4α-Methylisofucostanol	2.07	2.11
30. 24-Ethylcholest-22(*E*)-enol	1.43	1.47		(1.95)	(2.06)
31. 24-Methylcholest-7-enol	1.46	1.57	59. 4α-Methyl-24(*Z*)-ethylidenelathosterol	2.26	2.52
32. 24-Methylcholesta-5,24(25)-dienol	1.47	1.67		(2.12)	(2.44)
33. 24-Ethylcholesta-5,22(*E*),24(28)-trienol	1.50	1.60	60. Cycloartanol	1.71	1.67
34. 24-Ethylcholesta-5,23-dienol	1.53	1.67		(1.60)	(1.53)
35. 24-Ethylcholesta-7,22(*E*)-dienol	1.57	1.70	61. Cycloeucalenol	1.74	1.88
36. Fucosterol	1.59	1.76		(1.69)	(1.78)
37. 24-Ethylcholesterol	1.60	1.67	62. Cycloartenol	1.86	2.05
38. 24-Ethylcholestanol	1.63	1.69		(1.73)	(1.85)
39. Isofucosterol	1.66	1.85	63. 24-Methylenecycloartanol	2.14	2.28
40. Isofucostanol	1.69	1.88		(1.99)	(2.06)
41. 24-Ethylcholesta-5,24(25)-dienol	1.77	1.98			

[a]GLC was performed on a temperature-programmed (90°C 1 min, 15°C/min increase to 250°C) DB-1 fused silica capillary column (14 m × 0.25-mm i.d., 0.25-μm film) and on an isothermally operated packed glass column containing 2.0% OV-17 stationary phase.

[b]Values in parentheses are RRT of steryl acetates relative to cholesteryl acetate. Trivial and systematic names: cholesterol, cholest-5-en-3β-ol; cholestanol, 5α-cholestan-3β-ol; desmosterol, cholesta-5,24-dien-3β-ol; lathosterol, 5α-cholest-7-en-3β-ol; fucosterol, 24(*E*)-ethylidenecholest-5-en-3β-ol; isofucosterol, 24(*Z*)-ethylidenecholest-5-en-3β-ol; isofucostanol, 24(*Z*)-ethylidene-5α-cholestan-3β-ol; obtusifoliol, 4α,14-dimethyl-24-methylene-5α-cholest-8-en-3β-ol; cycloartanol, 4,4,14-trimethyl-9β,19-cyclo-5α-cholestan-3β-ol; cycloeucalenol, 4α,14-dimethyl-24-methylene-9β,19-cyclo-5α-cholestan-3β-ol; cycloartenol, 4,4,14-trimethyl-9β,19-cyclo-5α-cholest-24-en-3β-ol.

dienol). Therefore, we assumed that nematode metabolites would have the same orientation at C-24 and have described such metabolites in Tables 3–5 as having the same 24α-orientation. Because quantities of sterols isolated from plant-parasitic nematodes or host roots were insufficient for nuclear magnetic resonance (NMR), we could not assign such orientations to the compounds in Table 6.

FREE-LIVING NEMATODES

The first question investigated was whether free-living nematodes could be useful models for investigating phytosterol dealkylation by plant-parasitic nematodes. *Caenorhabditis elegans* successfully utilized several phytosterols to satisfy its nutritional requirement for sterol (12). When this free-living species was propagated in medium containing only sitosterol (5,13), the major metabolite was 7-dehydrocholesterol (Table 3). Other metabolites included cholesterol, lathosterol, cholesta-5,7,9(11)-trienol, and most interestingly, 4α-methylcholest-8(14)-enol. Identification of these metabolites indicated that *C. elegans* not only is capable of phytosterol dealkylation but also introduces a double bond at C-7 and saturates the Δ^5-bond of $\Delta^{5,7}$-sterols. The subsequent direct 4α-methylation of lathosterol to produce 4α-methylcholest-7-enol and (after isomerization of the Δ^7-bond)

TABLE 2

Mass Spectral Data for Nematode Sterols, Analyzed as Steryl Acetate Derivatives[a]

Steryl acetate	Mass spectrum (*m/z*, intensity relative to base peak)
Cholesta-5,7,9(11)-trienol	424 (5), 364 (100), 349 (33), 251 (31), 209 (64), 197 (43), 195 (52)
Cholest-8(14)-enol	428 (100), 413 (18), 368 (6), 353 (13), 315 (16), 288 (7), 273 (6), 255 (21), 229 (42), 213 (43), 81 (80), 55 (83)
Cholesterol	368 (100), 353 (14), 260 (15), 255 (13), 247 (18), 213 (14), 147 (48), 145 (37), 81 (74), 55 (71)
Cholestanol	430 (12), 415 (2), 370 (33), 355 (16), 316 (3), 276 (28), 275 (18), 257 (4), 230 (17), 215 (100), 201 (18), 147 (32), 81 (53)
Cholest-8(9)-enol	428 (89), 413 (18), 368 (5), 353 (16), 315 (9), 255 (23), 229 (37), 213 (46), 55 (100)
Cholesta-7,9(11)-dienol	426 (100), 411 (22), 366 (19), 351 (37), 313 (30), 286 (19), 271 (11), 259 (31), 253 (38), 211 (62), 199 (33), 55 (50)
Cholesta-5,7,9(11),24-tetraenol	422 (4), 362 (63), 347 (15), 251 (18), 249 (14), 209 (47), 195 (39), 69 (100)
Desmosterol	366 (51), 351 (11), 253 (23), 213 (10), 145 (24), 69 (100)
7-Dehydrocholesterol	426 (2), 366 (100), 351 (34), 253 (23), 211 (17), 199 (20), 158 (62), 143 (86), 55 (93)
24-Methylcholesta-5,22(*E*)-dienol	380 (100), 365 (5), 337 (3), 282 (7), 255 (49), 213 (11), 81 (78), 69 (82)
Cholest-24-enol	428 (4), 413 (8), 353 (4), 344 (12), 317 (3), 315 (70), 257 (6), 255 (43), 229 (3), 215 (17), 69 (100)
Lathosterol	428 (94), 413 (17), 368 (6), 353 (13), 315 (12), 288 (6), 273 (10), 255 (89), 229 (31), 213 (53), 81 (93), 55 (100)
24-Methylcholest-22(*E*)-enol	442 (18), 344 (29), 329 (3), 315 (36), 257 (57), 215 (14), 55 (100)
Cholesta-8(9),24-dienol	426 (23), 411 (10), 351 (8), 315 (1), 313 (3), 255 (3), 229 (11), 213 (32), 69 (100), 55 (51)
Cholesta-5,7,9(11),22(*E*),24-pentaenol	420 (1), 360 (14), 345 (1), 251 (14), 249 (7), 109 (100)
Cholesta-5,22(*E*),24-trienol	424 (1), 364 (1), 313 (8), 282 (24), 255 (6), 253 (16), 213 (2), 199 (1), 109 (100)
Cholesta-5,7,24-trienol	424 (3), 364 (98), 349 (20), 253 (16), 251 (10), 211 (15), 199 (13), 69 (100)
Cholesta-7,24-dienol	426 (11), 411 (11), 351 (4), 315 (3), 313 (100), 255 (12), 229 (7), 213 (23), 69 (98), 55 (54)
24-Methylcholesta-5,23-dienol	380 (28), 365 (3), 296 (3), 283 (34), 259 (4), 255 (6), 253 (24), 227 (4), 215 (6), 213 (8), 159 (27), 145 (37), 133 (54), 55 (100)
24-Methylcholesta-5,7,9(11)-trienol	438 (5), 378 (100), 363 (23), 251 (27), 225 (13), 209 (64), 195 (53), 158 (14), 143 (19), 55 (99)
24-Methylenecholesterol	380 (57), 365 (5), 296 (32), 281 (9), 253 (17), 213 (13), 81 (87), 55 (100)
Cholesta-5,7,22(*E*),24-tetraenol	422 (1), 362 (26), 347 (3), 280 (1), 253 (10), 251 (6), 157 (27), 143 (22), 109 (100)
24-Methylenecholestanol	442 (1), 427 (1), 358 (46), 343 (11), 315 (21), 298 (2), 283 (4), 255 (11), 215 (23), 55 (100)
24-Methylcholesterol	382 (100), 367 (14), 274 (15), 261 (12), 255 (17), 213 (16), 147 (63), 81 (60)
24-Methylcholestanol	444 (17), 429 (2), 384 (31), 369 (16), 330 (2), 317 (1), 315 (1), 276 (33), 275 (21), 257 (6), 230 (14), 215 (100), 201 (19), 147 (34), 81 (53)
24-Methylenecholesta-5,7-dienol	438 (1), 378 (45), 363 (6), 253 (8), 227 (1), 211 (5), 158 (30), 143 (29), 55 (100)
24-Ethylcholesta-5,22(*E*)-dienol	394 (92), 379 (3), 351 (6), 313 (2), 282 (6), 255 (35), 228 (4), 213 (6), 81 (87), 55 (100)
24-Methylcholesta-7,24(28)-dienol	440 (3), 425 (3), 365 (2), 356 (22), 315 (2), 313 (100), 255 (14), 253 (9), 227 (12), 213 (26)
24-Methylcholesta-5,7-dienol	440 (1), 380 (100), 365 (14), 313 (1), 253 (37), 211 (18), 199 (19), 158 (81), 143 (79)
24-Ethylcholest-22(*E*)-enol	456 (9), 353 (9), 344 (14), 329 (2), 317 (2), 315 (19), 257 (36), 255 (8), 215 (5), 55 (100)
24-Methylcholest-7-enol	442 (40), 427 (6), 382 (3), 367 (5), 315 (7), 255 (100), 229 (29), 213 (52), 81 (67)
24-Methylcholesta-5,24(25)-dienol	380 (27), 365 (5), 296 (53), 281 (17), 253 (19), 213 (17), 83 (100)
24-Ethylcholesta-5,22(*E*),24(28)-trienol	452 (1), 392 (12), 313 (18), 282 (89), 255 (24), 253 (78), 213 (8), 157 (14), 143 (14), 137 (48), 95 (100)
24-Ethylcholesta-5,23-dienol	394 (100), 379 (7), 283 (26), 253 (22), 213 (11), 81 (78), 55 (96)
24-Ethylcholesta-7,22(*E*)-dienol	454 (21), 439 (4), 411 (7), 394 (4), 379 (4), 351 (6), 342 (13), 315 (13), 313 (86), 255 (37), 229 (14), 213 (15), 81 (93), 55 (100)
Fucosterol	394 (23), 379 (2), 296 (100), 281 (14), 253 (8), 228 (6), 213 (13), 55 (92)
24-Ethylcholesterol	396 (100), 381 (14), 288 (13), 275 (13), 255 (19), 213 (17), 147 (52), 81 (56)
24-Ethylcholestanol	458 (18), 443 (2), 398 (32), 383 (13), 344 (3), 276 (23), 275 (21), 257 (6), 230 (15), 215 (100), 201 (17), 147 (34), 81 (49)
Isofucosterol	394 (12), 379 (1), 296 (100), 281 (19), 253 (8), 228 (7), 213 (11), 55 (87)
Isofucostanol	456 (2), 358 (83), 343 (15), 315 (7), 298 (9), 283 (10), 255 (8), 215 (24), 55 (100)
24-Ethylcholesta-5,24(25)-dienol	394 (32), 379 (6), 296 (100), 281 (23), 253 (18), 228 (8), 213 (9), 55 (81)
24-Ethylcholesta-5,7-dienol	454 (1), 394 (100), 379 (21), 253 (40), 211 (22), 199 (23), 158 (84), 143 (87)
24-Ethyllathosterol	456 (24), 441 (1), 396 (1), 381 (1), 315 (3), 255 (73), 229 (20), 213 (42), 81 (97), 55 (100)
24-Ethylcholest-24(25)-enol	456 (79), 441 (6), 358 (52), 343 (13), 315 (19), 283 (6), 255 (48), 229 (16), 213 (23), 55 (100)
24Z-Ethylidenelathosterol	454 (2), 439 (1), 394 (1), 356 (37), 313 (100), 296 (4), 281 (5), 255 (5), 253 (4), 213 (12), 55 (48)

(continued next page)

TABLE 2 *(continued)*

Steryl acetate	Mass spectrum (*m/z*, intensity relative to base peak)
4α-Methylcholest-8(14)-enol	442 (36), 427 (4), 382 (2), 367 (7), 329 (4), 269 (14), 243 (23), 227 (34), 95 (73), 55 (100)
4α-Methylcholestanol	444 (17), 429 (4), 384 (48), 369 (30), 355 (7), 343 (4), 329 (1), 316 (1), 290 (26), 289 (17), 262 (13), 244 (21), 229 (100), 215 (23), 95 (74), 55 (59)
4α-Methylcholesta-8(14),24-dienol	440 (19), 425 (6), 365 (5), 329 (1), 327 (2), 269 (2), 243 (9), 227 (16), 69 (100), 55 (57)
4α-Methylcholest-7-enol	442 (33), 427 (4), 382 (4), 367 (6), 329 (4), 269 (52), 243 (18), 227 (36), 95 (96), 55 (100)
4α-Methylcholesta-7,24-dienol	440 (1), 425 (3), 365 (1), 329 (1), 327 (67), 269 (4), 243 (2), 227 (10), 69 (100), 55 (68)
Obtusifoliol	468 (28), 453 (64), 393 (22), 369 (9), 343 (4), 309 (11), 287 (19), 227 (14), 55 (100)
4α,24-Dimethylcholest-8(14)-enol	456 (20), 441 (2), 396 (1), 381 (2), 329 (3), 269 (9), 243 (15), 227 (23), 95 (64), 55 (100)
4α,24-Dimethylcholestanol	458 (18), 443 (3), 398 (48), 383 (31), 369 (7), 290 (32), 289 (18), 271 (6), 244 (23), 230 (57), 229 (100), 215 (22)
4α,24-Dimethylcholest-7-enol	456 (26), 441 (2), 396 (2), 381 (3), 329 (4), 269 (53), 243 (14), 227 (33), 95 (93), 55 (100)
4α-Methyl-24-ethylcholest-22(*E*)-enol	470 (28), 455 (2), 427 (2), 367 (17), 358 (24), 343 (4), 329 (23), 271 (33), 229 (8), 55 (100)
4α-Methyl-24-methylenelathosterol	454 (6), 439 (5), 394 (4), 379 (3), 370 (22), 327 (100), 269 (12), 241 (23), 227 (20)
4α-Methyl-24-ethylcholestanol	472 (16), 457 (3), 412 (47), 397 (24), 383 (7), 290 (32), 289 (10), 271 (7), 244 (21), 230 (56), 229 (100), 215 (23)
4α-Methylisofucostanol	470 (4), 455 (1), 372 (100), 357 (12), 329 (6), 312 (4), 269 (3), 230 (27), 229 (18)
4α-Methyl-24(*Z*)-ethylidenelathosterol	468 (12), 453 (9), 408 (6), 370 (33), 327 (100), 267 (5), 241 (9), 227 (7), 55 (65)
Cycloartanol	470 (1), 455 (2), 410 (8), 395 (9), 367 (3), 357 (1), 355 (1), 341 (3), 297 (6), 288 (10), 273 (3), 55 (100)
Cycloeucalenol	468 (4), 453 (7), 408 (62), 393 (37), 300 (6), 283 (8), 281 (7), 55 (100)
Cycloartenol	468 (1), 453 (1), 408 (11), 393 (7), 365 (3), 339 (2), 297 (1), 295 (1), 286 (7), 271 (4), 257 (1), 255 (1), 243 (2), 69 (100)
24-Methylenecycloartanol	482 (1), 467 (1), 422 (9), 407 (6), 379 (4), 353 (1), 300 (4), 297 (2), 285 (1), 55 (100)

[a]Numbers in parentheses are relative abundances (%).

TABLE 3

Relative Percentages of Sterols from *Panagrellus redivivus, Turbatrix aceti* and *Caenorhabditis elegans* Propagated in Semidefined Medium Containing Sitosterol or Sitosterol Supplemented with 5 μg/mL 25-Azacoprostane Hydrochloride or 25 μg/mL *N,N*-Dimethyldodecanamine (C_{12} amine)

			C. elegans		
	P. redivivus	*T. aceti*	No inhibitor	Azacoprostane	C_{12} amine
Sitosterol[a]	6.7	4.5	49.6	29.8	19.6
Stigmastanol	0.1	—[b]	Trace[c]	—	—
Cholesterol	61.7	18.8	9.4	0.4	9.0
Cholestanol	20.3	1.1	Trace	—	—
7-Dehydrocholesterol	—	42.9	27.7	1.2	23.1
Lathosterol	0.4	9.4	3.3	0.2	7.7
Cholest-8(14)-enol	—	0.4	Trace	—	—
Cholest-8(9)-enol	—	0.5	—	—	—
Cholest-24-enol	—	—	—	0.1	—
Desmosterol	6.1	0.9	—	9.0	4.7
Cholesta-7,24-dienol	—	0.2	Trace	2.1	1.2
Cholesta-8(9),24-dienol	—	0.1	—	0.1	0.1
Cholesta-5,7,24-trienol	—	0.2	Trace	44.6	14.8
Cholest-5,7,9(11)-trienol	—	1.5	1.2	0.1	0.7
Cholesta-5,7,9(11),24-tetraenol	—	—	—	1.2	1.4
Fucosterol	0.1	0.3	Trace	2.3	0.4
Stigmasta-5,7-dienol	—	—	—	0.1	—
Campesterol	0.4	0.1	1.0	0.9	1.5
Campesta-7,24(28)-dienol	—	—	—	0.1	—
Campesta-5,7-dienol	—	—	—	0.1	—
4α-Methylcholest-8(14)-enol	Trace	10.4	7.6	0.2	4.1
4α-Methylcholestanol	4.2	6.3	—	0.1	—
4α-Methylcholest-7-enol	—	2.4	0.2	0.5	2.9
4α-Methylcholest-8(14),24-dienol	Trace	—	—	2.5	2.2
4α-Methylcholesta-7,24-dienol	—	—	—	3.1	1.7
Others (unidentified)	Trace	Trace	Trace	1.3	4.9

[a]Sitosterol contained 1.5% campesterol as an impurity. [b]Not detected. [c]Less than 0.05% of total sterol.

TABLE 4

Relative Percentages of Sterols from *Caenorhabditis elegans* Propagated in Semidefined Medium Containing Stigmasterol, Stigmastanol, or Stigmasterol Supplemented with 25 μg/mL *N,N*-Dimethyldodecanamine (C_{12} amine)

		Stigmasterol	
	Stigmastanol	No inhibitor	C_{12} amine
Stigmasterol	—[a]	20.8	17.8
Stigmastanol	15.4	—	—
Cholesterol	—	9.1	5.7
Cholestanol	3.9	—	—
7-Dehydrocholesterol	—	50.6	23.3
Lathosterol	65.5	4.8	4.4
Cholest-8(14)-enol	3.9	—	—
Cholest-5,7,9(11)-trienol	—	5.2	5.6
Desmosterol	—	—	4.2
Cholesta-5,7,24-trienol	—	—	14.8
Cholesta-5,7,9(11),24-tetraenol	—	—	3.2
Cholesta-5,22(*E*),24-trienol	—	—	3.0
Cholesta-5,7,22(*E*),24-tetraenol	—	—	4.1
Cholesta-5,7,9(11),22(*E*),24-pentaenol	—	—	0.6
Stigmasta-5,22(*E*),24(28)-trienol	—	—	0.9
4α-Methylcholest-8(14)-enol	10.6	9.2	5.0
4α-Methylcholest-7-enol	0.7	0.3	1.7
4α-Methylcholesta-8(14),24-dienol	—	—	1.8
4α-Methylcholesta-7,24-dienol	—	—	0.9
Others (unidentified)	—	—	3.0

[a]Not detected.

TABLE 5

Relative Percentages of Sterols from *Panagrellus redivivus* and *Caenorhabditis elegans* Propagated in Semidefined Medium Containing Campesterol or Campesterol Supplemented with 25 μg/mL *N,N*-Dimethyldodecanamine (C_{12} amine)

		C. elegans	
	P. redivivus	No inhibitor	C_{12} amine
Campesterol	29.5	38.9	60.0
Cholesterol	36.4	4.0	0.8
Cholestanol	14.9	—[a]	—
Lathosterol	0.7	3.4	0.7
7-Dehydrocholesterol	—	26.3	12.5
Cholesta-5,7,9(11)-trienol	—	1.5	0.8
Desmosterol	3.1	—	—
Cholesta-5,7,24-trienol	—	—	5.4
Campestanol	8.9	—	—
Campesta-5,23-dienol	4.1	1.8	1.2
Campest-7-enol	—	0.7	—
Campesta-5,7-dienol	—	13.9	12.5
Campesta-5,7,9(11)-trienol	—	0.9	0.6
24-Methylenecholesterol	—	1.4	0.7
Campesta-5,7,24(28)-trienol	—	1.1	0.2
4α-Methylcholest-8(14)-enol	—	4.6	1.6
4α-Methylcholest-7-enol	—	0.2	0.9
4α-Methylcholestanol	1.9	—	—
4α,24-Dimethylcholest-8(14)-enol	—	0.8	0.4
4α,24-Dimethylcholest-7-enol	—	—	0.3
4α,24-Dimethylcholestanol	0.5	0.5	0.3
4α-Methylcholesta-8(14),24-dienol	—	—	0.4
4α-Methylcholest-7,24-dienol	—	—	0.3
Others	—	—	0.4

[a]Not detected.

TABLE 6

Relative Percentages of Sterols in the Plant-Parasitic Nematodes *Heterodera zeae* (Hz), *Meloidogyne incognita* (Mi), *M. arenaria* (Ma), and *Pratylenchus agilis* (Pa) and Their Host Plants

Sterol	Hz	Corn[a]	Mi[b]	Ma[b]	Pa	Corn[c]
Cholesterol	9.0	0.5	5.6	11.6	0.4	0.2
Cholestanol	0.4	0.02	2.6	3.8	0.4	0.01
24-Methylcholesta-5,22(*E*)-dienol	0.6	0.1	—[d]	—	0.1	0.3
Lathosterol	0.3	0.1	0.2	0.3	0.1	0.04
24-Methylcholest-22(*E*)-enol	—	—	—	—	0.1	—
24-Methylcholesta-5,23-dienol	—	—	—	—	—	0.1
24-Methylenecholesterol	0.2	0.1	0.2	0.4	0.2	1.2
24-Methylenecholestanol	—	—	0.3	0.6	0.3	0.1
24-Methylcholesterol	15.2	16.7	8.6	11.7	2.9	14.2
24-Methylcholestanol	1.7	1.5	7.7	8.6	9.3	0.5
24-Ethylcholesta-5,22(*E*)-dienol	12.4	46.6	4.1	2.7	23.7	60.3
24-Ethylcholest-22(*E*)-enol	2.8	1.4	2.0	1.8	30.0	1.4
24-Methylcholesta-5,24(25)-dienol	—	—	—	—	—	0.1
24-Ethylcholesta-5,23-dienol	—	—	—	—	—	0.2
24-Ethylcholesta-7,22(*E*)-dienol	—	—	—	—	0.3	0.3
Fucosterol	—	—	0.1	0.2	—	0.1
24-Ethylcholsterol	51.2	12.7	38.7	35.0	4.6	9.0
24-Ethylcholestanol	4.6	1.0	23.8	18.2	10.7	0.6
Isofucosterol	0.8	0.3	3.0	2.2	1.9	4.3
Isofucostanol	—	—	1.0	1.0	5.2	0.1
24-Ethylcholesta-5,24(25)-dienol	—	—	—	—	—	0.2
24-Ethyllathosterol	0.5	0.3	0.3	0.3	0.5	0.1
24-Ethylcholest-24(25)-enol	—	—	—	—	0.1	—
24Z-Ethylidenelathosterol	0.3	0.5	0.2	0.2	1.4	0.2
Obtusifoliol	—	—	—	—	0.1	0.1
4α,24-Dimethylcholestanol	—	—	0.2	0.1	0.5	—
4α-Methyl-24-ethylcholest-22(*E*)-enol	—	—	—	—	0.4	—
4α-Methyl-24-methylenelathosterol	—	—	0.4	0.4	—	—
4α-Methyl-24-ethylcholestanol	—	—	0.4	0.1	0.5	—
4α-Methylisofucostanol	—	—	—	—	0.3	—
4α-Methyl-24(*Z*)-ethylidenelathosterol	—	—	0.2	0.2	0.1	0.1
Cycloartanol	—	1.5	0.1	0.2	—	0.3
Cycloeucalenol	—	—	—	—	0.1	0.4
Cycloartenol	—	16.4	0.2	0.2	4.8	3.9
24-Methylenecycloartanol	—	0.3	0.1	0.2	1.0	1.7

[a]Roots of *Zea mays* 'Pioneer 3184' from growth chambers.

[b]Host roots (greenhouse grown *Solanum melongena* 'Black Beauty') were not analyzed.

[c]Sterile root cultures of *Zea mays* 'Iochief'.

[d]Not detected.

4α-methylcholest-8(14)-enol was completely unexpected. This direct nuclear methylation of sterols is unique to nematodes; in other organisms, 4α-methylsterols are biosynthetic intermediates produced from 4,4,14α-trimethylsterols during *de novo* cholesterol biosynthesis (3). We considered the possibility that nematode 4α-methylsterols were artifacts, but in subsequent experiments with radiolabeled cholesterol, sitosterol, or desmosterol, the 4α-methylsterols isolated from *C. elegans* had the same specific activity as the dietary sterol. This result indicates that nematode 4α-methylsterols were not concentrated medium contaminants. Similarly, incubated nematode-free medium did not contain 4α-methylsterols. It remained possible that the 4α-methylsterols were the products of some external contaminant or internal symbiont, but supplementation of cultures with antimicrobial compounds (penicillin, streptomycin, and ketoconazole) failed to decrease the amount or composition of the 4α-methylsterols (Chitwood, D.J., unpublished).

Next, *C. elegans* was propagated in medium containing sitosterol plus an azasteroid, 25-azacoprostane hydrochloride (5β-cholan-24-dimethylamine hydrochloride) (5), an inhibitor of phytosterol dealkylation in insects (29). Excluding the dietary sitosterol, over 96% of the total sterol from *C. elegans* consisted of sterols with $\Delta^{24(25)}$- or $\Delta^{24(28)}$-bonds (Table 3), none of which normally occur in more than trace quantities. This accumulation of Δ^{24}-sterols and fucosterol indicated that the latter is a key intermediate in the phytosterol dealkylation pathway and that the azacoprostane was inhibiting the nematode Δ^{24}-sterol reductase enzyme system. Because growth and reproduction of *C. elegans* was strongly inhibited by 5 μg/mL of the azacoprostane·HCl, this experiment demonstrated the feasibility of using inhibitors of nematode sterol metabolism as model compounds for development of novel control agents for nematodes. Structurally related long-chain dimethylalkylamines (*e.g.*, *N,N*-dimethyldodecanamine) similarly inhibited growth, reproduction, and sterol metabolism of *C. elegans* (6).

Although inhibition of sterol metabolism by the alkyl-

amines was not as great as that with azacoprostane, the alkylamines were successfully used as metabolic probes to elucidate the pathways involved in the metabolism of other phytosterols by *C. elegans* (10). For example, treatment of stigmasterol-fed *C. elegans* with *N,N*-dimethyldodecanamine (Table 4) revealed that stigmasterol is dealkylated *via* a stigmasta-5,22,24(28)-trienol intermediate prior to reduction of the Δ^{22}-bond, which is then reduced prior to the Δ^{24}-bond. Analogous experiments with campesterol also resulted in an accumulation of Δ^{24}-sterols (Table 5). Interestingly, both control and inhibitor-treated cultures contained substantial proportions of campesterol metabolites with intact side chains but modified sterol nuclei, *i.e.*, $\Delta^{5,7}$-, Δ^{7}-, or $\Delta^{5,7,9(11)}$-bond systems, as well as $\Delta^{8(14)}$- and Δ^{7}-4α-methylcampestenols. This finding indicates that the nuclear modification enzymes in the *C. elegans* sterol metabolism pathway exclude 24-ethylsterols but may bind to 24-methylsterols. Lastly, use of stigmastanol-supplemented medium revealed that stigmastanol can be dealkylated by *C. elegans* (Table 4). Interestingly, cholesterol and 7-dehydrocholesterol were not detected, despite the fact that lathosterol was the major sterol metabolite. This finding revealed that *C. elegans* lacks the ability to introduce double bonds at C-5, a conclusion supported by additional investigations with dietary lathosterol and cholestanol (14).

Some comparative investigations with *Panagrellus redivivus* and *Turbatrix aceti* revealed that these free-living species also can dealkylate phytosterols (Tables 3, 5). The dealkylation pathway in *P. redivivus,* in contrast to *C. elegans,* does not result in the production of 7-dehydrocholesterol; instead, the major metabolite is cholestanol, with the principal 4α-methylsterol being 4α-methylcholestanol. *Turbatrix aceti* contains both pathways in that it produces both Δ^{0}- and $\Delta^{5,7}$-sterol metabolites.

PLANT-PARASITIC NEMATODES

The inability to culture plant-parasitic nematodes *in vitro* away from their plant hosts has restricted investigation of their sterol metabolic pathways to comparison of sterol compositions of host and parasite. Evidence for existence of C-24 dealkylation in plant-parasitic nematodes was initially discovered in the stem and bulb nematode, *Ditylenchus dipsaci,* which contained equal amounts of cholesterol and lathosterol and only traces of the phytosterols of its host, alfalfa callus (30). Although the major sterol of both cotton roots and the reniform nematode, *Rotylenchulus reniformis,* was 24-ethylcholesterol, cholesterol comprised 23% of the sterols of *R. reniformis* and only 0.6% of the sterols of the host roots (31). The major sterols of the tobacco cyst nematode, *Globodera tabacum solanacearum,* included 43% 24-methylcholestanol and 12% cholesterol (32). Although analysis of tobacco roots was not performed, the scarcity of stanols in higher plants led to the conclusion that this cyst nematode can hydrogenate plant sterols.

In our studies with a cyst nematode, *Heterodera zeae* (7), the major sterols of roots of its host (corn propagated in a growth chamber) were 24-ethylcholesta-5,22-dienol, 24-methylcholesterol, and 24-ethylcholesterol (Table 6). The predominant sterols of cysts (*i.e.,* adult females containing eggs) of *H. zeae* were 24-ethylcholesterol, 24-methylcholesterol, 24-ethylcholesta-5,22-dienol, and cholesterol. The abundance of cholesterol in this nematode in comparison to its host is suggestive of existence of C-24 dealkylation, although the possibility of selective uptake of cholesterol cannot be excluded. Similarly, the difference in ratios of 24-ethylcholesterol to 24-ethylcholesta-5,22-dienol in *H. zeae* and corn roots is suggestive of C-22 hydrogenation, but selective uptake remains a possibility, especially because *C. elegans* dealkylates stigmasterol before saturating the Δ^{22}-bond, as discussed previously. Another interesting finding in *H. zeae* was the detection of only a small relative percentage of stanols. Stanols of *G. tabacum solanacearum* comprised the majority of its sterols (32). The possibility that the two genera of cyst nematodes have different sterol saturation capabilities is a speculation worthy of further investigation.

Because the sterols of two different species of the same nematode genus had not been compared, we examined the sterols from the eggs of two different species of root-knot nematodes, *Meloidogyne incognita* and *M. arenaria* (15). The sterol compositions of the two species were very similar, with the principal sterols being 24-ethylcholesterol, 24-ethylcholestanol, 24-methylcholestanol, 24-methylcholesterol, and cholesterol (Table 6). The sterols of another race of *M. incognita* (race 3) were indistinguishable (15). We did not analyze sterols from the host roots (eggplant); because higher plants seldom contain more than traces of stanols, it is very likely that *M. incognita* and *M. arenaria* hydrogenate Δ^{5}-bonds. Cholesterol comprised a substantial percentage of the sterols (Table 6). Because roots of other Solanaceae contain as much as 10% cholesterol (33), it is uncertain whether *Meloidogyne* females or eggs can dealkylate phytosterols.

More recently, we have examined the sterols of mixed developmental stages of *Pratylenchus agilis* (Table 6), a nematode chosen because large quantities of vermiform stages can be obtained from sterile cultures of corn root explants. The only previous investigation of sterols in vermiform plant-parasitic nematodes was performed several years ago in *D. dipsaci* (33), as previously described. We had hoped to use *P. agilis* for studies involving dealkylation of radiolabeled phytosterols by phytoparasitic nematodes. A total of 28 sterols were identified; the major nematode sterols were 24-ethylcholest-22-enol, 24-ethylcholesta-5,22-dienol, 24-methylcholestanol, 24-ethylcholestanol, isofucostanol, and 24-ethylcholesterol (Table 6). The unexpectedly small amount of cholesterol (0.4% of the nematode sterol) indicates that *P. agilis* lacks the ability to dealkylate the sterols of corn roots. Instead, the four major metabolic products are 24-ethylcholest-22-enol, 24-methylcholestanol, 24-ethylcholestanol, and isofucostanol. As with *Meloidogyne* spp., *P. agilis* primarily saturates the phytosterol nucleus; our results indicate that *P. agilis* does not dealkylate the side chains of phytosterols.

Use of an on-column injector fitted to the gas chromatograph facilitated the identification in *P. agilis* of 9 sterols previously unidentified in nematodes: 24-methylcholest-22(*E*)-enol, 24-methylcholesta,5,24(25)-dienol, 24-ethylcholesta-5,23-dienol, 24-ethylcholesta-5,24(25)-dienol, 24-ethylcholest-24(25)-enol, 4α-methyl-24-ethylcholest-22-enol, 4α-methylisofucostanol, obtusifoliol, and cycloeucalenol. We are not aware of previous reports in *Z. mays* of 24-methylcholesta-5,24(25)-dienol, 24-ethylcholesta-

5,23-dienol, and 24-ethylcholesta-5,24(25)-dienol. Some Δ^{23}- and $\Delta^{24(25)}$-sterols have been implicated in biosynthesis of the major sterols of corn (34), although few of these have been detected previously in *Z. mays*.

Because of the uniqueness of the 4α-methylation pathway in nematodes, we were disappointed not to find any 4α-methylsterols in cysts of *H. zeae*. Small quantities of several 4α-methylsterols occurred in eggs of *Meloidogyne* spp., but the compounds identified had previously been reported in host plant seeds (23) or else were the stanol derivatives. In *P. agilis*, however, we detected very small quantities of 4α-methyl derivatives of the four major products of nematode sterol metabolism described above, *i.e.*, 4α-methyl-24-ethylcholest-22-enol, 4α,24-dimethylcholestanol, 4α-methyl-24-ethylcholestanol, and 4α-methylisofucostanol. Therefore, it is conceivable that *P. agilis* contains a slightly active 4α-methylation pathway. Whether our detection of these compounds in *P. agilis* and not other plant-parasitic nematodes has resulted from improved analytical equipment, greater analytical expertise, or existence of larger amounts of 4α-methylsterols in vermiform life stages is not known.

Compared to plant-parasitic and free-living nematodes, vertebrate-parasitic nematodes are less extensively investigated with respect to their sterol metabolism. As expected, cholesterol is the major sterol of these organisms, with digestive tract parasites also containing substantial amounts of phytosterols and stanols (8). In the only investigation of phytosterol metabolism in vertebrate-parasitic nematodes, *Ascaris* adults did not convert injected radiolabeled sitosterol to other sterols (35). Comparison of the sterol compositions of the insect-parasitic nematode *Steinernema feltiae* and its host, *Heliothis zea*, indicated that this nematode saturates Δ^5-bonds and introduces Δ^7-bonds (36), somewhat similarly to *C. elegans*, although 7-dehydrocholesterol was not detected in *S. feltiae*.

The diversity of phytosterol metabolism in nematodes is interesting. Particularly notable is the frequent abundance of phytosterols or their stanol derivatives in some plant-parasitic species. It is unknown whether these C-24 alkylsterols are utilized intact or else dealkylated by many of these nematodes prior to utilization, but the extremely low relative proportion of cholesterol in *P. agilis* indicates that this species probably utilizes phytosterols with a saturated nucleus in their intact form. Various species of nematodes are capable of saturation of Δ^5-, Δ^{22}-, and Δ^{24}-bonds, introduction of double bonds at C-7 and C-9(11), isomerization of a Δ^7-bond to a $\Delta^{8(14)}$-bond, 4α-methylation, C-24 dealkylation, and C-3 esterification. Exploration of other nematode taxa may reveal further metabolic capacities. Regardless, the fact that disruption of phytosterol metabolism has severely deleterious effects on the nematode life cycle (5,37) indicates that nematode steroid metabolism is a fruitful area for development of new approaches toward nematode control.

EXPERIMENTAL APPENDIX

Nematodes. Caenorhabditis elegans, Panagrellus redivivus, and *Turbatrix aceti* were propagated in an aqueous semidefined medium containing yeast extract, soy peptone, glucose, hemoglobin, Tween 80, and supplemented sterol (13). All of the medium components except soy peptone were extracted previously to remove endogenous sterol contaminants (5). Nematodes from late-logarithmic cultures were isolated by centrifugation, flotation on 40% sucrose, and repeated rinsing with distilled water.

The corn cyst nematode, *Heterodera zeae,* was propagated on roots of *Zea mays* 'Pioneer 3184' in growth chambers. Cysts (mature adult females containing eggs) were washed from roots and sand by collection on a sieve, floated on 40% sucrose, and rinsed thoroughly with distilled water (7). The root-knot nematodes *Meloidogyne incognita* race 2 and *M. arenaria* race 1 were propagated on eggplant (*Solanum melongena* 'Black Beauty') in a greenhouse (15). Eggs were released from egg masses, concentrated and thoroughly rinsed (38). The root lesion nematode *Pratylenchus agilis* was propagated on *Zea mays* 'Iochief' root sections on Gamborg's B5 medium formulated with 1.5% agar and 2.0% sucrose and lacking cytokinins and auxins (39). The agar was extracted previously with methanol and chloroform/methanol 2:1 (v/v, twice each) to remove the high amounts of endogenous cholesterol in commercial agar (40). The agar cultures were placed on top of a wet-strength paper tissue supported by a wire mesh screen in a funnel filled with water (*i.e.*, a modified Baermann funnel). Fifteen hours later, the mixed juvenile and adult nematodes that had settled at the bottom of the funnel were collected and cleaned as described above for the free-living species.

Sterol analysis. Lipids were extracted from lyophilized nematodes and fractionated into various lipid classes including steryl esters and free sterols by column chromatography on silica (7). The steryl ester and free sterol fractions were saponified separately, and the 4-desmethylsterols and 4-methylsterols isolated were further purified by Florisil column chromatography (15). For comparison of host and nematode sterols, uninfected roots of *Z. mays* 'Pioneer 3184' or root cultures of *Z. mays* 'Iochief' were analyzed identically as the nematodes, except the total lipid extract was saponified.

Sterols were tentatively identified by gas-liquid chromatography (GLC) on a DB-1 fused silica capillary column and an OV-17 packed glass column (15). The sterols were then acetylated, purified on Florisil columns, positively identified *via* gas chromatography-mass spectrometry (GC-MS), and, when necessary, fractionated on argentation columns (15). Instrumentation for mass spectrometry, liquid scintillation counting, and, when appropriate, UV spectrophotometry and 60 Mhz NMR spectroscopy, has been published previously (5,6,13).

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ARTICLE

Cloning and Disruption of the Yeast C-8 Sterol Isomerase Gene[1]

William H. Ashman[a], Robert J. Barbuch[b], Corinne E. Ulbright[c], Harry W. Jarrett[d] and Martin Bard[a]*
[a]Department of Biology, Indiana University-Purdue University at Indianapolis, Indianapolis, Indiana 46205, [b]Merrell Dow Research Institute, Cincinnati, Ohio 45215, [c]Veterans Affairs Medical Center, Indianapolis, Indiana 46202 and [d]Department of Biochemistry, University of Tennessee Medical School, Memphis, Tennessee 38163

The yeast *ERG2* gene codes for the C-8 sterol isomerase, an enzyme required for the isomerization of the Δ8 double bond to the Δ7 position in ergosterol biosynthesis. The *ERG2* gene was cloned by complementation of a C-8 sterol isomerase mutant strain (*erg2*). The complementing region of DNA required to restore ergosterol synthesis to *erg2* was limited to a 1.0 kb *StuI-BglII* fragment. In order to determine whether the *ERG2* gene was essential for yeast viability, a *LEU2* gene was inserted into the *NdeI* site (made blunt) of this 1.0 kb fragment. Transformation of a wild type diploid strain with the *ERG2* substituted DNA resulted in the generation of viable haploids containing the *erg2* null allele (*erg2-4::Leu2*). These results suggest that the C-8 sterol isomerase activity is not essential for yeast cell viability. This disruption represents the second ergosterol biosynthetic gene in the distal portion of the pathway to be disrupted without adversely affecting cell viability.
***Lipids* 26, 628-632 (1991).**

Two distinct roles for sterol molecules in the yeast *Saccharomyces cerevisiae* have been postulated. The "bulk membrane function" is fulfilled by the major amount of sterol and is involved in the regulation of membrane fluidity, permeability, and growth rates. The "sparking function" (1,2) or "sterol synergism" (3) has been postulated based on the observation that yeast cells require nanomolar levels of ergosterol to enter the S phase of the mitotic cycle (4). Dahl *et al.* (4) demonstrated that sterol-depletion in the ergosterol-auxotroph, GL7 (*erg12-1 hem3-6*) results in arrest at the unbudded G1 state. Cell-budding and proliferation are reinitiated upon addition of nanomolar levels of ergosterol. Ergosterol was found to stimulate a protein kinase antigenically related to $pp60^{v\text{-}src}$ enabling the cell through the G1 to S transition (4). Several investigators have suggested that *erg6* or *erg3* mutants are leaky as these enzymatic modifications, the sterol transmethylase or the C-5 sterol desaturase are essential for the formation of the sparking sterol (1,2,5,6). We have previously demonstrated through cloning, gene disruption, and allele replacement studies of *ERG6* that this gene is unessential for yeast cell viability (7). If methylated sterols are essential for the sparking sterol, perhaps a non-allelic methylase can perform this function.

The cloning and disruption of *ERG2* was undertaken to determine whether this gene is essential for yeast cell viability. *ERG2* is the putative structural gene for the C-8 sterol isomerase, which is responsible for the isomerization of the B ring double bond from the 8(9) to the 7(8) position (8). This isomerization is followed by the introduction of a second double bond at C-5 to give a conjugated double bond system in the B ring. In yeast, an *erg2* mutation leads to the accumulation of sterols containing the Δ8 double bond (ergosta-8-en-3β-ol, ergosta-8,22-dien-3β-ol, and ergosta-5,8,22-trien-3β-ol). Heme and cytochrome P450 mutants unable to remove the 14α methyl group of lanosterol accumulate only Δ8-containing sterols suggesting that the C-8 sterol isomerase enzyme requires the prior removal of the 14α methyl group (9,10). Finally, the partially purified rat liver enzyme isolated by Paik *et al.* (11) was found to have a M_r of approximately 80 kdal.

In this report, we demonstrate the cloning of the *ERG2* gene, its disruption and allele replacement, which suggests that the *ERG2* gene is not essential for yeast cell viability.

MATERIALS AND METHODS

Strains. The *S. cerevisiae* strain WAO which contained the *erg2-3* mutation was the result of a cross between CG379 (kindly supplied by C. Giroux) and *pol2*, a polyene resistant strain derived from the wild-type strain A184D (12). Strains WA1 and WA6 were the result of crosses using strains CG379 and WAO. Strain WA2 was the result of transforming WA1 with a plasmid (pIU405) containing *ERG2* DNA inserted into the integrating vector, Ylp5. The yeast strains used are further described in Table 1. The *Escherichia coli* strains HB101 and MC1066 were used for amplification and selection of plasmids.

Plasmids and transformations. To clone the wildtype *ERG2* gene, a library of genomic yeast DNA fragments ligated into the shuttle vector YCp50 (13) was used. Yeast transformations were accomplished using the method of Gaber *et al.* (7). The subcloning vectors were pRS314, pRS315 and pRS316 (14). YIp5 was used for directed integration and YEp13 was the source of the LEU2 gene. Calcium chloride treated competent *E. coli* were transformed as described by Maniatis *et al.* (15).

Media. YPD (1% yeast extract, 2% peptone, and 2% dextrose), YNB (0.67% yeast nitrogen base, 2% dextrose) were prepared as described by Sherman *et al.* (16). YNB was supplemented with appropriate amino acids and nitrogenous bases and, when required, with 0.06 μg of cycloheximide or 0.8 μg of nystatin per mL. *E. coli* complete media, TY, consisted of 0.5% NaCl,

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*To whom correspondence should be addressed at the Department of Biology, Indiana University-Purdue University at Indianapolis, 1125 East 38th Street, Indianapolis, Indiana 46205.

Abbreviations: Ergosterol, ergosta-5,7,22-dien-3β-ol; GC-MS, gas chromatography-mass spectrometry; YNB, yeast nitrogen base minimal medium; YPD, yeast extract, peptone, dextrose complete medium; zymosterol, cholesta-8,24-dien-3β-ol.

TABLE 1

Strains Used in This Work

Strain	Genotype	Source of Reference
pol2,	a *erg2-3*	Ref. 12
CG379,	α *ade5 his7-2 leu2-3,112 trpl-289 ura3-5*	C. Giroux
WA0,	a *his7-2 leu2-3,112 ura3-52 erg2-3*	This study
WA1,	α *ade5 his7-2 leu2-3,112 ura3-52*	This study
WA2,	α *ade5 his7-2 leu2-3,112 ura3-52/pIU4051*	This study
WA3,	a/α *ade5/+ his7-2/his7-2 leu2-3,112/leu2-3,112 ura3-52/ura3-52 ERG2*::pIU405/*erg2-3*	This study
WA6,	a *ade5 his7-2 leu2-3,112 ura3-52*	This study
WA1/6,	a/α *ade5/ade5 his7-2/his7-2 leu2-3,112/leu2-3,112 ura3-52/ura3-52*	This study
WA8,	WA1/6 *erg2Δ::LEU2/ERG2*	This study
WA10,	WA1/6 *erg2-4::LEU2/ERG2*	This study
WA10-3-1A,	a *ade5 his7-2 leu2-3,112 ura3-52 erg2-4::LEU2*	This study
WA10-3-1B,	α *ade5 his7-2 leu2-3,112 ura3-52*	This study

0.8% Bacto-tryptone and 0.5% Bacto-yeast extract. Ampicillin was added 50 μg per mL. *E. coli* minimal medium was made as described by Miller (17) and supplemented with ampicillin and/or appropriate amino acids.

Plasmid constructions. Standard recombinant DNA techniques were used to subclone the DNA fragments containing the *ERG2* gene from pIU402 and pIU403 (15). Frameshift mutations were introduced into various plasmids by complete digestion with the appropriate restriction endonuclease, followed by filling in of the protruding ends using the *E. coli* polymerase 1 large fragment (Klenow). The blunt ends were then ligated to recircularize the plasmid. For subcloning, gel-purified DNA was ligated into appropriately digested vectors. Disrupted constructions were generated by the one-step gene disruption method of Rothstein (18).

DNA manipulations. Large-scale plasmid DNA isolation from *E. coli* was prepared as described by Maniatis *et al.* (15); small-scale plasmid DNA was isolated from *E. coli* using the Miniprep Kit Plus (Pharmacia Inc., Piscataway, N.J.). Plasmid DNA from yeast and yeast genomic DNA were prepared as described by Sherman *et al.* (16). Hybridization probes for Southern blots were made using a random oligonucleotide primer kit from NEN (Boston, MA). The *ERG2* probe was prepared by gel purification of a 1.45 kb *Bgl*II-*Sca*I fragment and yeast genomic DNA was digested with *Xba*I. Southern analysis was carried out as described by Maniatis *et al.* (15) and the protocol using Nytran membranes supplied by Scheicher & Schuell (Keene, NH).

Sterol analysis. Nonsaponifiable sterols were extracted from stationary-phase yeast cells into n-heptane by the method of Molzahn and Woods (12) and scanned between 200 and 300 nm for absorption maxima. The gas chromatographic-mass spectral (GC-MS) analyses were done using either a Finnigan MAT Model 4600 or VG Trio-1 mass spectrometer system (Finnigan Corp., Sunnyvale, CA). The GC separations were performed on a 15 M × 0.32 mm Durabond DB-1 column with a 0.25 μm film thickness. The column oven temperature was programmed from 200–300°C at 15°C per min and the carrier gas was helium at a linear velocity of 50 cm/sec. The indicated MS ion source temperature was 120°C. The mass spectrometer was operated in the electron impact ionization mode at 70°V and was scanned from *m/z* 40 to *m/z* 600. Mass spectra were enhanced by subtraction when necessary.

RESULTS AND DISCUSSION

Isolation of the ERG2 *gene.* The *ERG2* gene was isolated using essentially the genetic transformation protocol of Gaber *et al.* (7). The gene was isolated from a yeast genomic library constructed in the shuttle vector, YCp50. Strain bearing *erg2* mutations exhibit a number of phenotypes including resistance to nystatin and sensitivity to cycloheximide and the *ERG2* gene was cloned on the basis of its ability to suppress the cycloheximide sensitivity of *erg2.* Four plasmids isolated—pIU401, pIU402, pIU403, and pIU404—suppressed the cycloheximide sensitivity of the *erg2—3* strain, WAO. This strain fails to grow on YNB medium containing 0.06 μg/mL cycloheximide, whereas *erg2-3* transformants containing plasmids pIU401–404 demonstrated wildtype growth levels on this medium. All transformants were also nystatin-sensitive.

Sterol analysis of erg2-3 *and transformants.* The sterol profile of *erg2* elucidated by Barton *et al.* (8) demonstrated that only sterols with Δ8 double bonds accumulated in this strain. No sterols with a Δ7 double bond were observed. The sterol profile of *erg2-3* grown on YNB medium contains three major sterols determined by GC-MS (Fig. 1): ergosta-5,8,22-trien-3β-ol (peak A); ergosta-8,24(28)-dien-3β-ol; and ergosta-8-en-3β-ol (peaks B and C). The sterol ratio for these three sterols are 22:15:60, respectively. These results are all consistent with our previous determination that *erg2* strains are deficient in C-8 sterol isomerase activity. The pIU401–404 transformants, however, are able to synthesize ergosterol as well as other wildtype sterols. *erg2*/pIU401 Accumulates zymosterol (peak D, 10%), ergosterol (peak E, 35%) as well as two other Δ7 sterols, ergosta-7,24(28)-dien-3β-ol and ergosta-7-3n-3β-ol (peaks F and G, both 4%). Ergosta-8,24(28)-dien-3β-ol and ergosta-8-en-3β-ol were present in the

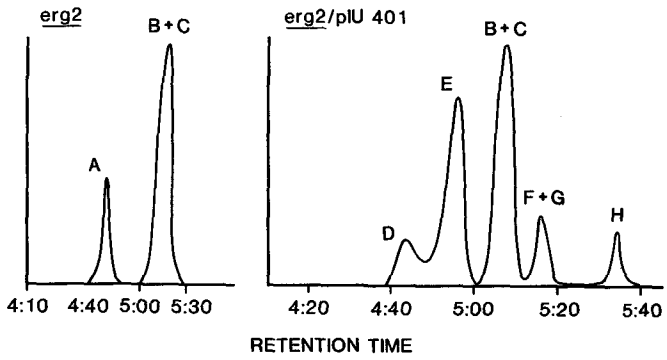


FIG. 1. Retention times (minutes) of sterols extracted from WA0 (*erg2–3*) and the same strain transformed with plasmid pIU401 (Erg$^+$). Peak A, ergosta-5,8,22-trien-3β-ol; peaks B & C, ergosta-8,24(28)-dien-3β-ol and ergosta-8-en-3β-ol, respectively; peak D, zymosterol; peak E, ergosterol; peaks F & G, ergosta-7,24(28)-dien-3β-ol and ergosta-7-en-3β-ol, respectively; and peak H, lanosterol. GC-MS was carried out using a Finnegan MAT Model 4600 or VG Trio-1.

transformed strains at 15% and 27%, respectively. Peaks not clearly separable by GC were distinguished by MS.

Subcloning and localization of the ERG2 *gene.* Plasmids pIU401 and pIU404 were identical containing inserts of 12.7 kb of genomic DNA. pIU402 and pIU403 Contained 6.4 and 6.6 kb of insert, respectively, and were identical except at their termini (Fig. 2). Plasmid pIU406 represents a subclone in which a 3.0 kb *Sac*I fragment was deleted from pIU403. Plasmid pIU407 was derived from pIU406 and contains a further 0.7 kb deletion at the left terminus of the insert. Plasmid pIU409 represents a subclone in which 3.1 kb of the pIU402 insert is deleted. All three plasmids complemented the *erg2–3* strain. pIU411 Contains a 2.7 kb *Kpn*I-*Sac*I fragment also able to complement *erg2–3*, whereas pIU412 deleted for a 1.2kb *Kpn*I-*Bgl*II fragment failed to complement the mutant strain. Two plasmids, pIU415 and pIU420, containing 1.9 kb *Stu*I-*Hind*III and 1.0 kb *Stu*I-*Bgl*II DNA frag-

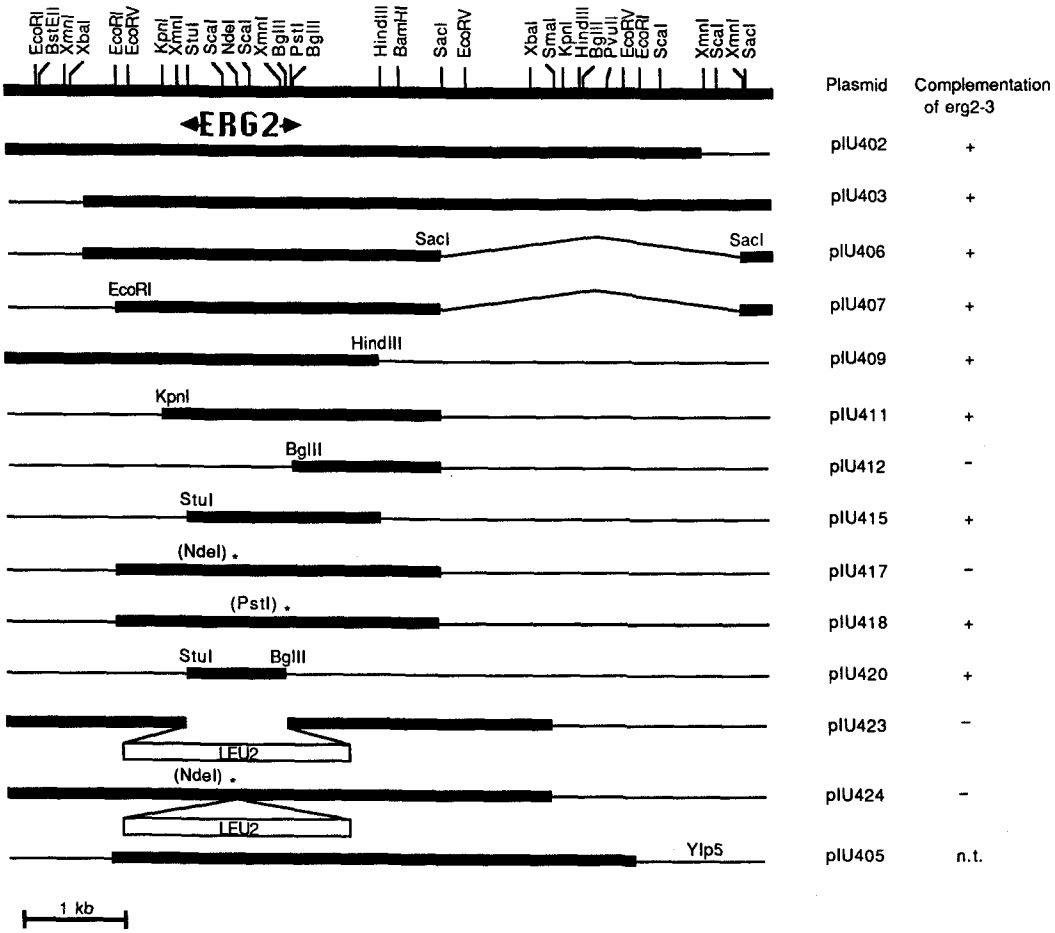


FIG. 2. Restriction endonuclease sites of the *ERG2* gene and construction of plasmid subclones. Genomic clones are pIU402 and pIU403. Subclones pIU406, pIU407, pIU409 are in YCp50; pIU405 is in YIp5. All others were constructed in the pRS vector series (14). The ability of each plasmid to complement *erg2–3* is indicated. Asterisked restriction sites indicate sites made blunt by DNA polymerase I(Klenow). n.t., not tested.

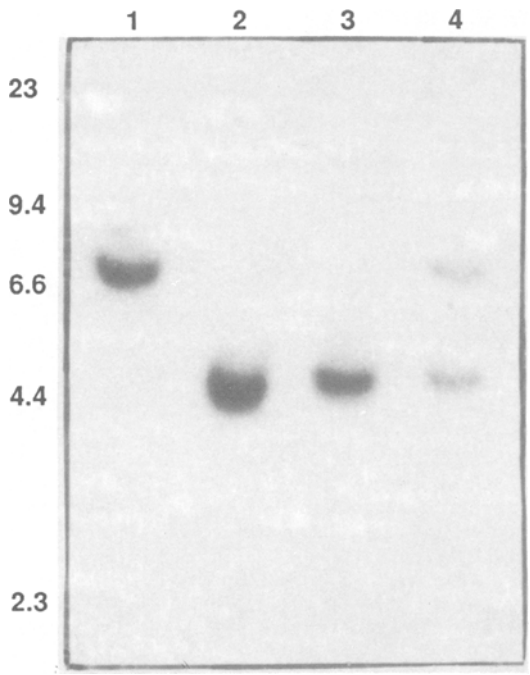


FIG. 3. Southern blot analysis of *Xbal* digested genomic DNA from haploid strains *erg2-4::LEU2* (WA10-3-1A, lane 1) and *ERG2* (WA10-3-1B, lane 2) probed with a 1.45 kb *Bgl*II-*Sac*I fragment from pIU 411. Lane 3, untransformed dipoid WA1/6 and lane 4, transformed diploid WA10, heterozygous for the disruption.

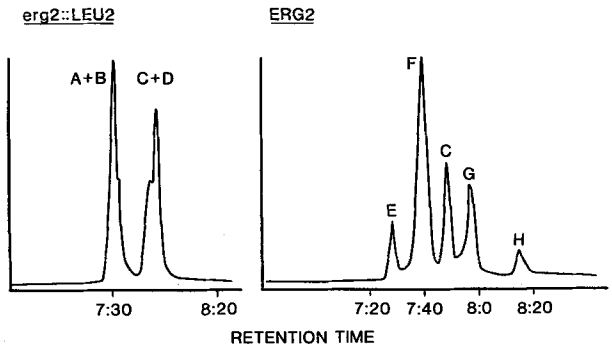


FIG. 4. Retention times (min) of sterols extracted from WA10-3-1A (*erg2-4::LEU2*) and WA10-3-1B (*ERG2*). Peaks A and B, ergosta-5,8,22-trien-3β-ol, and ergosta-8,22-dien-3β-ol, respectively; peaks C and D, ergosta-8,24(28)-dien-3β-ol, and ergosta-8-en-3β-ol, respectively; peak E, zymosterol; peak F, ergosterol; peak G, ergosta-7,24(28)-dien-3β-ol; and peak H, lanosterol.

ments, respectively, also complemented *erg2-3* (Fig. 2).

Frameshift mutagenesis of ERG2. The location of restriction endonuclease sites within the *ERG2* gene was determined through *in vitro* mutagenesis. Frameshift mutations were introduced at the *Nde*I and *Pst*I sites in pIU417 and pIU418, respectively, as described in Materials and Methods. Only pIU418 was able to complement *erg2-3*, suggesting that the *Nde*I site is within the coding region of *ERG2* (Fig. 2). The *Pst*I site is either not in the coding region, or if it is, it must be in a C-terminal portion of the gene.

Directed integration of ERG2 DNA into the yeast genome. In order to verify that the cloned DNA coded for *ERG2* and not an unlinked supressor gene, a directed integration experiment in which the 5.1 kb *EcoRI* fragment from pIU403 was subcloned into YIp5 to make the recombinant plasmid pIU405. pIU405 Was linearized by digestion with *Xba*I to enhance frequency of integration into the homologous chromosomal site. A *ura3-52* strain (WA1) was transformed with the linear plasmid to uracil prototrophy and one of the transformants (WA2) URA$^+$ ERG$^+$ mated to a *ura3-52 erg2-3* strain (WA0). The resultant diploid (WA3) was sporulated and asci were disected onto YPD agar plates. Twenty-two tetrads showed a 2 Ura$^+$ Erg$^+$: 2 Ura$^-$Erg$^-$ segregation pattern, demonstrating genetic linkage between the integrated plasmid containing the *URA3* marker and the *ERG2* locus. This indicates that the cloned DNA integrates at the *ERG2* locus, and is therefore the *ERG2* gene.

Disruption of the ERG2 *gene.* In order to determine whether the C-8 sterol isomerase gene is essential for yeast cell viability, null alleles of *ERG2* were generated. pIU423 Contained a 1.0 kb deletion of the *Stu*I-*Bgl*II region (previously shown to fully complement *erg2-3*) and in its place was inserted a 2.4 kb *Hpa*I-*Bgl*II fragment containing the *LEU2* gene isolated from YEp13. A 5.7 kb *Xba*I fragment derived from pIU423 encompassing this disrupted *erg2Δ::LEU2* allele was gel-purified and used to transform the diploid strain WA1/6 to leucine prototrophy. The recombination event to Leu$^+$ transformants involves the replacement of a wildtype *ERG2* allele with the deletion/substitution (disrupted) allele (18). The Leu$^+$ transformants were sporulated and, following asci dissection, showed a 2:0 segregation pattern with only Leu$^-$ spores growing. However, when Leu$^+$ transformants were sporulated, asci dissected, and grown anaerobically on ergosterol containing medium, the results were the same (only two viable Leu$^-$ colonies) suggesting that disruption resulted in the loss of non-*ERG2* chromosomal DNA required for viability. A second *ERG2* gene disruption was performed using plasmid pIU424. This plasmid contained the entire complementing *Stu*I-*Bgl*II 1.0 kb DNA fragment, in which the *Nde*I site was restricted, made blunt using DNA polymerase I (Klenow) followed by ligation of a 2.2kb *Hpa*I *LEU2* gene. A 6.7 kb *Xba*I fragment (derived from pIU425) encompassing this disrupted *erg2::LEU2* allele was obtained and again used to transform strain WA1/6. Leu$^+$ transformants were isolated, sporulated, and asci dissected. Among 27 tetrads, spore viability was 100%. Leu$^+$ Erg$^-$ and Leu$^-$ Erg$^+$ spores segregated 2:2 and showed complete linkage (parental ditype asci only), indicating that the disruption of *ERG2* did not result in cell inviability or a requirement for exogenous ergosterol. Southern blot analysis of genomic DNA prepared from the Leu$^+$ Erg$^-$ (Fig. 3, lane 1) and Leu$^-$ Erg$^+$ (lane 2) haploids confirmed that the former har-

bored the *erg2-4::Leu2* mutation as a result of gene disruption. Genomic DNA digested with *Xba*I and probed with a 1.45 kb *Bgl*II- *Sca*I DNA fragment gave rise to two hybridizeable bands—a non-disrupted 4.5 kb band and a 6.7 kb band (containing 2.2 kb of *LEU2* DNA). Figure 3 similarly demonstrates that the non-disrupted diploid, WA1/6 (lane 3) contains only the 4.5 kb hybridizeable band, whereas the transformed diploid WA10 (lane 4), heterozygous for the disruption, contains both the 4.5 and 6.7 hybridizeable bands.

Sterol analysis of the erg2 *disruption.* Figure 4 shows a sterol profile of a disrupted and a non-disrupted isolate from the same tetrad. WA10-3-1A (Leu^+ Erg^-) grown on YPD medium contains sterols with only Δ8 double bonds. These are peaks A and B, comprising 50% of the total sterol, ergosta-5,8,22-trien-3β-ol and ergosta-8,22-dien-3β-ol, and peaks C and D, also comprising 50% of total sterol containing ergosta8,24(28)-dien-3β-ol and ergosta-8-en-3β-ol. Within the limits of our detection (less than 1% of total sterol), we found no evidence of Δ7 sterols. Strain WA10-3-1B, a non-disrupted colony from the same tetrad (Leu^- Erg^+) gave the wildtype sterol spectrum with ergosterol (peak F, 76%) as the predominant sterol.

The molecular disruption of *ERG2* represents the second ergosterol biosynthetic gene which we found to be not essential for cell viability. Thus, it is still unclear whether there is a specific requirement for a particular sterol molecule. It is also unclear whether other non-allelic genes may be responsible for enzymes required for the formation of the sparking sterol since it is clear that the disruptions in *ERG2* and *ERG6* eliminate their participation in ergosterol formation. The yeast, *Saccharomyces cerevisiae*, exhibits flexibility with regard to lipid composition. For example, a *chol1-1 ino1-13* double mutant supplemented with inositol and choline is deficient in the synthesis of phosphatidylserine and a strain which overproduces inositol (*opi3-d*) is deficient in phosphatidylcholine synthesis (19). Similarly, viable yeast strains that are deficient in sphingolipids have been described (20). Thus, at least in *Saccharomyces cerevisiae*, there is unusual tolerance to major alterations in the phospholipid, sterol, and sphingolipid compositions.

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Sterol Pathway in Yeast. Identification and Properties of Mutant Strains Defective in Mevalonate Diphosphate Decarboxylase and Farnesyl Diphosphate Synthetase[1]

Christophe Chambon[a], Veronique Ladeveze[a], Madeleine Servouse[a], Laurence Blanchard[a], Catherine Javelot[b], Barbu Vladescu[b] and Francis Karst[a,*]

[a]Laboratoire de Biochimie et Génétique des Microorganismes Université de Poitiers, F-86022 Poitiers Cedex and [b]Laboratoire de Microbiologie, Centre de Recherches Pernod-Ricard, F-94015 Créteil, France

Yeast mutant strains auxotrophic for ergosterol and blocked in mevalonate diphosphate decarboxylase (*erg19*) and farnesyl diphosphate (FPP) synthetase (*erg20*) were isolated. The main feature of the mutants blocked in FPP synthetase is their ability to excrete prenyl alcohols, such as geraniol and farnesol. The isolation of the functional *ERG20* gene allowed us to show that farnesyl diphosphate synthetase could be a rate limiting enzyme in ergosterol biosynthesis in yeast.
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Saccharomyces cerevisiae cells contain 1-3% sterols depending on the growth medium. Ergosterol represents about 50% of the total sterols and is the major free sterol involved in membrane structure.

Three kinds of mutants blocked in the ergosterol pathway have been isolated and extensively studied. The first kind corresponds to mutations affecting late steps in the pathway, down stream of zymosterol; these mutants were isolated based on resistance to the polyene antibiotic nystatin and are not sterol-auxotrophic (1). The second type of mutants were isolated as requiring both ergosterol and an unsaturated fatty acid (2,3). It was shown that the pleiotropic requirements are related to deficiency in porphyrin biosynthesis; hematin enzymes are implicated in respiration, lanosterol demethylations, fatty acid desaturation and sulfate reduction. The physiological study of these mutants allowed us to isolate numerous mutant strains affected in enzymes from acetoacetyl-CoA thiolase to lanosterol C-14 demethylase. The main feature of the mutants of this third class is that they are generally temperature sensitive. Temperature sensitivity of mutants defective in early enzymes of the sterol pathway is easy to understand since the biosynthetic route provides, in addition to ergosterol, many essential molecules such as the side chain of ubiquinone, dolichol, and farnesyl groups of prenylated proteins.

We describe here the last mutations that we have isolated; *erg19* and *erg20*, affecting mevalonate diphosphate (MVAPP) decarboxylase and farnesyl diphosphate (FPP) synthetase, respectively. Furthermore, we present evidence that FPP synthetase may be a rate-limiting enzyme in sterol biosynthesis in yeast.

EXPERIMENTAL PROCEDURES

Strains and culture conditions. The strains used were derived from *Saccharomyces cerevisiae* FL100 (A.T.C.C. 28383) haploid, a mating type, or from an isogenic strain FL200 (A.T.C.C. 32119), haploid, α mating type. Culture conditions and media were those previously described (4). 5247 Was an *ura3 trp1* auxotroph. Plasmid pCC1 was a derivative of pUC 19 bearing a yeast chromosomal origin of replication (ARS), the CEN 6 DNA, the *TRP1*, and *ERG20* genes. pCC2 was a pUC 19 derivative bearing the yeast 2 μm DNA as replication origin, the *URA3*, and *ERG20* genes (5).

Enzyme preparation. A 12,000 $\times$ *g* cell-free supernatant was prepared as described (5).

MVAPP decarboxylase assay. [1,3,5-^{14}C]MVAPP was synthesized by an enzymatic method using a cell-free extract of mutant strain MN*erg19*. The standard assay mixture contained, in a total volume of 0.5 mL, 75 mM potassium phosphate buffer pH 7.4, 1.5 mM DTT, 10 mM ATP, 10 mM G6P, 1 mM NADP, 5 mM PEP, 0.25 mM CoA, 10 mM KF, 5 mM $MgCl_2$, 30 mM KCl, 2 units glucose-6-phosphate dehydrogenase, 2 units pyruvate kinase and [1-^{14}C]acetate 1 mM (2 mCi/mmol). The assay was initiated by addition of 5-mg of crude enzyme. The incubation period was 90 min at 30°C; the reaction was stopped by addition of 0.05N HCl in 75% ethanol. The mixture was warmed quickly to 60°C and kept at that temperature for 90 sec. The mixture was then cooled in ice, and the precipitate was sedimented by centrifugation. The supernatant was decanted and saved, and the precipitate was washed three times with 75% ethanol. The washings combined with the first supernatant were concentrated on a rotary evaporator at 50°C, and the residue was dissolved in a minimum amount of buffer pH 7.4. An aliquot was assayed for radioactivity by liquid scintillation counting. The remainder was applied to Silica Gel G plates and the plates were developed with n-propanol/NH_4OH/water (6:3:1, v/v/v). Distribution of the radioactivity incorporated was then determined using a Berthold 2382 thin-layer chromatography(TLC) Linear Analyzer (Berthold Analytical, Wildbad, Germany). The band containing [1,3,5-^{14}C](R)-5-mevalonate diphosphate (R_f 0.09) was scrapped into a small flask, and MVAPP was extracted by shaking with dilute aqueous ammonia; the preparation was 40 μM (6 mCi/mol). MVAPP was identified by two different methods: acid hydrolysis in 1N HCl for 10 min at 100°C led to the formation of mevalonate phosphate (MVAP) (R_f 0.15), and alkaline phosphatase hydrolysis released mevalonic acid (R_f 0.7).

[1]Based on a paper presented at the symposium on Plant and Fungal Sterols: Biosynthesis, Metabolism and Function, held at the AOCS Annual Meeting, Baltimore, MD, April 1990.

*To whom correspondence should be addressed at: Laboratoire de Biochimie et Génétique des Microorganismes Université de Poitiers, 40, avenue du Recleur Pineau, F-86022 Poitiers Cedex, France.

Abbreviations: DMAPP, dimethylallyl diphosphate; DTT, dithiothreitol; ERG, functional gene in ergosterol pathway; erg, gene defective in ergosterol pathway; FPP, farnesyl diphosphate; GC/MS, gas chromatography/mass spectrometry; GPP, geranyl diphosphate; HMG-CoA, hydroxymethylglutaryl-Coenzyme A; IPP, isopentenyl diphosphate; MVA, mevalonic acid; MVAP, mevalonate phosphate; MVAPP, mevalonate diphosphate; TLC, thin-layer chromatography; [], denotes plasmid-carrier state.

The assay of MVAPP decarboxylase activity was carried out in 3-mL Warburg flasks. The center well contained 0.2 mL of 40% KOH and frilled filter paper. The two side arms contained 0.1 mL of 6N HCl and 0.1 mL MVAPP (40 μM), respectively. The 0.4-mL incubation mixture in the main compartment consisted of 75 mM potassium phosphate buffer, pH 7.4, 5 mM $MgCl_2$, 10 mM KF, 1.7 mM DTT, 5 mM ATP, and about 3 mg protein. The substrate [1,3,5-^{14}C](R)-mevalonate diphosphate was added last to a concentration of 8 μM. The mixture was incubated with shaking at 30°C for 60 min, then the acid was tipped from the side arm into the main compartment and the incubation was continued for one hour. The filter papers were removed from the center well, kept overnight at room temperature and counted for radioactivity. Enzyme specific activity is expressed as nanomoles of CO_2 generated per mg protein per hour.

FPP synthetase assay. The assay was performed as previously described (5).

Phosphatases assays. The assays were performed as described by Torriani (6).

Analysis of excreted prenyl alcohols. The prenyl alcohols were analyzed by gas chromatography/mass spectrometry (GC/MS) after dichloromethane extraction, using methyl undecanoate as internal standard and a Fracto Vap 4160 (Carlo Erba, Milano, Italy) chromatograph (Carbowax 20M column) coupled to a Kratos MS25 (Kratos Analytical, Ramsey, NJ) mass spectrometer connected to a Data General Eclipse S120 computer. The compounds were identified by comparison to a library of reference spectra.

RESULTS AND DISCUSSION

Mutant isolation and general characteristics. Mutants bearing *erg19* and *erg20* mutations were isolated on the basis of resistance to the polyene antibiotic nystatin as previously described (7). Thus far we have isolated sterol auxotrophic mutant strains which belong to 14 complementation groups. Moreover, intragenic complementation was observed in the *ERG10* and *ERG12* genes, reflecting the oligomeric nature of these two proteins (Table 1).

The mutants are, in general, temperature sensitive, which means that they are unable to grow at high tempratures (36°C), even in sterol-supplemented medium. Four mutants are simple sterol auxotrophs, namely *FKerg7, FKerg17, FKerg9* and *VL134* (or *VL135*). We have shown (7) that mutant strains *FKerg7, FKerg9* and *FKerg17* bear an additional mutation (*aux30*) allowing sterol permeability, as *hem* mutations do. All other mutant strains are intrinsically permeable to exogenous sterols and do not require an additional mutation. One can assume that exogenous sterol uptake takes place as soon as the cells become sterol-requiring; the accumulation of oxidosqualene (*FKerg7* and *FKerg17*) or FPP (*FKerg9*) could neutralize the starvation signal.

The lack of sterol permeability in aerobiosis is not the reason why sterol auxotrophs must be isolated as conditional mutants. More likely, a complete enzymatic block in an early step of the sterol pathway would lead to a pleiotropic requirement not satisfied by exogenous supplementation. This has been shown by gene disruption experiments (8,9). Under standard aerobic growth conditions at 28°C the sterol auxotrophs present optimal growth at ergosterol supplementation of 2 mg/L. Under such conditions, the ergosterol level of the cells is about 0.25% of cell dry weight (*i.e.,* three-fold lower than in the wild type strain). With a high ergosterol supplementation (50 mg/L), the ergosterol level of the mutants is strongly enhanced by 6–10-fold; in spite of this high ergosterol content, enzyme activities such as acetoacetyl-CoA thiolase and HMG-CoA synthase remain induced (10).

TABLE 1

Gene-Enzyme Relation in Ergosterol Pathway in *Saccharomyces cerevisiae*

Gene	Enzyme
ERG10A, ERG10B	Acetoacetyl-CoA thiolase
ERG11, ERG13	HMG-CoA synthase
ERG12A, ERG12B	Mevalonate kinase
ERG8	MVAP kinase
ERG19	MVAPP decarboxylase
ERG20	FPP synthetase
ERG9	Squalene synthetase
ERG1, ERG18, ERG14	Squalene epoxidase
ERG7, ERG17	Oxidosqualene:lanosterol cyclase
ERG16	Lanosterol-14 demethylase

TABLE 2

MVAPP Decarboxylase and FPP Synthetase Activities in Wild Type, MN*erg19*, VL134 and VL135 Mutant Strains

Strains[c]	FPP synthetase[a] DMAPP	FPP synthetase[a] GPP	MVAPP decarboxylase[b]
WT	6.47	5.13	0.36
MN*erg19*	10.60	9.10	0.01
VL134	0.09	0.09	—[e]
VL135	ND[d]	ND	—[e]

[a]Results are expressed as nmoles [^{14}C]IPP converted per min per mg protein.

[b]Results are expressed as nmoles $^{14}CO_2$ formed per min per mg.

[c]Wild type strain and sterol auxotrophic mutants were grown in a complete medium supplemented with ergosterol (2 mg/L) at 28°C with shaking. Cells were harvested during the exponential phase.

[d]ND, not detected.

[e]Not determined.

Determination of the enzymatic lesions. Enzyme lesions were identified first by precursor accumulation and then by measurement of *in vitro* enyme actiities. Identification of *erg10* (acetoacetyl-CoA thiolase), *erg11* (HMG-CoA synthase), *erg12* (mevalonate kinase), *erg8* (MVAP kinase), *erg9* (squalene synthetase), *erg1, erg18* and *erg14* (squalene epoxidase), *erg7* and *erg17* (oxidosqualene lanosterol cyclase) and *erg16* (lanosterol demethylase) has been described previously (7,11).

Identification of defects in MVAPP decarboxylase (erg19) *and FPP synthetase* (erg20). [^{14}C]Acetate incubation in cell free extracts of MN*erg19* mutant strain showed accumulation of a compound more polar in TLC than MVAP. This compound was identified as MVAPP as described in the experimental section. Mevalonate diphosphate decarboxylase activity was measured in cell-free extracts of wild type and MN*erg19* mutant strains; no significant activity was observed in MN*erg19* extracts (Table 2).

[^{14}C]Mevalonic acid (MVA) incubation in cell-free extracts of VL134 and VL135 mutant strains showed the accumulation of a compound displaying the same strong polarity on Silica Gel G plates as dimethylallyldiphosphate (DMAPP), isopentenyl diphosphate (IPP) or MVAP. This compound was identified as DMAPP (5). Therefore, the two mutants were likely to be affected in FPP synthetase. This enzyme activity was measured in crude extracts of the wild type strain and VL134 and VL135 mutant strains using [^{14}C]IPP and either DMAPP or geranyl diphosphate (GPP) as substrates. Table 2 shows that no activity was detected in the extracts of VL135. A very low activity was detected in the extracts of VL134 with a high protein concentration. These results show that VL134 and VL135 were blocked in FPP synthetase.

Properties of mutant strains affected in FPP synthetase. Upon incubation of mevalonate with cell-free extracts of FK*erg9* (defective in squalene synthetase), VL134 and VL135, the major reaction products were FPP and DMAPP. We wondered whether the dephosphorylated forms of these sterol precursors are excreted into the culture medium during cell growth. Table 3 shows that, as expected, no prenyl alcohols were found in the culture medium of the wild type strain. By contrast, we noted the presence of isopentenol, dimethylallyl alcohol and farnesol in culture media of VL134, VL135 and FK*erg9*. The amounts of dimethylallyl alcohol and isopentenol are very high in VL134 and VL135 in agreement with the inactivation of FPP synthetase. The farnesol amount, however, is lower in VL134 and VL135 than in FK*erg9* blocked in squalene synthetase. Secondly we observed that, unlike the other strains tested, VL134 produces geraniol and linalool in addition to farnesol. These results suggest that the two mutant strains, VL134 and VL135, carry two different mutations in the FPP synthetase gene, *erg20-1* and *erg20-2*, respectively. However, the measurement of the genetic linkage between the two mutations showed less than 10^{-3} recombinants, strongly suggesting that VL134 and VL135 bear the same mutation in FPP synthetase gene. The genetic analysis of these two mutant strains detailed elsewhere (5) is summarized in Table 4. The results reveal that the two strains differ by additional mutations in the ergosterol pathway, affecting either squalene synthetase (*erg9*) or mevalonate kinase (*erg12-2*). One additional mutation is required for cell viability, for decreasing geraniol production. In the presence of both mutations, there is no geranoil formation. It is noteworthy that, by one UV light mutagenesis (10% survival) we obtained multiple mutations; this can be explained by the genetic instability of our haploid wild type strain FL100, but also by the selection procedure, which involves successive cell growths on different selective media.

We have measured phosphatase activities in cell-free extracts of wild type, FK*erg9*, VL134 and VL135 mutant strains. The results show that the specific activities of both acid and alkaline phosphatases are about 2-3-fold higher in mutant strains than in the parent strain (Table 5). Therefore, phosphatase activities may actually be involved in the dephosphorylation of FPP and GPP. As for the excretion itself, it probably proceeds by simple diffusion due to the hydrophobic nature of farnesol and geraniol.

Effect of the overexpression of FPP synthetase on the ergosterol level of yeast cells. We isolated the *ERG20* gene by complementation of the *erg20* mutation. The DNA fragment isolated from a yeast genomic library had a length of 5.7 kb. The restriction map was established and the insert could be reduced to 3 kb without loss of activity (5). The DNA fragment was identical to the yeast FPP synthetase gene isolated by hybridization of a synthetic oligonucleotide probe based on the NH_2 terminal sequence of the purified enzyme (12). This result shows that the *erg20* mutation affects the FPP synthetase structural gene. Therefore, it can be assumed that geraniol formation observed in VL134 is directly related to the properties of the modified FPP synthetase.

We constructed strains 5247 [pCC1] and 5247 [pCC2] carrying one copy or multiple copies of the *ERG20* gene, respectively, depending on the replication origins of the vectors used. Table 6 shows that in 5247 bearing pCC1 the addition of the extrachromosomal *ERG20* gene to the

TABLE 3

Terpenic Compound Excretion in Culture Medium from Wild Type and Mutant Strains VL134, VL135 and FK*erg9*[a]

	FL100	FK*erg9*	VL134	VL135
Isopentenol	ND[b]	0.05	3.32	2.90
Dimethylallyl alcohol	ND	0.69	6.62	6.20
Linalool	ND	ND	1.1	ND
Geraniol	ND	ND	1.48	ND
Farnesol	ND	0.74	0.18	0.01

[a]The strains were cultured in minimal medium supplemented with ergosterol (2 mg/L) for 72 hr at 28°C. Terpenic compounds were extracted and analyzed as described in the experimental section. The results are expressed in mg/L.

[b]ND, not detected.

TABLE 4

Genetic and Phenotypic Properties of *erg20* Mutant Strains

Strains	Genotype	Geraniol excretion
VL134	*ERG12, erg20-1, erg9*	+
VL135	*erg12-2, erg20-2, erg9*	ND[a]
AO527[b]	*erg12-2, erg20-2, ERG9*	+
Not viable	*ERG12, erg20-1, ERG9*	−
Not viable	*ERG12, erg20-2, ERG9*	−

[a]ND, not detected.

[b]AO527 was isolated from the progeny of the heterozygous diploid VL135/+.

TABLE 5

Phosphatase Activities in Wild Type and Mutant Strains[a]

Strains	Acid phosphatase[b]	Alkaline phosphatase[c]
FL100	9.6 ± 0.8 (3)	8.3 ± 0.9 (3)
VL134	19.2 ± 1.2 (3)	23.6 ± 1.7 (3)
VL135	18.3 ± 1.5 (3)	24.1 ± 1.2 (3)
FK*erg9*	20.2 ± 1.4 (3)	25.3 ± 1.8 (3)

[a]Phosphatase activities are expressed as nmoles *p*-nitrophenol formed per min per mg protein ± S.D. (number of independent experiments).

[b]Specific activity was measured at pH 4.3.

[c]Specific activity was measured at pH 10.

TABLE 6

FPP Synthetase Activity and Ergosterol Level of Yeast Cells

Strains	FPP synthetase specific activity[a]		Ergosterol level[b]
	DMAPP	GPP	
5247	6.4 ± 0.1 (3)	5.4 ± 0.2 (3)	0.65 ± 0.06 (3)
5247 [pCC1]	7.8 ± 0.1 (3)	6.9 ± 0.2 (3)	0.84 ± 0.07 (3)
5247 [pCC2]	23.0 ± 0.9 (3)	17.2 ± 1.1)3)	0.72 ± 0.09 (3)

[a]Specific activity is expressed as nmoles [^{14}C]IPP incorporated per min per mg protein ± S.D. (number of independent experiments).

[b]Expressed as % of mg cell dry weight ± S.D. (number of independent experiments).

chromosomal copy leads to an increase of about 25% in FPP synthetase specific activity; the ergosterol amount is correspondingly increased by 28% in comparison with the parent strain. This result strongly suggests that FPP synthetase is a rate-limiting enzyme in the sterol pathway. In 5247 [pCC2] increased more than three-fold the FPP synthetase specific activity is in comparison with the parent strain, owing to the high number of *ERG20* gene copies. However, the ergosterol level is not correspondingly increased, instead it is lowered as compared to 5247 [pCC1]. This last result can be explained by a regulatory mechanism in the sterol pathway. The high FPP synthetase activity in 5257 [pCC2] may increase the FPP pool. It is well known that mevalonate kinase activity is inhibited by an excess of FPP; squalene synthetase specific activity is 0.5 nmoles FPP converted/min/mg, and the FPP synthetase in 5247 [pCC2] produces about 7 nmoles FPP/min/mg. Further experiments need to be performed in order to evaluate the importance of mevalonate kinase feed-back regulation by FPP in the overall sterol pathway in yeast.

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Aspects Related to Mevalonate Biosynthesis in Plants[1]

Thomas J. Bach[a,*], Albert Boronat[b], Carmé Caelles[b], Albert Ferrer[b], Thomas Weber[a] and Annette Wettstein[a]

[a]Botanisches Institut (Pflanzenphysiologie und Pflanzenbiochemie), Universität Karlsruhe, D-7500 Karlsruhe 1, Germany and [b]Universitat de Barcelona, Facultat de Farmàcia, Unitat de Bioquimica, E-08028 Barcelona, Spain

We purified and characterized a membrane-associated enzyme system from radish (*Raphanus sativus* L.) that is capable of converting acetyl-CoA into 3-hydroxy-3-methylglutaryl-coenzyme A (HMG-CoA). The enzyme system apparently comprises acetoacetyl-CoA thiolase (EC 2.3.1.9) and HMG-CoA synthase (EC 4.1.3.5). Its activity *in vitro* can be strongly stimulated by Fe^{II}. When ferrous ions are applied chelated with ethylenediaminetetraacetate, citrate or adenosine 5'-triphosphate (ATP), the stimulation is further increased. Stimulation is due to a higher catalytic efficiency as indicated by an increase in V_{max}, whereas the affinity of the enzyme towards acetyl-CoA remains constant (K_m = 6 μM). A considerable portion of HMG-CoA lyase activity is associated with the same membranes. HMG-CoA lyase (EC 4.1.3.4) is also solubilized and partially co-purified. Its activity requires comparatively high concentrations of Mg^{2+}. The conversion of HMG-CoA to mevalonic acid is catalyzed by HMG-CoA reductase (EC 1.1.1.34) that is associated with the same membranes. By cDNA encoding the *Arabidopsis* HMG-CoA reductase, we isolated a corresponding gene from a cDNA library newly established from etiolated radish seedlings. This full-length cDNA, referred to as λcRS3, encodes a polypeptide of 583 amino acids with a molecular mass of about 63 kDa. The hydropathy profile suggests the presence of two hydrophobic membrane-spanning domains within the N-terminal 165 amino acids. The carboxy-terminal part, where the catalytic site resides, is highly conserved in all eukaryotic HMG-CoA reductase genes sequenced so far. *Lipids 26*, 637-648 (1991).

Plants are capable of synthesizing a myriad of isoprenoid derivatives which are biochemically functional in cells. The basic building block of all these compounds, despite their variety in structure and intracellular location, is derived from mevalonic acid (MVA, see refs. 1-3 for literature). The synthesis of MVA, which is generally considered the first step in isoprenoid biosynthesis, is catalyzed by the enzyme 3-hydroxy-3-methylglutaryl-coenzyme A reductase [HMGR, (*R*)-mevalonate:$NADP^+$ oxidoreductase (CoA-acylating), EC 1.1.1.34]. This particular enzyme has been the subject of a great number of studies, mainly because of its key role in the regulation of cholesterol biosynthesis (4,5). HMGR has recently become a target in the treatment of hypercholesterolemia, a prevalent risk factor of cardiovascular disease in Western societies (6-12).

In plants, HMGR is apparently regulated by various environmental factors and stimuli, including light (13-16), growth regulators and phytohormones (16-18), as well as herbicides (19). Regulation by feedback mechanisms (17), by wounding and infection with pathogens or by fungal elicitor treatment (20-25), and by Hg (26) have also been reported. The cell-free synthesis of MVA has been demonstrated in a variety of plant species and in tissue cultures (1,3). Generally, the reaction is catalyzed by a membrane-bound enzyme. Recently, the solubilization of HMGR from the membranes either by detergent treatment (3,27-29) or by partial trypsinization (30) and subsequent purification (3,27-30) was shown to be possible. The data available on plant HMGR suggest its occurrence in plastids and mitochondria, in addition to the cytoplasm. However, this model, originally based on incorporation experiments (31), is still a matter of debate (32). The model appears difficult to prove due to the low activity and the susceptibility of HMGR towards rapid inactivation *in vitro*. However, the results from inhibition studies (1,3,32-35) strongly support the existence of HMGR isozymes in plant cells. Further evidence in favor of this hypothesis can be gathered using the powerful techniques of molecular biology and immunology. Genes encoding HMGRs from several plants have been characterized (36-40); some of the latter findings will be summarized and discussed in this contribution.

Another problem to be addressed is how HMG-CoA synthesis is regulated in plants. HMG-CoA plays a pivotal role not only as a precursor of the isoprenoid pathway, but it additionally serves as a putative intermediate in the degradation of branched-chain amino acids. Until recently, surprisingly little had been known about the properties as well as the intracellular localization of the enzymes involved (cf. 2,3). The synthesis of HMG-CoA from three acetyl-CoA units comprises: i) a Claisen-type condensation that is catalyzed by acetoacetyl-coenzyme A thiolase (AACT, acetyl-coenzyme A acetyltransferase, EC 2.3.1.9); and ii) a subsequent aldol condensation catalyzed by 3-hydroxy-3-methylglutaryl-coenzyme A synthase [HMGS, (*S*)-3-hydroxy-3-methyl-glutaryl-coenzyme A:acetoacetyl-coenzyme A lyase (CoA acylating), EC 4.1.3.5]. The presence of HMGS was largely inferred from the formation of an uncharacterized HMG-derivative from acetate in a cell-free extract of flax seedlings (41), the same plant tissue from which HMG-acid had first been isolated (42). Lynen (43) determined the activity of several enzymes of the rubber biosynthetic pathway in the latex of *Hevea brasiliensis*, among them AACT and HMGS; their properties were not investigated further. The synthesis of a HMG-derivative, most likely HMG-CoA, from acetyl-CoA

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*To whom correspondence should be addressed at: Universität Karlsruhe, Botanik II, Kaiserstrasse 12, D-7500 Karlsruhe 1, Germany.

Abbreviations: AACT, acetoacetyl-CoA thiolase; ATP, adenosine 5-triphosphate; Brij W-1, polyoxyethylene ether Brij W-1; CoA, coenzyme A; DTE, dithioerythritol; EDTA, ethylenediaminetetraacetate; FPLC, fast-performance liquid chromatography; HMG-CoA, 3-hydroxy-3-methylglutaryl-coenzyme A; HMGL, HMG-CoA lyase; HMGR, HMG-CoA reductase; HMGS, HMG-CoA synthase; HPLC, high-performance liquid chromatography; MVA, mevalonic acid; PAGE, polyacrylamide gel electrophoresis; SDS, sodium dodecylsulfate.

in a cell-free system of sweet potato was reported (44). This biosynthetic capacity was found in cytosolic and microsomal preparations and did not require co-factors such as ATP, Mg^{2+}, HCO_3^- or NADPH (44). An iodoacetamide-sensitive AACT activity has been identified in vesicles from mature orange fruits (45); attempts to assay HMGS and HMGR activities were not successful. Quite recently, AACT activity has been found in plastid preparations from *Parthenium argentatum* and *Phaseolus radiatus* (46). The presence of HMGS activity in osmophores of the orchid *Stanhopea anfracta* was detected by a cytochemical approach (47). Our experience with cytosolic and membrane-associated AACT/HMGS activity (3,48) support the view of a variable intracellular location of these enzymes.

Once synthesized, HMG-CoA instead of entering the isoprenoid pathway can be cleaved by HMG-CoA lyase [HMGL, (*S*)-3-hydroxy-3-methylglutaryl coenzyme A lyase, EC 4.1.3.4] in a stereochemically controlled Claisen-type retrocondensation to yield acetoacetate and acetyl-CoA (49). This important reaction contributes to the formation of ketone bodies in vertebrate cells (50,51). Indeed, Hepper and Audley (52) provided some evidence for the presence of this enzyme activity in the latex of the rubber tree. An enzyme activity that interferes with the HMGR assay in membrane preparations from *Ipomoea batatas*, most probably HMGL, was also reported (53). Skrukrud *et al.* (54) have assayed HMGL in the soluble fraction of latex collected from *Euphorbia lathyris*. The presence of HMGL in latex explains, in part, the failure to detect any significant incorporation of acetyl-CoA, HMG-acid or of HMG-CoA into triterpenoids (55). HMGL might also be involved in the MVA shunt pathway (56,57), a cyclic retroconversion of MVA to HMG-CoA and a mechanism of routing away isoprene-units from their inclusion into the isoprenoid pathway. Convincing evidence has been presented of the MVA shunt existing in plants (58,59). We have started to characterize from plant tissue the enzymes that are involved in the synthesis and metabolism of HMG-CoA (3,48). Some improvements in the purification protocols and further *in vitro*-properties of these enzyme systems are reported here.

MATERIALS AND METHODS

Chemicals. [1-^{14}C]Acetyl-CoA, (*R,S*)-[3-^{14}C]HMG-CoA, [2-^{3}H]mevalonic acid and [^{32}P]GTP were purchased from Amersham (Braunschweig, Germany); [^{35}S]ATP was from New England Nuclear (Bad Homburg, Germany). Brij W-1 (polyoxyethylene ether), HMG-CoA, acetyl-CoA and molecular weight standards were from Sigma (Deisenhofen, Germany); HS-CoA, NADPH, NADH, glucose-6-phosphate, glucose-6-phosphate dehydrogenase, and a DNA random priming kit were from Boehringer (Mannheim, Germany). Superose 6 and oligo(dT) cellulose were from Pharmacia (Freiburg, Germany); Cellufine GC 200 and GC 700 were from Amicon (Witten, Germany). Fractogel materials (anion exchange and affinity chromatography) were purchased from Merck (Darmstadt, Germany); sorbitol and DTE from Serva (Heidelberg, Germany); and scintillation vials plus cocktails from Zinsser (Frankfurt, Germany). The sources of materials used for experiments in the cloning and expression of plant HMGR have been described elsewhere (38,60). All other chemicals purchased from commercial sources were of the highest purity available.

Plant cultivation and membrane isolation. Radish (*Raphanus sativus*, var. Saxa Knacker) seedlings were grown in tap water in complete darkness for four days. Membrane fractions (P 16,000 and P 140,000) and cytosol were isolated as described (16). Membranes were resuspended in small volumes of isolation buffer "A" (28). DTE was added to the suspended membranes to a final concentration of at least 20 mM. The suspensions were kept on ice until use or immediately deep-frozen and stored at −20°C. The enzyme activities determined remained stable under these conditions.

Enzyme assays. The assay of HMGR activity has been described in detail (28).

Assay of HMG-CoA synthesis from acetyl-CoA (coupled assay of AACT and HMGS) using a crude enzyme preparation. Fifteen μL Tris/HCl [pH 7.5, 0.2 M, containing 10 mM ethylenediaminetetraacetate (EDTA)] was mixed with 25 μL of enzyme solution (in 0.2 M K_xPO_4, 10 mM EDTA, 5 mM $MgCl_2$, 20 mM DTE, supplemented with 1% w/v Brij W-1) and kept on ice. After preincubation at 30°C for 5 min the reaction was started by addition of a 10 μL mixture containing 1.25 μL [1-^{14}C]acetyl-CoA (10 μCi/mL, specific activity 52 mCi/mmol), 1.2 μL acetyl-CoA (2 mM), and 7.75 μL 0.2 M Tris/HCl, pH 7.5, containing 10 mM Na_2 EDTA. The incubation at 30°C was continued for 10 min and the reaction was terminated by addition of 150 μL of 6N HCl. After incubation at 30°C for 30 min the reaction mixture was transferred into plastic scintillation vials (5-mL) that were kept at 105°C for more than 5 hr. Under such conditions, thioesters are hydrolyzed, acetate evaporates, and HMG acid remains in the vial (51,61). In order to avoid any contamination, the samples were evaporated in a closed metal box inside a drying oven. Vapors from the box were continuously removed by pumping and the radioactive material was trapped by passage through three wash bottles containing water, 1N NaOH and paraffin. Water (250 μL) was then added to the vials, and the samples were vigorously vortexed. Quickszint 2000 (4 mL) was added, and after vortexing the samples were assayed for radioactivity by liquid scintillation counting (Packard 2000 CA, Frankfurt, Germany). For time course and kinetic studies the enzyme assay volume was increased to 150 μL. At various time points, 50 μL aliquots of the reaction mixture were withdrawn, transferred to 150 μL 6N HCl and treated further as described above. The data were computed using spline functions to calculate best fit curves (62), which allowed calculation of approximate v_o values. In kinetic studies, substrate concentrations were varied as appropriate (substrate dilution, cf. 16,28) and data were plotted as described (16,28). When the separation of AACT and HMGS activities was attempted, assays were run in duplicate, either in the presence or in the absence of 50 μM acetoacetyl-CoA. HMGS activity in fractions not containing AACT should be measurable only in the presence of acetoacetyl-CoA.

Assay of partially purified AACT/HMGS (free of HMGL). Tris/HCl (25 μL, pH 7.5, 0.2 M) was mixed with 15 μL enzyme solution (in 50 mM K_xPO_4, 0.3 M sorbitol, 5 mM EDTA, 5 mM $MgCl_2$, 3 mM DTE, 0.3% Brij W-1). After preincubation at 30°C the reaction was started by addition of 10 μL of a mixture containing 1.25 μL

[1-^{14}C]acetyl-CoA (10 μCi/mL, 52 mCi/mmol), 0.6 μL acetyl-CoA (2 mM), and 8.2 μL 0.2 M Tris/HCl pH 7.5. Further processing of the assay mixtures was as described above. When the effects of various cations on the activity of AACT/HMGS were studied, the cation tested (if required, complexed in the presence of a double concentration of EDTA) was first added to the Tris buffer, immediately followed by addition of the enzyme solution.

Assay of HMGL activity. The standard assay system in a final volume of 50 μL contained 20 μL of enzyme solution, and 20 μL of 0.2 M Tris/HCl, buffer pH 7.5–8.0, supplied with 40 mM $MgCl_2$. After preincubation for 2 min at 30°C the reaction was started by addition of 10 μL of starting mixture: 1.25 μL (*R,S*)-[3-^{14}C]HMG-CoA (*ca.* 30,000 dpm, specific activity 52 mCi/mmol), 0.6 μL unlabeled (*R,S*)-HMG-CoA (4 mM stock solution in 50 mM KH_2PO_4), and 8.15 μL of 200 mM Tris/HCl pH 7.5). The final concentration of (*R,S*)-HMG-CoA thus obtained was *ca.* 50 μM. After incubation at 30°C the reaction was stopped by addition of 150 μL 6N HCl. The mixture was transferred to minivials and kept at 115°C for a minimum of 3–4 hr. Labeled acetoacetate evaporated and nonvolatile HMG-CoA remained in the vial (51,61). Further processing of samples was as described for the AACT/HMGS assay. Because HMGL cleaves only the (*S*)-isomer of synthetic HMG-CoA (49), a maximum of 50% of the initial radiolabeled material was used up by the enzyme.

Enzyme solubilization and purification. AACT/HMGS activity was solubilized from a heavy-membrane pellet (P 16,000) by thawing of frozen samples followed by incubation at 30°C for 30 min in the presence of 2% (w/v) Brij W-1. The turbid yellow suspension was homogenized by aid of a teflon homogenizer (15 to 20 strokes) and centrifuged at 100,000 × *g* for 45 min at 15°C. In the supernatant 80–90% of the initial enzyme activity was found. The clear solution was brought to a final concentration of higher than 10 mM with DTE. A 1.5-fold volume of −20°C cold acetone was added. After incubation on ice for 10 min, the turbid solution was centrifuged for 10 min at 10,000 × *g* (2°C). The resulting pellet was washed three times with cold acetone and resuspended in a buffer system consisting of 50 mM K_xPO_4 pH 7.5, 0.3 M sorbitol, 5 mM EDTA, 5 mM $MgCl_2$ and 0.3% Brij W-1, 3 mM DTE (buffer system "D") (28). The volume of resuspension buffer was 50% of the enzyme solution used for acetone precipitation. Material that could not be redissolved by 15 strokes with a teflon homogenizer was removed by centrifugation for 5 min at 5,000 × *g*. After this step about 80% of the initial enzyme activity was found in the supernatant.

Gel filtration. Superose 6 prep grade (90 cm^3) in an HR 16/50 column (Pharmacia-LKB, Freiburg, Germany) was equilibrated overnight with buffer system "D" with a biocompatible high-performance liquid chromatography (HPLC) system (Pharmacia-LKB) at a flow rate of 0.1 mL/min. Partially purified enzyme (3.3 mL, resuspended in buffer D) was loaded on the column and elution was continued with buffer D (0.75 mL/min). The fractions collected (2.25 mL) were tested for AACT/HMGS activity as described above. The column was calibrated with standard proteins with molecular masses from 29 to 150 kDa.

Anion exchange chromatography. Fractogel EMD TMAE 650 S (11 cm^3) in a Superformance glass column (Merck, Darmstadt, Germany) was equilibrated with 50 mL of buffer D at a flow rate of 1.5 mL/min by using the same HPLC system. The three peak fractions from the gel filtration with highest apparent AACT/HMGS activity were pooled, and 6.6 mL were loaded on the column. The column was washed with the same buffer system. AACT/HMGS activity eluted with the breakthrough fraction.

Determination of molecular weight by classical gel filtration. Cellufine 200 (or 700, 130 cm^3) in a C 16/70 column (Pharmacia-LKB) was equilibrated with 200 mL of modified isolation buffer A (0.05 M K_xPO_4, 0.35 M sorbitol, 10 mM Na_2EDTA, 5 mM $MgCl_2$, 3 mM DTE), containing 0.2% Brij W-1. Solubilized enzyme (7.5 mL; see above) was loaded and the column was eluted with the same buffer system. The fractions collected (2.25 mL) were tested for AACT/HMGS activity as described above. The column was calibrated with standard proteins with a molecular mass ranging from 29 to 150 kDa.

Protein assay. Proteins were assayed by a modified Lowry assay (63) with some additional modifications, as described elsewhere (28).

Molecular cloning of radish HMGR. Total RNA from either etiolated or green (light regime: 16 hr white light, *ca.* 4500 lux, 8 hr darkness, 22–24°C) four-day-old radish seedlings was isolated as described (64), with minor modifications (28). Poly(A)$^+$ RNA was purified by oligo(dT) cellulose chromatography according to Aviv and Leder (65). RNA samples were fractionated on 1% agarose/formaldehyde gels, transferred and UV-cross-linked to Zeta-Probe membranes and hybridized for Northern blot analysis as described (28). A cDNA library from mRNA isolated from etiolated radish seedlings was constructed by the aid of a cDNA synthesis kit (Bethesda Research Laboratories, Eggenstein, Germany) according to the manufacturer's manual and cloned into the expression vector λgt11 (*Eco*RI restriction site) following established protocols (66,67). The radish cDNA library was screened by hybridization with a ^{32}P-labeled cDNA probe encoding HMGR from *Arabidopsis thaliana* (38). Repeated screening yielded seven positive clones referred to as λcRS1–7. λcRS3 comprised the entire protein-coding transcription unit of a HMGR gene of radish.

DNA sequencing and analysis. For DNA sequencing, the DNA fragments generated by digestion of the *Eco*RI insert with various restriction enzymes were subcloned into appropriate Bluescript plasmids (Stratagene, San Diego, CA). The DNA was sequenced by the dideoxy chain termination method (68) as modified by Biggen *et al.* (69) by the aid of the T7-Sequencing kit (Pharmacia-LKB). Microgen software from Beckman (München, Germany) was used to analyze DNA sequences. Hydropathy profiles were calculated according to the algorithm of Kyte and Doolittle (70).

RESULTS

Solubilization and partial purification of plant AACT/HMGS. A considerable portion of the total activity capable of converting acetyl-CoA to HMG-CoA appears to be associated with a heavy-membrane pellet (P 16,000) isolated from four-day-old etiolated radish (48) and maize seedlings (3). Since the activity found in the cytosolic fraction was shown to be salt-sensitive, concentration and

fractionation by ammonium sulfate precipitation was not possible (48). However, in radish, the same membrane fraction (P 16,000) is the major source of HMGR activity (28) and was therefore used as a source of enzymes to further study the properties of AACT/HMGS. Previously, we successfully developed a protocol for solubilizing HMGR from membranes using the detergent Brij W-1 (27,28). For the enzyme system containing AACT and HMGS we used a similar, but simplified protocol. Prolonged incubation at 37°C was detrimental to the enzyme activity, whereas HMGR appeared to be quite heat-stable (3,28). Therefore, the temperature during the incubation in the presence of 2% Brij W-1 was lowered to 30°C. By application of the protocol, as outlined in Materials and Methods, the recovery of total AACT/HMGS activity was about 90% of the initial value (Table 1).

During the attempts to purify the enzyme(s) further, we always ran enzyme assays twice—either in the presence or absence of unlabeled acetoacetyl-CoA, in addition to [1-^{14}C]acetyl-CoA. If a protein fraction exclusively would contain HMGS, but not AACT activity, only if both substrates were available should we have been able to observe the incorporation of radiolabel into HMG-CoA. We have not separated AACT from HMGS activity as yet, even though we have developed different protocols, including precipitation with polyethylenimine (3,48) and free solution IEF (3,71). As already mentioned above, the salt-sensitivity of this enzyme system, in contrast to the properties of HMGR (28), prevented the use of salt gradients higher than 200 mM for the elution of enzyme activity from various column materials. An important factor in maintaining enzyme activity during purification is to speed up the procedures, which is possible by using fast-performance liquid chromatography (FPLC). In this way we have already achieved a considerable purification factor by combination of mild detergent solubilization, gel filtration, acetone precipitation, and anion exchange chromatography (Table 1). If cation chromatography is included in the purification protocol (where AACT/HMGS is passing through the column, whereas many contaminating proteins bind) an additional purification by 1.5–2-fold is possible. It was possible to separate AACT/HMGS activity from HMGL (cf. Fig. 1). This is especially important when kinetic properties have to be determined, where any interference of HMGL with AACT/HMGS has to be excluded.

Some properties of AACT/HMGS. Recently it has been shown that both enzymes appear to cooperate closely in a way so that no intermediate acetoacetyl-CoA is released into the medium from AACT before it can bind to the active site of HMGS (3,48). During the course of these experiments we have also proved that our enzyme-assay system allows for the conversion of acetyl-CoA to HMG-CoA, which is quantitatively converted to MVA in the presence of NADPH and highly purified yeast HMGR (3,48). Recrystallization from diethyl ether of an enzymatically formed and acid-hydrolyzed product, supplied with additional unlabeled HMG-acid, resulted in greater than 90% radiochemical purity (data not shown). Complex formation between both enzymes could easily explain why any attempt to separate AACT from HMGS was unsuccessful. The apparent molecular mass of the coupled enzyme system AACT/HMGS as deduced from conventional gel filtration on Cellufine 200 and 700 was about 54 kDa (3). This value was confirmed by chromatography of a crude solubilisate from radish membranes (P 16,000) using a FPLC system with Superose 6 prep grade. Although the properties of this material do not permit a good separation of proteins with a difference in molecular mass of less than 20 kDa, some separation from HMGL activity (68–72 kDa, cf. 3) was achieved (Fig. 2).

When the enzyme system was purified by anion exchange chromatography, especially after an additional passage through small PD-10 columns to remove low-molecular-weight molecules and ions, apparent activity was drastically decreased. Reconstitution of activity required the presence of bivalent cations in addition to Mg^{2+} (3). Fe^{2+} was the most active cofactor of various cations tested. Complex formation with EDTA led to an even higher efficacy of Fe^{2+} (Fig. 3) in stimulating AACT/HMGS activity *in vitro*. This stimulation of enzyme activity, *e.g.*, a doubling of enzyme activity requiring less than 50 μM of Fe^{2+}EDTA in the assay mixture, was apparently due to a V_{max} effect (Fig. 4) without change in the affinity towards the substrate acetyl-CoA. The k_m value was calculated to be 6 μM. Since EDTA cannot shield all six coordination sites of Fe^{2+}, additional Fe^{2+} complexes were tested for their ability to stimulate the *in vitro* conversion of acetyl-CoA to HMG-CoA. The data show that concomitant with a decrease in the dissociation constants of the complexes the stimulation disappeared (Fig. 3). Fe^{2+}, either complexed with CN^-,

TABLE 1

Solubilization and Purification of Radish AACT/HMGS[a]

Fraction	Specific activity (nmol × mg^{-1} × min^{-1})	Purification factor	Yield (%)	Total protein (mg)
Homogenate	0.29	1	100	4235
P 16,000	0.32	1.11	11.8	451.8
P 16,000 + Brij	0.68	2.33	13.6	247.3
Resuspended acetone precipitate	1.40	4.80	8.1	71.8
Gel filtration (peak fraction)	28.03	96.22	17.5	7.7
Anion exchange chromatography	70.22	240.9	12.3	2.2

[a]The homogenate was prepared from four-day-old etiolated seedlings, and membranes (P 16,000) were isolated as described (15,28). The enzyme activity was solubilized by incubation in the presence of 2% (w/v) Brij W-1 for 30 min at 30°C. Substrate concentration: 30 μM [1-^{14}C]acetyl-CoA (30,000 dpm).

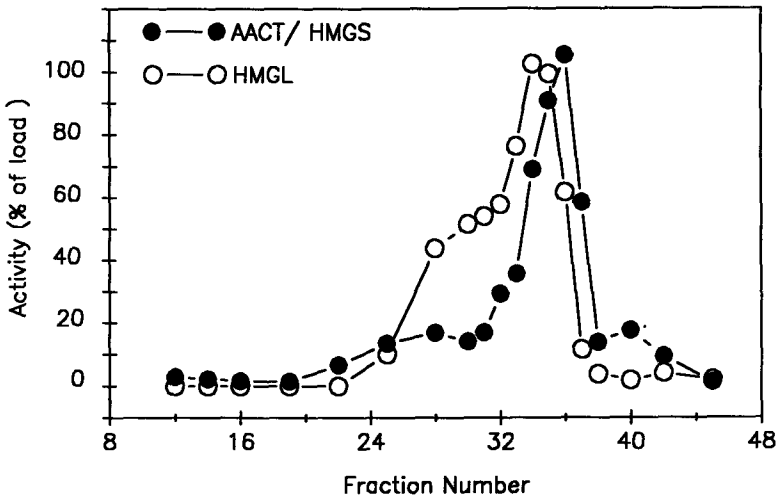


FIG. 1. Gel filtration on Superose 6 prep grade. Proteins from a heavy-membrane pellet isolated from four-day-old etiolated radish seedlings were solubilized in the presence of 2% (w/v) Brij W-1. Solubilized proteins were concentrated by acetone precipitation. The precipitate was resuspended, and insoluble proteins were removed by centrifugation. The clear solution (3.3 mL) was loaded onto the column and eluted as described in Materials and Methods. Volume activity is expressed as percent of loaded fraction. Assay conditions: a) AACT/HMGS: 5 min at 30°C, 25 μM [1-^{14}C]acetyl-CoA, 250 μM Fe^{2+} and 500 μM EDTA. Assay volume was 55 μL: 20 μL fraction, 25 μL Tris/HCl (0.2 M, pH 7.5), immediately mixed with EDTA (stock solution: 40 mM, pH 7.0) and $FeSO_4$ (stock solution: 20 mM, pH 2). The Tris/HCl-Fe-EDTA system was immediately added to the enzyme solution and after a preincubation of 2 min, 10 μL of start mix containing [^{14}C]acetyl-CoA were added. The reaction was stopped by addition of 6N HCl. b) HMGL: Similar to a), however, the Tris buffer was supplied with 30 mM Mg^{2+} instead of Fe-EDTA. Concentration of *R,S*-[3-^{14}C]HMG-CoA, 25 μM.

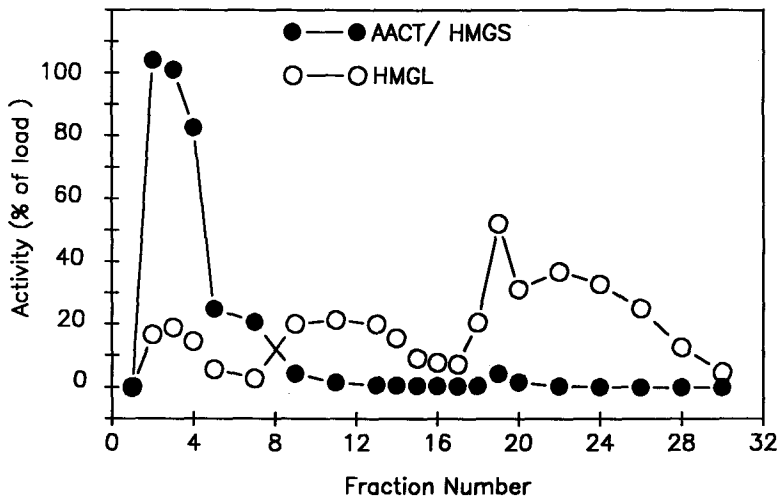


FIG. 2. Anion exchange chromatography on Merck Fractogel EMD TMAE 650. The four peak fractions from gel filtration (Fig. 1) were loaded and the column was eluted as described in Materials and Methods. The assay conditions for AACT/HMGS and HMGL, respectively, were described in the legend to Figure 1 with the exception that 25 μL of fraction was mixed with 20 μL of Tris buffer.

where all six coordination sites are occupied, or sterically shielded, as in the heme moiety of reduced cytochrome c (not shown), was inactive. When EDTA was replaced by either ATP or citrate, which may act as natural chelators of Fe^{2+} (72), the activation of AACT/HMGS was relatively efficient (Fig. 3). When ferrous ions were prevented from becoming oxidized by the presence of excess ascorbic acid (a natural reductant), an even better stimulation of *in vitro* activity occurred (Fig. 5). Hydroxyurea that is known to scavenge radicals (73), inhibited the activation by ferrous ions of gel-filtrated AACT/HMGS, but it was slightly inhibitory to the enzyme(s), even in the absence of Fe^{2+} (Table 2).

Molecular properties of radish HMGR. When HMGR

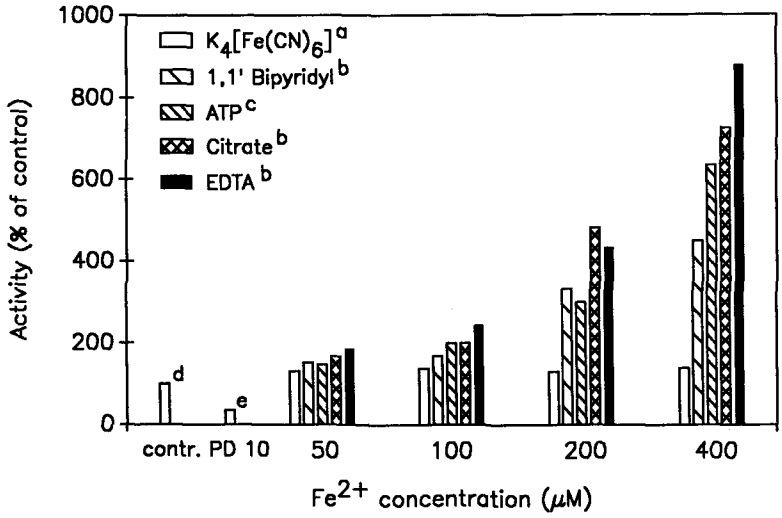


FIG. 3. Effect of ferrous ions on the *in vitro* activity of AACT/HMGS. a) Enzyme solution (15 μL) purified as summarized in Table 1 (control); or b) additionally stripped off from low molecular-weight components by a passage through a PD-10 column, was used per assay in a final volume of 50 μL (35 μL Tris buffer system, 0.2 M, pH 7.5). The sequence of mixing solutions was always kept identical (see legend to Fig. 1). After a preincubation of 2 min at 30°C, the reaction was started by addition of 10 μL start mix containing [1-^{14}C]acetyl-CoA (final concentration, 15 μM). After 5 min at 30°C, the reaction was terminated by addition of 6N HCl. a) No additional Fe^{2+} in the assay; b) concentration of chelator was two times [Fe^{2+}]; c) concentration of chelator was four times [Fe^{2+}]; d) no other reagent in the assay; e) after gel filtration (100 mM K_2PO_4). Stock solutions: Fe^{2+} in H_2O, pH 2; EDTA, $K_4[Fe(CN)_6]$, citrate, ATP in H_2O, pH 7; bipyridyl in 50% (v/v) MeOH (max. concentration in the assay: < 1%).

was detergent-solubilized from radish membranes and purified further (28), a band of 45 kDa was detected in the peak fractions from the last purification step when analyzed by SDS-PAGE. Sucrose-density centrifugation suggested a molecular weight of 180 kDa for the active, non-denatured enzyme. Thus, a tetramer of 45 kDa subunits was suggested to represent the functional entity (28). However, in the first peak fraction we also observed an additional band of less than 66 kDa, which was first interpreted as a typical contaminant appearing in SDS gels loaded with mercaptoethanol-treated protein samples (cf. 74). We have characterized a full-length cDNA clone, referred to as λcRS3 (39), from a newly established radish cDNA library. Northern blot analysis of poly(A)$^+$RNA isolated from etiolated and light-grown radish seedlings revealed a total length of 2.3 kb for the primary transcript (39). The cDNA consists of a 5′-noncoding region of 45 nucleotides, an open reading frame of 1749 bases, and a 3′-untranslated region with two potential polyadenylation signals. λcRS3 codes for a polypeptide of 583 amino acids with a molecular mass of 62,720 Da. The hydropathy profile of the amino-acid sequence (Fig. 6) suggests the presence of two membrane-spanning domains within the N-terminal 165 amino acids (residues 33 to 57, and 68 to 104) as was found for HMGR(s) from *Arabidopsis* (38), pea (40) and tomato (J.O. Narita and W. Gruissem, personal communication). These domains and the spacer region in between are highly conserved between the *Arabidopsis* and radish HMGR (Fig. 7). The linker region (residues 105 to 165) that joins the N- and C-terminal parts of radish HMGR contains a proline-rich "PEST" sequence (cf. 75), which is slightly longer than in *Arabidopsis* and is less conserved. The C-terminal part of radish HMGR (residues 165 to 571), where the catalytic site resides, is highly conserved in all plant and other eukaryotic HMGR genes sequenced so far (36–40,76–82), indicating a stringent evolutionary pressure onto this region (Fig. 8). Since proline-rich sequences serve as recognition sites for certain proteases, a cleavage product presumably has given rise to the formation of the 45 kDa fragment seen on the SDS gel, although the solubilization efficiency was not affected in the presence of protease inhibitors (28,83). For the HMGR isolated from latex of *Hevea brasiliensis*, by application of essentially the same protocol as was developed for radish, a subunit molecular mass of 44 kDa was determined by the aid of SDS-PAGE and, as deduced from native PAGE, a functional size of 176 kDa (29). The band of higher molecular mass as detected in the SDS gel loaded with purified HMGR from radish was most probably not caused by contamination, as was discussed earlier (28), but more likely represented undegraded, native HMGR protein.

DISCUSSION

Synthesis and metabolism of HMG-CoA. In mammalian systems it is well documented that regulation of HMGR is mediated by rapid processes both at the translational and the post-translational level (cf. refs. 4–6). HMGS appears to be regulated in parallel (84–92). Similar processes can be expected to occur in plant cells. A thorough study using plant tissue requires a profound knowledge of the properties of the enzymes involved in the formation of HMG-CoA.

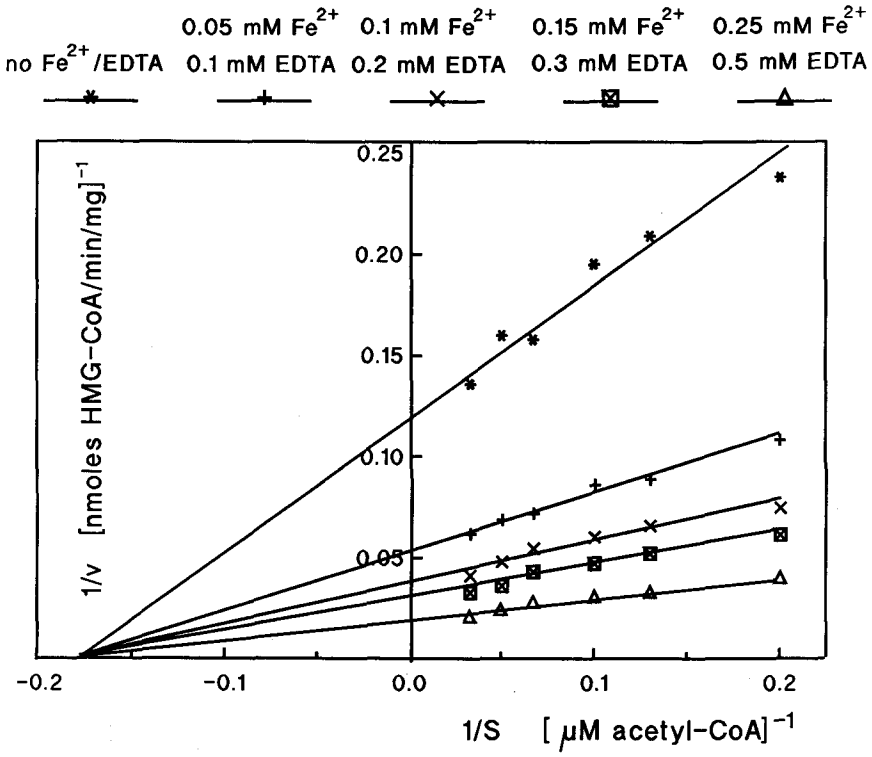


FIG. 4. Effect of ferrous ions on the apparent kinetic properties of partially purified AACT/HMGS. AACT/HMGS was purified as indicated in Table 1, followed by gel filtration on PD-10. Substrate was varied as indicated (isotope dilution). All other conditions were as described in the previous legends. The apparent K_m with respect to acetyl-CoA was calculated to be 6 μM.

TABLE 2

The Effect of Hydroxyurea on Fe^{2+}-Induced Activation of AACT/HMGS[a]

Treatment	[^{14}C]HMG-CoA synthesized[b] (dpm)	[^{14}C]HMG-CoA synthesized[c] (dpm)
Control	2,293 (100%)	12,779 (100 %)
+ 10 mM Hydroxyurea	1,839 (80.2%)	9,502 (74.4%)
+20 mM	1,607 (70.1%)	5,593 (43.8%)
+ 50 mM	1,602 (69.8%)	3,955 (30.9%)

[a]AACT/HMGS was solubilized and purified as indicated in Table 1, followed by gel filtration on a PD-10 column.

[b]Activity was determined in the absence of Fe^{2+}-EDTA.

[c]Activity was determined in the presence of 400 μM Fe^{2+} and 800 μM EDTA.

Our data (3,38) imply the formation of a tightly fitted complex between AACT and HMGS. The acetoacetyl-CoA arising from the AACT reaction was immediately transferred to the active site of the consecutive enzyme HMGS without any substantial diffusion into or exchange with the medium (3,38). We have some further evidence for this hypothesis since the antibiotic F-244 is completely inactive in our system (3). F-244 has been described as a highly specific inhibitor of HMGS both from yeast (93) and rat liver (94), with K_i values in the range of 100 nM. Through the formation of this enzyme complex, the active site of HMGS may somehow be shielded, preventing the binding of the inhibitor, but otherwise facilitating the direct transfer of acetoacetyl-CoA to enter the HMGS catalyzed reaction. In view of the numerous enzymes competing for the central intermediate acetyl-CoA (cf. 1), a directed flux of carbon units into the isoprenoid pathway makes sense. However, despite the differences in the reaction mechanisms in both condensations leading from acetyl-CoA to HMG-CoA, *viz.* Claisen *vs.* aldol condensation, it cannot be excluded that a single polypeptide catalyzes both partial reactions.

Separation of HMGL from AACT/HMGS has been achieved, *e.g.*, by means of free-solution IEF, where the enzyme appears to band at high activity with an apparent pI value of 7.0 (3,71), or by ion exchange chromatography. Considerable activity of HMGL is associated with the same heavy-membrane pellet (P 16,000) like AACT/HMGS (cf. 3,38). HMGL behaves rather unstably and loses ac-

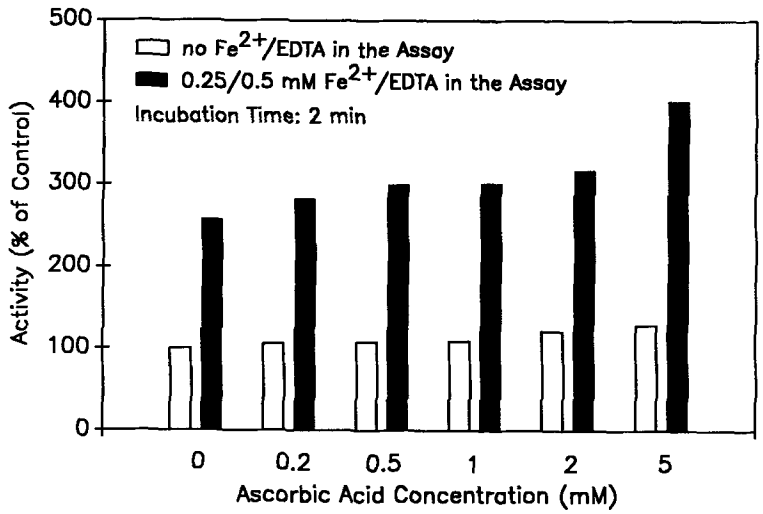


FIG. 5. Effect of ascorbic acid on the efficiency of AACT/HMGS activation by Fe^{2+}-EDTA. AACT/HMGS was purified as indicated in previous legends. The assay volume was 70 μL (30 μL of enzyme solution, 30 μL of Tris buffer system). Cofactors were freshly added to this buffer system (sequence: EDTA, ascorbate, Fe^{2+}). After preincubation at 30°C for 2 min the reaction was started by addition of 10 μL start mix (final concentration of 1-acetyl-CoA, 25 μM. Control 100%): without ascorbate (stock solution: 40 mM, pH 7.0) and Fe^{2+}-EDTA.

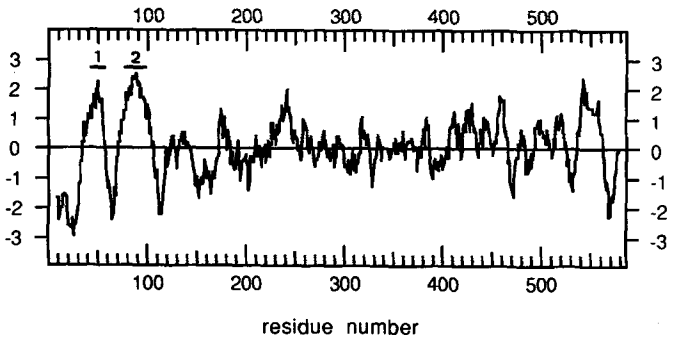


FIG. 6. Hydropathy index plot of radish HMGR. The algorithm of Kyte and Doolittle (70) was used with a window size of nine residues. Positive values indicate hydrophobic regions. Bars 1 and 2 indicate putative *trans*-membrane regions.

```
RS : DQPRTKASDALPLPLYLTNAVFFTLFFSVAYYLLRRWRDK
AT1: IAPPP.............................H.....
AT2: DP.LD.............WF.LS...ATV.F..S...E.
PSa: -KNEKHD.QSQ.-S.........G.......F......E.

     IRHSTPLHVMTITELGAILALVASFIYLLGFFGIDFV-QS
     ..YN.....V........I..I..............-..
     ..NS......DLS.IC.L.GFV.........C...LIFR.
     ..TS.....L..S.TV.LLS....IF..MA..S.S..LHP

     F-ISRADTNNEFE
     .-....-SGDAWD
     SSDDDVWVN.GMI
     .PV...VQI.EE.
```

FIG. 7. Amino-acid sequence alignment of the putative transmembrane regions of plant HMGRs. RS: *Raphanus sativus, HMG1*; AT1, AT2: *Arabidopsis thaliana*, corresponding to *HMG1* and *HMG2* (data from ref. 37,38,40); PSa: *Phaseolus vulgaris, HMG1*. Dots indicate residues identical to radish enzyme.

tivity within a few hr, even at 4°C, but can be kept active when stored frozen at −20°C. In our assay system, the radish HMGL had a pH optimum in the alkaline region (3), similar to values reported for the bovine enzyme (95,96); however, under such conditions the influence of artifact formation from the instability of thioesters cannot be excluded. For that reason, the activity of plant HMGL was routinely assayed at pH 8.0 (3). HMGL is rapidly inactivated above 30°C and requires the presence of Mg^{2+}. If rather crude fractions are examined, interference of HMGL with the HMGR assay can be excluded by incubation at 37°C in the presence of EDTA (standard conditions of the HMGR assay). Total cellular HMGL activity far exceeds that of HMGR. However, *in vivo* the interference of both enzymes competing for the same substrate might be prevented by differential compartmentalization, a subject of future research.

Gel filtration of solubilized and partially purified radish HMGL on Sephadex G-25 or G-10 to remove low molecular weight molecules resulted in inactivation (3). A series of cations have been tested for their ability to restore the initial activity. Mg^{2+}, Co^{2+}, Mn^{2+}, and Fe^{2+} were similarly effective in stimulating HMGL activity, whereas trivalent cations were inactive (3). However, it cannot be excluded that cations such as Ca^{2+} remain bound to the enzyme even after passage through a gel filtration column. Higgins *et al.* (97) compared the data obtained for several mammals and tissues as an enzyme source and assumed the enzyme to contain bound Ca^{2+}, which was not affected by chelators above pH 8.

The effects of cations have been studied in great detail for AACT/HMGS. Although required for some basal activity, Mg^{2+} alone stimulated enzyme activity *in vitro* only slightly. This was demonstrated when the enzyme system that was purified about 240-fold was additionally

```
RS1: SRFARLQSVKCTLAGKNAYVRFCCSTGDAMGMNMVSKGVQNVLEFLTDDF
AT1: ............I.............................Y.....
PSa: .......NI.A.I....L.I..T....................D..QS..
PSb: ......LGIQ.AM....L.M..T....................D..RN..
PSc: ........IQPAI....L.I..R....................D..QN..
Tom: .......GIQ.AI....L.M..S....................DY.QSEY

     PDMDVIGISGNFSSDKKPAAVNWIEGRGKSVVCEAVIRGEIVNKVLKTSV
     ............C.....................................
     ............C....A...................KE.V.K.....N.
     ...........YCA.......................KE...K.......
     F..E........C....A...................KE.V.Q.....N.
     ............C......................I.KEDV.K.....E.

     AALVELNMLKNLTGSAVAGSLGGFNAHASNIVSAVFIATGQDPAQNVELS
     ............A.....................................S.
     ES..............M..A..............IYL............S.
     ..............A...I..A..............I...........I.S.
     E...............I..A..............I.L...........S.
     ................M..A..............YL............S.

     QCITMMEAVNDGKDIHVSVTMPSIEVGTVGGGTQLASQSACLNLLGVKGA
     ........I.......I................................
     H.M...........L...................E...............
     H.............L.I.................................S
     H.............L.I.................................
     H.............L.I.................................

     STESPGMNSRTLATIVAGAVLAGELSLMSAIAAGQLVKSHMKYNRSSRDI
     ........A.R............................R...........
     .K....A...Q..I...AS.............................K.V
     .K....S...L.......S...................N........CK.V
     .KD...A...L.......S.................................
     NRDA..S.A.L.......S............S...........

     SGAMTATTT
     ...T.T...TT
     TKIAS
     TKISS
     .KVVS
```

FIG. 8. Amino-acid sequence alignment of the C-terminal region of plant HMGRs. RS: Radish (*HMG1*); AT: *Arabidopsis thaliana* (*HMG1*) (data from refs. 37,38,40); PSa, PSb, PSc: Pea, 3 related HMGR isozymes (data from ref. 40); Tom: tomato (*HMG1*) (data from ref. 36).

filtered through PD-10 columns previously equilibrated with 100 mM phosphate buffer without any Mg^{2+}. In previous experiments, it was shown that AACT/HMGS can be separated from HMGL by free-solution IEF, forming a peak at a rather acidic pH. The reason for this apparent acidic pI value may be due to complex formation of the enzyme system with phospholipids that are detergent-solubilized in parallel. Under such conditions AACT/HMGS lost most of its activity. A series of cations were tested for their ability to restore activity, with Fe^{2+} being the most effective (3). The even higher activity observed in the presence of a double concentration of EDTA (at pH 7.5 to 8.0) can be readily explained by stabilization of Fe^{2+} which, as a free cation, is rapidly oxidized to Fe^{3+} and does not stimulate enzyme activity. Which amino acids of the protein(s) are involved in the reaction of Fe^{2+}, presumably histidine residues (cf. 98), is not yet known.

When Fe^{2+} was added as a complex where all six coordination sites are occupied (*e.g.*, with CN^-) the enzyme

system was not activated. There remains the possibility that Fe^{2+} is involved in the formation of an organic free radical, as it is essential for ribonucleotide reductase activity (cf. 73, and literature cited therein). Addition of hydroxyurea, which is capable of scavenging such radicals, partially prevented activation of AACT/HMGS (Table 2).

Other bivalent cations such as Co^{2+}, Mn^{2+}, Sn^{2+} or Ni^{2+}, which are more stable than Fe^{2+}, were much less effective (3). When the ferrous ions were prevented from being oxidized because of the presence of ascorbic acid, activation of AACT/HMGS was increased further. Iodoacetamide capable of alkylating thiol groups did not drastically affect catalytic efficiency. A mechanism that only involves the shielding by Fe^{2+}-EDTA of reactive thiol groups from becoming oxidized seems unlikely. It was reported that Fe^{2+}-EDTA, but not unchelated ferrous and ferric ions, efficiently inhibited porcine brain lipoamidase, possibly due to interaction with the active center (99). Anaerobically induced NAD-linked glycerol dehydrogenase of *Klebsiella pneumoniae* was inactivated in the presence of O_2, Fe^{2+} and ascorbate (100). This was interpreted to mean that O_2 and ascorbate generated H_2O_2 and that H_2O_2 reacted with Fe^{2+} to produce an activated species of oxygen, which attacked the enzyme (100). In the cell Fe^{2+} possibly forms complexes with ATP or citrate (101). These complexes, capable of generating oxygen radicals, might lock plant AACT/HMGS into a conformation that results in a higher catalytic efficiency, *viz.* in an increased turnover rate, whereas the apparent affinity towards the substrate is not affected; V_{max}, but not k_m, changes. A doubling of apparent activity can already be achieved at concentrations less than 50 μM Fe^{2+}, which is well within physiological range.

Cloning and characterization of radish HMGR. Recently, by the aid of a cDNA encoding hamster HMGR (76), it has been possible to isolate a corresponding gene from *Arabidopsis thaliana* (38). The isolation of the corresponding gene from radish has been facilitated because of the close phylogenetic relationship between *Raphanus sativus* and *A. thaliana*, both belonging to the same family of *Brassicaceae*. The radish HMGR was the first enzyme that was purified from a plant source; its kinetic properties (28,83) and many aspects of regulation are already well known (15,16,19). That we have indeed cloned a radish HMGR gene has been proven through overexpression in *E. coli* of a truncated, but highly active protein referred to as HMGR45, comprising the soluble domain where the catalytic site resides (60). Because of a unique *Eco*RV restriction site close to the initial methionine codon, only the full-length radish cDNA clone (λcRS3), but not the corresponding *Arabidopsis* cDNA clone could be used for the construction of a plasmid and overexpression of a slightly truncated protein in *E. coli*, comprising nearly the entire amino-acid sequence (583 − 1) of the holoenzyme (60). The protein thus produced was, however, insoluble and catalytically inactive. This can be readily explained by the postulated presence of two membrane-spanning domains at the N-terminal end of the intact enzyme as suggested by the hydropathy profile. Incorrect folding of nascent protein chains and hydrophobic interactions within the non-bacterial protein molecule or between different molecules can lead to the formation of precipitates (inclusion bodies) without activity (cf. 102).

As far as we know, plant HMGRs contain only two membrane-spanning domains, whereas other eukaryotic HMGRs usually have seven and more (76–82). In mammalian cells the presence of the part anchored in the membrane is necessary for intact regulation (77,103,104). Soluble, truncated HMGR, lacking this moiety and reintroduced by transfection into a mutant cell line having lost HMGR is not feedback-regulated (103). In the yeast system, ultrastructural changes, formation of karmellae (105), can only be observed when HMGR arising from the *HMG1*, but not from the second *HMG2* gene, is overproduced. There is no experimental data available as yet that would ascribe a similar regulatory role to the membrane domain of plant HMGR.

Whereas animal cells apparently contain only one HMGR gene (76–78,80–82), yeast contains two related, but rather divergent genes referred to as *HMG1* and *HMG2* (79). From the cDNA library established from poly(A)$^+$RNA isolated from etiolated radish seedlings, we have isolated several positive clones (39). λcRS2, only about 1.6 kb in length, has a different restriction pattern than λcRS3, which has been completely sequenced. Thus, in radish seedlings at least two genes are actively expressed. In *Arabidopsis* two genes have been identified (38,40), which are also referred to as *HMG1* and *HMG2*. Since *HMG2* is apparently expressed at a very low level and only in certain parts of seedlings, in contrast to *HMG2*, its complete sequence has been determined from genomic, but not from cDNA clones (Enjuto, M., and Boronat, A., unpublished observations). In tomato, only a single HMGR gene had been initially isolated (36); however, a second HMGR gene also has been identified (Narita, J.O., and Gruissem, W., personal communication). Based on the presumably variable localization of HMGR isozymes in the plant cell, the presence of three HMGR genes has been postulated (1). Recently it has been shown that in the pea three closely related genes appear to be active (40).

The expression system we have used to produce radish HMGR45 in *E. coli* (60) should facilitate isolation of HMGR protein from other cDNA clones for identification of kinetic and molecular properties. Since this enzyme can be considered a promising target for plant growth regulators (1,33,34,106), such as the antibiotic mevinolin (lovastatin) and its natural and synthetic analogues (cf. 7), structural modeling requiring the crystallization of plant HMGR could be envisaged. It is possible to obtain greater than 95% pure radish HMGR45 by just two purification steps (Bach, T.J., unpublished observations).

Clear information on the differences of HMGR isozymes may finally provide us with the clue as to the intracellular location, and of the expression of the corresponding genes in space and time. Antibodies that are raised against oligopeptides comprising non-homologous parts of the isozymes could be used for immuno-cytochemical studies. The ultimate aim must be to isolate the genes coding for AACT, HMGS, HMGL and for enzymes beyond the HMGR step, to get a better understanding of how MVA biosynthesis and utilization is regulated in plants.

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On the Structure, Biosynthesis, Function and Phylogeny of Isoarborinol and Motiol

W. David Nes[a,*], Rosalind Y. Wong[b], Jane F. Griffin[c] and William L. Duax[c]

[a]Plant and Fungal Lipid Research, Microbial Products Research Unit, Richard B. Russell Research Center, Athens, Georgia 30613, [b]Western Regional Research Center, U.S. Department of Agriculture, Albany, California 94710 and [c]Medical Foundation of Buffalo, Buffalo, New York 14203

The solid-state conformations of the C-3 acetates of two isomeric hopanoids — 1, isoarborinol (D:C-friedo-B[1]:A[1]-3β,5α,8α,10β,13β,14α,17β,18α, 21β) and 2, motiol (D:C-friedo-B[1]:A[1]-neogammacer-7(8)-en-3β-ol[3β,5α,9α,10β,13α,14β,17-α,18β,21α])—have been determined by X-ray crystallography. The data show that whereas both molecules are planar, 1 orients into a chair-halfchair-chair-chair-halfchair conformation while 2 orients into a chair-sofa-twist-half-chair-halfchair conformation. To explain the biogenesis of 1 and 2 from squalene oxide, a step-wise mechanism is proposed which proceeds through the protosteroid cation (for 1) and dammarenyl cation (for 2). After ring enlargement from the corresponding 13(17)bond followed by concerted 1,2-migrations and loss of the 11β-H and 7β-H as protons, respectively, a 9,11-double bond (in 1) and a 7,8-double bond (in 2) is introduced into the nucleus. The mechanism is discussed in relation to the classical view of a non-stop cyclization process where, for example, squalene oxide folds in a chair-chair-chair-chair-boat conformation to give a cyclized product (motiol) presumably with the same conformational disposition as the cyclizing material. The three-dimensional geometry of 1 and 2 was found to be structurally dissimilar from sterols. For instance, 1 and 2 are shorter and volumetrically smaller molecules than cholesterol, and this may explain their diminished importance as membrane inserts compared with sterols in eukaryote evolution.
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Pentacyclic triterpenoids (PT) are a class of 30-carbon isopentenoid (isoprenoid) natural products which function in a manner analogous to sterols as structural components of membranes (1–3). Pentacyclic triterpenoids, specifically the hopanoids, are thought to have predated sterols in evolution perhaps having arisen with the primal prokaryotes in the precambrian anaerobic environments *via* the cyclization of squalene (4,5). As sufficient molecular oxygen became available for the genesis of squalene oxide, several new classes of triterpenoids, *e.g.*, pentacycles (hopanoids) and tetracycles (steroids), were synthesized by prokaryotes (1,3). Apparently, the genetic information for the varied cyclases was carried into the algae (6). However, selection mechanisms evolved during eukaryote development which caused PT production to become ontogenetically delayed relative to sterol synthesis in fungi (7) and vascular plants (8) suggesting a secondary metabolite role for pentacyclic triterpenoids. In the metazoan line, selection pressures led ultimately to their eradication (9).

The utility of developmental regulation of the isopentenoid pathway is not entirely clear, especially when we are led to believe that in their molecular dimensions, rigidity and amphiphilic properties, hopanoids are structural equivalents of sterols, thus suggesting functional equivalence as well (2,4,5). In search for an explanation to this anomaly, we were guided by two rather opposing ideas: architectural parity existed between PT and sterols so that random mutations which directed altered biosynthesis in bacteria led to the production of PT mimics (sterols) which replaced PT as membrane inserts. Because of this parity (functional equivalence), as mutations in PT formation occurred, life continued so long as compensatory mutations accrued to insure the formation of PT-like compounds, *e.g.*, sterols in eukaryotes. If this was so, then PTs should readily replace sterols as membrane bulk components in prokaryotes and eukaryotes. In support of this view, mycoplasmas are known to use PTs and sterols equally well as growth-support agents (10,11). However, this is not the case in fungal sterol auxotrophs. As we have recently demonstrated, PTs fail to serve in the bulk role at certain stages of the life cycle of either of two sterol auxotrophic fungi — in growth of *Saccharomyces cerevisiae* (8) or in oosporogenesis in *Phytophthora cactorum* (ref. 8, and refs. cited therein). The other possibility is that sterols and PTs are structurally and stereochemically dissimilar. The variations, while also arising by mutation, are then actively, rather than passively retained because they serve a purpose *e.g.*, in one or another temporally expressed function during development. If this is so, then we might expect that, in contrast to what is generally thought (2,4,5), sterols (which came after PTs in evolution) are alternatives to, rather than replacements of PTs. As such, they should possess distinguishing structural characteristics which can be shown to increase their fitness compared with PTs to promote survival and speciation.

In an attempt to shed further light on PT and sterol phylogenesis, we have examined by X-ray crystallography the three-dimensional structure of two PTs which may be considered geohopanoids (4) and which occur throughout the plant kingdom (3,9).

MATERIALS AND METHODS

Crystal and refinement data. X-ray crystallography was performed at the Western Regional Research Center (Albany, CA). $C_{32}H_{52}O_2$, isoarborinol acetate and [in brackets] motiol acetate: M = 468.9, monoclinic [orthorhombic], space group $P2_1$ (C_2^2, No. 4) [$P2_12_12_1$ (D_2^4, No. 19)], a = 11.039(2) [7.587(1)], b = 7.498(1) [17.792(6)], c = 17.451(4) [20.758(4)] (Å), β = 106.63(1) [90.0]°, V = 1384 [2802] Å^3, D_c = 1.12 [1.11] g cm^{-3}, Z = 2 [4], F(000) = 520 [1040], μ(Cu-K$_\alpha$) = 4.79 [4.73] cm^{-1}, R = 0.045 [0.080], R_w = 0.051 [0.079] for 2226 [2154] unique reflections with $|F_0| \geq 3\sigma|F_0|$ in the range of $3° \leq 2\theta \leq 114°$. Tables of anisotropic thermal parameters with their estimated standard deviations (e.s.d.) for the non-hydrogen atoms, positional and thermal parameters for the hydrogen atoms, and observed-calculated structure factors have

*To whom correspondence should be addressed.

Abbreviations: PT, pentacyclic triterpenoid; ^{1}H NMR, proton nuclear magnetic resonance spectroscopy.

been deposited at the Cambridge Crystallographic Data Centre.

Data collection and structure refinement. Intensity data were collected on a Nicolet R3 diffractometer with graphite monochromatized Cu-Kα radiation (λ = 1.5418Å) by the θ-2θ scan technique with variable scan speed (4-30°) min^{-1} at room temperature. The intensity data were corrected for background, Lorentz-polarization effects (Nicolet XTL Operation Manual (1980), Nicolet Analytical Instruments, Inc., Cupertino, CA 95014, but not for absorption or secondary extinction. The crystal structures were solved by direct methods and refined by a 'blocked-cascade' full-matrix least-squares procedure with the SHELXTL (12) program package. The function minimized was $\Sigma\omega(|F_o|-|F_c|)^2$, where $\omega = [\sigma^2|F_o|^2+0.001|F_o|^2]^{-1}$. Scattering factors were from *International Tables for X-ray Crystallography* (13); those of oxygen were corrected for anomalous dispersion. Atomic parameters of all non-hydrogen atoms were refined anisotropically, and all hydrogen positions were estimated but verified in subsequent difference Fourier maps are included with invariant idealized values in the respective structure-factor calculation.

Numbering system. The numbering system for the atoms in the arborane and fernane skeletons shown in Figure 1 follows IUPAC rules whereas the carbon atoms assigned numbers in the X-ray crystallographic study are of an arbitrary system. When discussion regarding hopanoid biosynthesis and function is presented, reference is to the numbering system shown in Figure 1.

RESULTS AND DISCUSSION

Sorghum bicolor leaves were the source of isoarborinol and motiol. In the leaves, two additional isomeric hopanoids are found. The isolation and separation methods, and chromatographic and spectral properties of the four hopanoids were given in an earlier report (14). Based on a proton nuclear magnetic resonance (^{1}H NMR) study of the sorghum hopanoids, we thought the plant synthesized a series of stereoisomers. Shortly after our study appeared, two Japanese groups provided an extensive tabulation of the ^{1}H NMR chemical shifts for numerous PTs including hopanoids (15,16). The data given by us for fernenol agreed with their data but our data for sorghumol, trematol and isoarborinol agreed with their data for isoarborinol, motiol (fern-7(8)-enol) and isomotiol (fern-8(9)-enol), respectively. The X-ray crystallographic studies which we now have obtained on what we thought was sorghumol and trematol are in fact of isoarborinol and motiol, respectively. Since we based our identification of trematol from mass spectral and ^{1}H NMR data given in the literature (17), we conclude 21-epifernenol has not been found in nature, although in the 3-desoxy series there is some evidence for its occurrence in ferns (18).

The atomic coordinates for isoarborinol acetate (1) and motiol acetate (2) are shown in Table 1. The torsion angles for 1 and 2 are shown in Figure 2, and the molecular conformations of 1 and 2 with their thermal motions and numbering scheme used in the X-ray investigation are shown in Figure 3. The average bond distances and angles

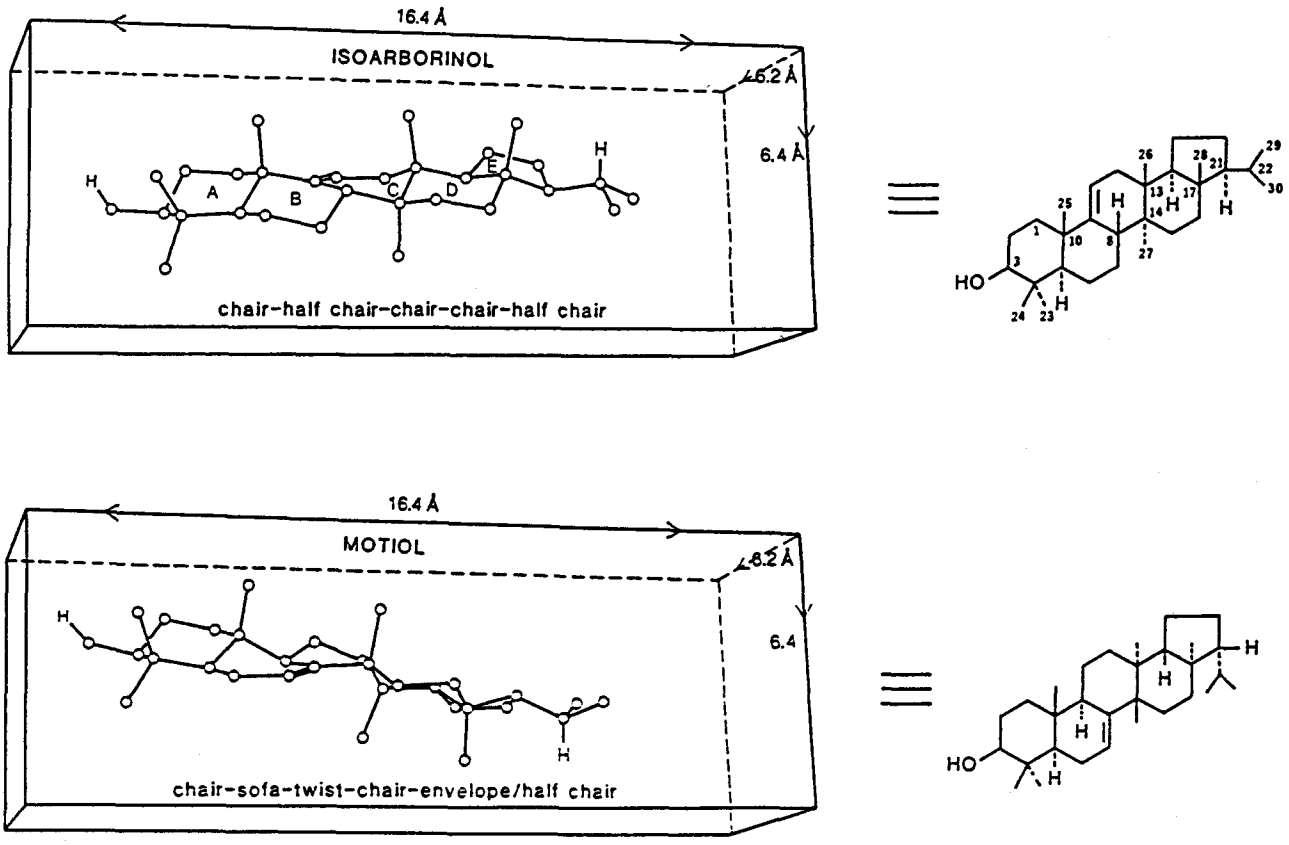


FIG. 1. Approximate molecular dimensions of isoarborinol and motiol as determined by X-ray crystallography.

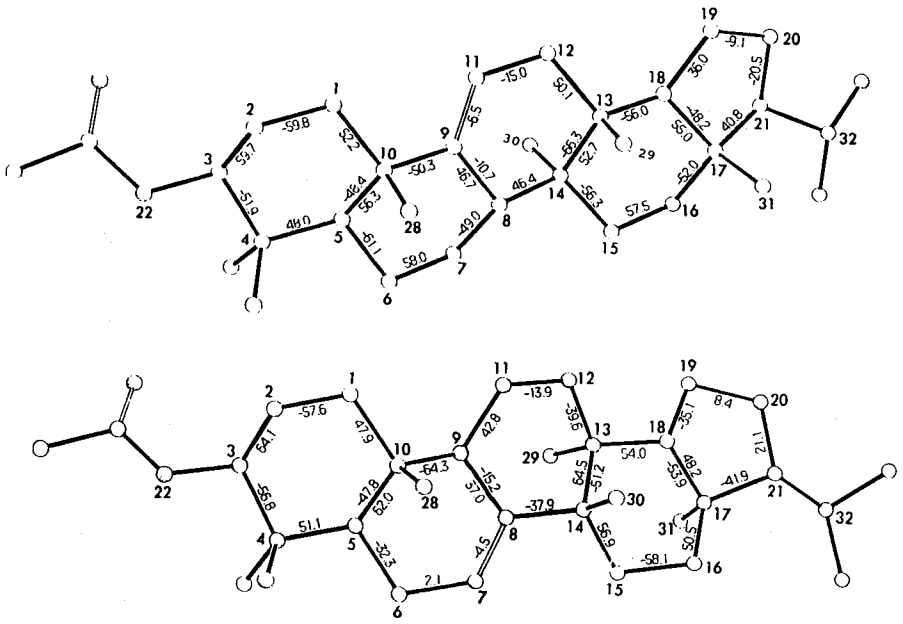


FIG. 2. Endocyclic torsion angles for isoarborinol acetate (top) and motiol acetate (bottom).

TABLE 1

Atomic Coordinates ($\times 10^4$) for Isoarborinol (1) and Motiol (2) Acetates

	1			2		
Atom	x	y	z	x	y	z
C-1	2424(3)	3670(4)	3655(2)	1590(8)	4566(3)	1382(3)
C-2	2333(3)	3629(5)	2764(2)	1625(9)	5409(4)	1453(3)
C-3	1249(3)	4791(4)	2297(2)	307(8)	5785(3)	1020(3)
C-4	1378(3)	6763(5)	2560(2)	−1604(9)	5581(3)	1191(3)
C-5	1620(3)	6794(4)	3492(2)	−1696(8)	4700(3)	1153(3)
C-6	1781(3)	8667(5)	3841(2)	−3573(8)	4391(3)	1280(4)
C-7	1621(3)	8651(4)	4682(2)	−3635(9)	3553(4)	1389(3)
C-8	2557(3)	7400(4)	5254(2)	−2215(8)	3097(4)	1400(3)
C-9	2567(2)	5565(4)	4876(2)	−437(7)	3431(3)	1247(3)
C-10	2646(3)	5526(4)	4019(2)	−283(8)	4239(4)	1526(3)
C-11	2611(3)	4086(4)	5312(2)	1090(8)	2897(4)	1444(4)
C-12	2782(4)	4031(4)	6192(2)	815(9)	2070(4)	1279(4)
C-13	3250(3)	5817(4)	6595(2)	−1047(7)	1819(3)	1090(3)
C-14	2350(2)	7309(4)	6095(2)	−2379(8)	2259(4)	1551(3)
C-15	2654(3)	9091(4)	6538(2)	−4228(8)	1940(3)	1474(3)
C-16	2557(3)	9049(4)	7411(2)	−4368(9)	1077(4)	1609(3)
C-17	3419(3)	7640(4)	7921(2)	−3149(8)	622(4)	1167(3)
C-18	3142(3)	5857(4)	7462(2)	−1303(7)	984(3)	1215(3)
C-19	3840(4)	4459(5)	8062(2)	−101(10)	424(4)	852(4)
C-20	3680(4)	5175(5)	8850(2)	−918(9)	−351(4)	1017(4)
C-21	3110(3)	7050(5)	8700(2)	−2693(8)	−191(3)	1374(3)
O-22	1174(2)	4749(3)	1453(1)	485(6)	6590(2)	1071(2)
C-23	665(3)	3281(5)	1041(2)	1633(11)	6912(4)	666(4)
O-24	209(3)	2088(4)	1312(1)	2570(9)	6589(3)	296(3)
C-25	776(4)	3388(6)	208(2)	1576(12)	7749(4)	754(4)
C-26	100(3)	7671(5)	2176(2)	−2782(10)	5911(4)	671(3)
C-27	2392(3)	7721(5)	2277(2)	−2154(10)	5921(4)	1839(3)
C-28	4030(3)	6103(5)	4070(2)	−580(9)	4239(4)	2258(3)
C-29	4613(3)	6068(5)	6566(2)	−1408(10)	2047(4)	385(3)
C-30	940(3)	6878(5)	6000(2)	−1879(9)	2187(4)	2270(3)
C-31	4795(3)	8286(5)	8133(2)	−3901(10)	595(4)	487(3)
C-32	3515(3)	8241(5)	9457(2)	−4031(9)	−829(3)	1282(3)
C-33	2972(4)	10095(6)	9324(2)	−5723(10)	−680(4)	1666(4)
C-34	3169(4)	7383(6)	10150(2)	−3256(12)	−1575(4)	1488(4)

for 1 are similar with those found in the structure 2α-bromoarbinone which was earlier examined by X-ray crystallography (19). The C–C distances in the nucleus range from 1.505 to 1.582 in 1 and from 1.505 to 1.598 in 2.

From Figure 1, it can be seen that 1 and 2 possess the same all-*trans*-anti-stereochemistry in the ring systems, a similar planarity and similar approximate molecular dimensions, as the free alcohols—164. × 6.2 × 6.4 Å. Examination of the torsion angles indicated that the rings of 1 orient into a chair-halfchair-chair-chair-halfchair conformation whereas the rings of 2 orient into a chair-sofa-twist-chair-halfchair conformation. The major conformational difference between 1 and 2 comes from the difference in the torsion angles shown in Figure 4 and indicates that ring C is distorted part way toward the twist (boat) conformation in 2. This results in a slight convex bending of the skeleton towards the α-side of the molecule. As also shown in Figure 1, atoms C-13 and C-14 in both molecules are appreciably out of the mean plane through the C-ring and point toward the opposite directions. Atoms C-13 and C-14 are displaced from their mean ring plane by +0.38 and −0.35Å in 1 and −0.41 and +0.40Å in 2, respectively. Presumably, the difference of the orientation in C-13, C-14, C-29 and C-30 on the C-ring accounts for the deviation of the relative positions and conformations of the D- and E-rings in both molecules. The comparative dihedral angle data between the plane normals of all the ring units in both structures are given in Table 2. The sofa-twist-chair (B-, C- and D-rings) arrangement in 2, a migrated hopanoid, is the favored conformation of lowest energy, although the molecule remains thermodynamically unstable and may undergo acid-induced molecular rearrangements to the more stable hopanoid skeleton, as in the rearrangement of friedelin to the amyrin skeleton or of the euphoid to the dammarenoid skeleton (20,21). Alternatively, due to the all-chair system present in isoarborinol acid treatment simply moves the double bond from the C-ring into the B-ring as in the acid-induced

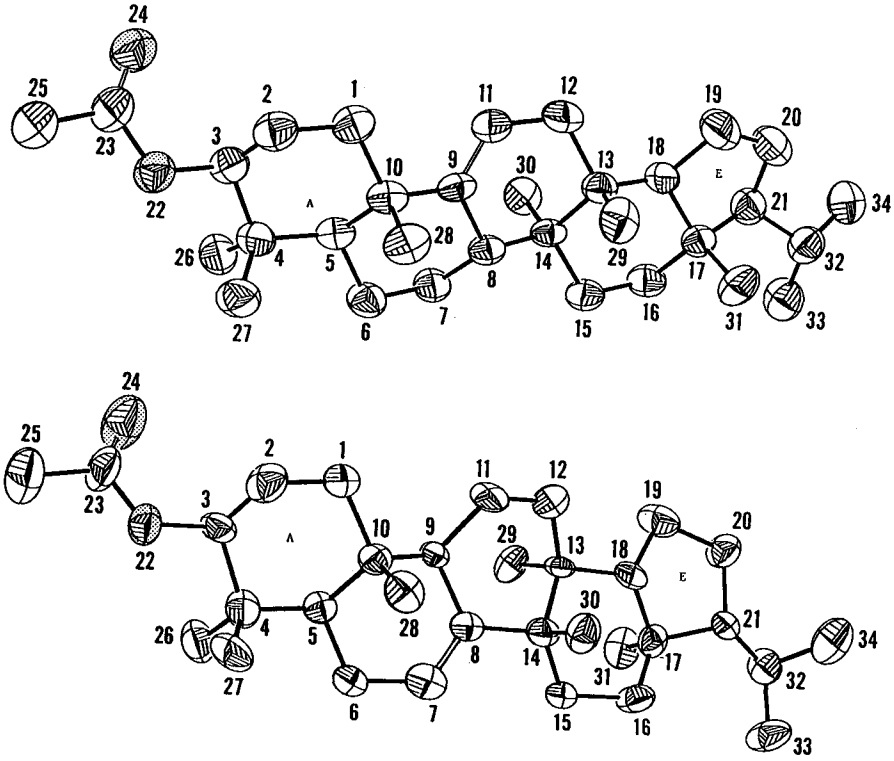


FIG. 3. Perspective view of isoarborinol acetate (top) and motiol acetate (bottom) with the crystallographic numbering system. The thermal ellipsoids are drawn at 50% probability level and the shaded ellipsoids represent oxygen (O-22 and O-24).

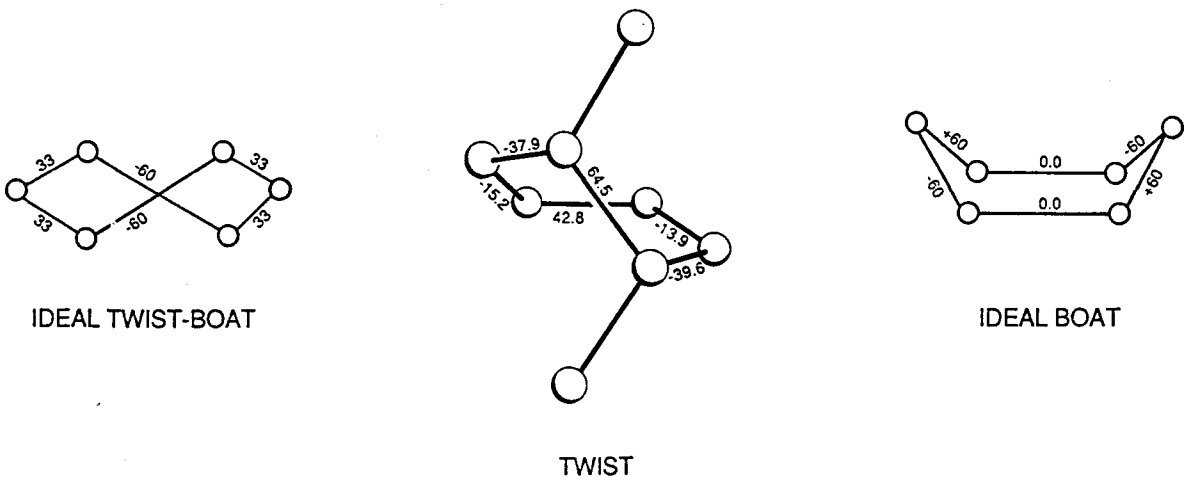


FIG. 4. Torsion angles of ideal twist-boat, ideal boat and the experimentally determined values in motiol ring C.

rearrangement of Δ^8- to Δ^7-steroids (21–23). In the usual schematic view of the molecule illustrated on the right side of Figure 1, the impression we are given is that the terminal isopropyl group in 1 and 2 may orient into different quadrants of space. In fact, certain hopanoids are known to possess 21α axial isopropyl groups (25). However, as seen in Figure 1, the terminal isopropyl group in 1 and 2 both orient into an equatorial position although C-22 in 1 is inverted compared with 2. Another interesting aspect of the geometry is that in 1 the molecule arcs up relative to the front end of the molecule while in 2 the molecule arcs down relative to the front (3-OAC) end. Here the isopropyl side chain is affected by long-range conformational transmission effects which mediate the

TABLE 2

Dihedral Angles (°) Between Plane Normals of Isoarborinol Acetate (1) and Motiol Acetate (2) with E.S.D.S. (mean) 0.5°

Plane	System	Planes	(1)	(2)
A	C-1-C-5, C-10	A-B	10.4	11.4
B	C-5-C-10	A-C	20.3	23.2
C	C-8-C-9, C-11-C-14	A-D	11.1	36.2
D	C-13-C-18	A-E	21.5	32.1
E	C-17-C-21	B-C	16.7	11.9
		B-D	6.9	25.0
		B-E	12.9	22.2
		C-D	10.3	14.5
		C-E	10.7	16.3
		D-E	10.9	10.4

orientation of the geminal methyl groups, C-29 and C-30. In 1, the geminal methyls point down whereas in 2 they point up. This is due to the 1,3-diaxial interactions between H-22 and C-28. Thus inversion of the configuration at C-21 resulted in a rotational preference of the side-chain methyls. That this conformation is likely maintained in solution is supported by NMR. For example, the chemical shifts and coupling constants for H-29 and H-30 are similar in 1 and 2 (14-16).

How these stereochemical similarities could have arisen based on previously described cyclization schemes to fernenol or arborenol is not clear. For the biosynthesis of fernenol, it has been thought that prior to cyclization, squalene oxide assumes a coiling pattern which rearranges into a chair-chair-chair-chair-boat conformation (21-23,25). After proton-initiated attack at C-3, the molecule then undergoes cyclization in a one-step non-stop process to an initial stable pentacyclic structure. The resulting hopanoid cation (consisting of five rings, four cyclohexanoid, and one cyclopentanoid), presumably with the same conformational disposition as the cyclizing material, is localized in the side chain at C-22 (Fig. 5). Additional 1,2-migration proceeds in a concerted manner to give rise to migrated hopanoids, *e.g.*, motiol. Other types of hopanoids, *e.g.*, tetrahymanol, are assumed to be formed by cyclization of squalene with no intramolecular rearrangements during the course of ring annulation (26). Thus, the cyclization of hopanoids has been envisaged as a completely concerted process, a view supported by the failure to trap or otherwise detect any of the cyclizing intermediates. The family of enzymes conducting cyclizations to produce hopanoids would have to be fundamentally different from the one(s) involved in the biogenesis of related PTs, such as, lupeol, the amyroids and epifriedelanol. For this group of PTs a step-wise pathway demonstrably operates by forming a series of intermediate frozen (27) mono- or bicyclic carbonium ion entities possessing boat-like conformations that pass through a key tetracycle, the dammarenyl cation (syn.—triterpenoid cation), before elaboration of the fifth ring (28-30). Several "missing links" have recently been identified as quasi intermediates between squalene oxide, the dammarenyl cation and β-amyrin which support the step-wise mechanism (31,32).

An alternative possibility that we now offer is that the hopanoid skeleton is formed by the same mechanistic pathway that proceeds from squalene oxide to other PTs using the dammarenyl cation, as shown in Figure 6. This would allow for a conservation of genetic material in PT cyclase evolution as speculated by Ourisson and Rohmer (4). For the biogenesis of 2, cyclization would be initiated by electrophilic attack by H^+ on the epoxy oxygen while the squalene oxide is held in the chair-chair-chair-boat-"left-handed" unfolded conformation on the active site of the enzyme. This leads to the formation of the dammarenyl cation (Fig. 6). The formation of the fifth ring involves ring enlargement from the 13,17-bond (steroid numbering system) so that C-13 is joined to C-20 producing a new cation at C-17 (4 in Fig. 6). If the terminal isoprene unit is folded as a boat, attack of the Δ^{22}-pi electrons (hopanoid numbering system) onto the electron-deficient site produced at C-17 would yield a new cation (5 in Fig. 6) which should be stabilized by hyperconjugation from the six H-atoms on C-29 and C-30, acting in part as a driving force for the formation of the cyclopentanoid ring. Remote

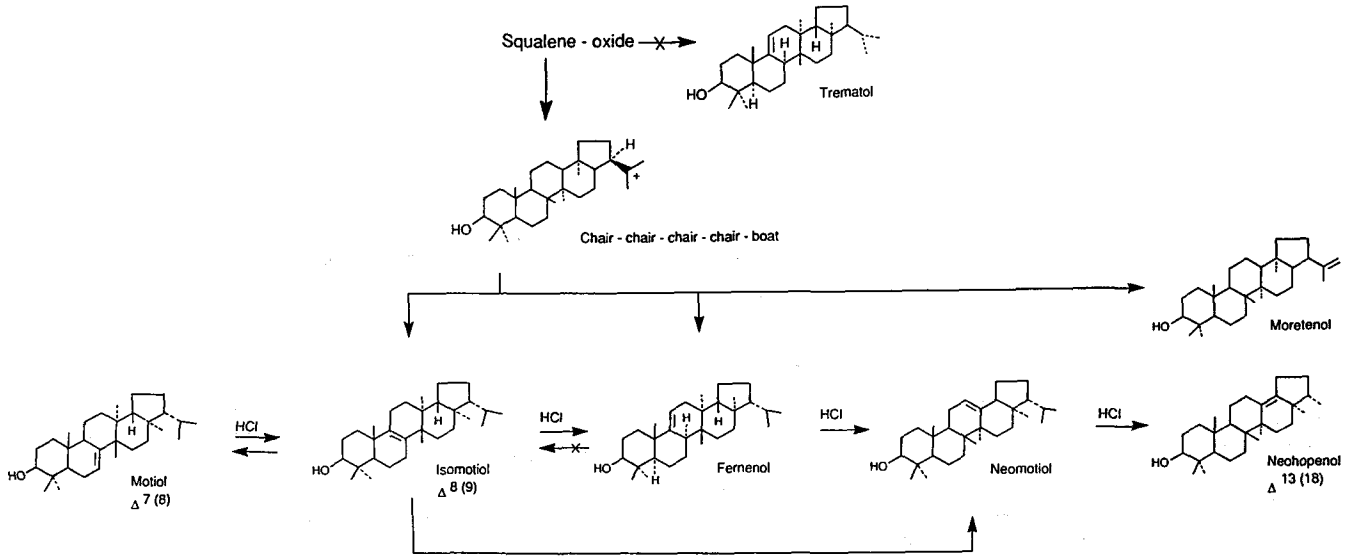


FIG. 5. Relationship of previously considered biogenetic mechanisms with acid-induced rearrangements in the formation of hopanoids (*cf.* 16,22-24,26).

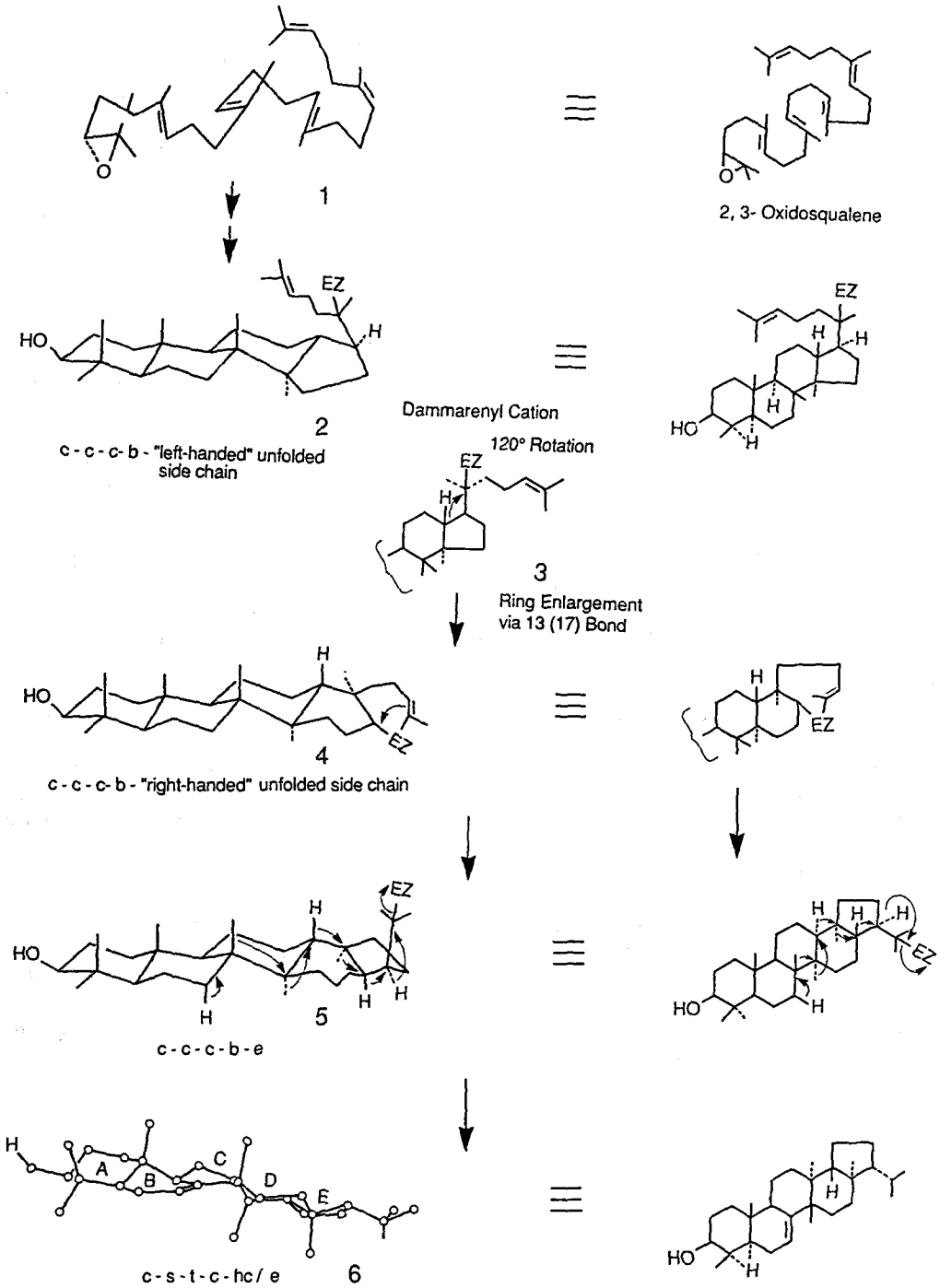


FIG. 6. Hypothetical cyclization mechanism for the conversion of squalene oxide to motiol.

deprotonation of the 7α-H atom would then induce a series of 1,2-migrations with inversion of the configuration at each of the carbons involved to produce motiol. The conformational driving force that affects the thermodynamics of inversion does not come from the all *trans*-anti arrangement of the intermediate cation 5; rather it comes from the steric compression in the boat-like D- and E-rings coupled with the eclipsed 17β-H and 21β-isopropyl group. These structural features confer the higher entropy to push for further backbone rearrangements that culminate in the migrated hopanoid structure with an *equatorial* 21α isopropyl group. Thus, as indicated by labeling studies (21) and now for conformational reasons, neohop-13(18)-enol, which assumably maintains an all-chair conformation, could not act as a legitimate precursor of fernenol, rather it is an end product of the synthetic sequence as shown in Figure 5. That boat-like cyclohexanoid rings are formed during cyclization and maintained in the cyclized product is supported by the conformation of some other PTs which possess a combination of chair

and boat rings in the completed cyclized structures. Most of the PTs were thought to be all-chair, but now some are known to possess one or more boat rings in the distal part of the molecule. The effect of introducing boat conformers is to produce a flat structure which then is maintained in solution and in the solid state. For instance, epifriedelanol is now known to obtain a flat structure by orienting into a chair-chair-chair-boat-boat conformation, rather than orienting into a bent conformer, as suggested earlier (33,34). On the assumption, for thermodynamic reasons, that chair-boat interconversions seem unlikely in the cyclized product, *i.e.*, to flip from the flat to the bent structure then in order to produce the three-dimensionally flat conformation, the original cyclization scheme to friedelin as suggested by others (29) requires modification. We have considered the possibility that elaboration of the dammarenyl cation and of the protosteroid cation, followed by ring enlargement from $\Delta^{13,17}$-bond may give rise to other triterpenoids, epifriedelanol, tetrahymanol and isoarborinol, respectively (8).

Another interesting aspect of the three-dimensional geometry of 1 and 2 is that they are somewhat shorter and volumetrically smaller than sterols. From the dimensions given in Figure 1 the length and volume for 1 and 2 are calculated to be 16.4 Å and 645 Å^3 while the corresponding data calculated for cholesterol are 19.1 Å and 853 Å^3, respectively (4). The size difference in the two groups of molecules is presumably the major reason for differences in their biological utilization rather than their conformation (flat compared with bent, *cf.* 8, for discussion) or location of angular methyl groups in the nucleus. Furthermore, we assume that the free isooctyl side chain in cholesterol contributes to the size differences due to its ability to sweep out a cone, *i.e.*, decreasing viscosity by increasing van der Waals' dispersion contacts (*cf.*, Fig. 7). Thus, this molecular feature should increase the volume of sterols compared to PTs and increase the suitability of sterols to function better than PTs as membrane components (8).

Regarding the phylogenetic arguments presented in the introduction, the results of our studies seem to support the second possibility: that is, sterols possess novel structural features which have allowed them to supersede the shorter, more rigid and volumetrically smaller PTs as architectural components of cell membrane inserts during the evolution of plant systems. PTs which continue to be synthesized by more advanced plants are produced presumably because they acquired additional new functions, *e.g.*, as components of the epicuticular wax (8). Thus we may now assume a non-conservation of architectural parity with evolutionary ascent.

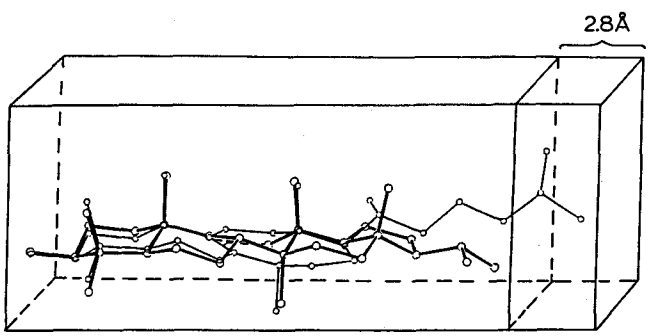


FIG. 7. Comparison of X-ray molecular dimensions of isoarborinol (this study) and 24,25-dihydrolanosterol (Griffin, J.F., and Nes, W.D., unpublished observations). Note the length of the sterol is approximately 3 Å greater than that of the pentacycle.

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Free and Combined Sterols of *Pavlova gyrans*[1]

Patricia K. Gladu[a,2], **Glenn W. Patterson***[,a], **Gary H. Wikfors**[b] and **William R. Lusby**[c]

[a]Department of Botany, University of Maryland, College Park, Maryland 20742, [b]NOAA, National Marine Fisheries Service, Northeast Fisheries Center, Milford Laboratory, Milford, Connecticut 06460, and [c]Insect Physiology Laboratory, Agricultural Research Service, U.S. Department of Agriculture, Beltsville, Maryland 20705

***Pavlova gyrans*, a unicellular alga of interest as food for oysters, was cultured axenically and examined for sterol composition. Desmethyl monohydroxy sterols, which are frequently seen in algae, made up 40% of the total sterols and were observed primarily in the free sterol fraction. The principal sterols of this group were 5-ergostenol, poriferasterol, and clionasterol, as well as some poriferast-22-enol and poriferastanol. Several "methyl" sterols with unusual structures made up 27% of the total sterols. The principal "methyl sterols" were 4α-methyl ergostanol, 4α-methyl poriferastanol, and 4α-methyl poriferast-22-enol. Methyl sterols were found primarily in the ester fraction. Also observed was a new class of dihydroxysterols composing 33% of the total sterols. These sterols are structurally related to the methyl and desmethyl sterols of *Pavlova* but contain an extra nuclear hydroxyl which can be acetylated when present on a desmethyl sterol, but which is nonreactive with acetic anhydride in 4α-methyl sterols. None of these sterols were observed in ester form but are concentrated in the acid-hydrolyzable, bound fraction. The unique nature of these sterols suggests potential taxonomic utility.**

***Lipids 26*, 656-659 (1991).**

Many marine invertebrates feed on unicellular algae, and it seems likely that the numerous exotic sterols found in these organisms might in fact be unchanged or modified dietary algal sterols (1,2). The commercial oyster, *Crassostrea virginica*, has been found to contain 39 sterols (3). Of these, only four appear to be produced by the oyster (4), although more recent work indicates that all oyster sterols are of exogenous origin (5).

Sterol composition has been found to be of interest in plant taxonomic considerations (6-8). The Prymnesiophyceae are a family of small, primarily marine protists characterized by the possession of a haptonema between two smooth flagella. Formerly, the organisms were considered a part of the chrysophyceae, until Christiansen (9) split them off into the Haptophyceae, a descriptive name. Subsequently, the name was changed to the Prymnesiophyceae (10).

Pavlova gyrans is a member of the Pavlovales in the Prymnesiophyceae and has been used for oyster culture (11, and Wikfors, G., and co-workers; manuscript in preparation). Ballantine *et al.* (12) found in the closely related species, *P. lutheri* (as *Monochrysis lutheri*), cholesterol, 24-methyl and 24-ethyl cholesterol and 22-dehydro-24-ethyl cholesterol as major sterols in cultures harvested in the exponential growth phase; however, when harvested in the stationary phase, this species contained an increased quantity of 4-methyl sterols which were present along with a C_{29} and a C_{30} dihydroxysterol. The specific structures of these sterols were not determined. In the present paper we report on the occurrence of four dihydroxysterols in stationary phase cells of *Pavlova gyrans*. Each compound was characterized by a saturated nucleus and contained two hydroxyl groups. We report here on the sterols of *P. gyrans* and *P. lutheri* and their occurrence in specific sterol fractions.

MATERIALS AND METHODS

Culture conditions. Pavlova gyrans Butcher, strain designation "93," utilized in this study, was obtained from the Milford Laboratory (Milford, CT) microalgal culture collection where it has been maintained for many years in enriched natural seawater medium, "E" formulation (13). At Milford, axenic test-tube cultures were increased in volume through a series of progressively larger flasks and finally inoculated into carboy assemblies (13). Carboy cultures were operated semi-continuously, with harvests of about one-third of the total culture volume (6 liters taken from 18 liters) on Friday of each week, followed immediately by replacement of harvested volumes with autoclave-sterilized "E" medium. Harvests and additions of growth medium were accomplished with sterile technique so that cultures remained axenic. Carboy cultures were routinely monitored for the presence of microbial contaminants at each harvest by inoculating 0.2 mL of algal culture into 5 mL of thioglycollate medium made up in 50% seawater. Direct observation with the fluorescence microscope using acridine orange confirmed that cell suspensions subjected to sterol analysis were, indeed, axenic.

Algae for sterol analysis were harvested five days after the most recent addition of medium. We consider these cells to be in the stationary phase of growth because no further increases in cell density occurred in carboy cultures after five days, and protein-nitrogen analysis of *P. gyrans* harvested on this schedule revealed that all nitrogen in the medium had been incorporated into algal biomass (Wikfors, G. H., unpublished observation). Harvested cultures were concentrated by cold centrifugation (1020 × *g* at 10°C for 20 min), and re-suspended in a minimal volume of isotonic NaCl. This concentrated algal cell suspension was volumetrically aliquotted into glass ampoules, with a small volume being retained for microscopic counting in an Improved Neubauer (Buffalo, NY) hemacytometer. Ampoules containing algal cell suspensions were then frozen in a rotating bath of cold methanol (−25°C) and lyophilized using a VirTis Unitrap 10-100 manifold-style freeze-dryer (VirTis, Gardiner, NY). Ampoule necks were melt-sealed immediately upon removal from the lyophilizer manifold, and algal samples were stored in the sealed ampoules until analyzed.

[1]Based on a paper presented at the Symposium on Plant and Fungal Sterols: Biosynthesis, Metabolism and Function, held at the AOCS Annual Meeting, Baltimore, MD, April 1990.

[2]Current address: Biology Department, Lindsey-Wilson College, Columbia, Kentucky 42728.

*To whom correspondence should be addressed.

Abbreviations: GC, gas chromatography; GLC, gas-liquid chromatography; NMR, nuclear magnetic resonance; RRT, relative retention times.

TABLE 1

Composition[a] of Sterols Isolated from *Pavlova*

Sterol	*Pavlova gyrans*[b] RRT	Ester	Free	Polar	Total	*Pavlova lutheri*[b] Total
Cholesterol (1A)[c]	1.00	*d*	*e*	*e*	*d*	*e*
Epibrassicasterol (1C)	1.10	*e*	*e*	*d*	*d*	*d*
5-Ergostenol (1B)	1.26	*d*	1	*d*	1	8
4α-Methylergost-22-enol (3C)	1.31	*e*	*e*	*e*	*e*	*d*
Poriferasterol (1D)	1.36	1	22	6	29	30
Poriferast-22-enol (2D)	1.38	*e*	6	1	7	*e*
4α-Methylergostanol (3B)	1.49	2	3	trace	5	*e*
Clionasterol (1E)	1.54	*e*	3	*e*	3	16
4α-Methylporiferast-22-enol (3D)	1.62	5	12	trace	17	16
24-Methylpavlovol (3B)[g]	2.06	*e*	2	3	5	22
4-Desmethyl-22-dehydropavlovol (2D)[f,g]	2.13	*e*	*e*	2	2	*e*
4-Desmethyl-22-dehydropavlovol (2D)[f,g]	2.22	*e*	*e*	7	7	4
24-Ethylpavlovol (3E)[g]	2.52	*e*	7	12	19	4
Total		10	59	31	100	100

[a]% of total sterol.

[b]Sterol content *P. gyrans*, 7 mg/g algal dry wt; *P. lutheri*, 9 mg/g algal dry wt.

[c]Refers to structures in Figure 1.

[d]Less than 1%.

[e]None detected.

[f]Apparently differ only in position or location of second hydroxyl.

[g]Structure also contains an additional hydroxyl on the nucleus.

Isolation of sterols. Lyophilized samples (7.6 g) of *P. gyrans* (#93) were extracted in chloroform/methanol (2:1, v/v) using a Soxhlet extractor (Fisher Scientific, Silver Spring, MD) for 8 hr. The crude lipid extract was partitioned by column chromatography using Unisil (100–200 mesh, Clarkson Chemical Co. Inc., Williamsport, PA). Hydrocarbons, sterol esters, free sterols, and polar sterols were eluted with 100 mL each of hexane, hexane/chloroform (50:50, v/v), chloroform, and ethanol, respectively. The polar sterol fraction was hydrolyzed in 6 N HCl/MeOH (1:5, v/v) at 80°C, for one hr; sterols were recovered by adding water and by partitioning into chloroform. The ester fraction was saponified with 7% KOH in methanol at 80°C for 1 hr, and sterols were recovered by adding water and by partitioning into diethyl ether. The ester and polar fractions from the Unisil column were further fractionated on an alumina column and eluted with 30, 40, 50, 60, and 75% diethyl ether in hexane, 100% diethyl ether, and 20% methanol/diethyl ether (v/v) to separate the methyl sterols from the desmethyl sterols.

Chromatography. Qualitative and quantitative analyses of sterols were performed by gas-liquid chromatography (GLC) on a Varian 3700 gas chromatograph with a hydrogen flame ionization detector and a 30 m × 0.25 mm i.d. SPB-1 capillary column (Supelco, Inc., Bellefonte, PA) at a column temperature of 255°C and an injector temperature of 265°C. Data were corrected for detector response with isolated dihydroxy sterols. Column flow was 1 mL/min with helium as the carrier gas. Peak area calculations were processed with a Varian 401 Chromatographic Data System. Relative retention times (RRT) of sterols are expressed relative to cholesterol.

Mass spectra of free sterols were obtained from a Finnigan-MAT Model 4512 gas chromatograph-mass spectrometer equipped with a 30 m × 0.32 mm i.d. fused silica capillary column with a 0.25 μm film of DB-1 (J&W Scientific, Folsom, CA). The column was operated at a head pressure of 13 psi of helium and the temperature was programmed, after a 2-min hold at 230°C, to 255°C at either 2 or 5°C/min. Electron impact mass spectra were measured at 70 eV and an ionization temperature of 150°C. Data were recorded and analyzed with an Incos Data Systems (Incos Finnigan Corp., San Jose, CA).

RESULTS AND DISCUSSION

Fractionation of the *P. gyrans* lipid extract into sterol esters, free sterols, and covalently-bound polar sterols permitted a more accurate description of the sterol composition of this alga than would be provided by analysis for total sterol alone. Sterols found in each fraction are summarized in Table 1. Sterol structures are seen in Figure 1. Sterols from *P. lutheri* were also analyzed, but data were obtained only for total sterols. The monohydroxy sterols identified possessed one of three types of sterol nuclei: monohydroxydesmethyl sterols (type 1 and 2 in Fig. 1), common in most living organisms, and monohydroxy-4-methyl sterols (type 3). Type 2 and type 3 sterol nuclei were also found with an extra hydroxyl in the sterol nucleus (Fig. 1). These dihydroxysterols are called pavlovols. Characterization of the dihydroxy sterols has been completed except for the location of the second hydroxyl. Work is continuing to identify the dihydroxy sterols. The monohydroxydesmethyl fraction consists principally of 5-ergostenol (1B) (Fig. 1), poriferasterol (1D), clionasterol (1E) and poriferast-22-enol (2D). Except for the last, these sterols are quite common in the algal division Chromophycota, to which *Pavlova* belongs (14,15).

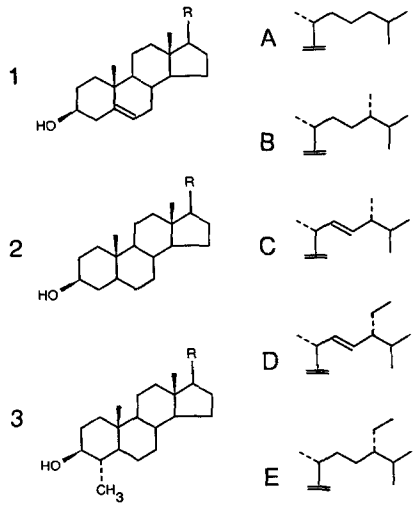


FIG. 1. Structures of sterols from *Pavlova*.

Small quantities of cholesterol (1A) and epibrassicasterol (1C) were also detected. Identifications were based on capillary gas chromatography (GC) relative retention times (Table 1) and on mass spectra, which matched those of authentic standards. Poriferasterol was identified as being 24β-ethyl (24R) by its melting point (156°C) and by ^{1}H nuclear magnetic resonance (NMR) (16–18). Other sterols in *Pavlova* are assumed to be 24β, based on the configuration at C-24 of poriferasterol and because the 24β-configuration is characteristic of most algae (6). The principal exception to the 24β configuration is epibrassicasterol which has been demonstrated to be 24α-methyl rather than 24β-methyl in all identified algae in the Chromophycota where such determinations have been made (7,8,19).

The monohydroxy-4-methyl sterols are, for the most part, unusual structures; their principal mass spectral characteristics are shown in Table 2. Each of these sterols corresponds structurally to one of the monohydroxy desmethyl sterols already identified, but with the retention of the 4-methyl group and the absence of the Δ^5-double bond. Sterols with a saturated nucleus and a 4-methyl group are quite rare, primarily occurring in marine animals where these sterols are presumed to be of dietary origin (20,21), although their nutritional role is not known. Although these sterols have also been isolated from the dinoflagellates *Heterocapsa* (22) and *Glenodinium* (23,24), they have not been found in other algal taxa. These sterols have not been reported to occur in *P. lutheri* harvested during logarithmic growth, but appear to be the methyl sterols detected by Ballantine *et al.* (12) and identified here. The quantities of sterols found in ester, free, and bound fractions are presented in Table 3. From these data it is apparent that various sterols are not distributed in each fraction in the 1:6:3 ratio found between quantities of ester, free, and polar sterols. For instance, monohydroxy-4-methyl sterols comprise 27% of total sterols but they account for nearly 90% of the ester sterols (Table 3). Dihydroxy sterols make up 33% of the total sterols but they make up over 77% of the polar sterols. Clearly, some mechanism is directing specific sterol structures preferentially toward certain

TABLE 2

Mass Spectral Data[a] for Some Unusual Sterols of *P. gyrans* and *P. lutheri*

	Sterol[b]				
Fragmentation	1	2	3	4	5
$[M]^+$	414(20)	414(32)	416(50)	428(25)	430(53)
$[M-CH_3]^+$			401(12)		415(10)
$[M-33]^+$			383(15)		397(10)
$[M-43]^+$	371(5)				
$[M-43-18]^+$	353(10)	353(7)		367(15)	
$[M-85]^+$					
$[M-(C_{22}-C_{29})]^+$	302(23)	316(32)		316(25)	
$[M-SC(+2H)]^+$	273(27)	287(37)	289(2)	287(37)	290(25)
$[M-SC+18(+2H)]^+$	257(25)	271(38)	271(2)	271(27)	271(5)
$[M-SC+D\ ring]^+$	233(5)	245(8)	247(78)	245(10)	247(75)
$[M-SC+60]^+$	215(10)	229(6)	229(95)	229(10)	229(90)
Base peak	55	69	95	55	95

[a]Reported as *m/z* (relative intensity).

[b]1 = poriferast-22-enol.
2 = 4α-methylergost-22-enol.
3 = 4α-methylergostanol.
4 = 4α-methylporiferast-22-enol.
5 = 4α-methylporiferastanol.

TABLE 3

Sterol Fractions[a] in *Pavlova gyrans*

Sterol type	Ester	Free	Polar	Total
Monohydroxy-4-desmethyl	1	32	7	40
Monohydroxy-4-methyl	9	18	[b]	27
Dihydroxy-4-desmethyl	[b]	[c]	3	3
Dihydroxy-4-methyl	[b]	9	21	30

[a]As % of total sterol.

[b]Less than 1%.

[c]None detected.

sterol fractions (ester, free, polar). Two-thirds of the total sterol in the *P. gyrans* culture examined here had a saturated nucleus. Therefore, direct algal synthesis as well as bacterial modification of sterols may be sources of marine stanols (25). Cholestanol is the principal sterol of several red algae (26) and several higher plants contain dominant saturated sterols (27,28), but in these cases the sterols were 4-desmethyl, in contrast to the 4α-methyl sterols in *Pavlova*.

Sterols have been suggested to have some chemotaxonomic value for algae because the occurrence of specific sterols in algae corresponds well with taxonomic placement (6). *P. gyrans* and *P. lutheri* belong to a newly erected algal class, the Prymnesiophyceae. Sterol compositions have been determined for this class for the orders Coccosphaerales (7,29,30) and Isochrysidales (8,17,29,30). In each of the above orders, epibrassicasterol was the principal sterol and sterols with a 9-carbon side chain were dominant. In a third order, Prymnesiales, sterols with 8-carbon side chains (mostly cholesterol) are dominant (30). In the order Pavlovales, to which *Pavlova* belongs, Ballantine *et al.* (12) have shown that sterols with

the 10-carbon side chain are dominant in both exponential and stationary cultures of *P. lutheri*. The present work confirms their findings with stationary-phase cultures of *P. lutheri*, and extends their results to include stationary-phase *P. gyrans*, in which about 90% of the sterols have a 10-carbon side chain. The order Pavlovales appears to differ sharply from the previously mentioned orders in this respect, although data on more species are needed.

Data on sterol composition must be viewed with some caution unless all sterol fractions from the organism are accounted for. The importance of including analyses for polar, bound sterols is demonstrated by the findings in this study, that they constitute 30% of the total sterol in *P. gyrans*. Several green algae showed an increase in total sterol when cultured photosynthetically *versus* heterotrophically (31), but the ratios between individual sterol components were rather constant. However, with *Emiliania huxleyi* (29) and *P. lutheri* (8), the phase of growth can markedly affect the ratio between various sterols. Compositional data between species cultured in different conditions must be evaluated with caution. However, it is apparent that sterol distributions within or between algal taxa can provide useful systematic information in support of the more traditional morphological characters (30).

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Sterols of *Kalanchoe pinnata*: First Report of the Isolation of Both C-24 Epimers of 24-Alkyl-Δ^{25}-sterols from a Higher Plant

Toshihiro Akihisa[a,*], W.C.M.C. Kokke[b], Toshitake Tamura[a] and Taro Matsumoto[a]

[a]College of Science and Technology, Nihon University, Tokyo 101, Japan, and [b]Department of Synthetic Chemistry, SmithKline Beecham Pharmaceuticals, King of Prussia, Pennsylvania 19406

Eighteen sterols were isolated from the aerial parts of *Kalanchoe pinnata* (Crassulaceae) including four novel sterols, *viz.* (24*R*)-stigmasta-5,25-dien-3β-ol (24-epiclerosterol), (24*R*)-5α-stigmasta-7,25-dien-3β-ol, 5α-stigmast-24-en-3β-ol, and 25-methyl-5α-ergost-24(28)-en-3β-ol. 24-Epiclerosterol and its Δ^7-analog occur together with their 24*S*/β-epimers in the same plant making this the first report of the isolation of both C-24 epimers of Δ^{25}-unsaturated 24-alkylsterols from a non-marine organism. Iodine-catalyzed isomerization of stigmasta-5,24-dien-3β-ol (24-ethyldesmosterol), the main sterol of *K. pinnata*, yielded 24-epiclerosterol among other products.
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The principal sterols of the great majority of higher plants are two 24α-ethylsterols, sitosterol (2e) (see Table 1 and Figure 1 for systematic names and structures) and stigmasterol (2g), and C-24 epimeric 24-methylsterols, campesterol (2a) and 22-dihydrobrassicasterol (2b) (1–5). There are, however, also plants which contain Δ^{25}-unsaturated 24-ethylsterols as the dominant sterols. Examples are several *Clerodendrum* species (Verbenaceae) (6,7,9,10; 8, and references cited therein), and *Kalanchoe* species (Crassulaceae) (4,5), which contain clerosterol (2n) and 22-dehydroclerosterol [(22*E*,24*S*)-stigmasta-5,22,25-trien-3β-ol, 2o]. The seeds of several cucurbitaceous plants (11–18; 19, and references cited therein) are the other examples which contain (24*S*)-5α-stigmasta-7,25-dien-3β-ol (3n) and (22*E*,24*S*)-5α-stigmasta-7,22,25-trien-3β-ol (3o), as their dominant sterols.

In this paper we report the sterol composition of the aerial parts of *Kalanchoe pinnata* (air plant; Crassulaceae). The sterols include both epimers at C-24 of stigmasta-5,25-dien-3β-ol (2m,n) and 5α-stigmasta-7,25-dien-3β-ol (3m,n). In all previous cases in which the stereochemistry at C-24 of Δ^{25}-unsaturated 24-alkylsterols of plants and their seeds has been carefully investigated (1–31), the stereochemistry at C-24 was found to be 24*S*/β The eighteen identified sterols of *K. pinnata* (Table 1) include four novel ones (1j, 1p, 2m, 3m) (*vide infra*).

MATERIALS AND METHODS

General methods. Crystallizations were from acetone/methanol. Melting points (m.p.) are uncorrected. Preparative thin-layer chromatographic (TLC) plates (silica gel) and argentation TLC plates (Silica Gel/$AgNO_3$, 4:1, w/w) were developed three times with hexane/EtOAc (6:1, v/v) and CCl_4/CH_2Cl_2 (5:1, v/v), respectively. High-performance liquid chromatography (HPLC) separations were performed using a Beckman (Altex) Ultrasphere ODS column (5μ, 25 cm × 10 mm i.d.) (Beckman Instruments, Inc., San Ramon, CA) with a refractive index detector and methanol/H_2O (98:2, v/v) as the mobile phase (flow 4 mL/min). A SCOT OV-17 glass capillary column was used for gas liquid chromatography (GLC) (30 m × 0.3 mm i.d., 260°C). Cholesterol acetate was the standard (RRT = 1.00) for the determination of relative retention times (RRT) in both HPLC and GLC. The electron ionization-mass spectrometry (EI-MS) spectra were taken on a Hitachi M-80B double focusing gas chromatography/mass spectrometry (GC/MS) (70 eV) (Hitachi Seisakusho Ltd., Tokyo, Japan). The MS data do not include peaks having *m/z* <200. The ^1H nuclear magnetic resonance (NMR) spectra (400 MHz) were recorded on a JNM GX-400 spectrometer (Japan Electron Optics Laboratory, Co., Tokyo, Japan) or on a Bruker AM400 instrument (Karlsruhe, Germany). The ^{13}C NMR spectra were recorded in $CDCl_3$ with $SiMe_4$ as internal standard; shifts (δ) are given downfield from $SiMe_4$. Acetylations were done in Ac_2O/pyridine at room temperature overnight. The aerial parts (leaves and stems) of *Kalanchoe pinnata* (Lam.)Pers. [*Bryophyllum calycinum* Salisb.; *B. pinnatum* (Lam.) Kurz.] were courteously donated by Mr. Yoshiya Satoh (Green Interia Co., Tokyo, Japan). Acetyl derivatives of 1n (32), and 2a, 2b, 2c, 2e, 2g, 2l, 2p, 3j, 3l, and 3n (19), 2d (26), 2j (33) and 2n (9) (see Table 1 for the systematic names of these sterols) were used as reference compounds. (24*E*)-Stigmasta-5,24(28)-dien-3β-ol (fucosterol, 2k) acetate was a gift from Kimitsu Chemical Industries, Co. (Tokyo, Japan).

Isolation of sterols. Leaves and stems of *K. pinnata* (15.4 kg) were air-dried and powdered (980 g) and then extracted with CH_2Cl_2 in a Soxhlet extractor. The extracted lipid (38.7 g) was saponified (5% KOH in methanol, reflux, 3 hr). The residue was partitioned between water and isopropyl ether giving neutral unsaponifiable lipids (6.9 g) which were fractionated over silica gel (200 g) using (i) hexane (1.0 L), (ii) hexane/ethyl acetate (EtOAc) (9:1, v/v; 3.0 L), (iii) hexane/EtOAc (7:3, v/v; 2.0 L), and EtOAc (1.0 L). Part of the fractions obtained from the hexane/EtOAc (9:1, v/v) eluate afforded the mixture of free sterols (750 mg). The mixture was acetylated, and the resulting acetate mixture (640 mg) was fractionated by argentation TLC. Five bands (referred to as bands 1–5 in order of polarity, beginning with the least polar) were scraped off and extracted. HPLC of the fraction from band 1 (15 mg, R_f 0.77–0.89) afforded 1j-acetate (2mg). The fraction from band 2 (184 mg, R_f 0.69–0.77) yielded 2e-acetate (74 mg) and a mixture of 2a- and 2b-acetates (13 mg) (The ratio of 2a and 2b in the mixture was estimated to be 60:40 by ^1H NMR spectroscopy.). The fraction from band 3 (136

*To whom correspondence should be addressed at College of Science and Technology, Nihon University, 1-8, Kanda Surugadai, Chiyoda-ku, Tokyo 101, Japan.

Abbreviations: ^{13}C NMR, carbon-13 nuclear magnetic resonance; EI-MS, electron ionization mass spectrometry; GC/MS, gas chromatography/mass spectrometry; GLC, gas-liquid chromatography; ^1H NMR, proton nuclear magnetic resonance; HPLC, high-performance liquid chromatography; RRT, relative retention time; TLC, thin-layer chromatography.

TABLE 1

Abundance (%), the Molecular ion (M^+), Mp, and Chromatographic Data of Sterols Present in *Kalanchoe pinnata*

Sterol[a]	Abundance (%)	Data for the acetyl derivatives			
		M^+(*m/z*)	Mp (°C)	RRT (HPLC)[b]	RRT (GLC)[b]
1j Stigmast-24-enol	1.4	456	102-104	1.21	2.02
1n (24*S*)-Stigmast-25-enol	0.6	456	123-125	1.08	1.65
1p 25-Methylergost-24(28)-enol	0.5	456	143-145	1.12	1.70
2a (24*R*)-Ergost-5-enol(campesterol)	1.7 }	442	143-145	1.14	1.31
2b (24*S*)-Ergost-5-enol(22-dihydrobrassicasterol)	1.1 }				
2c Ergosta-5,24(28)-dienol	0.1	380[c]	—	0.81	1.35
2d (24*S*)-Ergosta-5,25-dienol(codisterol)	0.3	380[c]	121-123	0.80	1.32
2e (24*R*)-Stigmast-5-enol(sitosterol)	27.4	396[c]	118-120	1.26	1.63
2g (22*E*,24*S*)-Stigmasta-5,22-dienol(stigmasterol)	1.8	394	145-146	1.06	1.43
2j Stigmasta-5,24-dienol(24-ethyldesmosterol)	30.2	454	139-141	1.00	1.95
2l (24*Z*)-Stigmasta-5,24(28)-dienol(isofucosterol)	11.8	454	135-137	1.01	1.81
2m (24*R*)-Stigmasta-5,25-dienol(24-epiclerosterol)	2.4	394[c]	125-127	0.93	1.65
2n (24*S*)-Stigmasta-5,25-dienol(clerosterol)	12.5	454	127-129	0.91	1.65
2p 25-Methylergosta-5,24(28)-dienol	0.5	394[c]	144-146	0.96	1.68
3j Stigmasta-7,24-dienol(peposterol)	2.0	454	—	1.01	2.30
3l (24*Z*)-Stigmasta-7,24(28)-dienol(avenasterol)	2.3	454	145-147	1.01	2.15
3m (24*R*)-Stigmasta-7,25-dienol	1.2	454	158-160	0.97	1.93
3n (24*S*)-Stigmasta-7,25-dienol	2.0	454	160-161	0.95	1.93

[a]All sterols have a C-3β hydroxyl group. Sterols with a ring system 1 (Δ^0) or 3 (Δ^7) are 5α-sterols.
[b]Retention time relative to cholesterol acetate.
[c]$[M - HOAc]^+$.

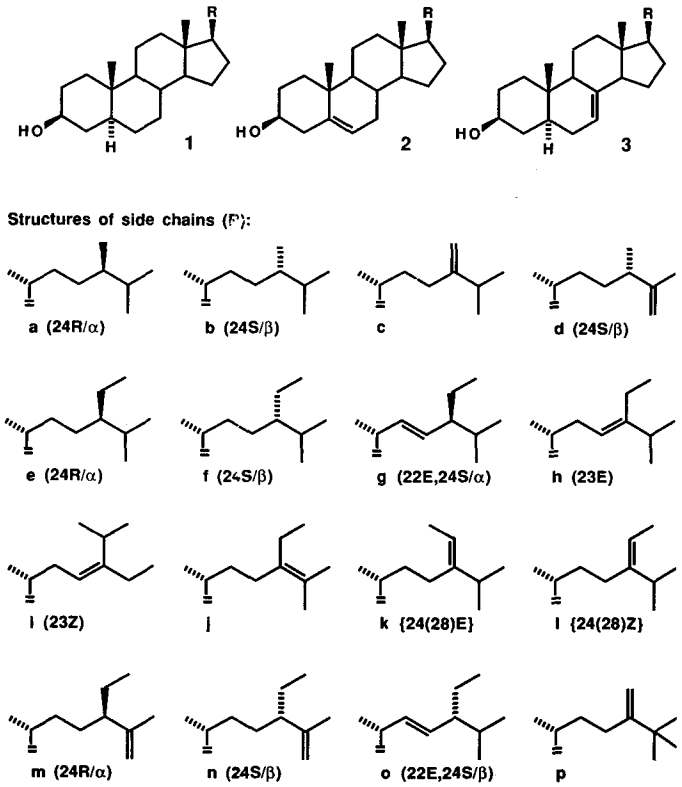


FIG. 1. Structures of sterols.

mg, R_f 0.56-0.69) was hydrolyzed, and the free sterol mixture obtained was separated by preparative TLC into two fractions, 3A and 3B. The faster moving band (fraction 3A, R_f 0.33) consisted mainly of Δ^5-sterols, and fraction 3B from the slower moving band (R_f 0.31) was composed mainly of Δ^7-sterols (18). The two fractions were acetylated and the resulting acetyl derivatives were fractionated by HPLC: fraction 3A-acetate afforded the acetates of **1n** (1 mg), **2g** (1 mg), and **2j** (76 mg); and fraction 3B-acetate gave **3j**-acetate (1 mg). The fraction from band 4 (116 mg, R_f 0.22-0.56) was hydrolyzed, and the resulting free sterol mixture was separated into two subfractions, 4A and 4B, in the same manner as described above, and subsequently acetylated. HPLC fractionation of 4A-acetate gave the acetates of **1p** (2 mg), **2d** (1 mg), **2l** (12 mg), **2m** (3 mg), and **2n** (5 mg), whereas HPLC fractionation of 4B-acetate yielded the acetates of **3l** (2 mg), **3m** (1 mg), and **3n** (1 mg). HPLC of the fraction from band 5 (17 mg, R_f 0.05-0.22) afforded the acetates of **2c** (1 mg) and **2p** (3 mg). Hydrogenation of **2m**- and **2n**-acetates in ethanol over PtO_2 at atmospheric pressure and room temperature overnight followed by HPLC purification gave an almost quantitative yield of sitosterol (**2e**) and clionasterol (**2f**) acetates, respectively.

5α-Stigmast-24-en-3β-ol (1j) acetate. MS, *m/z* (assignment, rel. int.) 456.3970 ($C_{31}H_{52}O_2$, $[M]^+$, 16, calcd. 456.3964), 441.3726 ($C_{30}H_{49}O_2$, 5), 396.3761 ($C_{29}H_{48}$, 8), 381.3568 ($C_{28}H_{45}$, 4), 358.2841 ($C_{24}H_{38}O_2$, 100), 343.2636 ($C_{23}H_{35}O_2$, 16), 315.2350 ($C_{21}H_{31}O_2$, 25), 302.2239 ($C_{20}H_{30}O_2$, 5), 298.2609 ($C_{22}H_{34}$, 4), 283.2403 ($C_{21}H_{31}$, 7), 276.2052 ($C_{18}H_{28}O_2$, 10), 275.1991 ($C_{18}H_{27}O_2$, 13), 255.2145 ($C_{19}H_{27}$, 13), 229.2008 ($C_{17}H_{25}$, 13), 216.1921 ($C_{16}H_{24}$, 15), 215.1801 ($C_{16}H_{23}$, 22), 203.1763 ($C_{15}H_{23}$, 6), 201.1666 ($C_{15}H_{21}$, 7).

25-Methylergost-24(28)-en-3β-ol (1p) acetate. MS, *m/z* (assignment, rel. int.) 456.3933 ($C_{31}H_{52}O_2$, $[M]^+$, 6), 441.3713 ($C_{30}H_{49}O_2$, 6), 381.3519 ($C_{28}H_{45}$, 5), 358.2840 ($C_{24}H_{38}O_2$, 100), 344.2672 ($C_{23}H_{36}O_2$, 8), 343.2670 ($C_{23}H_{35}O_2$, 9), 315.2310 ($C_{21}H_{31}O_2$, 27), 302.2219 ($C_{20}H_{30}O_2$, 5), 298.2662 ($C_{22}H_{34}$, 5), 285.2555 ($C_{21}H_{33}$, 6), 283.2409 ($C_{21}H_{31}$, 7), 275.1991 ($C_{18}H_{27}O_2$, 10), 255.2092 ($C_{19}H_{27}$, 13), 241.1954 ($C_{18}H_{25}$, 3), 229.1988 ($C_{17}H_{25}$, 15), 215.1822 ($C_{16}H_{23}$, 29), 213.1667 ($C_{16}H_{21}$, 10), 203.1776 ($C_{15}H_{23}$, 8), 201.1622 ($C_{15}H_{21}$, 10).

(24R)-Stigmasta-5,25-dienol (2m) acetate. MS, *m/z* (assignment, rel. int.) 394.3547 ($C_{29}H_{46}$, $[M - HOAc]^+$, 100,

calcd. 394.3596), 379.3385 ($C_{28}H_{43}$, 8), 352.3093 ($C_{26}H_{40}$, 1), 310.2676 ($C_{23}H_{34}$, 2), 296.2520 ($C_{22}H_{32}$, 2), 286.2650 ($C_{21}H_{34}$, 3), 281.2282 ($C_{21}H_{29}$, 4), 273.2565 ($C_{20}H_{33}$, 4), 255.2075 ($C_{19}H_{27}$, 7), 253.1912 ($C_{19}H_{25}$, 8), 228.1926 ($C_{17}H_{24}$, 8), 213.1640 ($C_{16}H_{21}$, 9), 211.1478 ($C_{16}H_{19}$, 3).

(24R)-5α-stigmasta-7,25-dien-3β-ol (3m) acetate. MS, *m/z* (assignment, rel. int.) 454.3797 ($C_{31}H_{50}O_2$, $[M]^+$, 34, calcd. 454.3807), 439.3577 ($C_{30}H_{47}O_2$, 22), 394.3611 ($C_{29}H_{46}$, 20), 379.3381 ($C_{28}H_{43}$, 5), 370.2883 ($C_{25}H_{38}O_2$, 2), 356.2662 ($C_{24}H_{36}O_2$, 3), 342.2515 ($C_{23}H_{34}O_2$, 5), 313.2140 ($C_{21}H_{29}O_2$, 100), 300.2120 ($C_{20}H_{28}O_2$, 5), 299.2057 ($C_{20}H_{27}O_2$, 5), 288.2100 ($C_{19}H_{28}O_2$, 9), 273.1873 ($C_{18}H_{25}O_2$, 9), 255.2092 ($C_{19}H_{27}$, 39), 253.1912 ($C_{19}H_{25}$, 9), 227.1819 ($C_{17}H_{23}$, 18), 213.1644 ($C_{16}H_{21}$, 32), 201.1617 ($C_{15}H_{21}$, 8).

Stigmasta-5,24-dienol (2j) acetate. ^1H NMR data. Assignment, δ (multiplicity, *J* in Hz): 3α-H, 4.604 (*m*); CO-CH_3, 2.028 (*s*); 4-H_2, 2.310 and 2.328 (each 1H and *m*); 6-H, 5.374 (*d* like, 5.0); 18-H_3, 0.682 (*s*); 19-H_3, 1.020 (*s*); 21-H_3, 0.970 (*d*, 6.6); 26-H_3 and 27-H_3, 1.627 and 1.631 (each *s*); 29-H_3, 0.932 (*t*, 7.7).

25-Methylergosta-5,24(28)-dien-3β-ol (2p) acetate. ^1H NMR data. Assignment, δ (multiplicity, *J* in Hz): 3α-H, 4.603 (*m*); CO-CH_3, 2.030 (*s*); 4-H_2, 2.309 and 2.328 (each 1H and *m*); 6-H, 5.372 (*d* like, 5.0); 18-H_3, 0.688 (*s*); 19-H_3, 1.021 (*s*); 21-H_3, 0.963 (*d*, 6.6); 26-H_3, 27-H_3, and 29-H_3, 1.057 (*s*); 28-H_2, 4.659 and 4.832 (each 1H and br. *s*).

(24R)-Stigmast-5-enol (2e) acetate prepared from (24R)-stigmasta-5,25-dienol (2m) acetate by hydrogenation. RRT 1.63 (GLC), 1.26 (HPLC). MS, *m/z* 396 $[M - HOAc]^+$. ^1H NMR data. Assignment, δ (multiplicity, *J* in Hz): 3α-H, 4.605 (*m*); CO-CH_3, 2.032 (*s*); 4-H_2, 2.310 and 2.328 (each 1H and *m*); 6-H, 5.375 (*d* like, 5.0); 18-H_3, 0.677 (*s*); 19-H_3, 1.020 (*s*); 21-H_3, 0.922 (*d*, 6.6); 26-H_3, 0.837 (*d*, 7.7); 27-H_3, 0.815 (*d*, 6.6); 29-H_3, 0.847 (*t*, 7.7).

(24S)-Stigmast-5-enol (2f) acetate prepared from (24S)-stigmasta-5,25-dienol (2n) acetate by hydrogenation. RRT 1.63 (GLC), 1.26 HPLC). MS, *m/z* 396 $[M - HOAc]^+$. ^1H NMR data. Assignment, δ (multiplicity, *J* in Hz). 3α-H, 4.604 (*m*); CO-CH_3, 2.031 (*s*); 4-H_2, 2.310 and 2.328 (each 1H and *m*); 6-H, 5.375 (*d* like, 4.7); 18-H_3, 0.678 (*s*); 19-H_3, 1.020 (*s*; 21-H_3, 0.926 (*d*, 6.6); 26-H_3, 0.831 (*d*, 7.1); 27-H_3, 0.812 (*d*, 6.9); 29-H_3, 0.855 (*t*, 6.6).

(24R)-5α-Stigmast-7-en-3β-ol (3e) acetate prepared from (24R)-5α-stigmasta-7,25-dien-3β-ol (3m) acetate by hydrogenation. RRT 1.94 (GLC), RRT 1.26 (HPLC). MS, *m/z* 456 $[M]^+$. ^1H NMR data. Assignment, δ (multiplicity, *J* in Hz): 3α-H, 4.696 (*tt*, 4.4, 11.5); CO-CH_3, 2.026 (*s*); 7-H, 5.147 (*dd* like, 2.7, 5.4); 18-H_3, 0.535 (*s*); 19-H_3, 0.811 (*s*); 21-H_3, 0.926 (*d*, 6.6); 26-H_3, 0.838 (*d*, 7.1); 27-H_3, 0.816 (*d*, 7.1); 29-H_3, 0.847 (*t*, 7.4).

(24S)-5α-Stigmast-7-en-3β-ol (3f) acetate prepared from (24S)-5α-stigmasta-7,25-dien-3β-ol (3n) acetate by hydrogenation. RRT 1.93 (GLC), RRT 1.26 (HPLC). MS, *m/z* 456 $[M]^+$. ^1H NMR data. Assignment, δ (multiplicity, *J* in Hz): 3α-H, 4.694 (*tt*, 4.4, 11.5); CO-CH_3, 2.026 (*s*); 7-H, 5.146 (*dd* like, 2.2, 4.4); 18-H_3, 0.533 (*s*); 19-H_3, 0.809 (*s*); 21-H_3, 0.931 (*d*, 6.6); 26-H_3, 0.832 (*d*, 7.1); 27-H_3, 0.812 (*d*, 6.6); 29-H_3, 0.855 (*t*, 7.4).

Reaction of stigmasta-5,24-dienol (2j) and (24E)-stigmasta-5,24(28)-dienol (2k) acetates with iodine. The isomerization reaction was performed in the same manner as described by Ikekawa *et al.* (34). A solution of 1.0 g fucosterol (**2k**) acetate and 70 mg iodine in 50 mL benzene was refluxed for 15 hr. After washing the solution with 1% $Na_2S_2O_3$ and water, the benzene layer was dried over anhydrous Na_2SO_4. Evaporation of the solvent gave 862 mg of crude product. Argentation TLC followed by HPLC of the product enabled the isolation of the following compounds as the acetyl derivatives (Percentages are abundances in the reaction mixture and not isolated yields.): **2h** [mp 120–121°C; RRT 1.61 (GLC) and 0.96 (HPLC); R_f 0.38; 21.6%], **2i** (mp 106–108°C; RRT 1.65 and 0.98; R_f 0.35; 17.0%), **2j** (mp 139–140°C; RRT 1.95 and 1.01; R_f 0.49; 39.8%), **2k** (mp 115–117°C; RRT 1.73 and 0.98; R_f 0.34; 13.3%), **2l** (mp 135–137°C; RRT 1.81 and 0.99; R_f 0.30, 4.3%), **2m** (mp 132–134°C; RRT 1.63 and 0.93; R_f 0.30; 1.4%), and **2n** (mp 130–131°C; RRT 1.63 and 0.91; R_f0.30; 2.6%). Isomerization products were identified by spectral comparison (MS, ^1H and ^{13}C NMR). See Table 2 for the ^{13}C NMR spectral data. The reaction of 24-ethyldesmosterol (**2j**) acetate with iodine yielded almost the same results as with **2k**-acetate described above (acetates of **2h**, 23.2%; **2i**, 12.1%; **2j**, 43.0%; **2k**, 13.4%; **2l**, 4.2%; **2m**, 1.3%; and **2n**, 2.8%). This work enabled us to isolate the acetates of **2h**, **2i**, **2j**, and **2m** in sufficient amount for ^{13}C NMR studies. They are known compounds but their ^{13}C NMR data had not yet been reported in the literature. Their spectra and those of **2l**- and **2m**-acetates were assigned by comparison with literature data (15,35,36).

RESULTS AND DISCUSSION

The abundances of all identified sterols of *K. pinnata* and the chromatographic data of their acetates are listed in Table 1. ^{13}C and ^1H NMR data of the acetates of the novel sterols **1j**, **1p**, **2m**, and **3m** are included in Tables 2 and 3 respectively. Fourteen of the eighteen compounds in Table 1 are known sterols. They were identified by comparison of their chromatographic (GLC, HPLC) and spectral data (400 MHz ^1H NMR, MS) with literature data (9,10,14–19) and with those of reference compounds.

Structure determination of the new sterols. A sample of crude (24*S*)- stigmasta-5,25-dien-34-ol (**2n**, clerosterol) acetate was obtained by reverse phase HPLC after fractionation of the acetylated *K. pinnata* sterols by argentation TLC, saponification of the acetates, separation of Δ^5- and Δ^7-sterols by silica gel TLC and re-acetylation. Purification of clerosterol (**2n**) acetate and isolation of the minor component of the crude clerosterol acetate sample was accomplished by repetitive HPLC fractionation. The eluate was divided into two subfractions (A and B), fraction A corresponding to the first half of the peak and fraction B corresponding to the second half. The residue of each of the fractions was reinjected, and, again, the peaks were divided into two fractions. This process was repeated several times until clerosterol (**2n**) acetate, which has the shorter retention time, and the acetate of the novel sterol (**2m**) which has the longer retention time, were pure by 400 MHz ^1H NMR.

The mass spectra of the acetates of **2m** and **2n** were indistinguishable and their ^1H NMR spectra very similar. There is only a 2–3 Hz difference in the shift of the 21-H_3 doublet and the 29-H_3 triplet (Table 2). Thus it appeared that **2m** and **2n** were stereoisomers.

The structure of **2n**-acetate, which had tentatively been assigned by comparison with reference compound, was confirmed by catalytic hydrogenation to give clionasterol

TABLE 2

^{12}C NMR Data (100.62 MHz, $CDCl_3$) of the Acetyl Derivatives of Some Side-Chain Unsaturated 24-Ethyl-Δ^5-sterols[a]

	Acetate						
Carbon	2h[b]	2i	2j	2k	2l	2m	2n
16	28.35	28.34	28.18	28.23	28.24	28.15	28.14
17	56.33	56.25	55.79	55.72	55.99	55.85	56.02
20	36.95	36.97	36.22	36.41	36.16	35.97	35.53
21	18.74	18.78	18.78	18.73	18.81	18.90	18.64
22	33.86	33.52	34.89	35.19	35.94	33.76	33.63
23	120.42	120.46	28.65	25.66	27.89	29.59	29.38
24	147.62	146.24	134.75	146.98	145.88	49.72	49.52
25	34.37	28.87	123.25	34.76	28.62	147.95	147.50
26	22.33[c]	20.96[c]	19.95	22.22	21.02[c]	18.08	17.78
27	22.36[c]	21.24[c]	20.10	22.11	21.10[c]	111.09	111.37
28	22.33	23.18	25.45	115.54	116.45	25.99	26.52
29	13.98	13.57	13.26	13.17	12.77	11.98	12.06

[a]Assignments were confirmed by DEPT experiments.

[b]Other signals: assignments (δ) C1(37.00), C2(27.78), C3(74.00), C4(38.13), C5(139.67), C6(122.65), C7(31.90), C8(31.88), C9(50.02), C10(36.60), C11(21.03), C12(39.68), C13(42.39), C14(56.69), C15(24.35), C18(11.90), C19(19.32), CO*Me*(21.46), *C*OMe(170.57). The carbon signals (C1-C15, C18, C19, *CO*Me) for the acetates of **2i**, **2j**, **2k**, **2l**, **2m**, and **2n** were almost identical with the corresponding signals of **2h**-acetate described above.

[c]Assignment interchangeable in each column.

TABLE 3

^{1}H NMR Data (400 MHz, $CDCl_3$) of the Acetyl Derivatives of Some Sterols[a]

	Acetate							
H	1j[b]	1p[b]	2m[b]	2m[c]	2n[b]	2n[c]	3m[b]	3n[b]
3α-H	4.683(*tt*) (5.0,11.0)	4.684(*tt*) (5.0,11.0)	4.601(*m*) (25)	4.602(*m*) (25)	4.603(*m*) (25)	4.601(*m*) (25)	4.692(*tt*) (4.7,11.0)	4.693(*tt*) (4.7,11.0)
CO−CH_3(*s*)	2.016	2.018	2.032	2.034	2.031	2.032	2.024	2.026
4-H_2 (*m*,1H each)	—	—	2.303 2.324	2.306 2.325	2.309 2.327	2.309 2.327	—	—
6-H(*d* like)	—	—	5.372(4.4)	5.373(4.4)	5.375(4.9)	5.372(4.9)	—	—
7-H(*dd* like)	—	—	—	—	—	—	5.142 (2.5,4.1)	5.144 (2.5,4.1)
18−H_3(*s*)	0.650	0.656	0.665	0.665	0.669	0.669	0.522	0.526
19−H_3(*s*)	0.818	0.818	1.016	1.016	1.017	1.016	0.807	0.809
21−H_3(*d*)	0.951(6.6)	0.946(6.3)	0.912(6.6)	0.912(6.6)	0.904(6.6)	0.904(6.6)	0.918(6.3)	0.910(6.6)
26−H_3	1.622(*s*)[d]	1.054(*s*)	1.568(*s*)	1.571(*s*)	1.565(*s*)	1.565(*s*)	1.572(*s*)	1.567(*s*)
27−H_3(*s*)	1.628[d]	1.054	—	—	—	—	—	—
27−H_2 (1H each)	—	—	4.636(*d*,2.4) 4.720(br.*s*)	4.635(*d*,2.0) 4.722(*dt*) (3.0,1.4)	4.641(*d*,2.2) 4.725(*dt*) (3.9,1.5)	4.641(*d*,2.2) 4.726(*dt*) (4.4,1.6)	4.637(br.*s*) 4.724(br.*s*)	4.644(br.*s*) 4.729(br.*s*)
28−H_2	1.993(*q*) (7.7)	4.656(br.*s*) 4.829(br.*s*)	—	—	—	—	—	—
29−H_3(*t*)	0.928(7.7)	1.054(*s*)	0.794(7.4)	0.796(7.4)	0.801(7.4)	0.801(7.4)	0.795(7.3)	0.802(7.4)

[a]Shifts are δ values. Internal standard TMS. Figures in parentheses denote *J* values (Hz) with the exception for the multiplet signals where the figures denote $W_{1/2}$ values (Hz).

[b]Isolated from *Kalanchoe pinnata* in this study.

[c]Semi-synthetic compound

[d]Assignment interchangeable.

(**2f**) acetate (9). Spectral similarities (*vide supra*) indicated that the structure of the minor component of the crude clerosterol (**2n**) acetate sample was (24*R*)-stigmasta-5,25-dien-3β-ol (24-epiclerosterol, **2m**) acetate. The structure of the minor component was confirmed by its catalytic hydrogenation to give sitosterol (**2e**) acetate which was identified on the basis of the ^{1}H NMR spectra. ^{13}C NMR data of **2m**- and **2n**-acetates are included in Table 2.

The repetitive HPLC peak fractionation was also used to obtain crude (24*S*)-5α-stigmasta-7,25-dien-3β-ol (**3n**) acetate, for its purification, and to obtain the minor component of that crude sample in pure form. This minor component was identified as (24*R*)-5α-stigmasta-7,25-dien-3β-ol (**3m**) acetate because of spectral similarities (^{1}H NMR, MS) between **3m**- and **3n**-acetates and because **3m**-acetate, upon catalytic hydrogenation, afforded (24*R*)-5α-stigmast-7-en-3β-ol (**3e**) acetate. The structure of the major component, **3n**-acetate, was confirmed because it yielded (24*S*)-5α-stigmast-7-en-3β-ol (**3f**) acetate upon hydrogenation.

The acetates of the novel sterols **1j** and **1p** had very similar mass spectra. Their molecular weight was 456 (acetate of a monounsaturated C_{29} sterol) and the presence of a diagnostic peak at *m/z* 315 [M − side chain 2H]$^+$ indicated that the sterols possess a saturated skeleton and a monounsaturated C_{10} side chain (37). Comparison of their ^{1}H NMR data (Table 3) with Zürcher values (38) for 5α-cholestan-3β-ol acetate (18-H_3 δ 0.650; 19-H_3 δ0.825) showed that both sterols had a normal (*i.e.*, 5α, 14α) skeleton. The diagnostic peak at *m/z* 358 (100%, [M − end of side chain]$^+$) due to a McLafferty rearrangement supported a structure with either a $\Delta^{24(25)}$ or a $\Delta^{24(28)}$ double bond (37). NMR comparison of **1j**-acetate (Table 3) and the acetate of 24-ethyldesmosterol (**2j**) (see Materials and Methods), the main sterol of *K. pinnata*, left no doubt that **1j** and **2j** had the same side chain. Thus the structure of **1j** is 5α-stigmast-24(25)-en-3β-ol.

The NMR spectrum of **1p**-acetate showed presence of two olefinic protons (δ 4.656, 1H, *s*; δ 4.829, 1H, *s*) and of a deshielded *t*-butyl singlet (δ 1.054), indicative of a *t*-butyl substituent at a double bond. In combination with the other information obtained from its mass and ^{1}H NMR spectra (*vide supra*), **1p** was shown to be 25-methyl-5α-ergost-24(28)-en-3β-ol. The proposed structure of **1p** was confirmed by NMR comparison of **1p**-acetate (Table 3) and **2p**-acetate (see Materials and Methods), a constituent of several higher plants (5,29,30,39).

Partial synthesis of both epimers at C-24 of stigmasta-5,25-dien-3β-ol (2m,n). 24-Ethyldesmosterol (**2j**) is the main sterol of *K. pinnata*. We felt that we had to address the question whether 24-epiclerosterol (**2m**) was indeed a natural product or whether it was an artefact formed during the isolation procedure from the main sterol *via* a carbonium ion intermediate. The iodine-mediated double bond isomerization of **2j**-acetate and fucosterol (**2k**) acetate has been reported to yield an equilibrium mixture of Δ^{23}, Δ^{24}, $\Delta^{24(28)}$ and Δ^{25} isomers (34). However, not all products were properly identified. We repeated this work (see Materials and Methods for details) and confirmed that essentially the same mixture was obtained starting with **2j**-acetate or **2k**-acetate. As expected, both epimers of Δ^{25}-sterols (**2m,n**) could be isolated. Δ^{23}-Sterols (**2h,i**-acetates, together ≈40%) and Δ^{24}-sterol (**2j**, ≈40%) are major components of the reaction mixtures. Thus if double bond isomerization had taken place during the isolation procedure, Δ^{23}-sterols would also have been formed. Careful analysis of the *K. pinnata* sterols did not result in the detection of Δ^{23}-sterols. Thus, no artefacts were formed during the isolation procedure.

^{1}H and ^{13}C NMR spectra of 24-ethyl-Δ^{25}-sterols. The 400 MHz ^{1}H NMR data of the two C-24 epimeric pairs of 24-ethyl-Δ^{25}-sterols (**2m,n** and **3m,n**) show that discrimination between the C-24 epimers is possible on the basis of the shift of the 21-H_3 and 29-H_3 signals (see Table 3). The 24*R*-epimers have their 21-H_3 doublet at lower field (*ca.* 3 Hz, 0.008 ppm) and their 29-H_3 triplet at higher field (2–3 Hz, 0.005–0.007 ppm) than the 24*S*-epimers. Differentiation between the epimers is also possible using the signals of C-17 and of the side-chain carbons (C-20–C-29) in the ^{13}C NMR spectra (see Table 2 for ^{13}C NMR data of **2m,n**-acetates).

Sterol side-chain biosynthesis and configuration at C-24. Some sterols of *K. pinnata* (Table 1) are sterically pure at C-24 (**2d, 2g**) whereas others occur as mixtures of epimers (**2a,b, 2m,n, 3m,n**). We have noticed this before in our detailed work on sterols of the *Cucurbitaceae* (11–19) and *Clerodendrum* species (Verbenaceae) (6–10). Apparently, *K. pinnata* is another example of a plant in which sterol biosynthesis has become complicated: different mechanisms for the formation of the same side chain seem to be operating parallel and at the same time. The Δ^{25}-sterols, **2m,n** and **3m,n**, are epimeric mixtures because different mechanisms do not necessarily result in the formation of the same epimer at C-24.

Other examples of 24-alkyl-Δ^{25}-sterols from higher plants. In cases where the configuration at C-24 has been firmly established, Δ^{25}-unsaturated 24-alkylsterols isolated from seed-bearing plants always were reported to have the 24*S*/β-stereochemistry (4–20,23–29,31). Thus, this study constitutes the first example of the isolation of 24*R*-alkyl-Δ^{25}-sterols, **2m** and **3m**, from the seed-bearing plants, and, also, the first demonstration of the co-occurrence of C-24 epimeric 24-alkyl-Δ^{25}-sterols (**2m/2n** and **3m/3n**) in a non-marine organism. Epimeric mixtures have been found in sponges (40,41). (Sponges do not do *de novo* synthesis of sterols but accumulate dietary sterols. Unless they modify dietary sterols they are thus an unlikely source of sterically pure 24-alkylsterols.) Some ferns (which are vascular plants) have been demonstrated to contain either 24*R*- or 24*S*- epimer of 24-alkyl-Δ^{25}-sterols. Thus, some polypodiaceous ferns contain (24*R*)-24-methyl-25-dehydrolophenol, 24-epicyclolaudenol, (24*R*)-24-ethyl-25-dehydrolophenol, and (24*R*)-cyclomargenol [(24*R*)-24-ethyl-9β,19-cyclo-5α-lanost-25-en-3β-ol] (42,43), whereas a dryopteridaceous fern contains polysthicol [(24*S*)-24-ethyl-9β,19-cyclo-5α-lanost-25-en-3β-ol], the 24*S*-epimer of (24*R*)-cyclomargenol (44).

Other examples of plant sterols alkylated at C-25. Sterol **1p**, a novel sterol, constituted the fourth 25-methylated 24-methylenesterol (with side chain **p**) from a natural source. The previously known sterols possessing a side chain **p** are: **2p** (5,28,29,39) isolated also in this study from *K. pinnata*, 25-methyl-5α-ergosta-7,24(28)-dien-3β-ol (**3p**) (45), and 24-methylene-25-methyllophenol (25-methylgramisterol) (46) isolated from some seed-bearing plants.

These are rare examples of sterols with quaternary carbon in the side chain. Sterols possessing an acyclic, quaternary carbon in the side chain are encountered more frequently in marine sponges (47–49).

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Occurrence and Levels of Ecdysteroids in Spinach[1]

Robert J. Grebenok, Perry V. Ripa and John H. Adler*
Department of Biological Sciences and Department of Chemistry, Michigan Technological University, Houghton, Michigan 49931

The dominant ecdysteroid in spinach, *Spinacia oleracea* L., is 20-hydroxyecdysone (2β,3β,14α,20R,22R,25-hexahydroxy-5β-cholest-7-en-6-one) with the presence of a smaller amount of polypodine B (2β,3β,5β,14α,20R,22R,25-heptahydroxycholest-7-en-6-one). Ecdysteroids are present in the seed embryo at 14 μg ecdysteroid/seed, and ecdysteroid levels increase in the plant during growth and development. During the onset of ecdysteroid production, the ratio of ecdysteroid to total sterol shifts from a value of one to about ten over the course of a week. Spinach may serve as a model to study ecdysteroid biosynthesis in plants.
Lipids 26, 666–668 (1991).

Since the first isolation of ecdysteroids from plants (1,2), these steroids were thought to serve as a defense against insects. Susceptibility of insects to these compounds varies depending upon the insect species and the structure of the ecdysteroid (2–4). The most commonly reported plant ecdysteroid, 20-hydroxyecdysone, when present at 50 parts per million (ppm) of an artificial diet, is effective on pink bollworm *(Pectinophora gossypiella)* causing a 95% ecdysis inhibition (3). The 20-hydroxyecdysone causes a 50% feeding inhibition for the aphid *(Schizaphis graminum)* at 650 ppm in the diet , but has no effect on feeding of corn earworm *(Heliothus zea)* at 3000 ppm in the diet (3). Alteration of the phytoecdysteroid structure from 20-hydroxyecdysone to ponasterone A by loss of the C-25 hydroxyl group produces at only 2 ppm a 95% ecdysis inhibition in pink bollworm (3). Polyphagous insects such as locust (2) are reportedly affected by dietary ecdysteroids to various degrees.

Well over one-hundred plant families have been reported to contain ecdysteroids (2,5), yet little is known about the production of phytoecdysteroids in plants. In perennials, phytoecdysteroids cycle within plant organs concurrently with the onset of developmental events, such as new shoot growth (6–10) and flowering (10). In the leaves of the perennial evergreen *Taxus cuspidata,* 20-hydroxyecdysone and ponasterone A levels increase as leaves mature during the first year's growth, and appear to be rapidly transported into newly developing leaves during the next flush of leaf growth (6). However, levels in two-year-old leaves do not decrease below a level that is physiologically effective (40 μg 20-hydroxyecdysone/g fresh weight (fr wt) and 20 μg ponasterone A/g fr wt) in altering insect development (3). In the herbaceous perennial *Rhaponticum carthamoides,* the underground parts cycle ecdysteroids to the newly developing shoots, and in autumn the ecdysteroid level of the underground parts rises (7). Similarly, in *Leuzea carthamoides* the young herbaceous shoots of spring have the highest ecdysteroid levels (8). The biosynthesis of ecdysteroids is difficult to study in perennial plants due in part to the large mass of these compounds that appear to be cycled in the plant. Annual plants, however, initially carry forward only the ecdysteroid present in the seed. In this paper we examine the occurrence, onset of production, and enhanced accumulation of ecdysteroids in the annual spinach. Spinach may serve as a model for studying phytoecdysteroid biosynthesis in seed crop plants.

EXPERIMENTAL

Spinacia oleracea (Spinach) variety Avon (Burpee #5133), used for analysis of ecdysteroid production at various developmental stages, was grown in the laboratory using 8-hr photoperiods. Seeds and leaves were extracted with ethanol for 72 hr. The material was ground, reextracted, evaporated to dryness, resuspended in 70% methanol/water and then partitioned against hexane to remove lipids and 4-desmethylsterols. The methanol/water fraction was evaporated to dryness, resuspended in water, and partitioned against butanol to remove ecdysteroids (6). The butanol fraction was evaporated to dryness, resuspended in methanol, and filtered through a 45-μm membrane. The filtrate was evaporated and analyzed at 254 nm using reverse phase high pressure liquid chromatography (RP-HPLC) on a C18 ODS, Supelco 4.6 mm × 25 cm, 5-μm particle column (Supelco, Bellefonte, PA). Elution was with methanol/water (45:55, v/v) at 1.0 mL/min at 33°C. Quantitation was based on a standard curve for 20-hydroxyecdysone. Fresh commercial spinach (226 g) was extracted as described above, to verify the presence of ecdysteroids by ^{1}H nuclear magnetic resonance spectroscopy (NMR). The butanol fraction after evaporation was resuspended in a minimal amount of methanol/ethyl acetate (1:3, v/v). The resuspended fraction was applied to a 15% deactivated neutral alumina column, and was eluted utilizing 7.5 mL of each of the following: methanol/ethyl acetate (1:3, v/v); methanol/ethyl acetate (1:1, v/v); methanol; and water. 20-Hydroxyecdysone eluted with the 1:1 methanol/ethyl acetate fraction. Polypodine B, when present, eluted with the methanol and water fractions. Thin-layer chromatography (TLC) on 0.3 mm layers of Silica Gel G (Sigma, St. Louis, MO) was utilized to identify the ecdysteroids in various column fractions. The solvent system chloroform/ethyl acetate/ethanol/acetic acid (50:25:20:5, by vol) was used which gave the following R_f values for authentic standards: 20-hydroxyecdysone, 0.20; polypodine B, 0.23; makisterone A, 0.25; and ecdysone, 0.26. Ecdysteroids were made visible on the TLC plates by spraying with a solution of 3% vanillin and 2% H_2SO_4 in ethanol and subsequent heating for 8 min at 110°C (2).

^{1}H NMR spectra were measured at 200 MHz in perdeuteropyridine at 22°C using tetramethylsilane (TMS) as internal standard. The ^{1}H NMR spectrum of 20-hydroxyecdysone isolated from spinach showed the following signals (in ppm from TMS) for protons on the

[1]Based on a paper presented at the symposium on Plant and Fungal Sterols: Biosynthesis, Metabolism and Function, held at the AOCS Annual Meeting, Baltimore, MD, April 1990.

*To whom correspondence should be addressed.

Abbreviations: fr wt, fresh weight; NMR, nuclear magnetic resonance; ppm, parts per million; RP-HPLC, reverse phase high pressure liquid chromatography; TLC, thin-layer chromatography, TMS, tetramethylsilane.

carbons designated: 3H at C-18(s) 1.23, 3H at C-19(s) 1.08, 3H at C-21(s) 1.60, 6H at C-26,27(s,s) 1.37. The ^{1}H NMR spectrum of polypodine B isolated from spinach showed the following chemical shifts: 3H at C-18(s) 1.23, 3H at C-19(s) 1.17, 3H at C-21(s) 1.60, 6H at C-26,27(s,s) 1.37. All ^{1}H NMR values are in agreement with those of authentic standards run under identical conditions and with reported values (2,6,11,12).

The sterols were isolated from the hexane fraction. The hexane fraction was evaporated to dryness and the residue saponified with 5% KOH in ethanol/water (7:3, v/v). The sterols were extracted with diethyl ether, and the diethyl ether was subsequently washed with water to neutrality. The neutral lipids present in the diethyl ether fraction were chromatographed on 10% deactivated alumina by eluting with 7.5 mL of each of the following: hexane; hexane/toluene (1:1, v/v); toluene; diethyl ether; diethyl ether/methanol (1:1, v/v); and methanol (13). The fractions were analyzed by gas-liquid chromatography on 1% XE-60 on Chromosorb Q (100–120 mesh) at 235°C. Desmethylsterols that were separated by silica gel TLC with the solvent system benzene/ethyl acetate (9:1, v/v) were analyzed by analytical RP-HPLC on a Supelco C18 ODS column (25 cm × 4.6 mm) using acetonitrile/isopropyl alcohol (8:2, v/v; 1 mL/min) at 45°C and were detected at 210 nm. The chromatographic characteristics of the three major sterols, spinasterol, 22-dihydrospinasterol and avenasterol were in agreement with reported values (13–15).

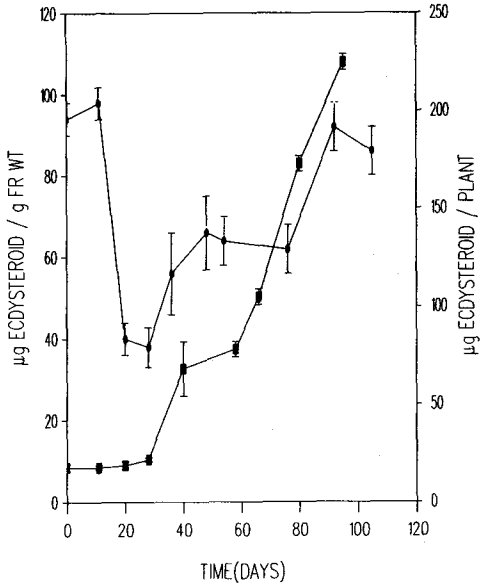


FIG. 1. Time course of ecdysteroid content in spinach plotted on a per plant and fresh weight basis. Plot of μg ecdysteroid per g fresh weight (●) and μg ecdysteroid per plant (■) from three independent analyses of spinach plants planted on different start dates. Data for day zero were from seeds on the day of planting which started the time clock for all analyses. Plants typically emerged from the soil between days 3 and 4. The bars represent the range of values from an average of the 3 data sets.

RESULTS AND DISCUSSION

20-Hydroxyecdysone (2β,3β,14α,20R,22R,25-hexahydroxy-5β-cholest-7-en-6-one) and polypodine B (2β,3β, 5β,14α, 20R,22R,25-heptahydroxycholest-7-en-6-one) from spinach were characterized by chromatography and identified by ^{1}H NMR. The data confirm previous reports on 20-hydroxyecdysone (16,17) in spinach and furthermore demonstrate the occurrence of polypodine B in this plant. Polypodine B and 20-hydroxyecdysone were present at a ratio of approximately 1:4 in mature leaves of commercially grown spinach as well as throughout the developmental stages of spinach examined in this study. The mixture(s) of 20-hydroxyecdysone and polypodine B will be referred to as ecdysteroid(s).

Laboratory-grown spinach from three different plantings was analyzed for ecdysteroid content. The levels of ecdysteroid (μg ecdysteroid/g fr wt) showed reproducible changes during growth (Fig. 1). Seeds contained approximately 90 μg ecdysteroid/g fr wt at day zero, and upon germination the level dropped to 40 μg ecdysteroid/g fr wt by day 20–27. This decrease on a μg ecdysteroid/g fr wt basis is a consequence of the gain in fresh weight as the seedling develops. During further growth, two cyclical increases in ecdysteroid levels occurred between days 27–44 and days 76–90. A relatively constant level of 20-hydroxyecdysone was found in spinach between days 44 and 76 at 65 μg ecdysteroid/g fr wt. Spinach plants at 90 days of age had reached a level of ecdysteroid sufficient to supply their seeds with ecdysteroid levels similar to those found in commercial seeds. Over the 100-day time course of our experiments, the level of ecdysteroid (μg ecdysteroid/g fr wt) maintained in the plants was sufficient to effectively control certain herbivorous insects (3). Although ecdysteroid levels (μg ecdysteroid/g fr wt) underwent cyclic changes in the course of plant growth, the amount of ecdysteroid on a per-plant basis (μg ecdysteroid/plant) showed a steady increase from day 24 onward (Fig. 1). This increase in ecdysteroid content per plant coincided with the increase observed on a μg ecdysteroid/g fr wt basis which may suggest the onset of ecdysteroid biosynthesis.

TABLE 1

Spinach Ecdysteroid and Sterol Content

Time (days)[a]	Plant mass (g fr wt)	Ecdysteroid (μg/g fr wt)	Sterol (μg/g fr wt)	Ratio ecdysteroid/ sterol
21	3.4	168	119	1.4
33	3.1	157	120	1.3
36	8.5	56	61	0.9
37	8.0	136	40	3.4
40	13	790	74	10.7
50	21	330	40	8.2

[a]All experiments were from the same day planting of *Spinacia oleracea* variety Avon, except that data for day 21 were from an independent planting.

Sterols have been shown to be the biosynthetic precursors of ecdysteroids in insects (18) and are presumed to serve a similar function in plants (19). Analyses of ecdysteroids and sterols from a single experiment are presented in Table 1. The predominant sterols were spinasterol (24α-ethylcholesta-7,22E-dien-3β-ol), 22-dihydrospinasterol (24α-ethylcholest-7-en-3β-ol) and avenasterol (24-ethylcholesta-7,24(28)Z-dien-3β-ol) which were present at 71%, 10% and 13% of total sterol content, respectively.

In Table 1, the levels of ecdysteroid and sterol are reported for the time period of the first cyclic increase in ecdysteroid content shown in Figure 1, *i.e.* for days 27–44. Throughout the time course, plant mass increased. Early in spinach development, the sterol levels at days 21 and 33 were elevated on a μg sterol/g fr wt of plant basis as compared to those at day 36 and onward. After day 36, an increase in μg ecdysteroid/g fr wt was observed. This increase in ecdysteroid levels in the plant coincides with the observed decreased sterol levels on g fr wt basis. The ecdysteroid-to-sterol ratio is maintained at approximately 1 from days 21 through 36, but dramatically increased from day 37 to 50 (Table 1). The timing of the shifts in sterol and ecdysteroid levels (Table 1) suggests a coordinated control of ecdysteroid and sterol biosynthesis and supports the presumption that plant ecdysteroids are derived from sterols. Interestingly, the levels of ecdysteroids and sterols in spinach were approximately equal (days 21–36) or the ecdysteroids were present in greater amounts than the sterols (days 37–50) during the early phase of plant growth (Table 1). Ecdysteroid-to-sterol ratios were substantially greater in this plant than those reported for insects. In insects the ecdysteroids are used hormonally and are present at a lower level than the sterols (18,19).

Spinach may be an ideal model system for the study of ecdysteroid biosynthesis in plants. The system has many advantages: the rather clear onset of phytoecdysteroid biosynthesis (Table 1); the relative lack of high background levels of ecdysteroids often present in perennial plants (6–8); the apparent regulation of ecdysteroid levels within physiologically effective dose ranges that deter some insect species (3,20); and the absence of interfering compounds in the chromatographic analysis of spinach ecdysteroids. Use of natural insect growth regulators instead of poisons is likely to take on greater significance in an environmentally aware world.

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Fatty Acid Metabolism in *Taphrina deformans* Treated with Sterol Biosynthesis Inhibitors[1]

J.D. Weete*, M. Sancholle[2], K.A. Patterson, K.S. Miller, M.Q. Huang[3], F. Campbell and M. Van den Reek
Department of Botany and Microbiology, Alabama Agricultural Experiment Station, Auburn University, Auburn, Alabama 36849

Changes in the unsaturated fatty acid content of the fungus *Taphrina deformans* as a function of growth, temperature, and sterol content were investigated. It was found that the highest growth rate was accompanied by a relatively high degree of fatty acid unsaturation (18:1 < 18:2 + 18:3) and low sterol (brassicasterol) content. Also, a substantial shift in the degree of unsaturation from mainly 18:2 + 18:3 to 18:1 occurred in the later stages (mid-linear) of culture development. Cells readily adapted from 18°C to 13°C, and exhibited a higher growth rate at the lower temperature after a period of acclimation. Growth was readily inhibited by the sterol biosynthesis inhibitors propiconazole and naftifine, which blocked brassicasterol biosynthesis at C-14 demethylation and squalene epoxidation, respectively. Growth was also inhibited by tunicamycin which did so without affecting sterol content. The shift in degree of fatty acid unsaturation did not occur in cells from cultures at reduced temperature or treated with any of the inhibitors. Since tunicamycin did not affect the sterol content, delay in the shift in the degree of fatty acid unsaturation was attributed to factors other than a reduction in sterol content.
***Lipids* 26, 669–674 (1991).**

There is a relatively large number of chemically diverse substances known collectively as sterol biosynthesis inhibitors (SBIs) that block the formation of ergosterol, or a functionally equivalent sterol, in fungi. For example, certain azoles (*e.g.* triazoles and imidazoles) prevent the conversion of lanosterol to ergosterol by inhibiting C-14 demethylation (1). Since sterols such as ergosterol are required for growth, many SBIs exhibit potent antifungal activity. We have worked with several triazoles and fungal species in this laboratory, but mainly with the propiconazole and *Taphrina deformans*, respectively. *T. deformans* proliferates by budding (as a yeast) in laboratory culture, produces C_{18} polyenoic fatty acids, and produces brassicasterol rather than ergosterol as the major sterol. Growth of this fungus is suppressed about 50% by 0.073 μg/mL propiconazole, and the sterol composition of the treated cells changes from approximately 95% brassicasterol to mainly C-14 methyl sterols such as 24-methylene-24,25-dihydrolanosterol, obtusifoliol and 14-methyl fecosterol (2,3).

This laboratory and others have reported that treatment of many fungi with certain azoles also results in altered fatty acid metabolism. This is characterized by increased free fatty acid content and changes in the relative proportions of fatty acids, *e.g.*, increased saturated fatty acids, particularly in the free fatty acid fraction at relatively high inhibitor concentrations, and an increase in the degree of fatty acid unsaturation mainly in the polar lipid fraction (1,4). We and others have interpreted these changes as adaptive or compensatory responses to inhibitor-induced alterations in sterol-related membrane fluidity, principally the reduction of ergosterol and/or increase in C-14 methyl sterols (ref. 1, and refs. cited therein). We began to question this interpretation of fungal responses to SBIs when the sterols and fatty acids in the fungus *Mucor rouxii* treated with these inhibitors were examined (5). About 80 μg/mL of propiconazole are required for 50% growth inhibition of *Mucor* compared to 0.05 to 2 μg/mL for most other fungi. Tolerance to the inhibitor was not due to inability of the inhibitors to suppress ergosterol biosynthesis because only about 2 μg/mL were required to substantially reduce ergosterol content and increase C-14 methyl sterols (5). Furthermore, the fatty acid responses observed in other fungi as described above did not accompany the change in sterol content.

In this study, we have investigated the changes in fatty acid composition in *T. deformans* as a function of culture age, temperature and inhibitor-treatment, including a non-sterol biosynthesis inhibitor to separate growth effects from sterol effects on fatty acid unsaturation. The results suggest that the inhibitor-induced changes in sterol content may not be directly responsible for the alterations in fatty acid composition observed in fungi treated with SBIs, at least not in *T. deformans*, and that the differences between control and treated cultures may be linked more closely to growth related factors than sterol content.

MATERIALS AND METHODS

Naftifine and propiconazole (Fig. 1) were gifts from Sandoz Forschungsinstitut (Vienna, Austria) and the Ciba Geigy Corporation (Greensboro, NC), respectively. Tunicamycin was purchased from Cal Biochem. (La Jolla, CA).

Fungal culture. Taphrina deformans (D1) was obtained as described previously (6) and cultured at 18°C or 13°C in 1% yeast extract/2% dextrose (YD) medium amended with inhibitors at concentrations given in the tables and figures. All cells used for inoculation, regardless of whether they were to be incubated at 18°C or 13°C, were cultivated 36 to 48 hr at 18°C. Except where growth was measured over time, cells were cultured for 48, 60 or 72 hr in 125-mL Erlenmeyer flasks containing 40 mL medium on a rotary incubator-shaker (150 or 176 cycles/min). In time-course experiments, cells were incubated with or without 0.073 μg/mL propiconazole for 72 hr in 2-L Erlenmeyer flasks containing 700 mL medium, and a

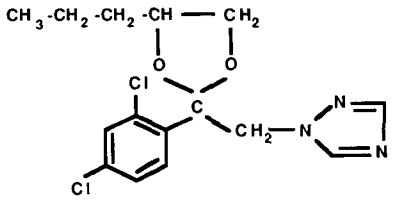
[1]Based on a paper presented at the Symposium on Plant and Fungal Sterols: Biosynthesis, Metabolism and Function, held at the AOCS Annual Meeting, Baltimore, MD, April 1990.

*To whom correspondence should be addressed.

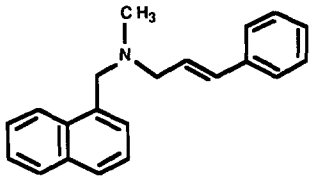
[2]Visiting scientist from the Laboratoire de Vegetale et Cryptogamie, Universite des Sciences et Techniques de Lille Flandres Artois Batiment SN2, 59655 Villeneuve d'Ascq Cédex, France.

[3]Visiting scientist from the South China Institute of Botany, Academia Sinica, Guangzhou, China.

Abbreviations: CoA, coenzyme A; ER, endoplasmic reticulum; FAME, fatty acid methyl esters; GLC, gas-liquid chromatography; GLC/MS, gas-liquid chromatography/mass spectrometry; NMR, nuclear magnetic resonance; SBI, sterol biosynthesis inhibitor; TLC, thin-layer chromatography.



PROPICONAZOLE



NAFTIFINE

FIG. 1. Structures of propiconazole (upper) and naftifine (lower).

portion of the cells was removed periodically for analysis. Cells were collected by centrifugation (1000 × *g*), washed with distilled water, and placed directly into the solvent for lipid extraction or stored at −15°C prior to analysis. Two or 5-mL portions of each culture were also taken at intervals (see tables and figures) and/or at the end of the incubation time for cell dry weight determination.

The values reported here are the average of 2 to 3 replicates with 10% or less variation. The experiments, except for the one involving tunicamycin which was performed once, have been conducted at least twice with essentially the same results each time.

Lipid extraction and hydrolysis. Lipid was extracted from the cells by the procedure of Bligh and Dyer (7). A portion of the total lipid or polar lipid (see below) was heated at 90°C for 120 min in 4.0 mL of 95% ethanol containing 0.4 mL 33% KOH (8). Sterols were obtained by washing the hydrolysate three times with hexane (nonsaponifiable fraction), and fatty acids were obtained in diethyl ether after adjusting the pH of the hydrolysate to approximately 2.

Thin-layer chromatography (TLC). Polar lipids, comprised mainly of phospholipids (2,3), were separated from neutral lipids by TLC on glass plates (20 cm × 20 cm) coated with a 250 μm layer of silica gel 60 (Merck, Darmstadt, Germany) developed twice in hexane/diethyl ether/acetic acid (79:20:4, v/v/v). Silica gel at the origin, which contained the polar lipids, was scraped from the TLC plates and washed with chloroform/methanol (1:1, v/v) containing 0.5% water to recover the polar lipids.

Gas-liquid chromatography (GLC). Sterols were analyzed either as the free alcohol or acetates as described previously (2), except that a 25-m fused silica capillary column coated with SE-54 (Applied Science Laboratories, State College, PA) was used. Acetates were prepared by heating the sterols in pyridine/acetic anhydride (1:1, v/v) at 60°C for 15 min. Steryl acetates were recovered in hexane. In experiments with naftifine, the inhibitor and squalene were both present in the nonsaponifiable fraction and analyzed with the sterols. Sterols were quantified using the internal standard method with either cholesterol or 5-dihydrocholesterol, or their respective acetates, as standards. The identification of brassicasterol has been confirmed by GLC/mass spectrometry (MS) and nuclear magnetic resonance (NMR) (2,9) and 24-methylene-24,25-dihydrolanosterol has been identified by GLC/MS (2). Squalene was detected in the nonsaponifiable fraction of cells treated with naftifine, and was tentatively identified by comparison of its GLC retention time with that of an authentic standard purchased from Sigma Chemical Co. (St. Louis, MO).

Fatty acid methyl esters (FAME) were prepared from the polar lipid fraction by transesterification using 0.3% sodium methoxide in methanol (Applied Science Laboratories), or from the fatty acids obtained from the hydrolysate by heating in 5% conc. HCl in methanol for 60 min at 80°C. FAME were analyzed by GLC as described by Weete *et al.* (2).

Miscellaneous. Lipid phosphorus was measured by the method of Bartlett (10).

RESULTS

Propiconazole. The growth of *T. deformans* was progressively reduced from 7% to 37% by 0.0125 to 0.05 μg/mL propiconazole (Table 1). The reduction of brassicasterol did not follow a similar linear decline as a function of increased inhibitor concentration. Instead, there was a sharp reduction (58%) of this sterol at 0.025 μg/mL propiconazole and no further reduction at higher inhibitor concentrations (Table 1). C-14 demethylation was reduced at the lowest inhibitor concentration, and inhibition of this reaction increased progressively as a function of propiconazole concentration based on changes in 24-methylene-24,25-dihydrolanosterol from 0.3 μg/mg at 0.0125 μg/mL to 0.9 μg/mg at 0.05 μg/mL.

The principal changes in polar lipid fatty acid content in propiconazole-treated cells involved the C_{18} unsaturated fatty acids. There was progressively less 18:1 and more 18:2 and 18:3 with increasing inhibitor concentration (Table 2). For example, there was 16%, 66%, and 81% less 18:1 at inhibitor concentrations of 0.0125, 0.025 and 0.05 μg/mL, and 5.4%, 59% and 87% more 18:2 + 18:3, respectively. There was little change in the relative proportions or absolute amounts of the principal saturated fatty acids, 16:0 and 18:0. However, at higher inhibitor concentrations (0.035 to 0.05 μg/mL), there was a progressive increase in the amounts of these saturated fatty acids in the polar lipid fraction relative to cell dry weight but their relative proportions remained essentially constant (data not given).

Naftifine. The growth of *T. deformans* was progressively reduced 6% to 37% by 0.75 to 3.0 μg/mL naftifine (Fig. 2), and 10 μg/mL were required for 90% inhibition. When expressed on the basis of lipid phosphorus rather than cell dry weight, brassicasterol was only slightly reduced at 0.75 μg/mL naftifine, but it was progressively reduced by 1.5 and 3.0 μg/mL, respectively. As expected, considering the mode of action of this compound as a squalene epoxidase inhibitor (11), squalene accumulated in the cells from 0.11 to 0.35 μg/mg dry weight with increasing inhibitor concentration (data not given).

As with propiconazole, the major changes in polar lipid fatty acid composition in naftifine-treated cells involved the C_{18} unsaturated fatty acids (Fig. 2). As inhibitor concentrations increased, the polar lipid fraction contained

TABLE 1

Effect of Propiconazole on the Growth and Sterol Composition of *Taphrina deformans*[a,b]

Molarity ($\times 10^{-7}$)	Concentration (μg/mL)	Cell dry weight (μg/flask)	%I[b]	Brassicasterol[c]	24-Methylene-24,25-dihydrolanosterol[d]
0	0	367	—	1.2	ND[e]
0.36	0.0125	343	7	1.1	0.3
0.72	0.025	310	16	0.5	0.5
1.44	0.05	231	37	0.6	0.9

[a]Each value is an average of two replicates.
[b]%I, percent inhibition.
[c]All sterol values are expressed as μg sterol per mg dry weight of cells.
[d]Obtusifoliol was also detected in cells treated with 0.025 and 0.05 μg/mL.
[e]Not detected.

TABLE 2

Effects of Propiconazole on the Phospholipid-Fatty Acid Composition of *Taphrina deformans*[a,b,c]

Inhibitor concentration (μg/mL)	16:0		18:0		18:1		18:2		18:3	
	A	B	A	B	A	B	A	B	A	B
0	15.5	0.75	5.4	0.26	40.7	1.97	32.2	1.56	6.2	0.30
0.0125	12.9	0.58	6.9	0.31	36.8	1.66	35.9	1.62	7.5	0.34
0.025	14.2	0.85	7.0	0.32	14.6	0.67	49.5	2.27	14.8	0.68
0.05	14.8	0.72	6.4	0.31	7.6	0.37	51.5	2.51	19.7	0.96

[a]A, relative percent; B, μg/mg cell dry weight.
[b]Cells were cultured at 18°C and harvested 72 hr after inoculation.
[c]These data are representative of those obtained in several experiments. Also see Weete *et al.* (2).

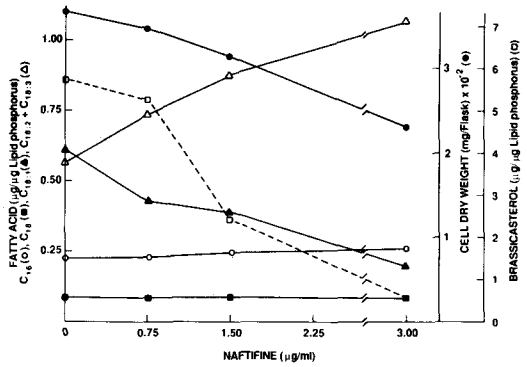


FIG. 2. Effects of naftifine on growth, and sterol and fatty acid composition of *Taphrina deformans* as a function of inhibitor concentration.

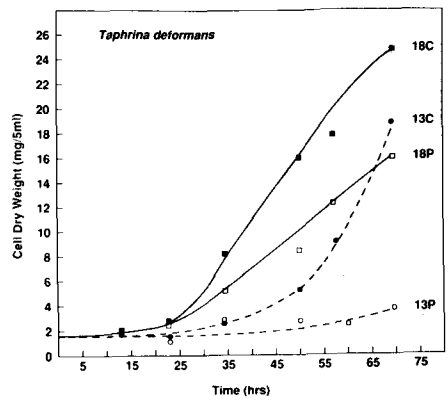


FIG. 3. Effects of propiconazole (P) on the growth of *Taphrina deformans* cultured at 13°C and 18°C (C, Control).

less 18:1 and more 18:2 + 18:3. The saturated fatty acid content remained essentially the same over the range of inhibitor concentrations used.

Effects of propiconazole and temperature on growth. There was a relatively short period of logarithmic growth in the control and inhibitor-treated cultures incubated at 18°C, and an extended linear growth phase. In this experiment, 0.073 μg/mL propiconazole resulted in about 40% growth inhibition after 60-hr incubation when the inhibitor was added at inoculation (Fig. 3). The initiation of growth was delayed slightly by the inhibitor, but the growth rate was reduced by about 30%. By contrast, control cultures incubated at 13°C had an extended log phase that spanned the incubation time (72 hr) of the experiment (Fig. 3). The growth of these cells was delayed approximately 12 hr past the time rapid cell proliferation had begun in control cultures at 18°C. Very little growth was observed in cultures containing propiconazole at 0.073 μg/mL and at 13°C.

Effects of propiconazole and temperature on fatty acid unsaturation. There was very little growth in the cultures at 18°C during the first 23.5 hr of incubation. In the

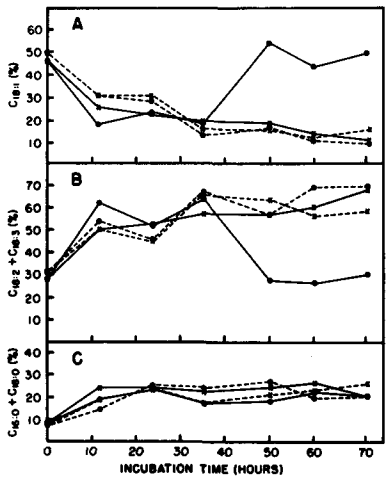


FIG. 4. Effects of propiconazole and temperature on changes in polar lipid unsaturated fatty acids as a function of time. Fatty acid content is expressed as a percentage of polar lipid fatty acids. 18°C: Control ●—●; Propiconazole-treated (0.073 mg/mL) □—□. 13°C: Control ●– – –●; Propiconazole-treated (0.073 mg/mL) □– – –□.

control culture at 13°C, growth was delayed 35 hr, and very little growth occurred in the inhibitor-treated culture at this temperature (Fig. 3). However, changes in the polar lipid fatty acid composition were detected during that time as reflected in changes in the degree of fatty acid unsaturation (Δ/mol). Cells comprising the inoculum culture contained relatively high amounts of 18:1 (45 to 50%) and low amounts of 18:2 + 18:3 (28 to 30%) (Fig. 4). There was a reciprocal shift in these fatty acids during the first 11.5 hr of incubation in all cultures resulting in an increase in the degree of unsaturation. The relatively high Δ/mol persisted during the first 35-hr incubation (Fig. 4). The degree of fatty acid unsaturation in control cells cultured at 18°C and 13°C was reduced 10% and 13%, respectively, between 11.5- and 23.5-hr incubation (Table 3). There was no difference in the Δ/mol in control and treated cells cultured at either temperature for 23.5 hr, but the degree of unsaturation of cells cultured at 13°C was only 8% lower than that of cells incubated at 18°C.

There was a second shift in the fatty acid composition of the control cells cultured at 18°C that involved an increase in the relative amount of 18:1 from 18.4% at 35 hr to 54.2% at 50 hr, and a corresponding decrease in 18:2 + 18:3 (Fig. 4), all of which occurred during the linear growth phase (Fig. 3). This shift in fatty acid composition did not occur in cells treated with propiconazole or cultured at 13°C (Fig. 4). After 35-hr incubation, the degree of fatty acid unsaturation was relatively high and similar in the inhibitor and cold temperature-treated cultures (Table 3). The Δ/mol in propiconazole-treated cells cultured at 18°C increased slowly from 1.31 to 1.60 over the 72-hr incubation period. There was little growth in inhibitor-treated cells at 13°C (Fig. 3).

When the degree of fatty acid unsaturation in cells cultured at 18°C with or without propiconazole is considered as a function of biomass accumulation rather than incubation time, it was found that attaining equal amounts of cells on a weight basis in the two treatments did not result in the same degree of fatty acid unsaturation. For example, control flasks contained 17 mg/mL dry

TABLE 3

Effects of Propiconazole and Temperature on the Degree of Unsaturation of Polar Lipid in *Taphrina deformans* as a Function of Culture Age[a]

Culture age (hr)	Δ/mol[a,b,c]			
	18C	18P	13C	13P
11.5	1.51	1.31	1.44	1.36
23.5	1.36	1.36	1.25	1.25
35.0	1.58	1.43	1.61	1.59
50.0	1.13	1.41	1.48	1.53
60.0	1.00	1.43	1.64	1.34
72.0	1.15	1.60	1.64	1.42

[d]

[a]Cells were cultured in medium containing 0.073 μg propiconazole/mL.

[b]$\Delta/\text{mol} = \frac{\%\ 18{:}1 + 2(\%\ 18{:}2) + 3(\%\ 18{:}3)}{100}$

[c]C, control, P, propiconazole-treated, and the number (18 or 13) refers to the cultivation temperature in °C.

[d]Little or no growth had occurred in cultures at the sampling times above this line.

TABLE 4

Effects of Propiconazole and Temperature on the Degree of Polar Lipid Fatty Acid Unsaturation in *Taphrina deformans* as a Function of Culture Biomass[a,b]

Biomass (mg/mL)	Δ/mol		
	18 C	18 P	13 C
1.5 to 2	1.51 (11.5 hr)	1.31 (11.5 hr)	1.44 (11.5 hr)
5	—	1.42 (35 hr)	1.39 (50 hr)
9 to 10	1.58 (35 hr)	1.41 (50 hr)	1.64 (60 hr)
17	1.13 (50 hr)	1.60 (72 hr)	—
20 to 21	1.00 (60 hr)	—	1.64 (72 hr)

[a]See Table 3 for calculation of Δ/mol.

[b]18 and 13 refer to the incubation temperature. C, control; P, propiconazole.

weight of cells having fatty acids with a Δ/mol of 1.13, *i.e.* after the shift from relatively high 18:2 + 18:3 to 18:1 (Table 4). When the inhibitor-treated cells reached the same biomass, which required an additional 22 hr, the Δ/mol was relatively high at 1.60.

Effects of tunicamycin on growth, sterol content and the relative proportion of total fatty acids. The growth of cells cultured in medium containing 0.02 μg/mL tunicamycin was inhibited 45% compared to controls, and there was no difference in the brassicasterol content of cells from either treatment at 1.1 μg/mg cell dry weight. As before, fatty acids from cells cultured for more than 50 hr at 18°C had a relatively low degree of unsaturation, *i.e.* Δ/mol of 1.27 with a high 18:1 content relative to 18:2 + 18:3 (Table 5). By contrast, cells from cultures

TABLE 5

Effects of Tunicamycin on the Total Fatty Acid Composition of *Taphrina deformans*[a]

Fatty acid	Control	Tunicamycin-treated
16:0	10.9	13.9
18:0	6.8	6.4
18:1	43.2	25.5
18:2	32.9	40.2
18:3	6.1	14.1
Δ/mol	1.27	1.48

[a]The cells were cultured as described in the Materials and Methods section, except that tunicamycin at 0.02 μg/mL was added to the cultures, and cells were harvested for analysis after 72-hr incubation.

containing tunicamycin did not exhibit the shift in fatty acid composition and their lipid had a relatively high degree of fatty acid unsaturation of 1.48 (Table 5).

DISCUSSION

As expected from previous studies, propiconazole is a potent inhibitor of growth (6) and lanosterol C-14 demethylation in *T. deformans* (2). At the relatively low levels of inhibitor used in this study, 24-methylene-24,25-dihydrolanosterol accumulation in the cells, rather than the depletion of brassicasterol, appeared to be a better reflection of dose-related inhibition of C-14 demethylation inhibition, and an indirect manifestation of the suppression of brassicasterol biosynthesis. This might be explained by the relatively slow turnover of brassicasterol in this fungus (Weete, J.D., unpublished data), and formation of small amounts of the sterol at the low inhibitor doses used.

Naftifine was also an effective inhibitor of *T. deformans* growth, showing approximately 10 times more activity against this fungus than several human pathogenic fungi previously tested (11), but over 40 times less potent than propiconazole. Also, the reduction in brassicasterol content of *T. deformans* confirmed naftifine as an inhibitor of sterol biosynthesis, and the accumulation of squalene indicated that squalene epoxidation is the metabolic step blocked by naftifine as observed in other fungi (11). The total amount of squalene, and C-14 methyl sterols in propiconazole-treated cells (see above), that accumulated with naftifine-treatment was greater than the amount of total sterols present in untreated cells. This has been observed in fungi treated with other SBIs (1). The increase in carbon partitioned into the isoprenoid pathway was possibly due to a reduction in feedback regulation of sterol biosynthesis that resulted from the decrease in brassicasterol synthesis.

Tunicamycin is a potent inhibitor of *T. deformans* growth with 0.02 μg/mL as the ED_{45} concentration, compared to 30 to 50 μg/mL as the minimal inhibitory concentration for several yeasts and 1 μg/mL for *Piricularia oxyzae* (12).

Under the batch culture conditions typically employed for *T. deformans,* there are two reciprocal changes in the polar lipid fatty acid fraction that involve the C_{18} unsaturated fatty acids. The first favors a greater degree of unsaturation (18:1 → 18:2 + 18:3) and occurs during logarithmic growth, and the second favors less unsaturation (18:2 + 18:3 → 18:1) and begins during mid-linear growth. Treatment of the cells with sterol biosynthesis inhibitors prevented the shift to less unsaturation. The difference in fatty acid composition between the control and inhibitor-treated cells did not appear to be due simply to differences in the stage of cell growth resulting from growth suppression. However, maintenance of the relatively high degree of unsaturation is not sufficient to overcome growth suppression, and does not appear to be a factor in the growth inhibitory action of propiconazole or probably other sterol biosynthesis inhibitors. Further evidence that fatty acid composition *per se* is not involved in growth suppression by propiconazole is that neither exogenous C_{18} unsaturated fatty acids nor palmitic acid will overcome the growth inhibitory effects of propiconazole in *T. deformans* (13). It is also unlikely that suppression of the shift to less unsaturation is the result of a direct action of the inhibitor since the two chemically distinct inhibitors (triazole *vs* allylamine) used in this study would not be expected to elicit the same response.

T. deformans readily adapts from 18°C to 13°C, and even to 8°C (data not shown), incubation temperature. In fact, after a 12-hr acclimation period, the cells have a higher growth rate at the reduced temperatures. Polar lipid from cells cultured at 13°C had a higher degree of fatty acid unsaturation than that from cells grown at 18°C after 50-hr incubation. These cells exhibit the first shift to higher relative amounts of 18:2 + 18:3 as in cells at 18°C, but rather than undergoing the shift to favor increased levels of 18:1, they maintain the high degree of unsaturation throughout the 72-hr incubation period. Ferrante *et al.* (14) have reported similar results for *Candida lipolytica, i.e.* sequential reciprocal changes in 18:1 and 18:2, first in favor of 18:2 and then 18:1, and prevention of the latter shift to maintain a relatively high degree of fatty acid unsaturation throughout the incubation period at relatively low cultivation temperatures. They also demonstrated that the high 18:2 levels were correlated with an increased maximal specific activity of 18:1-CoA desaturase.

It is well-known that relatively high levels of lipid unsaturation are associated with acclimation to cold temperature in poikilothermic organisms (15). While indeed this was true in *T. deformans,* timing of the increase in unsaturation did not correlate with the onset of growth by cells at 13°C. For example, the 18:1 → 18:2 + 18:3 shift began during the first 11.5-hr incubation, but measurable growth occurred only after about 23.5-hr incubation. Perhaps this might be explained by the fact that a certain degree of lipid unsaturation necessary for growth was not achieved until 23 hr, but it also suggests that there may be adaptive changes other than fatty acid unsaturation in the acclimation process that are required for growth of *T. deformans* at the reduced temperature.

The early proposal that alterations in fatty acid composition in fungi treated with azoles are due to changes in sterol content of the membranes was based on the following: i) One of the mechanisms of modulating membrane fluidity by poikilothermic microorganisms in response to environmental signals is by changing the acyl chain lengths and the degree of fatty acid saturation/unsaturation in membrane lipid (15), and ii) the specific sterol available to sterol-auxotrophic yeast cells influences

the fatty acids that best support growth (16). In addition, by analogy with acclimation to cold temperature, where a relatively high degree of fatty acid unsaturation is associated with reduced cultivation temperature, reduction in sterol content may alter fluidity such that a compensatory response manifested by increased fatty acid unsaturation results. However, the results of this study suggest that the high degree of fatty acid unsaturation in *Taphrina* cells treated with SBIs may be coincident with, but not the direct result of, reduced brassicasterol and/or elevated C-14 methyl sterols. Maintenance of the relatively high degree of fatty acid unsaturation could result from growth suppression *per se* rather than a decrease in sterols, as was suggested by incubating the cells with tunicamycin, an inhibitor of glycoprotein biosynthesis (12). In this case, growth was inhibited 45% with no effect on sterol content, but the 18:2 + 18:3 content remained relatively high in the inhibitor-treated cells compared to the corresponding controls. Further evidence that the sterol content of membranes and desaturase activity are not directly related is that Mucorales fungi such as *Mucor rouxii* and *Rhizopus stolonifer,* which are relatively tolerant to the triazoles with respect to growth, show inhibition of C-14 demethylation without a change in the degree of fatty acid unsaturation (5). The endoplasmic reticulum (ER) is believed to be the site of both the Δ^{12} and Δ^{15} fatty acid desaturases and enzymes in the latter stages of sterol biosynthesis, and has a relatively low sterol content. If indeed activities of the desaturases are responsive to changes in membrane fluidity, the apparent unresponsiveness to reduction in sterol content might be explained on the basis of the low levels that may occur in specific domains spatially separated from the desaturase or, perhaps more likely, the ER is geared for high sterol turnover and the sterols are rapidly removed through rapid membrane differentiation or transport. Therefore, sterols may not play an important role in fluidity modulation of the ER and hence fatty acid unsaturation as it may be influenced by membrane fluidity.

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COMMUNICATION

A Facile Chemical Synthesis of Cholest-4-en-3-one. Carbon-13 Nuclear Magnetic Resonance Spectral Properties of Cholest-4-en-3-one and Cholest-5-en-3-one[1]

Edward J. Parish,* Hiroshi Honda, Sarawanee Chitrakorn and Peter Livant
Department of Chemistry, Auburn University, Auburn, Alabama 36849

This paper presents a simplified method for the chemical synthesis of cholest-4-en-3-one, which is a naturally occurring steroid and a salient intermediate in steroid chemistry. Pyridinium chlorochromate (PCC) in refluxing benzene has been found to be an effective and convenient reagent for the oxidation and concomitant isomerization of cholest-5-en-3β-ol (cholesterol) to cholest-4-en-3-one in high yield. Also described are the carbon-13 nuclear magnetic resonance spectral properties of cholest-4-en-3-one and of the isolated reaction intermediate cholest-5-en-3-one.
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The cholesterol derivative cholest-4-en-3-one (1) is a naturally occurring substance found in both plant and animal tissues. Its presence may result from biosynthesis or be due to autoxidation of cholesterol (1,2).

In steroid chemistry, 1 is a key intermediate in the modification of the A-ring (including alkylations at C-4) and in further chemical transformations of the steroid nucleus (2–10). Moreover, the conversion of cholest-5-en-3-β-ol (cholesterol) to 1 is an important reaction of basic steroid chemistry and in the synthesis of many cholesterol derivatives (9–12; and references herein).

As a result of our continuing interest in selective steroid oxidation reactions, we have developed a simplified method for the chemical synthesis of 1 from commercially available cholesterol. The one-step synthesis reported herein is applicable to both small- and large-scale preparations and represents an improvement in methodology over previously reported procedures.

Also described are complete carbon-13 nuclear magnetic resonance (^{13}C NMR) spectral assignments for 1 its reaction intermediate 2.

EXPERIMENTAL

General methods. Procedures for recording of melting points (m.p.) and of infrared (IR), ^1H NMR ^{13}C NMR, mass (MS), and ultraviolet (UV) spectra were those used previously (13,14). Similarly, details concerning the use of gas-liquid (GLC), thin-layer (TLC), and column chromatography have been described (13,14). Solvent systems for TLC analysis were: SS-1, 2% ethyl acetate in toluene; SS-2, 10% diethyl ether in toluene; SS-3, 10% diethyl ether in hexane (by volumes). Details concerning distortionless enhancement polarization transfer (DEPT) experiments and carbon-hydrogen (C-H) correlation have been presented (13). Commercial cholesterol (Sigma Chemical Co., St. Louis, MO) was purified by multiple recrystallization from acetone water. Samples of authentic 1 and 2 were prepared by the method of Fieser (15,16). Pyridinium chlorochromate (PCC) was obtained from Aldrich Chemical Co. (Milwaukee, WI).

General oxidation procedures. Cholest-5-en-3β-ol-(cholesterol, 3.80 g, 0.01 mol) was dissolved in benzene (100 mL) containing molecular sieves (0.5 g, type 3A). Pyridinium chlorochromate (PCC, 53.90 g, 0.25 mol) was added, and the stirred reaction mixture was gently refluxed under a nitrogen atmosphere for 5 hr. The benzene solution was decanted, and the remaining contents of the reaction flask were washed several times with diethyl ether. The combined extracts were washed with a saturated NaCl solution, dried over anhydrous magnesium sulfate and evaporated to dryness under reduced pressure. TLC analysis (SS-1) indicated the presence of essentially one component of ca. 95% purity. The residue was recrystallized from acetone/water to yield cholest-4-en-3-one (1, 3.42 g, 89%): m.p., 80.5–82°C; lit. (16), 81–82°C; IR λ_{max} was identical with that of an authentic sample (C=O, 1671 cm^{-1}); ^1H NMR 0.71 (*s*, 3H, C-18 CH_3), 1.18 (*s*, 3H, C-19 CH_3), 5.72 (*s*, 1H, C-4 H); ^{13}C NMR: (see Table 1); MS, *m/e* (rel. int.): 384 (M; 67%), 369 (M−CH_3; 7.5%), 242 (22%), 271 (M − side chain; 7.7%), 299 (6.8%), 261 (25%), 229 (39%), 124 (100%). UV λ_{max} (ethanol) 242 nm, ε = 16,500, lit. (14) 242 nm. GLC analysis indicated the presence of a single major component of 98% purity, and TLC analysis in three solvent systems (SS-1, SS-2 and SS-3) confirmed that 1 consisted of a single component. GLC and TLC chromatographic mobilities corresponded to those of the authentic compound.

When the preceding reaction was discontinued after 15 minutes at reflux, TLC analysis (SS-2) showed that the reaction product consisted of ca. 60% cholest-4-en-3-one(1) and ca. 40% cholest-5-en-3-one(2). The residue was then subjected to column chromatography using a solvent gradient of diethyl ether in toluene. The purified products were recrystallized from acetone/water to yield 1 (2.08 g, 54%), described above, and cholest-5-en-3-one (2, 1.38 g, 36%): m.p., 125–127°C; lit. (17), 125–127°C; lit. (15) 117–125°C; lit. (16) 126–127°C; IR λ_{max} was identical with that of an authentic

[1]Based on a paper presented at the Symposium on Plant and Fungal Sterols: Biosynthesis Metabolism and function held at the AOCS Annual Meeting, Baltimore, MD, April 1990.
*To whom correspondence should be addressed.

Abbreviations: COSY, correlation spectroscopy; DEPT, distortionless enhancement polarization transfer; GLC, gas-liquid chromatography; IR, infrared spectrometry; MS, mass spectrometry; NMR, nuclear magnetic resonance; PCC, pyridinium chlorochromate; SS, solvent system; TLC, thin-layer chromatography; UV, ultraviolet.

TABLE 1

^{13}C Chemical Shifts for Cholest-4-en-3-one (1) and Cholest-5-en-3-one (2)[a]

Carbon atom	1	2
1	35.43	36.84
2	33.64	37.52
3	198.55	209.42
4	123.43	48.23
5	170.68	138.52
6	32.60	122.67
7	31.78	31.78
8	35.31	31.92
9	53.56	49.18
10	38.23	36.88
11	20.76	21.35
12	39.37	39.69
13	42.08	42.35
14	55.60	56.59
15	23.88	24.25
16	27.90	28.20
17	55.85	56.17
18	11.69	11.89
19	17.06	19.13
20	35.47	35.76
21	18.39	18.73
22	35.86	36.18
23	23.57	23.85
24	39.21	39.50
25	27.68	27.95
26	22.31	22.55
27	22.56	22.79

[a]In ppm downfield from tetramethylsilane using $CDCl_3$ as a solvent.

sample (C=O, 1725 cm^{-1}); ^1H NMR 0.73 (*s*, 3H, C-18 CH_3), 1.24 (*s*, 3H, C-19 CH_3), 5.37 (*m*, 1H, C-6 H); ^{13}C NMR: (see Table 1); MS, *m/e* (rel. int.): 384 (M; 100%), 369 (M−CH_3; 28%), 271 (M − side chain; 19%), 229 (55%). TLC analysis in three solvent systems (SS-1, SS-2 and SS-3) indicated the presence of a single component.

RESULTS AND DISCUSSION

The presently prevailing methods for the synthesis of cholest-4-en-3-one(1) include the Oppenauer oxidation of cholest-5-en-3β-ol (cholesterol) (18–20) and the acid-catalyzed isomerization of cholest-5-en-3-one (2) (15,16,19). The Oppenauer oxidation involves the initial formation of the corresponding β,γ-unsaturated ketone 2, *in situ*, which is immediately isomerized (in basic medium) to the α,β-unsaturated ketone 1 (19,20). Cholest-5-en-3-one (2) was originally obtained by a three-step sequence involving protection of the Δ^5 double bond of cholesterol by bromination, followed by chromate oxidation to the 5,6-dibromo-3-ketone, and finally debromination with zinc in acetic acid. The ketone 2 was then isomerized to 1 by oxalic acid in ethanol (15,16). Other methods used to prepare 1 from cholesterol include the use of trifluoromethanesulfonic anhydride/dimethyl sulfoxide, which gave 1 in 51% yield (21), and trifluoroacetic acid/dimethyl sulfoxide, which gave Δ^5-3-one (2) and Δ^4-3-one (1) a 2:1 ratio (22). In addition, the conversion of cholesterol to 1 can be achieved using cholesterol oxidase derived from microbial sources (2,23).

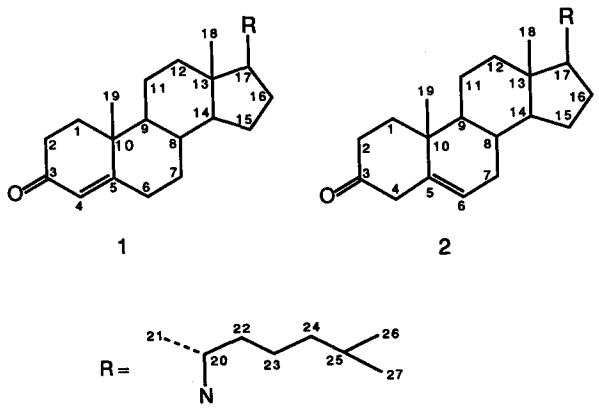
In the present communication, we report that pyridinium chlorochromate (PCC) in refluxing benzene can convert, in one step and in high yield, cholesterol to the corresponding Δ^4-3-ketosteroid 1. The present study (represents an extension of our previous work in which we focused on the selective oxidation of cholesterol to the unconjugated Δ^5-3-ketone 2 (17,24). Using PCC in methylene chloride, we found that the inclusion of powdered $CaCO_3$ in the reaction mixture effectively prevented isomerization of the Δ^5 double bond during reaction and aqueous work-up. Previous workers obtained a mixture of Δ^4- and Δ^5-3-ketones under their reaction conditions (25). In the present case, using PCC in refluxing benzene, the acidic properties of PCC (26) are thought to be responsible for the complete isomerization of the Δ^5 double bond during reaction to give the final Δ^4-3-ketone product. When the reaction time was shortened, isolation of the reaction intermediate 2 was possible.

Commercially available PCC has recently become widely used in organic synthesis for the oxidation of primary and secondary alcohols (27). This reagent, in methylene chloride containing pyridine (28), other aromatic amines (29), pyrazole (30), 3,5-dimethylpyrazole (31), or benzotriazole (32) was reported to effect the selective oxidation of allylic alcohols and to bring about allylic and benzylic oxidations in refluxing methylene chloride (33) and benzene (13,34). Other recent studies have found PCC useful for the oxidation of carbon-boron bonds in organoboranes to produce aldehydes (35) and ketones (36).

Carbon-13 nuclear magnetic resonance (^{13}C NMR) spectroscopy can usually provide more information concerning the structure of steroids than can proton (^1H) NMR, since most carbons produce separately resolved signals that can be assigned to specific carbon atoms (37). As an extension of our synthetic studies, we have completed the ^{13}C NMR assignments for the carbon atoms in cholest-4-en-3-one (a) and cholest-5-en-3-one (2) (Table 1, Scheme 1). The carbon assignments of 1 represent a revised and corrected interpretation of the original reported spectra (38), using improved spectroscopic methods. Supporting data for the assignments of the ^{13}C resonances in these compounds were derived from spectral assignments made previously (13,39).

A DEPT (11) experiment made it possible to distinguish the carbon resonances of secondary carbons (down signals) from those of primary and tertiary carbons (up signals). Once carbon assignments were made, a C-H correlation (13) spectrum facilitated the correct assignment of the proton chemical shifts. Among the more surprising results was the vast separation between the non-equivalent protons on C-12 of cholest-4-en-3-one (2.03 ppm and 1.16 ppm) and of cholest-5-en-3-one (2.05 ppm and 1.21 ppm). The correlation spectroscopy (COSY) spectrum (40,41) of cholest-4-en-3-one revealed a clear cross peak connecting one of the two protons on C-1 (the more upfield peak) with the methyl protons of C-19.

Distinguishing C-9, C-14, and C-17 is problematic since all three signals usually fall in the 50–57 ppm region. In cholest-4-en-3-one, the signals appeared at 53.6, 55.6 and 55.9 ppm, respectively. The 55.9 ppm peak correlated with the proton chemical shift of 1.10



SCHEME 1. Steroid structures of 1 and 2.

ppm, the 55.6 ppm peak with the 1.00 ppm shift, and the 53.6 peak with the 0.91 ppm shift. The 1.10 ppm signal exhibited a clear cross peak in the COSY spectrum with one proton on C-16, thus establishing the 55.9 ppm peak as due to C-17. Similarly, the 0.91 ppm signal showed a cross peak in the COSY spectrum connecting to the proton on C-8 at 1.53 ppm, which established the 53.6 ppm peak as being due to C-9, and, by default, the 55.6 peak as being due to C-14. In cholesterol, C-9, C-17 and C-14 appear at 50.0, 56.1 and 56.6 ppm respectively, which is very close to their positions in cholest-5-en-3-one (49.2, 56.2 and 56.6 ppm). In cholest-4-en-3-one, however, C-9 shifts downfield by 3.6 ppm to 53.6 ppm and C-14 and C-17 reverse order. Accompanying the downfield displacement of C-9 is C-8, which appears 3.5 ppm downfield of the position of C-8 in cholesterol. Other peaks displaced downfield of their positions in cholesterol are C-10 (1.8 ppm displacement) and C-2 (2.1 ppm displacement). An upfield displacement is observed for C-19 (−2.1 ppm displacement).

In summary, we describe a new and facile synthesis of 1 which expands the scope of synthetic transformations known to be accomplished by PCC. Additionally, we have presented the complete ^{13}C NMR assignments for the carbon resonances in 1 and its synthetic intermediate 2.

ACKNOWLEDGMENTS

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REVIEW

Lipoprotein(a): Nonhuman Primate Models

Kazuhiko Makino[1] and **Angelo M. Scanu***
Department of Medicine, Biochemistry and Molecular Biology, and Lipoprotein Study Unit, University of Chicago, Chicago, Illinois 60637

Lipoprotein(a) [Lp(a)] is a low density lipoprotein which has apo(a) disulfide-linked to apoB100. Apo(a) has recently been shown to have a striking homology with plasminogen, a knowledge that has stimulated a lot of interest in the mechanism of atherogenicity and thrombogenicity of this lipoprotein particle. Several studies have documented the presence of Lp(a) in nonhuman primates with particular reference to the rhesus monkeys and baboons. The Lp(a) of rhesus monkey is structurally very similar to that of humans, except for the absence of kringle V and the amino acid composition of the catalytic region. The Lp(a) of nonhuman primates, like their human counterparts, exhibit a wide range of interindividual plasma levels and also a wide size polymorphism of apo(a). Nonhuman primates appear to represent a good model for the study of the structure and biology of Lp(a).
Lipids 26, 679-683 (1991).

Lipoprotein(a) [Lp(a)], first described by Berg (1) as a genetic variant of low density lipoprotein (LDL), is considered to be an independent risk factor for atherosclerotic cardiovascular disease, (ACD) (2-5). The distinguishing feature of Lp(a) is to have apoB100 bound to apo(a) by a disulfide linkage (6,7). This protein has recently been shown to have a striking homology with plasminogen (8,9), which is a serine protease zymogen that promotes clot lysis on activation to plasmin. This knowledge has stimulated a lot of interest in the mechanism of atherogenicity and thrombogenicity of this lipoprotein particle.

Lp(a) is heterogeneous in size and density due to differences in protein/lipid mass ratio, apo(a) size polymorphism and apoB100/apo(a) molar ratio (10,11). Apo(a) size isoforms are under the control of a single gene which is localized in the long arm of chromosome 6 in proximity of the plasminogen gene (12,13). Family studies have shown a linkage between apo(a) and plasminogen genes (14).

The studies conducted thus far favor the concept that apo(a) is under a strict genetic regulation, a notion that is in keeping with the insensitivity of plasma Lp(a) to diets and hypolipidemic agents (15). However, data are emerging that post-translational events may be contributing factors in determining the plasma levels of Lp(a) (16). Most of the studies on Lp(a) have been carried out in human subjects. Lp(a) has been reported absent in the plasma of common laboratory animals (*e.g.*, dogs, rats, mice, etc.). On the other hand, several studies have documented the presence of Lp(a) in nonhuman primates. The purpose of this article is to provide an overview on these studies with particular emphasis on the Old World monkeys.

GENERAL OBSERVATIONS

Lp(a) immunoreactive materials were identified by Berg (17) in serum samples from rhesus monkey, baboon, chimpanzee and orangutan. Several reports have also identified Lp(a) in the plasma of patas monkeys (18,19), pig-tailed monkeys (19), mangabey monkeys (20) but not in common marmosets (21) which belong to the New World monkeys.[2]

Makino *et al.* (23) carried out a systematic, genealogical study on some of the immunological characteristics of Lp(a) or Lp(a)-like material in the serum samples of 77 nonhuman primates of 24 species (see Table 1). The immunological cross-reactives of Lp(a) or Lp(a)-like materials were studied by Ouchterlony's double diffusion using a rabbit anti-human Lp(a) serum (Fig. 1). Sera from great apes (orangutan and chimpanzee) exhibited a reaction of complete identity with human sera (Fig. 1D, E). Sera from Old World monkeys (savannah, patas, Assamese, rhesus, Formosan, pig-tailed, crab-eating, Japanese monkeys and hamadryas baboon) generated spurs when tested in adjacent wells against human sera, indicating that Lp(a) species of Old World monkeys are relatively deficient in antigenic determinants as compared with human Lp(a).

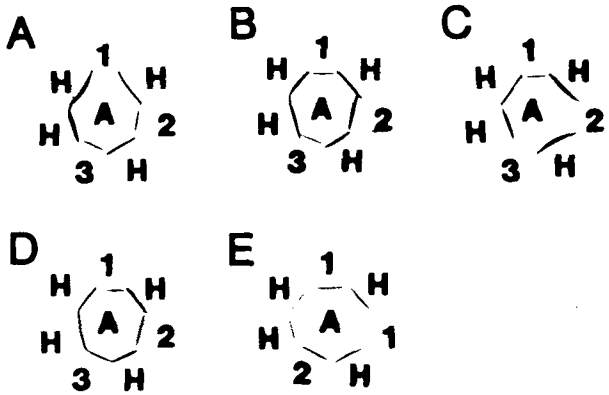


FIG. 1. Ouchterlony's double diffusion tests. The anti-human serum is in the central well (H, human serum). A: 1, Common marmoset; 2, savannah monkey; 3, patas monkey. B: 1, Assamese monkey; 2, Formosan monkey; 3, rhesus monkey. C: 1, pig-tailed monkey; 2, night monkey; 3, gibbon. D: 1, Japanese monkey; 2, crab-eating monkey; 3, chimpanzee. E: 1, Chimpanzee; 2, orangutan.

[1] Permanent address: Department of Laboratory Medicine, Gifu University School of Medicine, Gifu 500, Japan.
*To whom correspondence should be addressed.

Abbreviations: ACD, atherosclerotic cardiovascular disease; FH, familial hypercholesteremia; Lp(a), lipoprotein(a); LDL, low density lipoprotein; LDL-R, low density lipoprotein receptor.

[2] A very recent study, which appeared after this article had completed the review process, reported the presence of Lp(a) in the plasma of the common marmoset (Callithrix jacchus). A single apo(a) isoform the size of apoB100 was observed and associated with variable plasma Lp(a) levels (22).

TABLE 1

Species and Numbers of Nonhuman Primates Used in the Studies and Lp(a) Detection[a]

Species		No.	Lp(a)
Prosimiae:	Prosimians:		
Lemuridae			
Lemur catta	Ring-tailed lemur	3	ND
Galagidae			
Galago crassicaudatus	Grand galago	3	ND
Lorisidae			
Nycoticebus coucang	Slow loris	1	ND
Simiae:	Simians:		
Platyrrhina	New World monkeys		
Callithricidae			
Callithrix jacchus	Common marmoset	1	ND
Sanguinus oedipus	Cotton-headed tamarin	5	ND
Cebidae			
Aotes trivirgatus	Night monkey	2	ND
Cebus capucinus	White-throated capuchin	2	ND
Cebus apella	Tufted capuchin	3	ND
Ateles geoffroyl	Central American spider monkey	2	ND
Cattarrrhina	Old World monkeys		
Cercopithecidae			
Cercopithecus aethiops	Savannah monkey	3	D
Erythorcebus patas	Patas monkey	3	D
Macaca assamensis	Assamese monkey	4	D
Macaca mulatta	Rhesus monkey	5	D
Macaca cyclopis	Formosan monkey	3	D
Macaca arctoides	Stump-tailed monkey	4	D
Macaca nemestrina	Pig-tailed monkey	4	D
Macaca radiata	Bonnet monkey	3	D
Macaca fascicularis	Crab-eating monkey	5	D
Macaca fuscata	Japanese monkey	4	D
Papio hamadryas	Hamadryas baboon	4	D
Hylobatidae	Lesser apes		
Hylobates lar	White-handed gibbon	2	D
Hylobates agilis	Agile gibbon	1	D
Pongidae	Great apes		
Pongo pygmaeus	Orang-utan	1	D
Pan troglodytes	Chimpanzee	8	D

[a]ND, not detected; D, detected.

On the other hand, Lp(a) was undetectable in sera of Prosimians (ring-tailed lemur, grand galago and slow loris) and New World monkeys (common marmoset, cotton-headed tamarin, night monkey, white-throated capuchin, tufted capuchin and Central American spider monkey) by both the double diffusion method and a sensitive ELISA. It is worth noting that the New World monkeys are believed to have diverged from the Old World monkeys about 40 million years ago, which is the same time frame that has been estimated for a divergence of the apo(a) gene from the plasminogen gene (9).

STUDIES IN THE RHESUS MONKEY

Early studies by Fless *et al.* (24) showed that plasma rhesus monkey Lp(a) is heterogenous in size and density and that such heterogeneity can also occur within individual monkeys. Those studies also showed that the concentration of Lp(a) in plasma is unaffected by diets, although some remodeling of the Lp(a) core components (*i.e.*, cholesteryl esters and triglycerides) can occur (25). From the structural standpoint, rhesus monkey Lp(a), like that of humans, represents an LDL-like particle having as a protein moiety apoB100 covalently linked to apo(a) (26,27). Tomlinson *et al.* (28) cloned and sequenced the 3′ 4.5kb region of the apo(a) cDNA from an individual rhesus monkey and found it to share 93% nucleotide homology and a predicted 90% amino acid identity with its human counterpart. The rhesus sequence was also composed of multiple, tandem repeats encoding plasminogen kringle IV-like sequences, but not a kringle V-like domain (Fig. 2).

Scanu and co-workers (29,30) recently described a rhesus monkey pedigree with members exhibiting a spontaneous hypercholesteremia associated with a low density lipoprotein receptor (LDL-R) deficiency (Fig. 3), assessed by [^{125}I]LDL binding to cultured skin fibroblasts, ligand and immunoblot analyses. By using the polymerase chain reaction in subsequent studies, they showed that the affected monkeys are heterozygous for a nonsense mutation (TGG→TAG) in position 284 of exon 6 of the LDL-R gene, potentially resulting in a truncated 283 (31) (Fig. 4). Using this *Spe I* site as a marker, this mutation was shown to

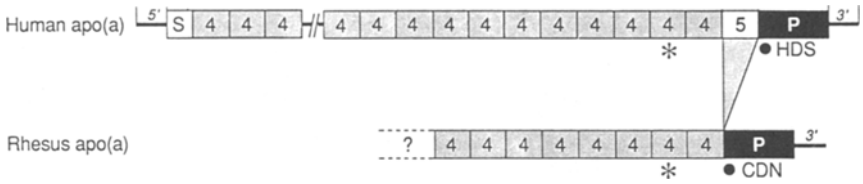


FIG. 2. Comparative representation of the structure of human and rhesus monkey apo(a). The sequence domains indicated are 5′ untranslated, signal peptide(S), plasminogen kringle IV- and V-like sequences of human and rhesus monkey apo(a), protease(P), and 3′ untranslated. Also indicated are the extra cysteine residue in kringle 36 that is a candidate for covalent linkage to apoB100 (*), the substitution of serine for the activation site arginine residue of plasminogen (●), and the catalytic triad of plasminogen (HDS) that is retained in human apo(a) and changed to CDN in rhesus monkey apo(a) (From Lawn, *et al.*; ref. 27).

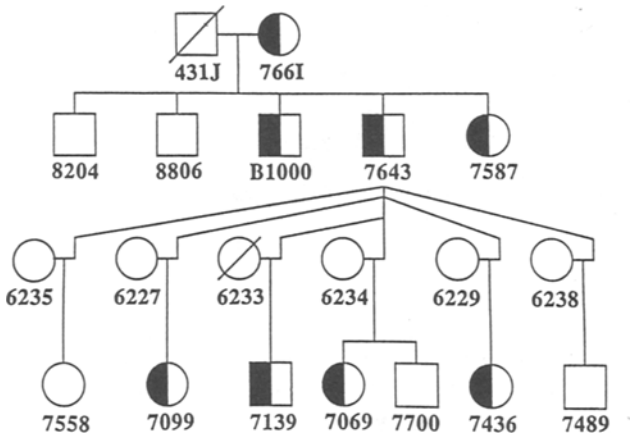


FIG. 3. Pedigree of the rhesus monkey family. Half-filled symbols indicate monkeys heterozygous for the mutation in exon 6 in the LDL-R gene as determined by the presence of an *Spe I* restriction site. Circles, females; squares, males; slashed lines, deceased animals.

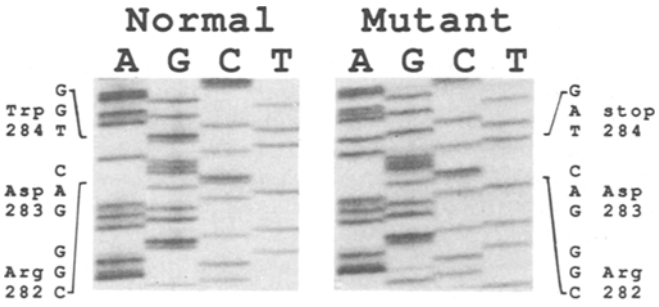


FIG. 4. Identification of the mutation in exon 6 of the rhesus monkey LDL-R gene. Exon 6 was amplified from genomic DNA of monkey 7643. PCR products were clones into M13 and sequenced. Five of thirteen clones had the sequence TGG at a position corresponding to amino acid 284 of the human protein (left). The remaining clones had a G→A transition which causes replacement of a tryptophan residue with a translation termination codon (right).

be present in all of the hypercholesteremic members of the pedigree and absent in unaffected members through three generations. Quantitative analyses of RNA obtained from liver biopsies showed that the abundance of the LDL-R RNA of the affected monkeys was also reduced by about 50% (Fig. 5).

All of the animals studied had levels of plasma Lp(a) protein ranging between 1.0 mg/dL and 57.5 mg/dL that were only weakly correlated with total plasma cholesterol, LDL cholesterol and apoB. LDL-R deficiency correlated with plasma LDL but not Lp(a). A seven-week fat challenge (16.5% lard, 0.64% cholesterol) that markedly raised the plasma LDL levels had no effect on plasma Lp(a) (30), suggesting that in the rhesus monkey the LDL receptor plays either only a limited or no functional role in the metabolism of Lp(a).

As in the human model, rhesus monkey apo(a) exhibits size polymorphism. A total of six isoforms were detected in all animals studied and classified by relating the R_f of each isoform to that of the apoB100 band. However, each animal presented either one or two isoforms (Fig. 6). About 25% of the animals studied exhibited a single fast band with an R_f of 1.13. About 5% of the animals were homozygous for the slow apo(a) isoform with an R_f of 0.80. The majority of the animals were heterozygous; the most frequent phenotypes were 0.64/1.13 and 0.80/1.13. The apo(a) isoforms with a migration faster than apoB100 (low molecular weight) had a preference for affiliation with the lighter lipoprotein particles and *vice versa*. As shown in Figure 7, animals with phenotype 1.13/1.13 had a tendency to have the highest plasma levels of Lp(a), whereas those with phenotype 0.80/0.80 had the lowest plasma concentrations. However, in spite of the same apo(a) phenotype, the plasma Lp(a) levels varied widely, supporting the concept that factors other than apo(a) phenotype participate in the regulation of these levels. In animals that were heterogeneous of the apo(a) isoforms the relation with plasma Lp(a) levels was equivocal, indicating that in the presence of heterozygosity it is difficult to assess the role that the apo(a) gene plays in the overall biology of Lp(a).

More recent studies showed the accumulation of Lp(a) in atherosclerotic arteries of rhesus monkeys with diet-induced atherosclerosis (32). Immunohistochemistry with monospecific Lp(a) antisera revealed striking accumulation of Lp(a) in atherosclerotic coronary artery lesions, whereas no Lp(a) was detected in the normal, nonatherosclerotic arteries. Analysis of paired tissue and serum samples from 17 male hyperlipoproteinemic monkeys revealed a significant correlation between the concentrations of aortic wall Lp(a) and serum Lp(a) levels. The serum cholesterol level failed to correlate with either aortic Lp(a) or serum Lp(a). These results add further evidence for the potential role of Lp(a) in the pathogenesis of atherosclerosis.

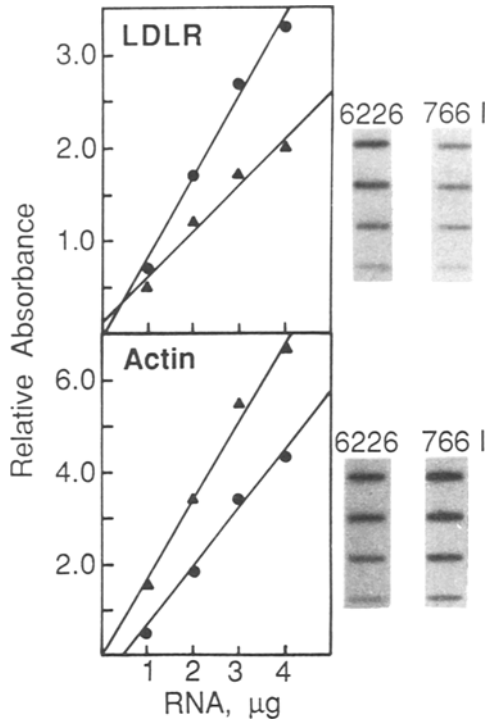


FIG. 5. Slot blot analysis of total cell RNA isolated from 766I, a hypercholesteremic monkey, and 6226, a normocholesteremic control. RNA (1, 2, 3 or 4 µg) from each monkey was applied to Zeta-Probe filter in triplicate. The filter was hybridized to a human LDL-R cDNA probe and scanned by laser densitometry. The average value for each point was calculated and the slope was determined by linear regression. One set of these data is shown at the right. The slopes are 0.50 (r=0.98) and 0.88 (r=0.99) for 766I and 6226, respectively. The filter was stripped and rehybridized with a mouse β-actin cDNA probe. Slopes of 1.77 (r=0.99) and 1.29 (r=0.99) were calculated for 766I and 6226, respectively. ●, 6226; ▲, 766I.

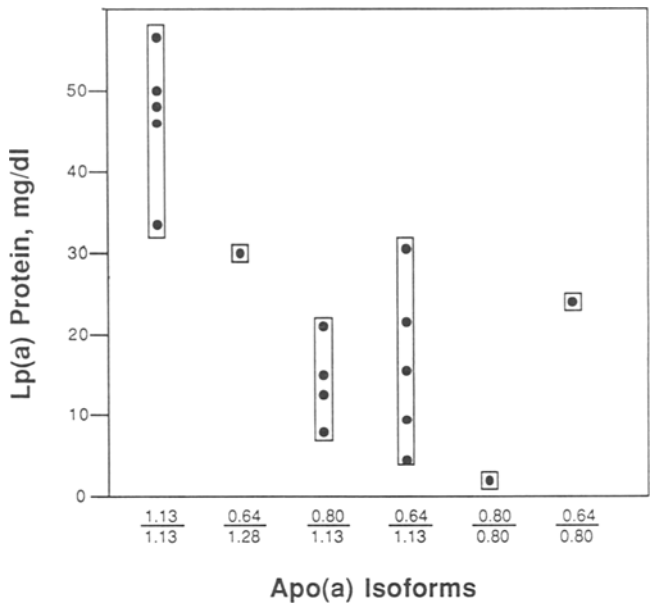


FIG. 7. Relation between apo(a) isoform phenotypes and plasma Lp(a) protein levels.

STUDIES IN THE BABOON

Studies by Rainwater and colleagues (33-38) have demonstrated that baboons possess Lp(a) that is similar to human Lp(a) and that the gene encoding apo(a) is closely linked to plasminogen (34). The mean serum level of 80 unrelated baboons was 4.7 mg/dL, with the distribution skewed toward the lower levels (35). Moreover, primary baboon hepatocytes were found to synthesize Lp(a) (36).

Baboon apo(a) exhibited at least nine isoforms distinguishable by size (36). Thirty-one different apo(a) isoform phenotypes were detected in a population of 165 unrelated baboons and found to be unaffected by changes in diet. The analysis of two large families suggested that apo(a) isoform patterns and serum Lp(a) concentrations are inherited. Putative parental alleles responsible for specific Lp(a) concentration was estimated to be 0.95 ± 0.04.

In a survey of 22 baboons, Hixson *et al.* (16) found a correlation between size of liver apo(a) transcripts (5.2∼11.2 kilobases) and size of serum apo(a) isoforms (r^2=0.996, $p<0.001$). However, the correspondence between length of the liver apo(a) messages and relative mobility of each specific isoform by gradient gel electrophoresis was not always observed, suggesting that post-transcriptional events might affect apo(a) production.

In more recent studies, twelve different size isoforms (including the null) of apo(a) were identified across the five subspecies (*Papio hamadryas hamadryas, P.h. cynocephalus, P.h. ursinus, P.h. papio* and *P.h. anubis*) and these isoforms acted as alleles (38). Significant differences in apo(a) isoform frequencies were found between subspecies. Plasma levels of Lp(a) also differed among subspecies. The genetic distances between the baboon

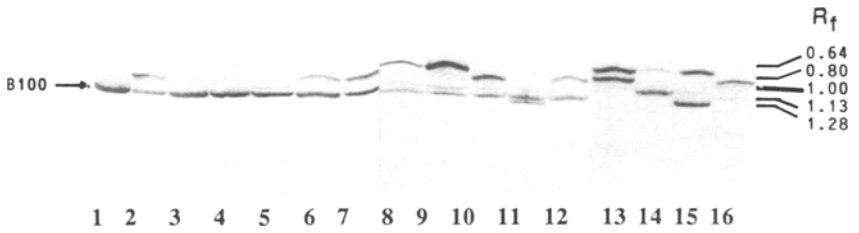


FIG. 6. Apo(a) isoform patterns of animals from a rhesus monkey pedigree with FH (see text). Some animals exhibited a single band and some two main bands. Each isoform was classified as ratio between their R_f and that of apoB100 by 2-16% polyacrylamide gel electrophoresis. 1 #766, 2 #431, 3 #1000, 4 #7643, 5 #8204, 6 #8806, 7 #7587, 8 #7099, 9 #7139, 10 #7436, 11 #7489, 12 #7558, 13 #6229, 14 #6234, 15 #6238, and 16 #7587.

subspecies based on the apo(a) isoforms were consistent with geographic relationships between the subspecies and previously assessed genetic marker information. However, the genetic distances based on apo(a) isoforms did not exhibit the same pattern of between-subspecies variation as phenotypic distances based on Lp(a) levels, suggesting that multiple genes might be involved in the determination of Lp(a) levels.

GENERAL COMMENTS

The results of the studies reviewed in the previous sections indicate that the Lp(a) of rhesus monkeys and baboons has structural properties very close to those of humans. It is apparent that like in human subjects, the plasma Lp(a) levels and apo(a) isoforms of these nonhuman primates are highly inherited traits, although this does not rule out the action of other factors, either genetic or environmental. Significant in this regard are the studies of Scanu and colleagues (29,30), showing that members of the same rhesus monkey family, although exhibiting the same fast apo(a) phenotype, had varying plasma levels of Lp(a). The studies on rhesus monkeys have also indicated that the plasma levels of Lp(a) do not closely correlate with LDL-R function, implying that the LDL receptor might have a very limited or no role in Lp(a) degradation. This conclusion is in accord with turnover studies showing a plasma residence time for Lp(a) significantly longer than that of LDL (unpublished data) and also with the dietary experiments documenting diverging effects on the plasma levels of Lp(a) and LDL.

While recognizing that nonhuman primates represent a good model for the study of the structure and biology of Lp(a), it is also important to stress that nonhuman primates offer unique features complementary to those in man. A case in point is absence of kringle V in rhesus monkey apo(a) that should permit an assessment of the functional significance of this kringle domain in Lp(a). Moreover, nonhuman primates offer the possibility of a comparatively wider experiment latitude like turnover studies, performance of serial liver biopsies, testing new pharmacological agents or potential drug toxicity. Not of lesser importance is the possibility of breeding studies to further elucidate the genetic basis of the inheritance of the apo(a) isoforms and also permit a careful analysis of the effect of development on plasma Lp(a) levels and distribution. These are just a few advantages that the nonhuman primate model offers in the study of Lp(a). An additional dimension is offered by the rhesus monkey family with FH described by Scanu and colleagues (29,30), since the members of this family provide an ideal setting for examining the potential role of the LDL receptor on Lp(a) metabolism.

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ARTICLE

ApoA-I Secretion by Rabbit Intestinal Mucosa Cell Cultures

Ting L. Carlson and Bruce A. Kottke*
Atherosclerosis Research Unit, Mayo Clinic and Foundation, Rochester, Minnesota 55905

Lipid and apolipoprotein (apo) A-I concentrations in different density fractions of New Zealand White (NZW) and Watanabe (WHHL) rabbit plasma were studied. Aside from the low plasma apoA-I and high density lipoprotein (HDL) cholesterol levels in WHHL rabbits, the distribution of apoA-I was also different between the two rabbits. ApoA-I was concentrated in both the HDL_2 and HDL_3 fractions of NZW rabbits but was found primarily in the HDL_3 fraction of WHHL rabbits. ApoA-I secretion in these two rabbits was further studied *in vitro* by using intestinal and hepatocyte cell cultures. ApoA-I secretion was highest from cultures of the duodenum and the proximal end of the jejunum; whereas, cell cultures of the distal end of the small intestine secreted very little apoA-I into the medium. Intestinal cell cultures from WHHL rabbits secreted less, but significant amounts of, apoA-I compared to that of NZW rabbits. ApoA-I was most concentrated in the density range of 1.12–1.21 (HDL_3) fraction in medium containing 10% fetal calf serum (FCS). Serum-free medium promoted apoA-I secretion by intestinal cell cultures that was mostly found in the d>1.21 (lipoprotein-deficient) fraction. Hepatocytes isolated from the same rabbits by collagenase perfusion secreted little apoA-I, and it was found only in the d>1.21 fraction. The addition of oleic acid into the culture medium with 10% FCS decreased the secretion of total apoA-I and HDL by intestinal cell cultures and increased the secretion of very low density lipoprotein (VLDL) and intermediate density lipoproteins (IDL). The results indicate that intestinal cells, not hepatocytes, are responsible for the secretion of apoA-I and HDL_3 in rabbits, and that the secretion may be regulated under different nutritional conditions. ***Lipids* 26, 684–688 (1991).**

HDL has been recognized as an important factor in assessing the risk of coronary artery disease (CAD) in humans (1,2). The higher the HDL cholesterol levels, the lower the risk of CAD. The major apolipoprotein in the HDL particles is apoA-I (3) which is synthesized both by the liver (4) and by the intestine (5) in humans. Under fed conditions, the intestine may contribute as much as 50% of the apoA-I circulating in plasma (6). Efforts in raising HDL cholesterol levels and lowering the low density lipoprotein (LDL) cholesterol by diet or medications have not been fruitful. Unsaturated fat that lowers LDL cholesterol levels also lowers HDL levels in humans (7). Similarly, saturated fat not only increases the levels of HDL cholesterol but also increases all the other plasma lipid levels (8). The difficulties in obtaining human hepatocyte and intestinal cell cultures further hinder the understanding of the regulation of apoA-I and HDL synthesis and secretion. By mRNA hybridization techniques, it was found that the majority of apoA-I was synthesized by the small intestine, but not the liver, in rabbits (9,10). Thus, rabbits can serve as an excellent model to study the regulation of apoA-I and HDL synthesis by intestinal cells under different nutritional conditions. Intestinal mucosa cells have been successfully isolated and cultured for a variety of studies from different animals (11–13). In this study, rabbit intestinal mucosa cells were cultured to study the secretion of apoA-I in different culture media. Furthermore, intestinal cells from WHHL rabbits (14) were also used in an attempt to understand if there are any other defects that may lead to the low plasma HDL cholesterol levels in these hypercholesterolemic rabbits.

MATERIALS AND METHODS

Materials. $Na^{125}I$ (carrier-free) was purchased from Amersham Corp., Arlington Heights, IL. Type IV collagenase was purchased from Sigma Chemical Co., St. Louis, MO. All tissue culture supplies were purchased from Grand Island Biological Co., Grand Island, NY. The Hep G2 cell line was generously provided by Dr. Barbara B. Knowles of the Wistar Institute, Philadelphia, PA.

Animals. WHHL rabbits were obtained from the National Institutes of Health, Bethesda, MD. NZW rabbits were obtained from the Institute Hills Farms of Mayo Clinic, Rochester, MN. WHHL rabbits were sacrificed at 2–3 yrs of age, and NZW rabbits were used at 6 mon to 1 yr of age.

Isolation of intestinal mucosa cells and hepatocytes. Fresh hepatocytes were isolated using the collagenase perfusion techniques as described by Attie *et al.* (15) with slight modification. Rabbits were anesthetized by ketamine hydrochloride (Ketaset; Aveco Co., Inc., Fort Dodge, IA) injection (20 mg/kg of body weight) and xylazine (Rompun; Mobay Corp., Shawnee, KS) administered (2 mg/kg of body weight) through the ear vein. An incision was made to expose the portal vein and inferior *vena cava.* A 13-gauge catheter was inserted into either vessel and secured. The liver was perfused first with preperfusion buffer (8.3 g NaCl, 0.5 g KCl, and 2.4 g HEPES per liter, pH 7.4) at a flow rate of 40 mL/min for 10 min. A collagenase perfusion buffer (3.9 g NaCl, 0.5 g KCl, 0.7 g $CaCl_2 2H_2O$, 24 g HEPES, and 1 g collagenase per liter, pH 7.1) was used afterwards for 30 min at a flow rate of 40 mL/min. The blanched liver was removed and cut into small pieces with a surgical blade. Loose hepatocytes were obtained by shaking the liver pieces vigorously in a small amount of collagenase buffer at 37°C. The viability of the hepatocytes was checked by Trypan blue exclusion under an inverted microscope. The small intestine was removed and cut into 10-cm segments starting from the ileocecal valve. A total of 34 segments were obtained. All the segments were washed in ice-cold saline and divided into four pieces. The mucosa was removed from each piece of 2.5 cm using a surgical blade and cultured in a 24-well Falcon tissue culture plate (American Scientific Products,

*To whom correspondence should be addressed.

Abbreviations: apo, apolipoprotein; BSA, bovine serum albumin; CAD, coronary artery disease; FCS, fetal calf serum; HDL, high density lipoprotein; IDL, intermediate density lipoprotein; LDL, low density lipoprotein; LPDP, lipoprotein deficient plasma; LPDS, lipoprotein deficient serum; NZW, New Zealand White; PBS, phosphate buffered saline; VLDL, very low density lipoprotein; WHHL, Watanabe.

McGaw Park, IL) with 2 mL of medium for 12 hr. Medium (250 μL) was withdrawn at time 0, 3 hr, 7 hr, and 12 hr. For the other experiments, 10 to 20 cm of the small intestine of the duodenal, jejunal, and ileal sections were incised. The intestine was washed four times with phosphate buffered saline (PBS), and the mucosa cells were scraped off with a surgical blade. Mucosa cells were further washed three times in PBS and precipitated by low speed centrifugation (1,000 rpm) at 4°C.

Cell culture. Hepatocytes (10^6 cells) were seeded into culture dishes (100 × 15 mm) in Dulbecco's Modified Eagle's Medium with 100 units/mL penicillin, 100 μg/mL streptomycin, 0.025 M HEPES buffer, pH 7.4 (medium A), and 10% fetal calf serum (FCS). Intestinal mucosa cells collected from each segment of 10–20 cm were suspended in medium A and divided into four culture dishes (100 × 15 mm with 10 mL of medium A and 10% FCS). Hep G2 cells (16) were grown in monolayers in medium A plus 10% FCS as above. Cells were incubated in a humidified incubator with 5% CO_2 in air at 37°C. Hepatocytes and intestinal cells were cultured for 48 hr after which the culture medium was collected and filtered through a 0.2 μ filter (Millipore, Corp., Bedford, MA) to remove cellular debris. The cells were collected by scraping with a rubber policeman, washed in PBS, and precipitated by centrifugation at 4°C. The cells were then used for protein analysis by the method of Lowry *et al.* (17). Oleic acid was prepared as the bovine serum albumin (BSA) conjugate as described by Van Harken *et al.* (18).

Fractionation of lipoproteins. The culture medium was fractionated into chylomicrons plus VLDL (d<1.006), IDL plus LDL (1.006–1.063), HDL_2 (1.063–1.12), HDL_3 (1.12–1.21), and lipoprotein deficient plasma (LPDP) (d>1.21) fractions by sequential centrifugation (35,000 rpm for 48 hr). One mL of standard buffer was layered on top of 8 mL of culture medium adjusted to each density with KBr before centrifugation. The top 1-mL layer was carefully withdrawn after centrifugation and used as the respective lipoprotein class. Each lipoprotein fraction was dialyzed overnight against the standard buffer.

Radioimmunoassays. Rabbit apoA-I was purified from pooled rabbit HDL as described for the isolation of human apoA-I (19). Purified apoA-I was used to immunize adult male goats to produce high titer goat anti-rabbit apoA-I antiserum (19). The antiserum was partially purified by ammonium sulfate precipitation (35% saturation) and by using a size exclusion column (Sephadex G-25, Pharmacia, Piscataway, NJ). The rabbit apoA-I assay was performed as described for the human apoA-I assay (20). A non-ionic detergent, Tween-20, was used to detect all the immunoreactivity of the apoproteins in lipoprotein particles (21). The assays were performed in 12 × 75 mm disposable glass tubes. All dilutions were made in radioimmunoassay (RIA) buffer containing 0.1% BSA, 0.1 M sodium borate, 0.01 M NaN_3, and 0.001 M EDTA, pH 8.5. The goat anti-apoA-I antiserum was titrated so as to bind 40–50% of the [^{125}I]apoA-I (1–2 ng with 20,000–30,000 dpm). A typical assay contained 100 μL [^{125}I]apoA-I, 100 μL anti-apoA-I serum (1:4,000 dilution in RIA buffer), 100 μL sample (1:10,000 dilution in RIA buffer), and 100 μL 1% Tween-20. The non-specific binding when no antibody was used was less than 2% of the total counts. The antibody-antigen complexes were equilibrated overnight at room temperature and precipitated with an excess of streptococcal protein A (diluted 3-fold with 0.1 M Tris buffer, pH 8.0). The precipitates were washed with diluted RIA buffer (1:5 dilution with saline) and counted. The linear range of this assay is 0.3125 ng–160 ng/100 μL sample. Pooled normal rabbit plasma (Calbiochem Corp., San Diego, CA) was used as a reference in every assay to correct for daily variations.

RESULTS

The plasma of four WHHL or four NZW rabbits were combined, and the lipids and lipoprotein levels were studied (Table 1). WHHL rabbits have elevated cholesterol and triglyceride levels as compared to NZW rabbits. Most of the cholesterol is carried in the LDL fraction. A significant amount of cholesterol was also found in the VLDL (d<1.006) fraction in WHHL rabbits. Plasma apoA-I levels were significantly lower in WHHL rabbits than those of NZW rabbits as expected. Further fractionation of HDL into HDL_2 (1.063<d<1.12) and HDL_3 (1.12<d<1.21) fractions indicated that most of the apoA-I (60%) was associated with the HDL_3 fraction in both NZW and WHHL rabbits. Little apoA-I was found in the HDL_2 fraction of WHHL rabbits, whereas 24% apoA-I was found in the HDL_2 fraction of NZW rabbits. Furthermore, a significant portion of apoA-I was found in the LPDP (d>1.21) fraction of both NZW and WHHL rabbits which presumably exists as free apoA-I and is not associated with lipids. The ratio of cholesterol to triglycerides of different lipoprotein classes indicated that VLDL of WHHL rabbits is enriched in cholesterol, and HDL of the same rabbits is enriched in triglycerides as compared to those of NZW rabbits.

The difference in plasma HDL levels and in the distribution of apoA-I in HDL_2 and HDL_3 fractions between NZW and WHHL rabbits suggested that the secretion of apoA-I by the intestines of these two breeds of rabbits may be different. The small intestine of a NZW rabbit was divided into 34 segments of 10 cm each, and the mucosa cells were isolated and cultured in medium A with 10% FCS as described in Materials and Methods. The secretion of apoA-I by the mucosa cell cultures into the culture medium was followed by RIA. The results shown in Figure 1 suggest that most of the secretion of apoA-I was observed in segments proximal to the entry of the common bile duct. Very little apoA-I was found in mucosa cells derived from the ileal end of the small intestine. The intestinal mucosa cells of representative duodenal, jejunal, and ileal sections of intestine of both NZW and WHHL rabbits were cultured in a larger amount of medium (10 mL) for 48 hr to maintain the viability of the mucosa. The results shown in Table 2 suggest that duodenal mucosa cells secreted the most apoA-I/mg of cellular mucosa protein within 48 hr. Jejunal cells also secreted significant amounts of apoA-I, whereas ileal cells secreted less than half the amount secreted by either duodenal or jejunal cells. The amount of apoA-I secreted by intestinal cells of the WHHL rabbits was less than that by cells of NZW rabbits. Fractionation of the culture medium of mucosa cell cultures by density gradient centrifugation indicated that apoA-I was most concentrated in the HDL fraction (Table 3). The secretion of apoA-I by jejunal cells of a NZW rabbit was further studied in medium with and without 10% FCS. As shown in Table 4, slightly more apoA-I was

TABLE 1

Plasma Lipid and Lipoprotein Levels in Typical NZW and WHHL Rabbits (6 mon old)[a]

		Chol	TG	Chol/TG	ApoA-I
NZW	Plasma	76[b]	41[b]	1.85	113.37[b]
	VLDL	2 (3%)[c]	8 (24%)	0.25	0.004 (0.005%)[c]
	LDL	29 (45%)	0 (30%)	2.83	0.05 (0.06%)
	HDL_2	14 (22%)	4 (12%)	3.5	18.9 (24%)
	HDL_3	14 (22%)	5 (15%)	3.07	47.94 (60%)
	LPDP	5 (8%)	6 (18%)	0.83	13.07 (16%)
	Recovery	84%	80%		71%
WHHL	Plasma	736[b]	414[b]	1.78	66.77[b]
	VLDL	180 (28%)[c]	118 (30%)	1.52	0.05 (0.1%)[c]
	LDL	445 (69%)	223 (62%)	2	0.13 (0.3%)
	HDL_2	4 (0.6%)	4 (1.1%)	1	0.83 (2%)
	HDL_3	9 (1.4%)	5 (1.4%)	1.8	27.36 (61%)
	LPDP	4 (0.6%)	9 (2.5%)	0.44	16.15 (30%)
	Recovery	87%	87%		67%

[a]Different classes of lipoproteins were isolated from rabbit plasma by sequential ultracentrifugation; VLDL (d<1.006), LDL (1.006–1.063), HDL_2 (1.063–1.12), HDL_3 (1.12–1.21), LPDP (d>1.21), total cholesterol, triglyceride, and apoA-I levels were measured in each fraction.

[b]mg/dL.

[c]% of total recovery.

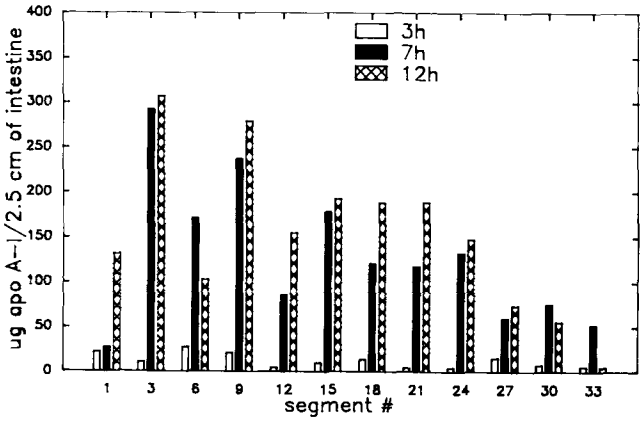


FIG. 1. Secretion of apoA-I by intestinal mucosa cells of a NZW rabbit. The small intestine of a NZW rabbit (10 mon old) was removed and divided into 34 segments numbered 1–34 consecutively from the proximal to distal ends. The mucosa cells were scraped off and cultured in 2 mL of medium A with 10% FCS. ApoA-I secretion into the medium was measured by RIA. Each value represents the average of eight determinations.

TABLE 2

Secretion of ApoA-I by Intestinal Cell Cultures in Medium Containing 10% FCS[a]

		10 Min	24 Hr	48 Hr
NZW	Duodenum	0[b]	22.73[b]	182.69[b]
	Jejunum	0	56.28	151.97
	Ileum	0	9.05	42.18
WHHL	Duodenum	0	15.76	104.1
	Jejunum	0	87.14	96.98
	Ileum	0	0.21	0.79

[a]Mucosa cells were scraped off different segments of small intestine and cultured in medium A with 10% FCS. ApoA-I concentration in the culture medium was measured after 10 min, 24 hr, and 48 hr incubations at 37°C.

[b]Net increase in μg/mg of total cellular protein.

TABLE 3

ApoA-I Concentration in Lipoprotein Fractions Secreted by Intestinal Mucosa Cell Cultures of NZW and WHHL Rabbits[a]

	Duodenal ApoA-I		Jejunal ApoA-I		Ileal ApoA-I	
	NZW	WHHL	NZW	WHHL	NZW	WHHL
d<1.006	0.001	0.14	0.055	0.09	0.008	<0.001
1.006<d<1.063	0.055	0.51	0.011	0.1	0.007	0.009
1.063<d<1.21	9.44	4.69	1.6	2.63	1.05	0.02
d>1.21	0.47	0.91	0.8	0.14	1.31	0.03

[a]μg/mg of protein in the respective density fractions.

secreted by jejunal mucosa cell cultures in medium without FCS than in medium containing 10% FCS. However, apoA-I was mostly found in the lipoprotein-deficient (d>1.21) fraction in medium without FCS. Similar results were obtained with cultures from WHHL rabbits (data not shown).

Hepatocytes were isolated from a NZW rabbit by collagenase perfusion techniques and cultured in medium A with 10% FCS. Hepatocytes attached to the culture dish after 6 hr of incubation at 37°C. The remaining floating cells were then washed away with PBS. Fresh medium with and without 10% FCS was then introduced, and the incubation was continued for 48 hr. As shown in Table 4, hepatocytes secreted less than the 1% of apoA-I that was secreted by jejunal mucosa cell cultures under the same conditions. Fractionation of the medium into different lipoprotein density classes indicated that most of the apoA-I was found in the lipoprotein-deficient (d>1.21) fraction.

TABLE 4

Secretion of ApoA-I by NZW Jejunal Cell Cultures and Hepatocytes in Medium with and without 10% FCS[a]

	Medium A + 10% FCS	Medium A
	ApoA-I	ApoA-I
Jejunal cells:		
Total	151.97[b]	184.52[b]
d<1.063	0.173[c]	0.337[c]
1.063<d<1.21	0.806	0.798
d>1.21	0.437	1.448
Hepatocytes:		
Total	11.5[b]	N.D.
d<1.063	<0.01[c]	<0.01[c]
1.063<d<1.21	<0.01	<0.01
d>1.21	0.08	0.9

[a] Jujunal mucosa cells and hepatocytes isolated from a NZW rabbit were cultured in medium A with and without 10% FCS for 48 hr. The medium was separated into different density ranges by ultracentrifugation. Each density fraction was dialyzed and the level of apoA-I was determined.

[b] μg/mg of total cellular protein.

[c] μg/mg of total protein in the respective density fraction.

The effect of oleic acid/BSA conjugate on the secretion of different lipoprotein classes by NZW rabbit intestinal cells and hepatocytes was studied. The results are shown in Table 5. Intestinal mucosa cells secreted only 58% of apoA-I into the medium in the presence of 0.8 mM oleate/BSA in 48 hr. ApoA-I distribution in each density class suggests that there may be a shift toward secreting more VLDL (d<1.063) and less HDL into the medium containing oleic acid by intestinal cells. The human liver cell line, Hep G2 cells, was known to secrete both VLDL and HDL and apoA-I into the culture medium (16) and was used in the same experiment for comparison. The total amount of apoA-I secreted into the culture medium after 48 hr was decreased 25% in the presence of 0.8 mM oleate/BSA.

DISCUSSION

Watanabe rabbits were developed by Watanabe (14) for the study of hypercholesterolemia and atherosclerosis. This breed of rabbit has a defect in the LDL-receptor gene that reduces their ability to clear LDL and VLDL (22). WHHL rabbits, unlike type IIa hypercholesterolemic patients, have elevated plasma triglyceride levels and low HDL cholesterol levels. From the plasma lipid analysis, it was apparent that WHHL rabbits had high cholesterol levels that were mainly associated with the LDL particles. WHHL rabbits also had low HDL cholesterol levels, and the HDL particles were relatively depleted of cholesterol and enriched triglycerides. By contrast, VLDL particles from WHHL rabbits were enriched in cholesterol but not those of NZW rabbits. The difference may be due to the long residence time of VLDL in WHHL rabbits. Plasma apoA-I levels in WHHL rabbits were only 39% of that of NZW rabbits. Further fractionation of HDL particles into HDL_2 (1.063<d<1.12) and HDL_3 (1.12<d<1.21) fractions indicated that most of the apoA-I was concentrated in the HDL_3 fractions in WHHL rabbits. It has been demonstrated that HDL_2 is formed from HDL_3 by multiple processes that may involve lipoprotein lipase, lecithin:cholesterol acyltransferase, and cholesterol ester transfer protein (23,24). The lack of HDL_2 in WHHL rabbits suggests that there may be very little lipid transfer between VLDL and HDL. Whether this is related to the low apoA-I and HDL levels and high VLDL and LDL levels in

TABLE 5

The Effect of Oleic Acid on the Secretion of ApoA-I by Jejunal Cells and Hep G2 Cells[a]

		NZW Jejunal Cells	Hep G2 Cells
		ApoA-I	ApoA-I
Medium A + 10% FCS	Total	128.69[b]	21.36[b]
	d<1.006	0.055[c]	0.45[c]
	1.006<d<1.063	0.011	1.29
	1.063<d<1.21	1.6	53.73
	d>1.21	0.8	0.15
Medium A + 10% FCS + 0.8mM Oleate/BSA	Total	74.4[b] (58%)	16.08[b] (75%)
	d<1.006	0.12[c]	0.67[c]
	1.006<d<1.063	0.032	4.09
	1.063<d<1.21	0.9	41.45
	d>1.21	1.37	0.02

[a] Jejunal mucosa cells isolated from a NZW rabbit and the human hepatoma cell line, Hep G2 cells, were cultured in medium containing 10% FCS with and without 0.8 mM oleate/BSA conjugate for 48 hr. The medium was separated into different density classes by ultracentrifugation. The level of apoA-I was determined in each fraction.

[b] μg/mg of total cellular protein.

[c] μg/mg of total protein in the respective fraction.

WHHL rabbits is not known. By using intestinal mucosa cell cultures and RIA for apoA-I, we demonstrated that apoA-I was secreted primarily from the intestinal cells, but not hepatocytes, of both NZW and WHHL rabbits (Table 2). Both duodenal and jejunal sections of intestinal cells secreted significant amounts of apoA-I into the culture medium, whereas ileal cells secreted very little apoA-I. Intestinal cells isolated from WHHL rabbits secreted approximately 60% the amount of apoA-I secreted by NZW rabbits. This difference may partly explain the low plasma apoA-I and HDL levels in WHHL rabbits. As expected, most of the apoA-I secreted was associated with particles in the HDL density fraction ($1.063<d<1.21$) of the culture medium. However, significant amounts of apoA-I were also found in the lipoprotein-deficient fraction ($d>1.21$) which may be nascent apoA-I particles without significant association with lipids. Use of serum-free medium further promoted the secretion of apoA-I into the $d>1.21$ fraction by jejunal mucosa cells of a NZW rabbit (Table 4). The results indicated that newly secreted apoA-I can be incorporated into lipoprotein particles in the culture medium. Hepatocytes isolated from NZW rabbits secreted a very small amount of apoA-I into the culture medium which is in accordance with other reports (9,10).

It has been demonstrated that addition of oleic acid into the culture medium of Hep G2 cells increased the secretion of VLDL (16). Similarly, the addition of oleic acid into the medium may increase the secretion of VLDL and chylomicrons by intestinal mucosa cells. In the present study, we demonstrated that oleic acid greatly decreased the secretion of apoA-I by intestinal cells. A similar decrease in apoA-I secretion into the medium containing oleic acid was found with jejunal cells from WHHL rabbits (from 96.98 μg/mg protein to 61.95 μg/mg protein). It is possible that plasma apoA-I levels may also decrease after a fatty meal that contains oleic acid. It will be of interest to study the effect of other fatty acids on the secretion of apoA-I by intestinal cells.

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BW755C or Staurosporine Inhibits Collagen-Stimulated Phosphoinositide Phosphorylation in Platelets

Lisa M. Thomas and **Bruce J. Holub***
Department of Nutritional Sciences, University of Guelph, Guelph, Ontario, Canada N1G 2W1

Stimulation of platelets by collagen results in increased formation of the polyphosphoinositides, phosphatidylinositol phosphate (PtdIns*P*) and phosphatidylinositol bisphosphate (PtdInsP_2) through stimulation of phosphoinositide kinase activities. We investigated a possible regulatory role of endogenous thromboxane formation and protein kinase C (PKC) activation in the induction of phosphoinositide phosphorylation following collagen stimulation, as well as following stimulation by the thromboxane mimetic, U-46619. Human platelets were prelabeled with [^{3}H]inositol and stimulated with collagen (2 μg/mL) or U-46619 (1 μM), in the absence or presence of either the cyclo-oxygenase/lipoxygenase inhibitor, BW755C, or staurosporine, a putative inhibitor or PKC. Collagen stimulation resulted in a time-dependent increase in [^{3}H]inositol-labeled PtdIns*P* and PtdInsP_2 which was completely inhibited in the presence of BW755C. Addition of U-46619 to BW755C-treated, collagen-stimulated platelets restored the increased polyphosphoinositide formation. Stimulation of platelets with U-46619 alone also resulted in increased formation of [^{3}H]PtdIns*P* and [^{3}H]PtdInsP_2, but this was not affected by the presence of BW755C. These results suggest that the collagen-induced activation of phosphoinositide kinases was dependent upon thromboxane formation, but that U-46619-induced phosphoinositide formation was rather independent of further thromboxane production. Pretreatment of platelets with staurosporine, prior to agonist addition, completely blocked the collagen-stimulated rise in radiolabeled PtdIns*P* and the U-46619-induced PtdIns*P* and PtdInsP_2 generations, suggesting that protein kinase, possibly PKC, may play a role in the activation of phosphoinositide kinases by these agonists.
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The levels of the phosphoinositides, phosphatidylinositol (PtdIns), phosphatidylinositol phosphate (PtdIns*P*) and phosphatidylinositol bisphosphate (PtdInsP_2) in resting platelets are controlled *via* phosphoinositide kinase and phosphomonoesterase activities (1). Activation of platelets by thrombin (2–5) or collagen (6,7) results initially in the hydrolysis of phosphoinositides followed subsequently by increased formation of PtdIns*P* and/or PtdInsP_2. Little information is available on the regulation of the phosphoinositide kinases in platelets. Platelet PtdIns kinase activity, *in vitro*, requires magnesium and ATP and has been shown to be inhibited in the presence of calcium (8,9). Although corresponding data in human platelets are not available, erythrocyte PtdIns*P* kinase also requires magnesium and ATP for activity and is inhibited by PtdInsP_2 (10). Stimulation of intact platelets with dibutryl cyclic AMP or cyclic AMP-elevating agents has been shown to increase polyphosphoinositide levels (11,12). Similarly, treatment of platelets with a diacylglycerol analog or phorbol ester also resulted in PtdIns*P* and PtdInsP_2 formation (13–15). This suggests that cyclic AMP-dependent protein kinase and protein kinase C (PKC) may somehow regulate phosphoinositide kinase activity. Recent evidence also suggests that the phosphoinositide kinases may be regulated by a guanine nucleotide binding protein (16–18).

Stimulation of platelets with collagen has been shown to cause changes in the levels of [^{32}P]orthophosphate-labeled phosphoinositides (5–7,19). Early collagen stimulation results in decreases in both PtdInsP_2 and PtdIns*P*, presumably due to increased phospholipase C activity, while stimulation for longer periods results in the formation of these polyphosphoinositides. The increased levels of polyphosphoinositides are considered to result from stimulated PtdIns and PtdIns*P* kinase activities (5,7). Recent evidence suggests that polyphosphoinositide phosphorylation may play important roles in mediating platelet and other cellular reactivity (20–23). Many collagen-induced responses in platelets, such as aggregation, secretion, arachidonic acid release, and calcium mobilization are dependent upon the production of endogenous thromboxane (24–27). Therefore, we investigated a possible thromboxane-dependency of the collagen-stimulated, as well as thromboxane mimetic-induced, polyphosphoinositide production in [^{3}H]inositol-labeled human platelets. In addition, we investigated a possible involvement of PKC in agonist-induced phosphoinositide formation by pretreating platelets with staurosporine, a putative PKC inhibitor, prior to collagen or thromboxane mimetic, U-46619 (9,11-dideoxy-11α,9α-epoxymethanoprostaglandin $F_{2\alpha}$), stimulation.

MATERIALS AND METHODS

Materials. The *myo*-[2-^{3}H]inositol (10–20 Ci/mmol) was purchased from Amersham Canada, Ltd. (Oakville, Ontario, Canada) and Aquasol-2 was from New England Nuclear (Lachine, Quebec, Canada). Collagen was obtained from Hormon Chemie (München, Germany) and U-46619 was from Upjohn Scientific Co. (Kalamazoo, MI). The U-46619 was evaporated under nitrogen and dissolved in ethanol for storage below 0°C. Staurosporine was donated by Dr. G. Murphy of Sandoz, Canada, Inc. (Whitby, Ontario, Canada) and was dissolved in Me_2SO for storage below 0°C. The 3-amino-1-(3-trifluoromethylphenyl)-2-pyrazoline (BW755C) was a gift from Dr. R. Ippersiel of the Wellcome Research Laboratories (Kirkland, Quebec, Canada), and was stored in powder form below 0°C. Heparin, phosphoinositide standards, and 8-anilino-1-naphthalenesulfonic acid (ANS) were from

*To whom correspondence should be addressed.

Abbreviations: ANS, 8-anilino-1-naphthalenesulfonic acid; BW755C, 3-amino-1-(3-trifluoromethylphenyl)-2-pyrazoline; lysoPtdIns, lysophosphatidylinositol; PKC, protein kinase C; PtdIns, phosphatidylinositol; PtdIns*P*, phosphatidylinositol phosphate; PtdInsP_2, phosphatidylinositol bisphosphate; U-46619, 9,11-dideoxy-11α,9α-epoxymethanoprostaglandin $F_{2\alpha}$.

Sigma Chemical Co. (St. Louis, MO), and bovine serum albumin (fraction V, fatty acid free) was from Boehringer Mannheim Canada (Dorval, Quebec, Canada). The method of Molnar and Lorand (28), modified by dialyzing the final material against 0.9% sodium chloride, was utilized to isolate apyrase from potatoes. Siliconized glassware or polypropylene tubes were used for the isolation and incubation of the platelets. All solvents and chemicals were of analytical grade.

Platelet isolation and labeling procedures. Platelets were isolated from healthy donors who denied taking anti-inflammatory drugs for the past 10 days. Blood was drawn into 1/6 volume of acid-citrate-dextrose anticoagulant (29) and the platelets isolated at 37°C according to the method of Mustard *et al.* (30). Platelets were labeled with [^{3}H]inositol by incubation for 2 hr at 37°C with 20–25 μCi of [^{3}H]inositol per 10 mL of the first washed platelet suspension [Tyrodes buffer containing 60 μg/mL apyrase, 50 units/mL heparin, 0.35% bovine serum albumin and 5 mM Hepes (30)]. In some experiments the volume of the first platelet suspension was decreased to 5 mL, which provided an approximate doubling of the incorporated radioactivity. In addition, the 1 mM $MgCl_2$ in the Tyrodes buffer was substituted with 2 mM $MnCl_2$ and the glucose concentration was decreased from 5.5 mM to 0.55 mM to increase the incorporation of [^{3}H]inositol into platelet phosphoinositides, as described by Culty *et al.* (31). Less than 1% of the labeled inositol was incorporated into the platelet phosphoinositides. Subsequently, the platelets were washed free of radiolabel and resuspended in a magnesium-containing Tyrodes buffer (with 5.5 mM glucose) containing 3 μg/mL apyrase and 0.35% bovine serum albumin (30). This final platelet suspension was adjusted to a concentration of 5×10^8/mL using a Coulter Counter Model ZM (Coulter Electronics of Canada Ltd., Burlington, Ontario, Canada).

Incubations. One-mL platelet suspensions were pre-incubated (with stirring) at 37°C for 1 min in an aggregometer (Payton Scientific Inc., Buffalo, NY) before incubation for 2 min with either 1 μM staurosporine (or the vehicle, Me_2SO, 0.5%, v/v) or 120 μM BW755C (or the vehicle, water). Collagen (2 μg/mL) or U-46619 (1 μM) was then added and the stirred suspensions incubated for various time periods. The platelet phosphoinositides in the resulting aggregates were extracted three times using an acidified Bligh and Dyer (32) method as described by Allan and Michell (33).

Thin-layer chromatographic separation of phosphoinositides. PtdIns*P*, PtdInsP_2 and lysophosphatidylinositol (lysoPtdIns) standards were added as carrier to the platelet lipid extracts prior to spotting on Merck pre-coated thin-layer chromatographic plates (BDH Chemicals Canada Ltd., Toronto, Ontario, Canada). The phosphoinositides were separated in a one-dimensional system described previously (8). Following detection using a 0.1% ANS solution (34) and viewing under ultraviolet light, the phosphoinositide bands were scraped into vials, 1.5 mL of water and 13.5 mL of Aquasol-2 were added, and the vials were vortexed. The radioactivity associated with the phosphoinositides was counted on a Beckman LS3801 scintillation counter (Beckman Instruments, Fullerton, CA).

Statistical analyses. For most experiments, the responses following agonist stimulation are expressed as a percentage of the unstimulated values due to the variability in the incorporation of the radioactive label into the phospholipids between individual subjects. To facilitate the statistical analysis, the radioactive data were first log transformed and analyzed using an analysis of variance, following which specific differences for preplanned comparisons between treatment means were determined using a paired Student's *t*-test (35). For some experiments, where the data is expressed as the mean (± SEM) in radioactive dpm values, statistical significance was determined using an unpaired Student's *t*-test (35).

RESULTS

Effect of BW755C on collagen-stimulated phosphoinositide metabolism. Figure 1 shows the changes in [^{3}H]inositol-labeled phosphoinositide levels following stimulation with collagen (2 μg/mL) for various time periods and the effect of a 2 min pre-incubation with the combined cyclo-oxygenase/lipoxygenase inhibitor, BW755C (120 μM) (36–38). Changes in the levels of phosphoinositides can occur through stimulation of phospholipase C and A_2, and kinase and/or phosphomonoesterase activities. Collagen stimulation for 30 seconds in the absence of BW755C (closed circles) resulted in decreases in both [^{3}H]inositol-labeled PtdIns and PtdInsP_2, consistent with phosphoinositide hydrolysis by phospholipase C. At this time period, there were no significant changes in the levels of [^{3}H]PtdIns*P* or [^{3}HlysoPtdIns. At later times of collagen stimulation (90 and 180 seconds) in the absence of BW755C, there were significant rises in the polyphosphoinositides, PtdIns*P* and PtdInsP_2, that were indicative of phosphoinositide kinase activity; however, the concurrent involvement of phospholipase C and/or phosphomonoesterase activities cannot be excluded. Pretreatment with BW755C (open circles) had no significant effect on the levels of PtdIns and PtdInsP_2 at 30 seconds of collagen stimulation, suggesting that the hydrolysis of these phosphoinositides by phospholipase C at this time period was eicosanoid-independent. However, the rises in PtdIns*P* and PtdInsP_2 at later times of stimulation with collagen were completely blocked by the presence of BW755C, suggesting the involvement of eicosanoids in this collagen-induced response. There were further decreases in PtdIns at 90 seconds and 180 seconds of collagen stimulation and an increase in lysoPtdIns at 180 seconds of collagen stimulation which were also markedly inhibited by the presence of BW755C. This suggests the involvement of eicosanoids in the further depletion of PtdIns by the action of PtdIns kinase and/or *via* the hydrolysis by phospholipase C and/or phospholipase A_2.

Effect of simultaneous stimulation of BW755C-treated platelets with collagen and U-46619. To investigate the eicosanoid-dependency of the collagen-stimulated changes in phosphoinositides, the effect of adding a thromboxane mimetic (U-46619) to platelets pre-incubated with BW755C and stimulated with collagen was determined. Platelet suspensions were first pretreated with the dual cyclo-oxygenase/lipoxygenase inhibitor, BW755C, and then were stimulated simultaneously with collagen and U-46619 for 3 min. The results are shown in Table 1. Collagen stimulation for 3 min caused a significant decrease in PtdIns and significant increases in the polyphospho-

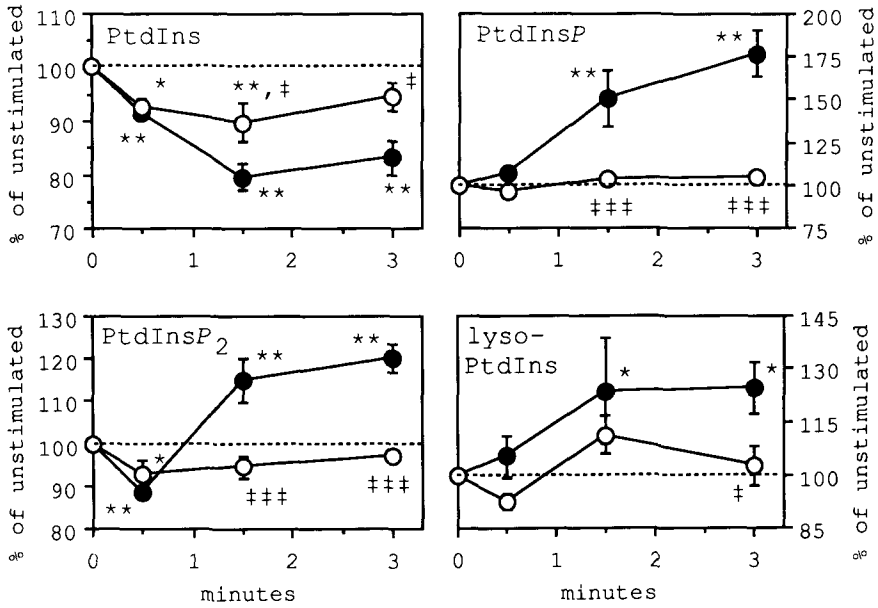


FIG. 1. Effect of BW755C on collagen-induced phosphoinositide metabolism in [^{3}H]inositol-labeled platelets. Platelet phosphoinositides were prelabeled with [^{3}H]inositol as described in Materials and Methods. One-milliliter samples of platelet suspensions (5 × 10^8/mL) were incubated at 37°C for 2 min in the absence (●) or presence (○) of 120 μM BW755C, prior to incubation with collagen (2 μg/mL) for the times indicated. Data are the mean (± S.E.M.) from three separate experiments and are expressed as the percentage of the unstimulated controls. Average unstimulated radioactivity associated with PtdIns, PtdIns*P*, PtdInsP_2 and lysoPtdIns, respectively, were 12665, 1204, 1455 and 830 dpm in the absence of BW755C and 12737, 1209, 1410 and 852 dpm, respectively, in the presence of BW755C. There was no significant effect of BW755C on the unstimulated dpm values associated with these phosphoinositides. The symbols * and ** indicate significant difference from the unstimulated value ($p < 0.05$ and $p < 0.01$, respectively); while the symbols ‡ and ‡‡‡ indicate significant difference from the collagen-stimulated value in the absence of BW755C ($p < 0.05$ and $p < 0.001$, respectively).

TABLE 1

Effect of Simultaneous Stimulation of BW755C-Treated Platelets with Collagen and U-46619 on Phosphoinositide Metabolism[a]

Phosphoinositide	Unstimulated	Collagen		
		Alone	+ BW755C	+ BW755C, + U-46619
	[dpm ± S.E.M.]		[dpm ± S.E.M. (%)]	
PtdIns	21847 ± 293	17857 ± 741 (82)**	21605 ± 118 (99)	20433 ± 259 (94%)*
PtdIns*P*	1583 ± 121	2264 ± 49 (143)**	1702 ± 8 (108)	2725 ± 121 (172%)**
PtdInsP_2	1318 ± 90	1594 ± 53 (121)[b]	1135 ± 4 (86)	1853 ± 65 (141%)*

[a]Platelet phosphoinositides were prelabeled with [^{3}H]inositol as described in Materials and Methods. One milliliter samples of platelet suspensions (5 × 10^8/mL) were incubated at 37°C for 2 min in the presence or absence of 120 μM BW755C prior to incubation with collagen (2 μg/mL) or collagen and U-46619 (1 μM) for 3 min. Data are the mean (± S.E.M.) radioactivity of three determinations. The numbers in parentheses express the values as a percentage of the unstimulated control. The symbols, * and **, indicate significant difference from the unstimulated values ($p<0.05$ and $p<0.01$, respectively).

[b]Indicates a p-value of 0.057 as compared to the unstimulated value.

inositides, PtdIns*P* and PtdInsP_2. The presence of BW755C significantly inhibited these responses to collagen. Simultaneous addition of collagen and U-46619 to BW755C-treated platelets restored the collagen-induced degradation of PtdIns and formation of polyphosphoinositides. These results suggest that the inhibitory effect of BW755C on the collagen-stimulated phosphoinositide metabolism (Fig. 1) was due to the inhibition of thromboxane production (*via* blockade of cyclo-oxygenase activity).

Effect of BW755C on U-46619-induced phosphoinositide metabolism. The effect of incubation of platelets with U-46619 alone or incubation of BW755C-treated platelets with U-46619 is shown in Table 2. A 3-min incubation with this thromboxane mimetic resulted in a decrease in the level of PtdIns as well as increases in PtdIns*P* and PtdInsP_2, thereby suggesting that thromboxane receptor occupancy alone could induce both the depletion of PtdIns and polyphosphoinositide generation. Pretreatment with BW755C, however, did not influence the U-46619-induced

TABLE 2

Effect of BW755C on U-46619 Induced Phosphoinositide Metabolism in [^{3}H]Inositol-Labeled Platelets[a]

Phosphoinositide	Radioactivity in unstimulated	U-46619	U-46619 + BW755C
	(dpm)	(% of unstimulated)	
PtdIns	17086	89 (±1)***	89 (±3)***
PtdIns*P*	1434	208 (±19)***	209 (±14)***
PtdInsP_2	1600	152 (±8)***	151 (±9)***
LysoPtdIns	1040	120 (±13)	112 (±9)

[a]Platelet phosphoinositides were prelabeled with [^{3}H]inositol as described in Materials and Methods. One-milliliter samples of platelet suspensions (5×10^8/mL) were incubated at 37°C for 2 min in the absence or presence of 120 μM BW755C prior to incubation with U-46619 (1 μM) for 3 min. Data are the mean (± S.E.M.) from four separate experiments and are expressed as the percentage of the unstimulated controls. The symbol *** indicates a significant difference from the unstimulated value ($p<0.001$).

TABLE 3

Effect of Staurosporine on the Levels of PtdIns*P* and LysoPtdIns in Collagen-Stimulated Human Platelets[a]

Phosphoinositide	Radioactivity in unstimulated	Collagen	Collagen + staurosporine
	(dpm)	(% of unstimulated)	
PtdIns*P*	683	183 (±23)**	99 (±3)‡‡
lysoPtdIns	586	122 (±7)**	103 (±5)‡‡

[a]Platelet phosphoinositides were prelabeled with [^{3}H]inositol as described in Materials and Methods. One-milliliter samples of platelet suspensions (5×10^8/mL) were incubated at 37°C for 2 min in the presence of 1 μM staurosporine or the vehicle, Me_2SO (0.5%, v/v) prior to incubation with collagen (2 μg/mL) for 3 min. Data are the mean (± S.E.M.) from four separate experiments and are expressed as the percentage of the unstimulated controls. The symbol ** indicates a significant difference from the unstimulated value ($p<0.01$), while the symbol ‡‡ indicates a significant difference from the collagen-stimulated value in the absence of staurosporine ($p<0.01$).

phosphoinositide metabolism, indicating that further endogenous eicosanoid production (*i.e.*, thromboxane) was without effect in platelets stimulated by U-46619 at this concentration.

Effect of staurosporine on collagen- and U-46619-induced phosphoinositide metabolism. The effects of staurosporine, a PKC inhibitor (39,40), on collagen-induced and U-46619-induced phosphoinositide metabolism are shown in Tables 3 and 4, respectively. Pretreatment of platelets with staurosporine (1 μM) for 2 min significantly blocked the collagen-induced formations of PtdIns*P* and lysoPtdIns. The data for PtdInsP_2 were too inconsistent to determine the effect of staurosporine in collagen-stimulated platelets. In U-46619-stimulated platelets, the loss in PtdIns and generation of PtdIns*P* and PtdInsP_2 were also significantly suppressed in the presence of staurosporine. These data suggest that PKC may be involved in collagen and U-46619-induced phosphoinositide metabolism, particularly the increased formations of polyphosphoinositides observed upon stimulation.

DISCUSSION

We investigated collagen- and U-46619-stimulated phosphoinositide metabolism in [^{3}H]inositol-labeled human platelets. It has been assumed that PtdIns, PtdIns*P* and PtdInsP_2 had the same specific radioactivity and that these phosphoinositides exist in a metabolically homogenous pool within platelets (41). Labeling with inositol in this study produced an average distribution of radioactivity between PtdIns, PtdIns*P* and PtdInsP_2 of 85, 7 and 8%, respectively. This is similar to the mass distribution of phosphoinositides described by Tysnes *et al.* (41).

The stimulated formations of polyphosphoinositides were studied in the presence or absence of either the cyclooxygenase/lipoxygenase inhibitor, BW755C, or staurosporine, a putative inhibitor of PKC. Caution must be exerted during the interpretation of data utilizing staurosporine since it has been demonstrated to be a non-specific inhibitor of protein kinases (42,43). However, the possible involvement of PKC in agonist-induced phospholipid alterations (40,44) and accumulation of inositol phosphates (45,46) in platelets has been investigated in the past using staurosporine. Both thrombin-induced and dioctanoylglycerol-induced phosphorylation of the 47 kDa protein, the main PKC substrate in platelets, were found to be completely inhibited by staurosporine at a concentration of 1 μM (40).

Following stimulation of [^{3}H]inositol-prelabeled human platelets with collagen, evidence for the depletion of radiolabeled PtdInsP_2 at 30 seconds was followed, at later times of stimulation, by increased formations of both

TABLE 4

Effect of Staurosporine on U-46619 Induced Phosphoinositide Metabolism in Human Platelets[a]

Phosphoinositide	Unstimulated	U-46619	
		− Staurosporine	+ Staurosporine
	[dpm ± S.E.M.]	[dpm ± S.E.M.(%)]	
PtdIns	18822 ± 526	14268 ± 607 (76)**	17989 ± 332 (96)‡‡
PtdIns*P*	1524 ± 127	3135 ± 241 (206)**	1570 ± 158 (103)‡‡
PtdInsP_2	1730 ± 196	2367 ± 188 (137)[b]	1415 ± 132 (82)‡

[a]Platelet phosphoinositides were prelabeled with [^{3}H]inositol as described in Materials and Methods. One-milliliter samples of platelet suspensions (5 × 10^8/mL) were incubated at 37°C for 2 min in the presence of 1 μM staurosporine or the vehicle, Me_2SO (0.5%, v/v) prior to incubation with U-46619 (1 μM) for 3 min. Data are the mean (± S.E.M.) radioactivity from three determinations, with qualitatively similar results obtained in a separate experiment. The numbers in parentheses express the values as a percentage of the unstimulated control. The symbol ** indicates a significant difference from the unstimulated value ($p<0.01$), while the symbols ‡ and ‡‡ indicate significant difference from the U-46619-stimulated value in the absence of staurosporine ($p<0.05$ and $p<0.01$, respectively).

[b]Indicates a p-value of 0.08 compared to the unstimulated value.

the polyphosphoinositides, PtdIns*P* and PtdInsP_2 (Fig. 1). This agrees with previous studies employing [^{32}P]orthophosphate-labeled platelets which showed a similar hydrolysis of PtdInsP_2 and increased polyphosphoinositide formation following collagen stimulation (6,7). However, in contrast with these investigators, we did not observe a decrease in PtdIns*P* using [^{3}H]inositol labeling, but we did observe a depletion of PtdIns. These differences may be due to the different radiotracers utilized or the time points taken for analysis of phosphoinositides. We also observed an increase in lysoPtdIns with collagen (2 μg/mL) stimulation. This agrees with previous studies employing very high levels of collagen that measured lysoPtdIns formation in [^{3}H]glycerol-labeled (47) and unlabeled (48) platelets. In addition, we recently observed a collagen-induced formation of [^{14}C]stearoyl-labeled lysoPtdIns (49). In [^{3}H]inositol-labeled platelets stimulated by U-46619, we also observed increased polyphosphoinositide formation above basal levels (Table 2) which was not observed in [^{32}P]orthophosphate-labeled platelets (50). The longer incubation period with U-46619 and/or the different radiotracer we utilized may have accounted for the differences.

In our experiments involving BW755C, we obtained opposite effects of this inhibitor in collagen- and U-46619-stimulated platelets. The collagen-induced rise in lysoPtdIns and the polyphosphoinositides, PtdIns*P* and PtdInsP_2, were found to be dependent upon endogenous eicosanoid production as was the further loss of PtdIns at later times of collagen stimulation (Fig. 1). These collagen-induced, eicosanoid-dependent changes in phosphoinositides are most likely explained as a thromboxane dependency. Platelets pre-incubated with BW755C and then simultaneously stimulated with collagen and the thromboxane mimetic, U-46619, showed a reversal of the inhibitory effect of BW755C on the collagen-stimulated phosphoinositide metabolism (Table 1). In addition, other collagen-induced responses, such as arachidonic acid release, calcium mobilization, secretion and aggregation, have been shown to be dependent upon endogenous thromboxane formation (24–27). Other investigators have demonstrated the thromboxane dependency of the collagen-stimulated loss in PtdIns (26,51) and gain in lysoPtdIns (48) in platelets treated with aspirin or indomethacin. Previously, we demonstrated that the formation of [^{14}C]stearoyl-labeled lysoPtdIns in collagen-stimulated platelets was inhibited by pretreatment with BW755C (49). We confirm these results in [^{3}H]inositol-labeled platelets and also show that the collagen-stimulated formation of PtdIns*P* and PtdInsP_2 are likely dependent upon thromboxane formation.

The failure of BW755C treatment to affect the phosphoinositide changes observed following U-46619 stimulation (Table 2) suggests that the level of thromboxane mimetic employed (1 μM) was sufficient to cause phosphoinositide phosphorylation without a dependency on further production of endogenous thromboxane. It cannot be determined from the present work whether thromboxane activates both PtdIns kinase and PtdIns*P* kinase, or only one of them. U-46619 stimulation caused a greater than two-fold increase in PtdIns*P* over PtdInsP_2 (Table 2), suggesting a greater activation of PtdIns kinase as compared to PtdIns*P* kinase. It is possible that thromboxane primarily acts on PtdIns kinase with the resulting increase in PtdIns*P* stimulating the formation of PtdInsP_2 *via* PtdIns*P* kinase.

Phorbol ester treatment of platelets has been shown to stimulate increase formation of PtdIns*P* and PtdInsP_2 with a resulting decrease in PtdIns, in [^{32}P]orthophosphate-labeled or unlabeled platelets (13,14), suggesting the stimulation of PtdIns and PtdIns*P* kinase activities by PKC. We investigated a possible involvement of PKC in agonist-stimulated phosphoinositide phosphorylation through the use of the protein kinase inhibitor, staurosporine. Pretreatment with staurosporine blocked the collagen-induced rise in PtdIns*P* (Table 3) and the U-46619-induced formation of PtdIns*P* and PtdInsP_2 (Table 4). These results suggest a role of protein kinase, possible PKC, in the collagen- and U-46619-stimulated phosphoinositide kinase activities. We recently observed a possible PKC-dependency of the collagen-stimulated release of arachidonic acid, formation of lysophospholipids, including lysoPtdIns, and production of thromboxane (52). Since thromboxane production appeared to be necessary for phosphoinositide phosphorylation in collagen-stimulated platelets (Fig. 1), we suggest that suppressed thromboxane production with staurosporine pretreatment may account for the inhibited PtdIns

kinase activity seen in staurosporine-treated, collagen-stimulated platelets. However, in U-46619-stimulated platelets, phosphoinositide phosphorylation was independent of further thromboxane formation, yet was inhibited in the presence of staurosporine. Therefore, staurosporine appears to inhibit U-46619-stimulated polyphosphoinositide formation by a mechanism not involving thromboxane formation. The ability of PKC to cause the sequestration of calcium in platelets (53) may account for the stimulated PtdIns kinase activity, since this enzyme activity appears to be inhibited, *in vitro*, in the presence of calcium (9). Alternatively, PtdIns kinase may be phosphorylated by PKC resulting in its activation. Endogenously produced thromboxane or thromboxane analog may activate PtdIns kinase through the activation of PKC (50).

Very recently, novel phosphorylated PtdIns compounds, PtdIns(3,4)P_2 and/or PtdIns(3,4,5)P_3, have been identified in platelets stimulated by either thrombin, U-46619 or phorbol 12,13-dibutyrate (54–57). It was suggested that these products resulted from stimulated PtdIns 3-kinase activity on PtdIns(4)P and PtdIns(4,5)P_2, respectively (54), or from stimulated PtdIns 4-kinase activity on PtdIns(3)P (55). The basal level of radioactivity associated with PtdIns(3)P in platelets has been reported to be <2% of the total PtdIns$P$, and PtdIns(3)P does not appear to increase upon stimulation (54–57). Therefore, the increased PtdIns$P$ formation we observed with stimulation is most likely due to PtdIns 4-kinase activity. Similarly, PtdIns(3,4)P_2 has been shown to be a minor component (<1%) of the total PtdInsP_2 in resting platelets (54–56). Although increases in PtdIns(3,4)P_2 in platelets have been observed upon stimulation, it represents a very minor component of the increase in total PtdInsP_2 (54–57). From the data of Kucera and Rittenhouse (54), it can be calculated that >93% and >98%, respectively, of the total increases in PtdInsP_2 following thrombin or phorbol ester stimulation were due to the PtdIns(4,5)P_2 isomer. Therefore, the majority of the increase in PtdInsP_2 which we report herein with collagen or U-46619 stimulation is most likely due to an increase in PtdIns(4,5)P_2.

It has been suggested that the activation of phospholipase C and the resulting decrease in PtdInsP_2 may remove the product inhibition on PtdIns$P$ kinase, thus stimulating the phosphorylation of PtdIns to PtdIns$P$ to PtdInsP_2 (58,59). Previously, it was shown that PtdIns$P$ kinase activity, *in vitro*, was inhibited by the presence of PtdInsP_2 (10,58,59). However, we find that in the presence of BW755C, the collagen-stimulated decrease in PtdInsP_2 was unaffected, while the formation of PtdIns$P$ and PtdInsP_2 at later times of stimulation were blocked. This suggests that a decrease in PtdInsP_2 may not be an efficient stimulus for phosphoinositide kinase activity in collagen-stimulated platelets. Recently, it was suggested that increased phosphoinositide kinase activity may precede or occur concurrently with activation of phospholipase C in thrombin-stimulated platelets (60).

The significance of polyphosphoinositide accumulation following collagen or U-46619 stimulation remains to be determined. However, PtdIns and PtdIns$P$ kinase activities have been associated with the platelet cytoskeleton (21) and PtdInsP_2 has been shown to interact specifically with the cytoskeletal proteins profilin and gelsolin (61,62), resulting in increased actin polymerization. Recently, PtdInsP_2 has been shown to be capable of activating PKC isolated from brain (22) and in adrenal chromaffin cells, the polyphosphoinositides may be required for secretion, independently of being substrates for the production of diacylglycerol (23). In addition, in a platelet mixed vesicle (endoplasmic plus plasma membranes), both PtdIns$P$ and PtdInsP_2 induced a rapid release of calcium which was distinguishable from that produced by inositol triphosphate (20). Increased polyphosphoinositide formation may result in the opening of a calcium channel in the plasma membrane allowing the influx of external calcium (63). The thromboxane dependent increase in PtdIns$P$ and PtdInsP_2 upon collagen stimulation may be associated with an influx of extracellular calcium, since the collagen-stimulated rise in cytoplasmic calcium is highly dependent upon thromboxane production (26,27).

In conclusion, the present work suggests an involvement of eicosanoids (presumably thromboxane) and protein kinase, possibly PKC, in the collagen-stimulated formation of polyphosphoinositides in human platelets. Furthermore, the induction of polyphosphoinositide phosphorylation using U-46619 as agonist appears to be mediated by protein kinase, possibly PKC, activation but not by the further production of thromboxane.

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Decrease in Platelet Reduced Glutathione Increases Lipoxygenase Activity and Decreases Vitamin E

Catherine Calzada, Evelyne Véricel and Michel Lagarde*
Institut National de la Santé et de la Recherche Médicale U.205, Chimie Biologique INSA, 69621 Villeurbanne, France

Unstimulated normal human blood platelets were treated with azodicarboxylic acid *bis*(dimethylamide) (diamide), a thiol-oxidizing agent. Oxygenated arachidonic acid (AA) metabolites, malondialdehyde (MDA), and tocopherols were then quantified by high-performance liquid chromatography (HPLC). Diamide treatment partially decreased the amount of reduced glutathione (GSH) content and induced a subsequent decrease in peroxidase activity. However, formation of 12-hydroxy-eicosatetraenoic acid (12-HETE), the end-product of lipoxygenation of AA, increased. Formation of MDA, a marker of overall lipid peroxidation, was also enhanced. Furthermore, platelet α-tocopherol, but not γ-tocopherol, significantly decreased. These results indicate that enhanced "basal" lipoxygenase activity, as a marker of specific AA oxygenation, may be linked to decreased platelet antioxidant status. *Lipids 26*, 696-699 (1991).

Platelet hyperactivity is of concern in atherosclerosis and thrombosis and has been linked to the high incidence of vascular diseases observed in pre-thrombotic states such as aging and diabetes. During agonist-induced platelet activation, arachidonic acid (AA) is liberated from membrane phospholipids and is subsequently oxygenated *via* the cyclooxygenase and lipoxygenase pathways (1). The major oxygenated end-products of the former pathway are thromboxane B_2 and 12-hydroxy-heptadecatrienoic acid (12-HHT). The latter pathway forms an unstable primary oxygenated product, 12-hydroperoxy-eicosatetraenoic acid (12-HPETE). 12-HPETE is rapidly reduced to its corresponding hydroxy derivative, 12-hydroxy-eicosatetraenoic acid (12-HETE), by a glutathione-dependent peroxidase (GSH-Px) (2) which is involved in the antioxidant defense of the cells. GSH-Px is tightly coupled to the hexose monophosphate shunt through reduced nicotinamide adenine dinucleotide phosphate (NADPH) which restores reduced glutathione (GSH) *via* the GSH-reductase. 12-HPETE is known to cause a marked stimulation of its own biosynthesis (3) as well as of the lipoxygenation and cyclooxygenation of some other polyunsaturated fatty acids (4). Its transitory accumulation may be important in pro-aggregatory prostanoid formation. However, platelets possess an efficient system of defense against lipid peroxidation. This system mainly consists of peroxide scavenger enzymes, such as GSH-Px, the glutathione redox system and vitamin E, the latter being the most important lipid-soluble antioxidant. During oxidative damage, enhanced lipid peroxidation occurred and the antioxidant system may be altered. Indeed, platelets from elderly people and from diabetic people synthesized higher amounts of oxygenated metabolites of AA. Vitamin E was decreased in such platelets (5-7).

The present study was undertaken to determine whether there is a link between increased lipoxygenase activity (leading to increased lipid peroxidation) and a decrease in vitamin E content. Unstimulated platelets were subjected to oxidative stress by using azodicarboxylic acid *bis*(dimethylamide) (diamide), a GSH-depleting agent. The effects of a moderate concentration of diamide on platelet lipid peroxidation and antioxidant status were investigated.

MATERIALS AND METHODS

Preparation of platelet suspensions. Venous blood was drawn from healthy adults who had not taken any drugs for at least ten days before venipuncture, and platelets were isolated as previously described (8). Briefly, after centrifugation of the blood at 200 × *g* for 20 min, platelet-rich plasma was acidified to pH 6.4 with 0.15 M citric acid and centrifuged at 900 × *g* for 10 min. Platelets were then resuspended into a Tyrode-HEPES buffer without external calcium (137 mM NaCl, 2.7 mM KCl, 11.9 mM $NaHCO_3$, 0.41 mM NaH_2PO_4, 1 mM $MgCl_2$, 5 mM HEPES, 5.5 mM glucose; pH 7.35). The platelet suspensions were incubated in the absence or presence of 10^{-6}M diamide for one hr at 37°C. Different parameters of the antioxidant status and of lipid peroxidation were then analyzed.

Glutathione measurement. For GSH determinations, platelet suspensions were extracted with methylene chloride in order to lyse platelets and remove diamide. After centrifugation, the upper aqueous layer was removed and assayed for total and reduced glutathione according to a modification of the method of Neuschwander-Tetri and Roll (9). For total glutathione measurement, disulfides were reduced with dithiothreitol in a first step. GSH was derivatized with *ortho*-phthaldialdehyde (OPT) and the GSH-OPT complex was quantified after reverse phase high-performance liquid chromatography (rp-HPLC) by measuring its ultraviolet (UV) absorbance at 340 nm (Fig. 1 A).

Measurement of the reduction of hydroperoxides. The ability of platelets to reduce hydroperoxides to hydroxy derivatives was investigated. The hydroperoxy derivative of AA used was 15-HPETE. 15-HPETE has been reported to inhibit 12-lipoxygenase activity (10); in addition, 15-HPETE is a physiological substrate of GSH-dependent peroxidase. Briefly, platelet suspensions were incubated with or without 10^{-6}M diamide for one hr at 37°C. The reaction was initiated by the addition of 10 μM [^{14}C]15-HPETE, which was obtained by incubating soybean lipoxygenase with [^{14}C]AA (11). The assay was terminated after one min at 37°C by extraction of the com-

*To whom correspondence should be addressed.

Abbreviations: AA, arachidonic acid; BHT, butylated hydroxytoluene; GSH, reduced glutathione; GSH-Px, glutathione-peroxidase; (12 or 15)-HETE, (12 or 15)-hydroxy-eicosatetraenoic acid; 12-HHT, 12-hydroxy-heptadecatrienoic acid; (12 or 15)-HPETE, (12 or 15)-hydroperoxy-eicosatetraenoic acid; MDA, malondialdehyde; NADPH, reduced nicotinamide adenine dinucleotide phosphate; OPT, *ortho*-phthaldialdehyde; rp-HPLC, reverse phase high-performance liquid chromatography; TBA, thiobarbituric acid; TLC, thin-layer chromatography; UV, ultraviolet.

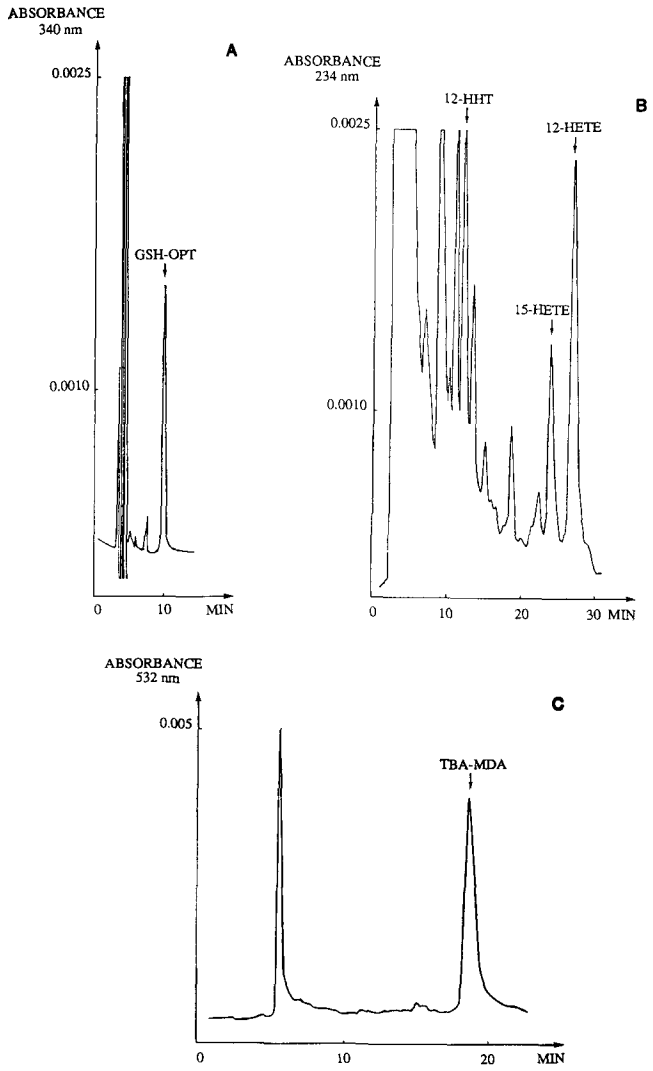


FIG. 1. Typical rp-HPLC separations of different parameters obtained from diamide-treated platelets. (A) GSH (*i.e.* GSH-OPT complex) was separated on a 25-cm column packed with Nucleosil C_{18} (5μm) and was eluted with 0.15M sodium acetate, pH 7/methanol (92.5:7.5, v/v) at a flow rate of 0.8 mL/min. (B) Monohydroxylated fatty acids (12-HHT and 12-HETE) and 15-HETE, the internal standard, were separated on a 25-cm column packed with Nucleosil C_{18} (5μm). The mobile phase, methanol/water (pH 3, with acetic acid) (75:25, v/v), was pumped at 0.7 mL/min. (C) MDA (*i.e.* TBA-MDA complex) was separated on a 15-cm column packed with Nucleosil C_{18} (5μm) and was eluted with water/methanol (80:20, v/v) at a flow rate of 1 mL/min.

pounds at pH 3 with diethyl ether. The hydroperoxy compound was separated from the hydroxy derivative by thin-layer chromatography (TLC) using the solvent mixture diethyl ether/hexane/acetic acid (85:15:0.1, v/v/v). Peroxidase activity was evaluated by determining the amount of [^{14}C]15-HETE formed in one min per 10^9 platelets using a Berthold TLC scanner (Elancourt, France). The classical measurement of GSH-Px activity according to Paglia and Valentine (12) could not be used in our model. The conventional assay was performed in the presence of an excess GSH (mM concentration) and of the recycling system, GSH-reductase and NADPH. The addition of an excess GSH to diamide-treated platelets would restore the capacity of cells to reduce hydroperoxides, *i.e.* peroxidase activity.

Basal metabolism of endogenous AA. The platelet lipids were extracted twice with chloroform/ethanol (2:1, v/v) containing 5×10^{-5}M butylated hydroxytoluene (BHT) as an antioxidant, in the presence of 15-HETE as an internal standard. After separation by TLC with the eluent mixture hexane/diethyl ether/acetic acid (60:40:1, v/v/v) (13), and extraction from the silica-gel with diethyl ether, 12-HHT and 12-HETE were analyzed by rp-HPLC and measured at 234 nm (14) (Fig. 1 B).

Malondialdehyde (MDA) determination. MDA was determined as an index of overall lipid peroxidation by rp-HPLC according to the method of Therasse and Lemonnier (15). Briefly, the platelet suspensions were incubated for one hr at 95°C at low pH with 10 mM thiobarbituric acid (TBA) in phosphate buffer (0.1 M, pH 3) and in the presence of BHT. The TBA-MDA complex was extracted with ethyl acetate and quantified by rp-HPLC at 532 nm (Fig. 1 C).

Vitamin E determination. Vitamin E determinations were done according to a previously reported method (7). The platelet suspensions were mixed with two volumes of ethanol/water (1:1, v/v) containing 1% ascorbic acid as an antioxidant and tocol as an internal standard. After extracting twice with three volumes of hexane, the vitamin E extract was analyzed by rp-HPLC and measured at 292 nm (16).

Statistics. Student's paired t-test was used to compare the different groups of data.

RESULTS

Diamide, a well-known thiol-oxidizing agent, has been reported to lower the level of GSH (17), a cofactor of the peroxidase activity associated with platelet lipoxygenase (2). Treatment of platelets with 10^{-6}M diamide for one hr at 37°C resulted in a substantial and significant decrease (28%) in the primary antioxidant, GSH (Table 1), whereas total glutathione was not significantly depressed (data not shown). In order to evaluate the ability of the cells to convert hydroperoxides to the corresponding hydroxylated fatty acids *via* the lipoxygenase cascade, *e.g.* to determine the peroxidase activity *in situ*, 15-HPETE was used as a physiological substrate and its reduction rate to 15-HETE was determined. Diamide treatment impaired the reduction of 15-HPETE to 15-HETE by one-third (Table 1) corresponding to the lower ability of cells to reduce hydroperoxides.

The effect of 10^{-6}M diamide on platelet lipid peroxidation, in the absence of agonist-specific stimulation, is shown in Table 2. The basal metabolism of platelet endogenous AA, *via* cyclooxygenase and lipoxygenase pathways, was modified after diamide treatment. The formation of the lipoxygenase end-product, 12-HETE, increased significantly in GSH-depleted platelets (40.4 pmol/10^9 in diamide-treated platelets *versus* 32.9 pmol/10^9 in controls). Simultaneous measurement of 12-HHT, a cyclooxygenase by-product of AA, revealed that its formation tended to increase under diamide treatment, although not significantly (data not shown). The overall lipid peroxidation was also affected by diamide treatment; the formation of MDA increased by 58% in GSH-depleted platelets compared to controls.

TABLE 1

Effect of 10^{-6}M Diamide on Different Parameters of Platelet Antioxidant Status

Diamide (M)	GSH[a]	[^{14}C]15-HETE formation[b] (peroxidase activity)	α-Tocopherol[c]
0	16.3 ± 4.5	9.2 ± 3.0	1.01 ± 0.08
10^{-6}	11.6 ± 3.2[d]	6.1 ± 1.9[d]	0.77 ± 0.08[d]

[a]Results are expressed in nmol/10^9platelets as means ± SEM of six determinations.

[b]Results, corresponding to the rate of reduction of [^{14}C]15-HPETE (10 μM for 1 min incubation at 37°C) by platelet peroxidase, are expressed in nmol/min/10^9 platelets as means ± SEM of eight determinations.

[c]Results are expressed in nmol/10^9 platelets as means ± SEM of six determinations.

[d]Values significantly different from control ($p<0.05$).

TABLE 2

Basal Lipid Peroxidation in Control and Diamide-Treated Platelets[a]

Diamide (M)	12-HETE	MDA
0	32.9 ± 4.9	122.7 ± 38.3
10^{-6}	40.4 ± 5.1[b]	194.8 ± 53.4[b]

[a]Results, in pmol/10^9 platelets, are means ± SEM of six determinations for 12-HETE derived from endogenous AA and of eight determinations for the TBA-MDA complex.

[b]Values significantly different from control ($p<0.01$).

The effect of 10^{-6}M diamide on platelet vitamin E content is shown in Table 1. α-Tocopherol concentration in diamide-treated platelets was significantly reduced when compared to control platelets (0.77 ± 0.08 nmol/10^9 in diamide-treated platelets *versus* 1.01 ± 0.08 nmol/10^9 in controls). No corresponding decrease in γ-tocopherol concentration was observed (0.037 ± 0.005 nmol/10^9 in diamide-treated platelets *versus* 0.038 ± 0.004 nmol/10^9 in controls).

Higher concentrations of diamide (10^{-5}M and 10^{-4}M) did not lead to either an increase in lipoxygenase activity or a further decrease in α-tocopherol concentration (data not shown).

DISCUSSION

It has been reported that the platelets from elderly populations demonstrate a moderate decrease in their vitamin E content (6,7). This has also been associated with increased peroxidation of AA (18). We have explored the mechanism of this relationship by treating normal platelets *in vitro* with diamide. The diamide induced oxidative stress by depleting the platelets of GSH.

Incubation of platelets, in the presence of 10^{-6}M diamide, for one hr at 37°C, without specific stimulation, resulted in a partial and significant decrease of GSH but not of total glutathione, suggesting that complete GSH regeneration did not occur in diamide-treated platelets under these conditions. As a result of a lesser recycling of oxidized glutathione, the hexose monophosphate shunt, which provides NADPH for the replenishment of GSH, may not have been significantly stimulated by the low concentration of diamide. In contrast, the hexose monophosphate shunt may have been involved in higher concentrations of diamide where no additional effect was noticed.

In accordance with the lowered GSH level, the peroxidase activity, evaluated by using 15-HPETE as a substrate, was depressed in diamide-treated platelets, which may have been due to the decrease of the enzyme cofactor, GSH. The 12-HPETE peroxidase is assumed to be a glutathione-dependent peroxidase requiring selenium at the active site (19,20).

The partial depletion of both the GSH level and peroxidase activity might have led to a transitory enhancement of 12-HPETE (2). 12-HPETE has been shown to markedly stimulate its own biosynthesis from the endogenous pool of non-esterified AA (3). In agreement with a presumed increase of lipoxygenase activity by 12-HPETE, we have indeed observed enhanced formation of the end-product of the lipoxygenase pathway, 12-HETE, in diamide-treated platelets. The increase of 12-HETE appears somewhat paradoxical on account of the lower ability of diamide-treated platelets to reduce hydroperoxides. However, we assume that the remaining peroxidase (around two-thirds of the total activity) is high enough to reduce the unstable 12-HPETE entirely. Moreover, the formation of a cyclooxygenase by-product of AA, 12-HHT, tended to be stimulated. This also agrees with previous results showing that platelet cyclooxygenase is less responsive to 12-HPETE than lipoxygenase (4,21). It is important to point out that the levels of the AA metabolites present in unstimulated platelets were very low (around 50 pmol/10^9 platelets) compared with the levels of AA metabolites in agonist-stimulated platelets. It corresponds with the very low amount of unesterified AA in unstimulated platelets (22,23) compared with the amount of free AA pool in activated platelets. More generally, MDA, which is formed either by the physicochemical degradation of lipid peroxides or by the cleavage of the cyclopentane ring of cyclic prostaglandin endoperoxides (24), was increased after diamide treatment, which means that 12-HPETE may not be the only peroxide responsible for the enhanced AA oxygenation. Although other peroxides might have contributed to the stimulation of oxygenases, we suggest that the transient accumulation of 12-HPETE could have been responsible for the increased oxygenase activities which are thought to be stimulated by peroxides (25).

Finally, platelet α-tocopherol, which is the most important antioxidant, was depressed after diamide treatment, whereas the much less effective γ-tocopherol (26) re-

mained unchanged. This is in good agreement with the results of Vatassery (27) who reported that diamide, in final concentrations of 2–5 mM, decreases platelet α-tocopherol by partially converting it to α-tocopherolquinone. The most probable explanation for the reduction of α-tocopherol in GSH-depleted platelets is the increased peroxide level which may have led to oxidation of vitamin E acting as a scavenger of peroxyl radicals. Such a reduction may also be considered as a marker of an elevated peroxide level in the cell.

Overall, the decreased platelet antioxidant status, of which vitamin E, GSH and peroxidase activity are some parameters, may be linked to an enhanced lipoxygenation of endogenous AA. We presume that the transient accumulation of 12-HPETE might lead to an increased formation of pro-aggregatory eicosanoids (responsible for at least part of the platelet hyperactivity).

We conclude from this study that lipoxygenase activity and α-tocopherol may be linked through a redox cascade. Although further studies are needed to demonstrate this, it is tempting to speculate that 12-HETE might be a biochemical marker of platelet lipid peroxidation in pre-thrombotic states (5,7).

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Characterization of the Coronary Vascular Responses to Platelet-Activating Factor in the Isolated Perfused Heart

Weimin Hu, Patrick C. Choy and Ricky Y.K. Man*
Department of Pharmacology and Therapeutics, and Department of Biochemistry, Faculty of Medicine, University of Manitoba, Winnipeg, Manitoba, Canada R3E OW3

Platelet-activating factor (PAF) is a potent phospholipid mediator with diverse *in vivo* and *in vitro* coronary vascular effects. In the present study, the coronary vascular responses to bolus injections of PAF were compared in rat hearts perfused under constant flow and under constant pressure. Low levels of PAF (1 pmol) produced vasodilatation only, while higher PAF concentrations (100 pmol) produced initial vasodilatation which was followed by a vasoconstriction under both experimental conditions. To determine species differences in PAF action, the effect of PAF was tested on perfused guinea pig hearts. Unlike in perfused rat hearts, only a dose-dependent vasoconstrictor response was observed in perfused guinea pig hearts following a bolus injection of 1 fmol to 10 pmol of PAF. The results from repeated injections of PAF indicated that depletion of vasoactive mediators induced by PAF or receptor desensitization may explain a failure of a second injection of PAF to initiate a vasoconstrictor response. After PAF injection, the coronary vascular response to leukotriene was not altered, indicating that the reduced vasoconstrictor effect of a second injection of PAF cannot be due to a reduced ability of the smooth muscle to constrict. The study demonstrates that similar coronary vascular responses to PAF are observed in perfused rat hearts under either constant flow rate or constant pressure and that some of the variable coronary vascular responses reported may be due to the difference between animal species.
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Platelet-activating factor (PAF) is a phospholipid mediator with vasoactive properties (1-3). The diverse cardiovascular effects of PAF in coronary circulation have been shown in a number of studies *in vivo* and *in vitro*. Vasoconstrictor effects of PAF were observed in guinea pig and rat hearts perfused at a constant perfusion pressure (4) and at a constant flow rate (5). In the anesthetized dog, Sybertz *et al.* (6) reported a vasoconstrictor response after an intracoronary injection of PAF while a vasodilator response, as indicated by an increase in coronary flow, was reported by Jackson *et al.* (7). These differences may be due to different experimental conditions. A biphasic response characterized by vasodilatation followed by vasoconstriction after intracoronary injection of PAF was shown in other studies (8-11). We have previously demonstrated that depending on the experimental conditions, coronary vasodilatation, vasoconstriction, or a combination of both responses can be observed following bolus injections of PAF in the isolated rat heart perfused at a constant flow rate (12).

*To whom correspondence should be addressed at the Department of Pharmacology and Therapeutics, University of Manitoba, 770 Bannatyne Avenue, Winnipeg, Manitoba, Canada R3E 0W3.

Abbreviations: LTC_4, leukotriene C_4; PAF, platelet-activating factor.

Depending on experimental conditions, changes in vascular tone by a vasoactive compound in the perfused heart can result in changes in the flow rate if the perfusion pressure is kept constant, or in changes in perfusion pressure if the flow rate is kept constant. In the present study, we sought to demonstrate a similar response to PAF in isolated rat hearts perfused at a constant pressure as compared to using a constant flow rate. The platelets of various animal species have been reported as having different sensitivities to PAF (13). Therefore, we also investigated the effects of PAF in guinea pig hearts to determine whether a similar vasodilator and/or vasoconstrictor response could be observed following bolus injections of PAF. It has been reported that in *in vitro* studies repeated injections of PAF did not elicit a vasoconstrictor response (5,12,14). Possible explanations include desensitization of PAF receptors, depletion of vasoactive mediators induced by PAF, or loss of vascular smooth muscle response to PAF or its mediators. In the present study, we attempted to further investigate the possible mechanisms for the lack of coronary vascular response to repeated injections of PAF.

MATERIALS AND METHODS

Materials. PAF (1-*O*-alkyl-2-acetyl-*sn*-glycero-3-phosphocholine, prepared from bovine heart) and bovine serum albumin were purchased from Sigma Chemical Co. (St. Louis, MO). Leukotriene C_4 (LTC_4) was a gift from Merck Frosst Canada Inc. (Montreal, Canada). All other chemicals and reagents were obtained from Fisher Chemical of Canada (Edmonton, Canada) and Canlab Scientific (Winnipeg, Canada).

Heart perfusion. Male Sprague-Dawley rats (250-300 g) and Hartley guinea pigs (300-400 g) were killed by cervical dislocation 15 min after heparin (1000 μ/kg, i.p.) was administered. Hearts were rapidly excised and placed in ice-cold Krebs-Henseleit solution gassed with 95% O_2/5% CO_2. The Langendorff technique was used to perfuse hearts with Krebs-Henseleit solution which was maintained at 37 $\pm$ 0.5°C and gassed with 95% O_2/5% CO_2. The composition of Krebs-Henseleit solution was as follows (mM): NaCl 120, KCl 4.7, NaH_2PO_4 1.18, $MgSO_4$ 1.18, $CaCl_2$ 1.25, $NaHCO_3$ 25, and glucose 5.5. The heart was allowed to beat spontaneously and was perfused at a constant flow rate or under a constant perfusion pressure. The perfusion pressure was measured by attaching a pressure transducer (Bentley Trantec Model 800, Bentley Laboratories of Canada, Markham, Canada) to a side arm of the aortic cannula. To measure changes in flow rate under constant pressure perfusion, the flow rate was measured by a electromagnetic flow meter (Carolina Medical Electronics, King, North Carolina). Both perfusion pressure and flow rate were recorded on a Gould chart recorder and monitored with a digital display of the perfusion pressure and flow rate. The procedures to determine the coronary vascular effects of PAF in the perfused rat heart have been described previously (12).

Drug preparation and administration. Hearts were equilibrated for 20 min using Krebs-Henseleit solution. In some experiments, the flow rate was adjusted to obtain a perfusion pressure of 65–75 mm Hg during the equilibration period and was kept constant for the rest of the experiment. In experiments in which the perfusion pressure was kept constant, the pressure was set at 70 mm Hg. PAF was prepared in saline (0.9% NaCl) solution containing 0.25% bovine serum albumin. LTC_4 was diluted in saline to the appropriate concentration. All stock solutions were made fresh daily and refrigerated between experiments. All bolus injections of PAF and LTC_4 were given a volume of 0.1 mL over a one-second period, into the perfusion line 5–6 cm proximal to the aortic cannula. In some experiments, a second injection was administered 10 min after the first injection. Changes in perfusion pressure or flow rate and the time at which maximum changes occurred were recorded.

Statistical analyses. The data were analyzed by Student's t-test, or one-way analysis of variance followed by Duncan's multiple range test where appropriate. Values were expressed as means and standard deviations (S.D.). A P value less than 0.05 was considered statistically significant.

RESULTS

Comparison of the coronary vascular responses to PAF in rat hearts using constant flow or constant pressure perfusion. Comparisons of the parameters of constant flow perfusion with those of constant pressure perfusion are shown in Tables 1 and 2. There was no significant difference in the flow rates required to attain a control pressure between 65–75 mm Hg using constant flow perfusion from those used at a constant perfusion pressure of 70 mm Hg. Bolus injections of 1 pmol PAF produced only vasodilatation (a decrease in perfusion pressure and an increase in perfusion flow rate in hearts using constant flow and constant pressure perfusion, respectively). With 100 pmol PAF (bolus injection), a biphasic response characterized by an initial vasodilatation followed by a vasoconstriction was observed. These results are summarized in Tables 1 and 2. The time to reach a peak response for the initial vasodilatation and the subsequent vasoconstriction when 100 pmol PAF was administered were similar in hearts perfused under constant flow and constant pressure conditions ($P > 0.05$).

The effect of PAF in perfused guinea pig hearts. To determine the coronary vascular effect of PAF in another animal species, experiments were conducted using guinea pig hearts. The results of bolus injections of PAF in guinea pig hearts using constant flow perfusion are summarized in Table 3. An injection of PAF ranging from 1 fmol to 1 pmol resulted in only a vasoconstrictor response. No vasodilator response was observed with any of the amounts of PAF tested in perfused guinea pig hearts. The magnitude of the vasoconstriction was dose-dependent. Serial dilution was used to obtain the very small amounts of PAF used for injections. The recovery after the dilution was determined by using [^{3}H]PAF. The accuracy of

TABLE 1

The Effects of PAF Under Constant Flow Perfusion[a]

Amount of PAF	Control flow rate (mL/min/g heart)	Decrease in perfusion pressure: Maximum (mm Hg)	Decrease in perfusion pressure: Time to peak (s)	Increase in perfusion pressure: Maximum (mm Hg)	Increase in perfusion pressure: Time to peak (s)	n
1 pmol	12.8 ± 1.7	6.3 ± 2.5	22 ± 7	—[b]	—[b]	6
100 pmol	12.1 ± 0.9	6.0 ± 4.1	9 ± 3	27.5 ± 8.2	60 ± 15	6

[a]Rat hearts were perfused as described in Materials and Methods. In these experiments, the flow rate was kept constant and changes in perfusion pressure were detected by a pressure transducer. Values represent mean ± S.D.

[b]Denotes no detectable change.

TABLE 2

The Effects of PAF Under Constant Pressure Perfusion[a]

Amount of PAF	Control flow rate (mL/min/g heart)	Increase in perfusion flow: Maximum (mL/min/g heart)	Increase in perfusion flow: Time to peak (s)	Decrease in perfusion flow: Maximum (mL/min/g heart)	Decrease in perfusion flow: Time to peak (s)	n
1 pmol	13.0 ± 3.7	2.3 ± 1.2	27 ± 7	—[b]	—[b]	6
100 pmol	12.8 ± 2.8	2.0 ± 1.4	13 ± 4	6.1 ± 2.5	45 ± 10	6

[a]Rat hearts were perfused as described in Materials and Methods. A constant perfusion pressure of 70 mm Hg was used in these experiments and changes in flow rate were detected by an electromagnetic flow meter. Values represent mean ± S.D.

[b]Denotes no detectable change.

TABLE 3

The Effects of a Bolus Injection of PAF in Perfused Guinea Pig Hearts[a]

Amount of PAF	Control perfusion pressure (mm Hg)	Decrease in perfusion pressure[b]		Increase in perfusion pressure		n
		Maximum (mm Hg)	Time to peak (s)	Maximum (mm Hg)	Time to peak (s)	
1 fmol	73 ± 1	—	—	8.2 ± 2.7[c]	64 ± 37	5
10 fmol	72 ± 2	—	—	12.0 ± 5.3[c]	83 ± 33	10
1 pmol	73 ± 2	—	—	21.4 ± 7.9[c]	56 ± 35	5

[a]Guinea pig hearts were perfused using a constant flow rate as described in Materials and Methods. Values represent mean ± S.D.

[b]Denotes no detectable change.

[c]$P < 0.05$ when compared to controls (injections of 0.1 mL saline).

the recovery was limited by the specific radioactivity of the PAF used (81 Ci/mmol). The lowest amount of PAF that we were able to measure reliably was 1 fmol. Hence further dilution to obtain a quantity smaller than 1 fmol was not used.

The effects of repeated injections of PAF in rat hearts. After a bolus injection of 100 pmol of PAF in the perfused rat heart, a second injection of 100 pmol of PAF did not produce any significant change in the perfusion pressure as reported previously (5,12). Figure 1 shows the coronary vascular effect of PAF was transient and the perfusion pressure returned to baseline level within 10 min (67 ± 3 mm Hg before PAF injection and 69 ± 6 mm Hg 10 min after PAF injection, n = 8). The second injection of PAF was therefore given after 10 min of the first injection in the rest of this study. The coronary vascular responses to a second injection of PAF was dependent on the amount of PAF given in the first injection. These results are summarized in Table 4. When 1 pmol PAF was administered first, a second injection of 10 or 100 pmol PAF resulted in an initial vasodilatation followed by a vasoconstriction. After an initial injection of 1 pmol PAF, the vasodilatation produced by 10 pmol PAF was significantly greater while the vasoconstriction was greatly reduced (Table 4). The vasoconstriction produced by a second injection of 100 pmol PAF was also reduced when compared to that produced by an initial injection of 100 pmol PAF (22.1 ± 6.2, n = 9 and 29.1 ± 12.4, n = 11 respectively, $P > 0.5$). When 10 pmol PAF was given in the first injection, a second injection of 10 pmol PAF did not produce a significant vasoconstrictor response while a second injection of 100 pmol PAF resulted in a significant vasoconstriction (Table 4). However, the vasoconstriction produced by a second injection of 100 pmol PAF was reduced (18.8 ± 10.3, n = 6, $P < 0.05$ when compared to the effect of an initial injection of 100 pmol PAF). In contrast, if 100 pmol PAF was given in the initial injection, a second injection of PAF (100 and 10,000 pmol) did not elicit any significant vasoconstriction (Table 4).

To determine whether the tachyphylaxis after an injection of 100 pmol PAF was time-dependent, a second injection of 100 pmol PAF was given after 60, 90 or 120 min after a first injection of 100 pmol PAF in separate experiments. The vasodilatation and vasoconstriction by a second injection of 100 pmol PAF was 6.0 ± 1.7 and 0 mm Hg respectively after 60 min (n = 3), 7.7 ± 2.1 and 0.7 ± 1.2 mm Hg respectively after 90 min (n = 3), and 5.7 ± 2.1 and 0.7 ± 0.6 mm Hg, respectively, after 120 min (n = 3). These effects were not statistically different from those obtained with a second injection of 100 pmol PAF, 10 min after the first injection of 100 pmol PAF. The results indicate that there was no time-dependent recovery of the vasoconstrictor response to PAF.

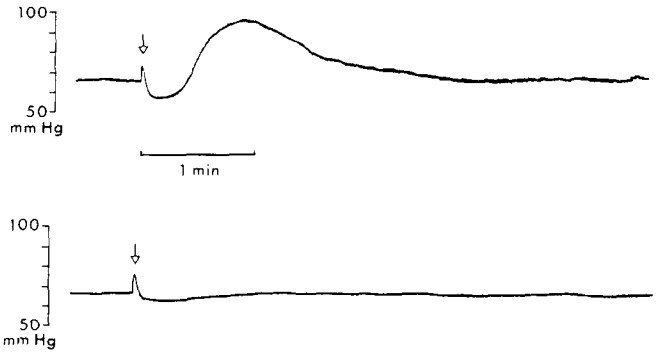


FIG. 1. Original tracings showing the changes in perfusion pressure in a perfused rat heart when 100 pmol PAF was injected first (top tracing) and 10 min later (bottom tracing). The heart was perfused using a constant flow rate as described in Materials and Methods. Arrow indicates the time when 100 pmol PAF was given as a bolus injection (a small injection artifact is displayed on the tracing). A first injection of 100 pmol PAF produced an initial vasodilatation followed by a vasoconstriction (top tracing). Perfusion pressure returned to preinjection level within approximately 5 min after the first injection of PAF. No significant change in perfusion pressure was observed when a second injection of 100 pmol PAF was administered 10 min later (bottom tracing).

The coronary vascular responses to bolus injections of PAF and LTC_4 in the same isolated perfused rat heart. To determine the response of the perfused rat heart to repeated exposure to different vasoactive compounds, PAF and LTC_4 were administered. The coronary vascular responses of the same hearts to PAF and LTC_4 are summarized in Table 5. There was no significant difference in the coronary vascular response to an injection of 100 pmol PAF in hearts with and without a previous administration of 50 pmol LTC_4. The vasoconstriction produced by 50 pmol LTC_4 was also similar in hearts with or without a previous administration of 100 pmol PAF (Table 5).

TABLE 4

The Effects of a Second Bolus Injection of PAF on the Perfusion Pressure of Rat Hearts[a]

Injection	Amount of PAF (pmol)	Control pressure (mm Hg)	Decrease in perfusion pressure		Increase in perfusion pressure		n
			Maximum (mm Hg)	Time to peak (s)	Maximum (mm Hg)	Time to peak (s)	
(A)							
First injection	1	70 ± 4	5.3 ± 4.0	23 ± 5	—[b]	—[b]	
Second injection	10	71 ± 1	12.7 ± 7.4[c]	25 ± 5	8.0 ± 9.8[d]	94 ± 8	3
First injection	1	70 ± 2	6.8 ± 3.8	25 ± 13	—[b]	—[b]	
Second injection	100	68 ± 4	5.8 ± 4.1	10 ± 3	22.1 ± 6.2[e]	65 ± 29	9
(B)							
First injection	10	67 ± 1	8.3 ± 2.9	14 ± 5	20.9 ± 9.9	95 ± 10	
Second injection	10	66 ± 2	6.0 ± 2.2	11 ± 3	1.5 ± 2.1[e]	93 ± 12	9
First injection	10	70 ± 2	8.3 ± 9.3	15 ± 4	25.0 ± 10.6	83 ± 15	
Second injection	100	70 ± 2	6.5 ± 6.2	15 ± 8	18.8 ± 10.3	83 ± 21	6
(C)							
First injection	100	67 ± 3	5.4 ± 3.9	10 ± 5	28.3 ± 12.6	76 ± 19	
Second injection	100	69 ± 6	4.1 ± 4.1	26 ± 17	1.3 ± 1.9[e]	[f]	7
First injection	100	67 ± 1	6.0 ± 8.0	11 ± 3	30.5 ± 9.3	53 ± 3	
Second injection	10,000	71 ± 4	5.5 ± 3.3	20 ± 4	3.3 ± 1.5[d]	71 ± 12	4

[a]Hearts were perfused using a constant flow rate. Values represent mean ± S.D.

[b]Denotes no detectable change.

[c]$P < 0.05$.

[d]$P < 0.01$ and [e]$P < 0.001$ when compared to the values after the first injection of PAF.

[f]Increases in perfusion were small and the time to peak effect could not be accurately measured.

TABLE 5

The Effects of Injection Order of PAF and LTC_4 on the Perfusion Pressure of Rat Hearts[a]

Compound	Control perfusion pressure (mm Hg)	Decrease in perfusion pressure		Increase in perfusion pressure		n
		Maximum (mm Hg)	Time to peak (s)	Maximum (mm Hg)	Time to peak (s)	
First injection PAF (100 pmol)	69 ± 3	12.7 ± 12.5	13 ± 6	22.9 ± 9.0[b]	76 ± 22	
Second injection LTC_4 (50 pmol)	69 ± 6	—[c]	—[c]	41.0 ± 31.2[d]	9 ± 1	3
First injection LTC_4 (50 pmol)	69 ± 6	—[c]	—[c]	41.2 ± 26.0	17 ± 11	
Second injection PAF (100 pmol)	69 ± 4	5.0 ± 4.2	10 ± 1	29.4 ± 24.4	44 ± 21	5

[a]Hearts were perfused using a constant flow rate. Values represent mean ± S.D.

[b]No statistical difference in the increase in perfusion pressure was detected when PAF was injected first or when PAF was injected second, 10 min after a first injection of LTC_4.

[c]Denotes no detectable change.

[d]No statistical difference in the increase in perfusion pressure was detected when LTC_4 was injected first or when LTC_4 was injected second, 10 min after a first injection of PAF.

DISCUSSION

In a previous study, we have demonstrated that bolus injections of PAF resulted in coronary vasodilatation, vasoconstriction, or the combination of both responses in isolated perfused rat heart (12). Perfusion flow was kept constant in these experiments and any change in vascular resistance was reflected by a change in the perfusion pressure. Alternatively, perfusion pressure can be kept constant and changes in vascular resistance can be measured as changes in flow rate. However, changes in vascular resistance by a vasoactive compound, in particular intense vasoconstriction, could produce relative ischemia which might change the subsequent coronary vascular response. In *in vivo* studies where a small amount of PAF was administered by an intracoronary injection, the perfusion pressure or the systemic blood pressure was usually not affected and alterations in vascular resistance resulted in changes in coronary blood flow. Hearts perfused under a constant pressure would mimic more closely the situation of *in vivo* studies. In the present study, the coronary vascular responses to PAF were similar under constant flow and constant pressure perfusion. Our data therefore suggest that different modes of perfusion are not a factor in the diverse results of previous studies on the coronary vascular effects of PAF.

In the present study, PAF only elicited a vasoconstrictor response, and the vasodilator response was not observed in the perfused guinea pig heart. The lowest amount of PAF tested (1 fmol) was still capable of producing a significant vasoconstrictor response in the perfused guinea pig heart. However, our previous study showed that 10 pmol of PAF was needed to elicit a significant coronary vasoconstrictor effect in the rat heart (12). These results are consistent with the report that the guinea pig heart was more sensitive to the cardiac action of PAF than the rat heart (14-16). In this regard, rat platelets had been shown to be insensitive to PAF due to a lack of PAF receptors while platelets from other animals including rabbit, dog, guinea pig and sheep were sensitive to PAF (13,17). Our results suggest that species differences may be a contributing factor to the reported diverse coronary vascular responses to PAF.

Repeated injections of PAF did not elicit a vasoconstrictor response in the perfused heart (5,12,14). Possible explanations include desensitization of PAF receptors, depletion of vasoactive mediators produced by PAF, and loss of vascular smooth muscle response to PAF or its mediators. In the present study, we found that the vasoconstrictor response to a second injection of PAF was dependent on the amounts of PAF given in the first and second injections. If the initial injection of PAF was small (1-10 pmol), a second injection of 10 pmol PAF produced only a small vasoconstrictor response while 100 pmol PAF could still produce a significant vasoconstrictor response. However, 100 pmol PAF in the initial injection would abolish or greatly diminish the vasoconstrictor response to PAF irrespective of the amount of PAF in the second injection. This indicates that depletion of vasoactive mediator induced by the initial injection of PAF may be the mechanism for the reduced coronary vascular response to subsequent injections of PAF. Alternatively, the exposure to 100 pmol PAF in the initial injection may cause receptor desensitization. This leads to a reduction or elimination of subsequent response to PAF in the perfused rat heart. The exposure to a smaller amount of PAF may produce only partial or incomplete desensitization. Thus a reduction of the coronary vascular effect to a second injection of PAF would be expected. In contrast, repeated intracoronary injections of PAF produced similar vascular responses *in vivo* (8-11). It is therefore unlikely that receptor desensitization occurred in *in vivo* studies. Alternatively, it can be speculated that depletion of vasoactive compounds after an initial injection of PAF only occurs in perfused hearts, while these vasoactive compounds can be replenished quickly *in vivo* to enable the heart to respond to subsequent injections of PAF.

It has been suggested that the coronary vasoconstrictor effect of PAF was indirectly mediated by the release of LTC_4 (5), and leukotrienes including LTC_4 have been shown to possess vasoconstrictor properties. The role of LTC_4 in mediating the coronary vasoconstrictor effect of PAF was further confirmed by the detection and quantitation of LTC_4 in the coronary effluent after the administration of PAF in the perfused heart (5). In order to determine whether the reduced response to a second injection of PAF was due to an altered coronary vascular response to leukotrienes, the effect of LTC_4 was examined. Our results indicated that there was no change in the coronary vascular responses to PAF or LTC_4 which were injected to the same perfused rat heart irrespective of the injection order of the two compounds. In spite of a significant loss of vasoconstrictor response to a second administration of PAF after a first injection of PAF in the perfused rat heart, our data show that the heart was still capable of generating a normal vasoconstriction in the presence of LTC_4. Therefore, the mechanism for a reduction or elimination of the PAF response after an initial injection of PAF cannot be due to a failure of the coronary vascular smooth muscle to constrict in response to LTC_4.

In summary, we have shown that diverse coronary vascular responses to PAF can be observed in two animal species and that the diverse responses are not due to the different perfusion models used in the isolated heart studies. Our data indicate that depletion of vasoactive mediator induced by PAF and/or receptor desensitization could be the reason why the vasoconstrictor response fails after repeated injections of PAF into perfused hearts. However, the mechanism(s) for the desensitization of the PAF receptor or for the depletion of vasoactive mediators after exposure to PAF remains to be elucidated.

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Modulation of Polyunsaturated Fatty Acid Content of Triglycerides in Rat Pre-Adipocytes in Culture

Grace R. Gavino and Victor C. Gavino*
Department of Nutrition, Université de Montréal, Montréal, Québec Canada H3C 3J7

Rat peri-renal and epididymal pre-adipocytes in culture undergoing triglyceride (TG) accumulation were incubated with oleic (18:1), linoleic (18:2), α-linolenic (18:3ω3), arachidonic (20:4) and 4,7,10,13,16,19-docosahexaenoic (22:6ω3) acids in the presence of 0.8 μM insulin. The fatty acids were incorporated in cellular TG with relative enrichments over control from 1.4-fold for 18:1 to greater than 40-fold for 18:3ω3. Greater than 80% of fatty acids taken up were incorporated into cellular TG. The balance was distributed, in decreasing amounts, into phospholipids, unidentified intracellular constituents, and ketone bodies. The P/S ratio of cellular TG was at least an order of magnitude lower than that of the external milieu for both cell types and for all treatment groups, including controls. Doubling the concentration of treatment fatty acid increased its incorporation into cellular TG. However, it did not affect the accumulation of the other fatty acids in TG. Epididymal cells consistently acquire a higher proportion of treatment fatty acids in cell TG than peri-renal cells. Pre-adipocytes with polyunsaturated fatty acids (PUFA)-enriched TG is a potential model for the study of PUFA metabolism in these types of cells.
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Rat peri-renal or epididymal-derived pre-adipocytes in culture have been used as model systems for adipocytes and adipose tissue metabolism (1–3). An important aspect of adipose tissue metabolism is the control of the flux of various fatty acids as they are esterified into adipose triglyceride (TG) and released into the circulation as serum-bound non-esterified fatty acids. Adipose tissue contains a lower proportion of polyunsaturated fatty acids (PUFA) relative to other tissues and organs of the body (4–12), a phenomenon that has not yet been adequately explained. It has, however, been difficult to study PUFA metabolism in adipose tissue not only because of the low proportion of PUFA, but also because adipose TG fatty acid composition adapts slowly to the fatty acid profile in the diet (4,13). In this communication, we describe an *in vitro* method for the PUFA enrichment of accumulating TG in cultured pre-adipocytes. Pre-adipocytes with PUFA-enriched TG may serve as a useful model for the study of PUFA metabolism in these types of cells.

EXPERIMENTAL PROCEDURES

Cell culture. Pre-adipocytes were prepared from rat epididymal and peri-renal fat pads by collagenase digestion as described by Van and Roncari (1). The cells were cultured in Falcon 9.6 cm^2 6-well plates (Ingram and Bell, Montréal, Canada) as described by Wiederer and Loffler (14), using culture medium consisting of M199E (Gibco, Grand Island, NY), 10% (v/v) iron transferrin-enriched bovine calf serum (Hyclone, Logan, UT), and the antibiotics penicillin (100 U/mL) and streptomycin (0.1 mg/mL). After 2 population doublings, fat-filling of the cells was induced by replacing the culture medium with differentiation medium. The latter consisted of M199E plus penicillin and streptomycin, 1% (v/v) fetal bovine serum (Gibco), 4% (v/v) CPSR-1 (a delipidized serum replacer from Sigma, St. Louis, MO), 10 mM glucose, 0.8 μM bovine insulin (Sigma), 0.1 μM corticosterone (Boehringer, Montréal, Canada) and 0.5 mM methylisobutylxanthine (Sigma). After 48 hr, the culture medium was again replaced with differentiation medium minus methylisobutylxanthine (14), and supplemented with various concentrations of different non-esterified fatty acids (NEFA) (NuChek Prep, Elysian, MN) complexed to CPSR-1. As an antioxidant, 5 μM of 2,3-di-*tert*-butyl-4-methylphenol was added to the NEFA-supplemented differentiation medium. Differentiation was monitored by the induction of glycerol-3-phosphate dehydrogenase (G3PDH) (15). Fat-filling of the pre-adipocytes was assayed by quantitative gas-chromatographic analysis of intracellular TG (described below) and by the accumulation of visually-apparent intracellular lipid droplets in cultures stained with osmium tetroxide (16).

Fatty acid incubations. Pre-adipocytes were incubated with fatty acid-supplemented differentiation medium for 4 days. During this period, the differentiation medium was renewed every day in order to maximize the uptake of treatment fatty acids by the cells. [1-^{14}C]-Labeled fatty acids (Dupont, Montréal, Québec Canada) were used to monitor fatty acid uptake by the cells. Uptake was calculated as the disappearance of non-acid volatile radioactivity from the differentiation medium summed over the 4-day incubation period. Non-acid volatile radioactivity was determined by acidifying aliquots of differentiation medium with a tenth volume of 12N HCl followed by incubation at 37°C for 60 min before counting. This procedure completely expels CO_2 from the samples. Calculations of fatty acid uptake were corrected for the release of ketone bodies which are also non-acid volatile. Ketone body production was estimated in aliquots of differentiation medium as $HClO_4$-soluble radioactivity. Using radiolabeled fatty acids, we verified that NEFA co-precipitate completely with protein upon $HClO_4$ treatment (data not shown). Fatty acid uptake data derived from radiolabel experiments were verified by quantitative gas-chromatographic analysis of non-esterified fatty acid in the differentiation media (see below) before and after cell incubation. Total intracellular radioactivity was determined in cells that had been rinsed and digested with 0.2N NaOH in 50% aqueous methanol.

Lipid analyses. Lipids were extracted from cultured

*To whom correspondence should be addressed at Department of Nutrition, Université de Montréal, P.O. Box 6128 Station "A", Montréal, Québec Canada H3C 3J7.

Abbreviations: FAME, fatty acid methyl esters; G3PDH, glycerol-3-phosphate dehydrogenase; NEFA, non-esterified fatty acid; PL, phospholipid; PUFA, polyunsaturated fatty acids; TG, triglyceride, VLDL, very low density lipoprotein.

cells, fractionated into a TG and phospholipid (PL) fraction and analyzed as fatty acid methyl esters (FAME) by gas-chromatography as described previously (17). NEFA in the culture media was extracted and derivatized to FAME with diazomethane. Quantification of lipid classes was accomplished by the internal standard method using tri-heptadecanoylglycerol for the TG fraction, 1,2-diheptadecanoyl phosphatidylcholine for the PL fraction, and heptadecanoic acid for the NEFA fraction. Internal standards were added to the samples at the time of lipid extraction. FAME were separated using a Carbowax-bonded phase 30 m fused silica capillary column (Chromatographic Specialties, Brockville, Ontario Canada). Detection was by flame ionization. The gas chromatograph was calibrated using standard FAME mixtures (NuChek). In experiments involving the extraction and quantitation of [1-^{14}C]-labeled lipids, the corresponding ^{3}H-labeled lipids were used as recovery standards.

Miscellaneous. Cell counts were performed with a hemocytometer after release with trypsin. All solvents were redistilled before use. Chemicals were reagent grade or better.

RESULTS

G3PDH has been used as a late differentiation marker for pre-adipocytes (14). Figure 1 shows that the appearance of G3PDH activity was induced by the treatment of cultured pre-adipocytes with differentiation medium which had 1% fetal calf serum plus 4% lipid-poor serum replacer CPSR-1. This proportion between the serum supplements was found to be optimal for lipid-filling in the cultured pre-adipocytes. Various other proportions of fetal calf serum and CPSR-1 serum replacer were tried and found inadequate with respect to cell survival and/or differentiation (data not shown). Figure 1 shows that insulin concentration did not influence the rate nor the extent of G3PDH induction in epididymal pre-adipocytes. Figure 1 suggests, however, that G3PDH induction in peri-renal pre-adipocytes is facilitated with a high (0.8 μM) concentration compared to a low (0.01 μM) concentration of insulin.

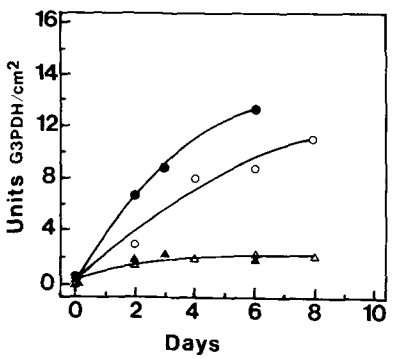


FIG. 1. Expression of glycerol-3-phosphate dehydrogenase activity during the differentiation of rat epididymal and peri-renal pre-adipocytes in culture. Batchwise enzyme assays were performed at the indicated time points for both epididymal and peri-renal cells. Each point is the mean of 2 assays. △, epididymal low insulin; ▲, epididymal high insulin; ○, peri-renal low insulin; ●, peri-renal high insulin.

Table 1 confirms the results of Figure 1 in that the cultured cells accumulate TG as is expected of differentiating pre-adipocytes. In the absence of treatment fatty acid, epididymal and peri-renal pre-adipocytes accumulated, respectively, 786 ± 99 and 3270 ± 490 nmol esterified fatty acid in TG per 9.6 cm² well. The difference in total TG accumulation is consistent with Figure 1 in that under our culture conditions, peri-renal pre-adipocytes developed more G3PDH activity and accumulated more TG than epididymal cells. Epididymal and peri-renal-derived cells that have undergone 12 population doublings and therefore have lost the ability to differentiate (14) did not accumulate TG under the same experimental conditions (data not shown).

The fatty acid treatments had no statistically significant effect on phospholipid content of either cell type (Table 1). However, the various fatty acid treatments at 50 μM concentration affected the total TG-accumulation by the cells. Depending on the treatment fatty acid, total TG-accumulation ranged from 0.7- to 1.5-fold of the corresponding controls (Table 1). Furthermore, the treatment fatty acid affected TG accumulation differently depending on the pre-adipocyte origin. In epididymal-derived pre-adipocytes, the highest TG-accumulation occurred in the 18:1 treatment group and the least in the 18:3ω3 treatment group. Peri-renal pre-adipocytes, in contrast, had the highest TG accumulation in the 18:2 treatment group and the least in the 22:6ω3 treatment group. These effects were statistically significant (Newman-Keuls procedure, 95% level).

At 50 μM, all the fatty acid treatments modulated the fatty acid profile of the accumulated TG (Table 2). For example, treatment with 22:6ω3 caused incorporation of this fatty acid into TG, whereas it was undetectable in control pre-adipocytes. The enrichment relative to control of other TG fatty acids by the corresponding exogenous treatment fatty acid ranged from 1.4 for 18:1 to more than 40 for 18:3ω3 (calculated from Table 2). Epididymal pre-adipocytes had higher proportions of treatment fatty acids in TG; however, peri-renal pre-adipocytes actually esterified more treatment fatty acids in TG based on absolute mass (calculated from Tables 1 and 2).

The fatty acid composition of accumulated TG in the control group of both peri-renal and epididymal pre-adipocytes (Table 2) did not match the fatty acid composition of the neutral lipid fraction (TG plus non-esterified fatty acids) present in the control differentiation medium (Table 3). The P/S ratio for intracellular TG was 0.018 and 0.043 for peri-renal and epididymal control pre-adipocytes, respectively, whereas it was 0.25 in the control differentiation medium. Furthermore, the change in the P/S ratio of TG in cells treated with PUFA did not increase in proportion to that in the fatty acid-supplemented differentiation medium. For example, the P/S ratio in peri-renal cells treated with 50 μM 18:3ω3 (calculated from Table 2) was 0.16 while that of the differentiation medium was 3.8 (calculated from Table 3).

In a separate series of experiments, peri-renal pre-adipocytes were incubated with either a high (80 μM) or a low (40 μM) concentration of 18:1 or 18:3ω3, respectively. The effect on total TG accumulation and fatty acid composition was determined and the results shown in Table 4. Treatment fatty acid concentrations above 100 μM resulted in a drop in the number of cells per culture

TABLE 1

Triglyceride and Phospholipid Accumulation in Pre-adipocytes Incubated with Different Fatty Acids[a]

	Tissue			
	Epididymal		Peri-renal	
Treatment	Triglyceride	Phospholipid	Triglyceride	Phospholipid
	nmol esterified fatty acid/9.6 cm^2 well (mean ± SEM, n = 3)			
Control	786 ± 99	168 ± 18	3270 ± 490	175 ± 22
18:1	1150 ± 87	159 ± 16	2780 ± 450	175 ± 49
18:2	977 ± 24	151 ± 3	4240 ± 370	174 ± 26
18:3ω3	677 ± 62	137 ± 11	2210 ± 240	221 ± 95
20:4ω6	873 ± 71	169 ± 24	3110 ± 460	185 ± 18
22:6ω3	809 ± 63	139 ± 22	2190 ± 290	161 ± 22

[a]Pre-adipocytes obtained from epididymal and peri-renal adipose tissue were incubated with differentiation media supplemented with 50 μM of the indicated fatty acids. Results are expressed as nmol esterified fatty acid in TG or PL per 9.6 cm^2 plate. *A posteriori* statistical tests were performed according to the Newmann-Keuls procedure subsequent to one-way analysis of variance, and statistical significance is declared at the 95% confidence level.

TABLE 2

Triglyceride Fatty Acid Profile of Cultured Pre-adipocytes Incubated with Different Fatty Acids[a]

	Treatment group (codes)							
Component fatty acid	Control (A)	18:1 (B)	18:2 (C)	18:3ω3 (D)	20:4ω6 (E)	22:6ω3 (F)	Scheffe multiple range test	Pooled SEM
Peri-renal cells								
16:0	56.2	52.6	58.9	56.7	66.4	62.6	BADCFE	0.5
16:1	13.2	11.1	10.3	8.1	5.0	4.6	FEDCBA	0.4
18:0	7.1	6.0	7.1	7.3	8.7	7.4	BCADFE	0.4
18:1	16.6	23.0	13.3	12.3	8.3	10.0	EFDCAB	0.4
18:2	0.82	1.1	5.9	1.3	0.92	1.7	AEBDFC	0.1
18:3ω3	0.2	0.2	0.2	8.1	0.2	0.6	BACEFD	0.2
20:4ω6	0.15	0.23	0.21	0.22	4.4	0.41	ACDBFE	0.04
20:3ω3				0.22				0.03
20:5ω3				0.13				0.02
22:4ω6					0.90			0.06
22:6ω3						6.9		0.2
others	5.7	5.8	4.1	5.6	5.2	5.8		
Epididymal cells								
16:0	52.9	42.5	45.1	41.2	50.8	49.9	DBCFEA	1.3
16:1	15.7	12.5	11.9	9.9	8.2	7.3	FEDCBA	0.4
18:0	4.7	3.4	3.8	4.2	4.9	4.8	BCDAFE	0.2
18:1	23.2	33.0	17.7	17.7	14.7	14.9	EFDCAB	0.6
18:2	2.0	2.0	14.5	2.9	2.3	2.9	BAEDFC	0.4
18:3ω3	0.1	0.2	0.2	17.2	0.2	0.5	ABCEFD	0.3
20:4ω6	0.4	0.5	0.8	0.7	10.9	0.7	ABDFCE	0.1
20:3ω3				0.6		0.3	NS	0.1
20:5ω3				0.52		0.29	p<0.001	0.01
22:4ω6			0.22 ± .05		2.1 ± 1.3		p<0.025	
22:6ω3						12.8		0.9
others	1.0	5.7	5.8	5.1	5.9	5.6		

[a]Pre-adipocytes from epididymal or peri-renal adipose tissue were incubated with 50 μM of the indicated fatty acids and 0.8 μM insulin for 4 days. TG fatty acid composition is expressed as relative percentage of fatty acid (n = 3) per treatment group. Data were analyzed by one-way analysis of variance using multiple range testing (Scheffe, 95% confidence level). The statistical test results are presented in ascending, ordered sequence. Statistically homogeneous groups are connected by an underscore. Student's t-test was used in cases where the component fatty acid is detectable only in two treatment groups.

well (data not shown), indicating toxic effects. Therefore, we used 80 μM fatty acid as the "high" concentration in order to provide a margin of safety for cell survival. Table 4 shows that doubling the treatment fatty acid concentration results in a minor increase in the total amount of TG accumulated by the cells over the 4-day incubation

TABLE 3

Fatty Acid Profile of Neutral Lipid in Differentiation Medium[a]

Fatty acid	Relative percentage
	mean ± SEM (n = 4)
16:0	26.5 ± 1.0
16:1	0.9 ± 0.9
18:0	23.7 ± 2.0
18:1	36.5 ± 0.8
18:2	10.5 ± 0.9
20:4ω6	1.9 ± 1.9

[a]Neutral lipid is TG plus NEFA. The total amount of neutral lipid fatty acid in the differentiation medium was 17.1 ± 3.7 nmole/mL.

period. Furthermore, the increase in total TG was due mainly to a rise in the incorporation of the treatment fatty acid. The data show that the absolute amounts of each of the other TG-esterified fatty acids were largely unaffected (Table 4).

The uptake and distribution of 18:1 and 18:3ω3 were further investigated in peri-renal pre-adipocytes using [1-^{14}C]-labeled fatty acids. The results (Table 5) show that the uptake was similar for these two fatty acids. Doubling the concentration of treatment fatty acid from 40 to 80 μM resulted in a 2.5-fold increase in 18:1 uptake and a 1.8-fold increase in 18:3ω3 uptake. Radioactive fatty acids taken up were accounted for by the accumulation of intracellular radioactivity. Only minor amounts (less than 2%) were metabolized to ketone bodies. The combined TG and PL fraction in turn accounted for at least 90% of the total intracellular radioactivity. Lastly, the radioactivity in the TG fraction was 92 to 96% of the total radioactivity in the combined TG plus PL fractions.

DISCUSSION

The results of these experiments demonstrate the feasibility of controlling the fatty acid composition of accumulating TG in differentiating, cultured pre-adipocytes. This model, while not intended to duplicate physiological conditions, does provide a way for inducing acute and rapid changes in TG fatty acid profile of cells destined for adipogenesis, which is otherwise difficult to accomplish in intact tissue. Pre-adipocytes enriched with PUFA would be useful as a model for the study of PUFA flux in these cells.

A significant difference between the culture conditions in this study and those of others (1,14) was the serum supplement used in the differentiation-medium. In general, proliferation and differentiation of pre-adipocytes are mutually exclusive (18). The low proportion of fetal calf serum in the differentiation medium was intended to minimize the concentration of mitogens while still providing the serum components essential for pre-adipocyte differentiation (19). The use of the lipid-poor serum replacer improved cell survival, while providing a vehicle for the fatty acid treatments. However, the low total serum content of the differentiation medium limits the maximum concentration of NEFA that may be used to treat the cells, due to toxic detergent effects.

Adipocytes obtain extracellular fatty acids for incorporation into adipose TG from the action of adipose capillary bed lipoprotein lipase on circulating very low density lipoprotein (VLDL) (20). Therefore, pre-adipocytes in culture, which also express lipase activity (21,22), should be able to hydrolyze serum TG normally present in culture medium, and incorporate the liberated fatty acids in intracellular TG. At the same time, since it is

TABLE 4

Effect of Treatment Fatty Acid Concentration on Triglyceride Fatty Acid Composition of Pre-adipocytes[a]

	Treatment fatty acid			
	18:1		18:3ω3	
	40 μM	80 μM	40 μM	80 μM
	nmole esterified fatty acid/9.6 cm^2 well mean ± SEM (n = 3)			
14:0	108 ± 10	113 ± 9	108 ± 10	114 ± 14
14:1	10 ± 10	14 ± .9	12 ± 1.8	12 ± 1.0
16:0	1210 ± 125	1330 ± 131	1320 ± 101	1531 ± 212
16:1	383 ± 34	347 ± 23	355 ± 42	338 ± 34
18:0	117 ± 10	139 ± 13	140 ± 8.0	176 ± 26
18:1	695 ± 52	1080 ± 89[b]	404 ± 43	387 ± 35
18:1ω7	68 ± 4.0	63 ± 2.4	67 ± 7.6	62 ± 4.4
18:2	25 ± 1.7	28 ± 2.0	35 ± 4.8	38 ± 2.8
18:3ω3	nd[c]	nd	255 ± 30	566 ± 48[b]
20:1ω9	2 ± 4	8.7 ± 1.5	nd	nd
20:3ω3	nd	nd	nd	13 ± 1.1
Total	2620 ± 240	3130 ± 270	2700 ± 242	3240 ± 370

[a]Peri-renal pre-adipocytes were treated with NEFA at the indicated concentrations and with 0.8 μM insulin for 4 days. TG fatty acid content is given as nmol esterified fatty acid.

[b]Statistically significant effect of NEFA treatment concentration ($p<0.005$, Student's t-test).

[c]Not detected.

TABLE 5

Uptake and Distribution of Radioactive 18:1 and 18:3ω3 by Peri-renal Preadipocytes[a]

	Treatment fatty acid			
	18:1		18:3ω3	
	40 μM	80 μM	40 μM	80 μM
	mean ± SEM (n = 3), dpm × 10^{-4}			
Uptake[b]	17.0 ± 0.5	42.4 ± 0.8	20.4 ± 0.2	35.7 ± 0.3
Ketone bodies[c]	0.19 ± 0.01	0.35 ± 0.01	0.34 ± 0.002	0.62 ± 0.03
Total intracellular radioactivity[d]	17.8, 17.7	37.2 ± 0.4	19.8 ± 0.5	37.4 ± 0.3
TG[e]	17.6 ± 1.4	34.2 ± 2.7	16.8 ± 0.9	31.2 ± 1.6
PL	0.72 ± 0.07	1.4, 1.5	1.5, 1.0	1.6 ± 0.2

[a]Pre-adipocytes were treated with [1-^{14}C]fatty acid (400-600 dpm/nmole).

[b]Culture medium was acidified with HCl to expel CO_2 before counting. Uptake was calculated as the decrease in non acid-volatile radioactivity in the medium, corrected for ketone body production.

[c]Ketone bodies were assumed to be $HClO_4$-soluble radioactivity. Radioactive NEFA co-precipitates completely with serum albumin in the culture medium upon $HClO_4$-treatment.

[d]Cells were rinsed and digested with 0.2 N NaOH/50% CH_3OH before counting.

[e]Intracellular lipid was extracted from intact cells and separated into a TG and PL fraction by thin-layer chromatography.

actually NEFA that cross the adipocyte membrane for incorporation into cellular lipid (23), exogenously added NEFA should also be incorporated into cellular TG, as was indeed the case.

The insulin concentration in our cultures, 0.8 μM, is within the saturation range of the insulin response curve for adipocytes (24). Since adipocytes internalize and degrade insulin (25), 0.8 μM is appropriate for a non-circulating, non-renewing system such as a cell culture, in order to maintain an effective concentration of insulin during the periods between culture media changes. Other investigators have used similarly high concentrations of insulin in pre-adipocyte cultures (25-27).

Several studies have shown that the fatty acid composition of adipose tissue adapts, albeit slowly, to the fatty acid profile of the diet (4,13,28). However, a closer analysis of published data reveals that the proportions of PUFA found in adipose tissue were always lower than the relative content of PUFA in the diet (4,9,29-33). The pre-adipocyte model described in this communication represents an extreme case where a high-concentration of PUFA is made directly available to the cell for incorporation into TG. Even under these conditions, the cell TG acquires a P/S ratio that is at least an order of magnitude lower than that in the NEFA supplemented differentiation medium. This is likely due to *de novo* synthesis and accumulation of 16:0, which appears to be unaffected by the presence of exogenous fatty acid. Interestingly, Nelson *et al.* (34) reported that rat adipose tissue can continue to synthesize fatty acids *de novo* even when the animal is consuming a high-fat diet. Taken together, the data suggest that pre-adipocytes have an innate ability to control their TG-fatty acid profile, and in particular, to favor a low P/S relative to the extracellular milieu. The mechanisms that control TG-fatty acid profile appear to be cell-type specific as peri-renal and epididymal pre-adipocytes differ in their capacity to incorporate exogenous fatty acids. The ability of epididymal pre-adipocytes to incorporate a higher proportion of PUFA *in vitro* is consistent with the reported *in vivo* differences between the fatty acid compositions of epididymal and dorsal fat (34).

In conclusion, the data indicate that TG accumulation in pre-adipocytes is not a simple and unspecific loading process but is likely to be subject to control mechanisms that determine which fatty acids are stored and in what proportion, and which ones are released to the external milieu. The pre-adipocyte model system with NEFA treatments permits a rapid and acute method for the modulation of the fatty acid composition of accumulating TG. This model may facilitate the study of PUFA metabolism in this type of cells.

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trans Fatty Acids. 4. Effects on Fatty Acid Composition of Colostrum and Milk

Jan Pettersen and **Johannes Opstvedt***
Norwegian Herring Oil and Meal Industry Research Institute, Bergen, Norway

trans **Isometric fatty acids of partially hydrogenated fish oil (PHFO) consist of *trans* 20:1 and *trans* 22:1 in addition to the *trans* isomers of 18:1, which are abundant in hydrogenated vegetable oils, such as in partially hydrogenated soybean oil (PHSBO). The effects of dietary *trans* fatty acids in PHFO and PHSBO on the fatty acid composition of milk were studied at 0 (colostrum) and 21 days *postpartum* in sows. The dietary fats were PHFO (28% *trans*), or PHSBO (36% *trans*) and lard. Sunflower seed oil (4%) was added to each diet. The fats were fed from three weeks of age throughout the lactation period of Experiment 1. In Experiment 2 PHFO or "fully" hydrogenated fish oil (HFO) (19% *trans*), in comparison with coconut oil (CF) (0% *trans*), was fed with two levels of dietary linoleic acid, 1 and 2.7%, from conception throughout the lactation period. Feeding *trans*-containing fats led to secretion of *trans* fatty acids in the milk lipids. Levels of *trans* 18:1 and *trans* 20:1 in milk lipids, as percentages of total *cis* + *trans* 18:1 and *cis* + *trans* 20:1, respectively, were about 60% of that of the dietary fats, with no significant differences between PHFO and PHSBO. The levels were similar for colostrum and milk. Feeding HFO gave relatively less *trans* 18:1 and *trans* 20:1 fatty acids in milk lipids than did PHFO and PHSBO. Only low levels of *cis* + *trans* 22:1 were found in milk lipids. Feeding *trans*-containing fat had no consistent effects on the level of polyenoic fatty acids but reduced the level of saturated fatty acids and increased the level of *cis* + *trans* monoenoic fatty acids. Increasing the dietary level of linoleic acid had no effect on the secretion of *trans* fatty acids but increased the level of linoleic acid in milk. The overall conclusion was that the effect of dietary fats containing *trans* fatty acids on the fat content and the fatty acid composition of colostrum and milk in sows were moderate to minor.**
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Increasing attention has been given to the nutritive value of human milk. As milk is the sole or main source of nutrients for an infant during the first six months of life, it is important that the nutrients in milk meet the substantial requirements of the rapidly developing infant.

The milk should contain sufficient quantities of essential fatty acids to meet the demand for synthesis of membrane tissues in the central nervous and vascular systems. The content of long-chain polyenoic fatty acids (*e.g.*, 22:6n-3) is particularly important for the development of the brain and the retina (1). Since *in vitro* studies have indicated that *trans* fatty acids may influence essential fatty acid metabolism, the effect on the fatty acid composition of human milk from ingestion of *trans*-containing partially hydrogenated fats has been of concern (2).

Although the transfer of *trans* fatty acids from diet and the adipose tissue into milk has been demonstrated in humans (3-8) and rats (9-11), no significant *trans* fatty acid effects on the content of essential fatty acids have been found. The studies used elaidic acid (*trans*-octadecenoic acid, *trans* 18:1n-9) or partially hydrogenated vegetable fats containing *trans* fatty acids with chain length of 18 carbon atoms or less. However, partially hydrogenated fish oils contain *trans* fatty acids with a chain length of 20 and 22 carbon atoms (12), and the biological effects of these acids are only poorly understood. We have previously reported the effects of dietary *trans* fatty acids in PHFO in comparison with PHSBO on brain weight, peroneal histology and nerve conduction velocity (13), and on the fatty acid profile of brain lipids and other organ lipids in sows (14) and offsprings (15). The present work was conducted to study the extent to which dietary *trans* fatty acids, and especially long-chain (*i.e.*, with 20 and 22 carbon atoms) *trans* monoenoic fatty acids, are incorporated into milk lipids under chronic dietary regimens (*i.e.*, when the contents of *trans* fatty acids have reached a steady state condition). It was further intended to find out if dietary *trans* fatty acids affect the overall fatty acid composition of milk lipids and essential fatty acid (EFA) content. Since an interrelationship between the metabolism of *trans* fatty acids and EFA has been indicated (16), the *trans* fatty acids were fed at two levels of dietary linoleic acid. Because of the many physiological similarities between pig and humans (17,18), we used pigs as models in these studies.

MATERIALS AND METHODS

General procedures. A composite sample of about 10 mL of milk was obtained from all lactating teats of each primiparous sow on day 0 (colostrum) and 21 days *postpartum*. Experiment 1 used four sows that had been fed 14 wt% of lard (control, diet 1), PHFO (partially hydrogenated capelin oil, mp 30-32°C, diet 2) or PHSBO (partially hydrogenated soybean oil, mp 40°C, diet 3) plus 4 wt% of SFO (sunflower seed oil, 65% linoleic acid) as additions to diets otherwise composed of solvent extracted soybean meal and cereals. The diets were fed from three weeks of age throughout the lactation period.

Experiment 2 used four sows that had been fed according to a 2 × 3 factorial design with 14 wt% of CF (coconut fat, diets 1.1 and 2.1), PHFO (diets 1.2 and 2.2) or HFO (fully hydrogenated fish oil, mp 50-52°C, diets 1.3 and 2.3), either without (diets 1.1, 1.2 and 1.3) or with (diets 2.1, 2.2 and 2.3) an extra 3 wt% addition of SFO to diets otherwise composed of solvent extracted soybean meal and cereals. The diets were fed from gestation at about six months of age throughout the lactation period.

*To whom correspondence should be addressed at: Norwegian Herring Oil and Meal Industry Research Institute, N-5033 Fyllingsdalen, Bergen, Norway.

Abbreviations: CF, coconut oil; EFA, essential fatty acids; GLC, gas-liquid chromatography; HFO, hydrogenated fish oil; HPLC, high-performance liquid chromatography; PHFO, partially hydrogenated fish oil; PHSBO, partially hydrogenated soybean oil; PUFA, polyunsaturated fatty acids; SFO, sunflower seed oil; VLDL, very low density lipoproteins.

In both experiments the sows were held in individual pens and offered the feed twice daily in amounts determined by their weights, stage of pregnancy and number of offspring (feeding standard). The design of the experiments and the fatty acid composition of the diets are shown in Table 1. Details of diets, feeding and management have been described earlier (13).

Chemical methods. Lipids from milk and colostrum were prepared by diethyl-ether extraction (19), and the lipid extracts were stored at −25°C pending further analysis. Total fat content was determined gravimetrically. Fatty acid composition was analyzed by capillary gas-liquid chromatography (GLC) using a 50-m fused silica capillary column (CP Sil 88, Chrompack, Middleburg, The Netherlands) as described earlier (14). The content of *trans* 16:1 was only analyzed in the dietary fats of Experiment 2 (Table 1).

Statistical methods. The statistical model used was a linear mixed model with diets as fixed, and sows as random effects. The various subgroups contained varying numbers of experimental units, making the statistical estimation and testing procedures numerically rather complex. The computer program selected among the few available at the time was General Mixed Model Analysis of Variance (BMDP P3V) (20). Estimation is Maximum Likelihood, and testing of effects uses chisquares in Likelihood Ratio Tests. Other methods used were simple, one-way analysis of variance models with varying group sizes. The pooled standard deviation, rather than the individual group standard deviations, was used in assessing the variability. Since testing of effects uses a one-way analysis of variance model, the underlying assumption that groups may vary according to location, but not in terms of spread, implies that the estimate of spread, found by MS error, comprises the total information on variability. Accordingly, in assessing differences in the group means the standard error of the different means were computed, making use of this common measure of spread for individual experimental units.

RESULTS

Experiment 1. Fat content and fatty acid composition of the milk at day 0 (colostrum) and 21 *postpartum* are shown in Table 2. Fat content of colostrum averaged 3.4 percentage units higher than that of milk. There were no major differences in fatty acid composition due to the stage of lactation. In group 1 and 2, but not in group 3, the level of linoleic acid decreased and the levels of *cis* monoenoic fatty acids increased from day 0 (colostrum) to day 21 of lactation. The effect of diets on the fatty acid composition of the milk was independent of the stage of lactation (*i.e.*, at 0 and 21 days *postpartum*).

At both sampling times, the *trans* 18:1 levels were

TABLE 1

Fatty Acid Composition (wt%) of the Dietary Fats (Total)[a] Fed to Sows[b]

	Experiment 1			Experiment 2					
Added SFO (%)	4			0			3		
Experiment fat	Lard	PHFO	PHSBO	CF	PHFO	HFO	CF	PHFO	HFO
Diet no.	1	2	3	1.1	1.2	1.3	2.1	2.2	2.3
8:0				7.1			10.5		
10:0				6.1			5.8		
12:0				40.9	1.0	0.2	34.7	2.1	0.2
14:0				16.7	8.0	8.0	13.0	7.2	6.7
16:0	22.6	16.3	11.2	10.8	15.5	20.5	9.1	14.8	17.6
18:0	14.8	5.6	13.2	3.2	4.2	14.3	3.0	4.7	12.2
20:0	0.3	2.2	0.6		2.6	13.2		2.4	10.6
22:0		2.0			2.1	10.5		2.0	8.5
24:0					0.1	0.6		0.1	0.5
16:1*cis*	1.5	1.1	0.6	0.2	2.9	0.5	0.2	2.7	0.6
16:1*trans*					4.8	1.9		3.9	1.9
18:1*cis*	34.6	14.1	29.1	6.8	10.3	4.2	8.4	9.8	6.6
18:1*trans*	0.3	6.8	24.3		7.7	3.1		5.8	2.9
20:1*cis*	0.7	3.9	0.2		5.8	2.0		5.2	2.2
20:1*trans*		6.0			9.5	5.9		7.9	5.3
22:1*cis*		5.7			6.0	2.0		5.2	3.2
22:1*trans*		5.3			6.7	4.4		5.5	3.6
24:1					0.5	0.6		0.4	0.5
18:2n-6	20.8	20.1	18.3	5.2	5.5	5:9	13.2	14.4	13.7
18:3n-3	1.8	2.2	1.9	2.2	1.6	1.4	1.8	1.1	1.3
Others	2.6	8.7	0.6	0.8	5.2	0.8	0.3	4.8	1.9
Sum PUFA	22.6	22.3	20.2	7.4	7.1	7.3	15.0	15.5	15.0
Sum monoenoic	37.1	42.9	54.2	7.0	54.2	24.6	8.6	46.4	26.8
Sum saturated	37.7	26.1	25.0	84.8	33.5	67.3	76.1	33.3	56.3
Sum *trans*	0.3	18.1	24.3	0.0	28.7	15.3	0.0	23.1	13.7
P/S[c]	0.6	0.85	0.81	0.09	0.21	0.11	0.20	0.47	0.27

[a]CF, coconut fat; HFO, hydrogenated fish oil; PHFO, partially hydrogenated fish oil; PHSBO, partially hydrogenated soybean oil; SFO, sunflower seed oil.

[b]Fed from three weeks of age through pregnancy in Expt. 1, from gestation through pregnancy in Expt. 2.

[c]Relative amount of sum of PUFA (P) to sum of saturated fatty acids (S).

TABLE 2

Fatty Acid Composition (wt%) and the Total Fat Content (wt%) of Colostrum and Sows' Milk[a] (three-week *postpartum*) in Experiment 1

	Colostrum					Sows' milk				
Added SFO (%)	4					4				
Experimental fat	Lard	PHFO	PHSBO	Pooled	Significant experimental	Lard	PHFO	PHSBO	Pooled	Significant experimental
Diet no.	1	2	3	std. dev.[b]	factor[c]	1	2	3	std. dev.	factor
Total fat content	7.4	6.1	8.2	1.7		4.1	4.2	3.0	1.4	
16:0	24.7	22.6	13.0	1.0	C	23.9	21.5	12.1	0.9	C
18:0	7.6	4.9	6.2	0.4	C	7.8	5.1	6.4	1.8	C
20:0	0.1	0.3	n.d.	0.1	C	0.1	0.4	n.d.	0.2	C
22:0	n.d.[d]	n.d.	n.d.			n.d.	n.d.	n.d.		
16:1*cis*	2.1	2.9	0.8	0.3	C	3.2	4.2	1.2	1.0	C
18:1*cis*	36.9	22.3	37.3	1.6	C	41.2	24.2	36.6	3.1	C
18:1*trans*	trace[e]	7.2	15.8	1.4	C	trace	7.0	17.0	1.3	C
20:1*cis*	trace	3.0	1.2	0.5	C	trace	4.4	1.3	0.7	C
20:1*trans*	n.d.	1.7	n.d.	0.2	C	n.d.	2.9	n.d.	0.2	C
22:1*cis*+*trans*	trace	1.3	n.d.	0.3	C	0.1	2.2	n.d.	0.4	C
18:2n-6	23.7	27.8	21.5	1.4	C	18.0	22.8	20.7	3.5	
20:2n-6	0.4	0.5	0.2	0.2		0.5	trace	0.2	0.2	C
20:4n-6	1.2	0.6	0.8	0.3		1.3	0.7	1.3	1.3	
22:4n-6	0.1	0.2	trace	0.1		0.5	0.2	trace	0.4	
18:3n-3	1.7	2.1	1.7	0.3		1.5	1.9	1.8	0.5	
20:5n-3	trace	n.d.	trace			n.d.	n.d.	n.d.		
22:5n-3	0.4	0.2	trace	0.2	C	0.2	trace	trace	0.1	
22:6n-3	0.2	trace	trace	0.1		0.7	trace	trace	0.8	
Sum PUFAn-6	25.4	29.1	22.5			20.3	23.7	22.2		
Sum PUFAn-3	2.3	2.3	1.7			2.4	1.9	1.8		
Sum PUFA total	27.7	31.4	24.2			22.7	25.6	24.0		
Sum monoenoic	39.0	38.4	55.1			44.5	44.9	56.1		
Sum saturated	32.4	27.8	19.2			31.8	27.0	18.5		
Sum *trans*	0.0	8.9	15.8			0.0	9.9	17.0		
P/S[f]	0.85	1.13	1.26			0.71	0.95	1.30		

[a]The sows were fed these diets from three-week of age through pregnancy until three-week *postpartum*, see legend for Table 1 for explanation of abbreviations.

[b]Pooled standard deviation, (error mean square)$^{1/2}$.

[c]Significance means ($p<0.05$) C, Effects of experimental fats.

[d]Not detectable.

[e]Trace, *i.e.*, <0.05%.

[f]Relative amount of sum of PUFA (P) to sum of saturated fatty acids (S). Average of eight replicates.

significantly influenced by diet. The level of *trans* 18:1 in milk from sows fed PHFO was similar to the dietary level, while that from sows fed PHSBO was only half the dietary level. In contrast, the level of *trans* 20:1 in the milk fat of PHFO-fed sows was lower than that of the dietary fat. Our analytical technique did not separate the *cis* and *trans* isomers of 16:1 and 22:1 in milk lipids.

Compared with the lard-fed sows, feeding of PHFO increased and of PHSBO reduced the level of linoleic acid in colostrum. In milk, both fat sources led to an increase in linoleic acid that was not significant. Feeding PHFO and PHSBO reduced the level of 22:5n-3 as compared with the control (lard) (significantly in colostrum). The general effects of PHFO compared with lard was an increase in polyunsaturated fatty acids (PUFA) n-6 and total PUFA at both sampling times, while PHSBO reduced PUFA in colostrum and increased PUFA in milk compared with lard. Feeding PHFO increased the level of 16:1, reduced *cis* 18:1 and increased 20:1 and 22:1 as compared with lard at both sampling times. Sows fed PHSBO had reduced levels of 16:1 and increased levels of *cis* 20:1 at both sampling times compared with lard.

Fatty acids with less than 16 carbon atoms were not determined in Expt. 1. Compared with lard, feeding partially hydrogenated fat (PHFO and PHSBO) reduced the level of 16:0 and 18:0. PHFO increased the level of 20:0 and PHSBO decreased the level of 20:0, as compared with lard.

Experiment 2. The fat content and fatty acid composition of colostrum and milk are shown in Table 3. Fat content in colostrum was at the same level as that found in Expt. 1, but higher in milk than in Expt. 1. Thus, contrary to the findings found in Expt. 1, the differences in fat content between day 0 and day 21 were negligible. Despite large differences in average fat content between dietary groups the differences were not significant due to large within diet variation. As in the findings in Expt. 1, the level of linoleic acid and saturated fatty acids decreased, while that of *cis* + *trans* monoenoic fatty acids increased from day 0 to day 21 of lactation.

TABLE 3

Fatty Acid Composition (wt%) and the Total Fat Content (wt%) of Colostrum and Sows' Milk[a] (three-week *postpartum*) in Experiment 2

	Colostrum							
Added SFO%	0			3				
Experimental fat	CF	PHFO	HFO	CF	PHFO	HFO	Pool. std. dev.[b]	Sig. exp. fact.[c]
Diet no.	1.1	1.2	1.3	2.1	2.2	2.3		
Total fat cont.	7.0	6.5	6.8	8.4	5.6	6.0	2.0	
12:0	2.9	0.1	trace[d]	3.0	0.7	0.1	0.8	C
14:0	3.8	4.7	5.4	14.4	5.6	5.5	2.2	C
16:0	26.6	22.8	29.7	23.2	18.5	26.1	1.6	B,C
18:0	7.1	6.1	8.8	5.7	4.5	7.8	0.9	B,C
20:0	0.2	0.4	1.1	0.2	0.3	1.4	0.4	C
22:0	n.d.[e]	n.d.	0.2	n.d.	0.1	0.2	0.1	C
16:1	3.2	4.1	3.2	2.0	5.2	2.7	1.3	C
18:1*cis*	28.4	27.5	27.5	21.1	25.4	20.9	3.0	B
18:1*trans*	0.2	6.6	2.9	0.3	5.4	2.4	0.9	C
20:1*cis*	0.3	4.2	2.0	0.3	2.9	1.7	0.6	B,C
20:1*trans*	n.d.	2.8	1.3	n.d.	1.4	1.4	0.7	A,C
22:1*cis*+*trans*	trace	1.0	0.4	trace	1.3	0.7	0.5	C
18:2n-6	9.7	7.2	8.7	22.3	17.8	22.0	1.6	B,C
20:2n-6	0.3	0.3	0.2	0.4	0.4	0.3	0.1	
20:4n-6	0.9	0.6	0.6	1.2	0.5	0.9	0.2	C
22:4n-6	0.1	trace	0.1	0.3	trace	trace	0.1	C
18:3n-3	2.1	1.7	2.1	1.8	1.4	1.7	0.3	B,C
20:5n-3	0.4	0.2	0.4	0.1	0.1	0.2	0.1	B
22:5n-3	0.9	0.4	0.5	0.6	0.3	0.3	0.2	B,C
22:6n-3	0.3	0.4	0.2	0.3	0.1	0.1	0.2	
Sum PUFAn-6	11.0	8.1	9.6	24.2	18.6	23.2		
Sum PUFAn-3	3.7	2.7	3.2	2.8	1.9	2.3		
Sum PUFA total	14.7	10.8	12.8	27.0	20.5	25.5		
Sum monoenoic	32.1	46.2	37.3	23.7	41.6	29.8		
Sum saturated	50.6	34.1	45.2	46.5	29.7	41.1		
Sum *trans*	0.2	9.4	4.2	0.3	6.8	3.8		
P/S[f]	0.29	0.32	0.28	0.58	0.69	0.62		
	Sows' milk							
Total fat cont.	5.8	5.8	9.7	6.8	7.1	7.5	3.4	
12:0	10.0	0.3	0.2	10.0	1.7	0.2	1.7	C
14:0	14.0	6.1	6.4	12.7	6.5	5.3	1.9	C
16:0	25.6	20.0	24.9	23.3	18.7	22.3	2.3	B,C
18:0	4.7	3.8	5.7	4.3	4.5	6.0	1.0	C
20:0	0.1	0.8	2.1	0.1	0.5	1.5	0.3	B,C
22:0	n.d.	0.2	0.5	n.d.	0.1	0.3	0.1	C
16:1	7.4	7.2	7.2	5.6	5.6	4.9	1.1	B
18:1*cis*	26.2	24.2	27.7	23.3	24.5	26.0	3.7	
18:1*trans*	0.3	7.4	3.3	0.1	4.5	3.3	0.8	A,B,C
20:1*cis*	0.3	5.2	2.3	0.4	3.5	3.0	1.0	A,C
20:1*trans*	n.d.	5.7	3.1	n.d.	2.7	2.5	1.1	A,B,C
22:1*cis*+*trans*	n.d.	3.2	1.6	trace	1.6	1.4	0.8	C
18:2n-6	6.7	5.7	6.5	15.6	16.5	17.3	1.8	B
20:2n-6	0.2	0.4	0.2	0.3	0.3	0.3	0.1	
20:4n-6	0.5	0.4	0.5	0.7	0.5	0.4	0.2	
22:4n-6	0.1	trace	trace	trace	0.1	trace	0.1	
18:3n-3	1.8	1.7	1.8	1.5	1.4	1.4	0.2	B
20:5n-3	0.2	0.2	0.2	trace	0.2	trace	0.1	A,B
22:5n-3	0.2	0.2	0.3	0.2	0.2	trace	0.1	A,B
22:6n-3	0.1	0.2	0.2	0.1	0.1	trace	0.1	B
Sum PUFAn-6	7.5	6.5	7.2	16.6	17.4	18.0		
Sum PUFAn-3	2.3	2.3	2.5	1.8	1.9	1.4		
Sum PUFA total	9.8	8.8	9.7	18.4	19.3	19.4		
Sum monoenoic	34.2	52.9	45.2	29.4	42.4	41.1		
Sum saturated	54.4	31.2	39.8	50.4	32.0	35.6		
Sum *trans*	0.3	13.1	6.4	0.1	7.2	5.8		
P/S[f]	0.18	0.28	0.24	0.37	0.60	0.54		

[a]The sows were fed the diets from gestation through pregnancy until three-week *postpartum*, see legend for Table 1 for explanation of abbreviations.

[b]Pooled standard deviation (error mean square)$^{1/2}$.

[c]Significance means ($p<0.05$) A, Interaction of experimental fats and SFO-level; B, effect of SFO-level; C, effect of experimental fats.

[d]Trace, *i.e.*, <0.05%.

[e]Not detectable.

[f]Relative amount of sum of PUFA (P) to sum of saturated fatty acids (S). Average of eight replicates.

There were no consistent differences in the content of *trans* fatty acids between colostrum and milk. Contents of *trans* fatty acids in both colostrum and milk varied significantly between diets, and only trace levels of *trans* fatty acids were found in milk from sows fed the *trans*-free control diets. As in Expt. 1, the level of *trans* 18:1 in milk of sows fed PHFO or HFO was similar to that in the dietary fat, while that of *trans* 20:1 was about half of that in the dietary fat. *trans* 16:1 and 22:1 Were not identified in milk. Increasing the dietary level of linoleic acid reduced the level of *trans* 18:1 and *trans* 20:1 in both colostrum and milk, but the reduction was only significant in milk.

The main effects of the dietary fat sources (*i.e.*, lard, PHFO and HFO) were found in the level of several fatty acids in colostrum. Compared with CF, the feeding of PHFO reduced the level of 18:2n-6, 20:4n-6, 22:4n-6, 18:3n-3 and 22:5n-3. Further, the feeding of PHFO increased the level of 16:1, *cis* 20:1 and *cis* + *trans* 22:1 as compared with CF. The differences in the fatty acid composition of milk from CF *trans* 20:1 and PHFO *trans* 20:1 fed sows were smaller than those seen in colostrum, though the levels of *cis trans* 20:1 and *cis* + *trans* 22:1 were significantly increased in PHFO as compared with CF. Compared with CF, the feeding of PHFO reduced the contents of all saturated fatty acids except 20:0 and 22:0, which were increased in both colostrum and milk. The effects of HFO compared with CF were similar, although generally less than those found with PHFO. Addition of dietary linoleic acid (18:2n-6) significantly affected the level of several fatty acids in milk at both stages of lactation. This resulted in an increase in the level of 18:2n-6, while the levels of 18:3n-3, 20:5n-3, 22:5n-3 and 16:0 were reduced.

Compared with CF, the feeding of PHFO and, to a lesser extent, of HFO, reduced the level of PUFA n-6 and PUFA n-3 in colostrum but not in milk. Feeding PHFO and HFO increased the level of monoenoic and decreased the level of saturated fatty acids as compared with CF at both sampling times.

Significant interactions between dietary fat sources and level of linoleic fatty acid in the diet, in addition to those in *trans* fatty acids (already pointed out), were also found for *cis* 20:1, 20:5n-3 and 22:5n-3 in milk. Thus, the level of *cis* 20:1 was higher when PHFO was fed at low dietary levels of linoleic acid compared with results at high levels.

DISCUSSION

Our experimental diets contained appreciable levels of *trans* isomers of 16, 18, 20 and 22 carbon monoenoic fatty acids and substantial levels of *trans* 18:1 and *trans* 20:1 were found in milk lipids from the *trans*-fed sows. In agreement with previous results in rats (10) the level of *trans* fatty acids in milk was not affected by the stage of lactation. As in earlier results with rats (2), the levels of *trans* 18:1 and *trans* 20:1 in the milk lipids were clearly related to the dietary levels.

We have previously (14,15) proposed the use of the discrimination factor (*i.e.*, the relation of the percentage *trans* isomers of the total monoene content of 18:1 or 20:1 in the synthesized lipids as compared to that of the dietary lipids) as a tool to evaluate the utilization of *trans* fatty acids for lipid synthesis. Table 4 shows the discrimination factors in colostrum and milk for the different diets in the two experiments. In Expt. 1 the discrimination factors were similar for milk and colostrum. They were also fairly similar for *trans* 18:1 and *trans* 20:1, and the differences between the two fat sources (*i.e.*, PHFO and PHSBO) were negligible. In Expt. 2 the discrimination factors were more variable. Thus, relatively more *trans* 20:1 than *trans* 18:1 were incorporated in milk lipids, regardless of dietary source and stage of lactation. Further, a relatively smaller amount *trans* 18:1 was incorporated into milk lipids from HFO as compared with PHFO, while there were apparently no differences between the two fat sources with regard to *trans* 20:1. As demonstrated earlier with regard to the level of *trans* 18:1 in milk from rats (11), there was no consistent effect on the incorporation of *trans* fatty acids as a result of dietary level of linoleic acid. On average, the discrimination factors for *trans* 18:1 in PHFO, PHSBO and HFO were 0.56, 0.68 and 0.27, respectively.

The value for PHSBO compares well with the discrimination factor of 0.66 calculated for milk by these authors from data from PHSBO-fed rats in the study of Wickwire *et al.* (11), but is lower than the discrimination factor of 0.77 calculated for milk from partially hydrogenated corn oil-fed rats in the data of Ross *et al.* (10). The value in Table 4 for PHSBO is also fairly similar to the discrimination factor of 0.61 that may be calculated for transfer of *trans* 18:1 from PHSBO into human milk (2). Our discrimination factors for milk are similar to those previously determined for blood lipids and adipose tissues in sows of Expt. 1 (14). Therefore, it appears that the incorporation of dietary *trans* monoenoic fatty acids into milk fat is 30–40% less than that of the homolog *cis* fatty acids.

The ratio of monoenes, saturated and polyunsaturated fatty acids in colostrum and milk relative to that of the dietary fats are shown in Table 5. The results show that the variation in fatty acid compostion in the diets

TABLE 4

Ratio of the Percentage of *trans* Isomers of the Total Monoene Content of Colostrum and Milk Lipids to that of the Respective Dietary Fats[a]

	Expt. 1		Expt.2			
Added SFO	4%		0%		3%	
Experimental fat	PHFO	PHSBO	PHFO	HFO	PHFO	HFO
18:1 *trans*						
Colostrum	0.75	0.65	0.45	0.22	0.47	0.34
Milk	0.69	0.70	0.55	0.25	0.42	0.37
20:1 *trans*						
Colostrum	0.60	-	0.64	0.53	0.54	0.64
Milk	0.66	-	0.84	0.77	0.72	0.64

[a]See legend of Table 1 for explanation of abbreviations.

TABLE 5

Ratio of Contents of Monoenoic, Saturated and Polyunsaturated Fatty Acids (PUFA) of Colostrum and Milk to that of the Respective Dietary Fats[a]

	Experiment 1			Experiment 2					
Added SFO	4%			0%			3%		
Experimental fat	Lard	PHFO	PHSBO	CF	PHFO	HFO	CF	PHFO	HFO
Colostrum									
Monoenoic	1.05	0.90	1.02	4.59	0.85	1.52	2.76	0.90	1.11
Saturated	0.86	1.07	0.77	0.60	1.02	0.67	0.61	0.89	0.73
PUFA	1.23	1.41	1.20	1.99	1.52	1.75	1.80	1.31	1.70
Milk									
Monoenoic	1.20	1.05	1.04	4.89	0.98	1.84	3.42	0.91	1.53
Saturated	0.84	1.03	0.74	0.64	0.93	0.59	0.66	0.96	0.63
PUFA	1.00	1.15	1.19	1.32	1.24	1.33	1.23	1.24	1.29

[a]See legend of Table 1 for explanation of abbreviations.

were moderated somewhat by the process of lactation. This is demonstrated by the milk fat from the CF fed-sows, which contained 50% saturated fatty acids as compared to 80% in the dietary fat. However, although the fatty acid composition of the dietary lipids had some influence, it seems natural to conclude from Table 5 that the lipid composition of colostrum and milk tends to remain fairly constant. This conclusion is in general agreement with those of previous studies on human milk (21). However, our results show that a restricted EFA level of the diets clearly reduced the total EFA level of colostrum and, in particular, of milk.

Milk lipids are derived from blood chylomicron and very low density lipoproteins (VLDL) (fatty acids with 16 or more carbon atoms) and from the *de novo* fatty acid synthesis in the mammary gland (fatty acids with 16 or less carbon atoms) (22).

The blood lipids are of dietary origin and from mobilized adipose tissue. The relative contribution of these two sources is dependent somewhat on species and energy status of the animals (7). *De novo* fatty acid synthesis in the mammary gland, which provided 10–12% of the total milk lipids in humans (23), would cause a dilution of total *trans*. Further, synthesis of *cis* 18:1 (24), and possibly of *cis* 20:1, in the mammary gland would reduce the ratio of *trans* to *cis* monoenes. Adipose tissue contains less *trans* fatty acids than dietary fat (14). Thus, the increasing contribution of adipose tissue to blood lipids would reduce content of *trans* fatty acids in milk fat. The reduced content of *trans* fatty acids in the milk lipids may also be due to discrimination of *trans* fatty acids in VLDL synthesis in the liver, or to reduced uptake from the blood in the mammary gland, *i.e.*, the *trans* fatty acids being transported in blood lipids other than chylomicrons and VLDL. Moreover, *trans* fatty acids may be discriminated against in the synthesis of milk lipids in the mammary gland.

From the data presented in this work, it is not possible to decide by which mechanism the reduction in the content of *trans* fatty acids in the milk occurred. It is, however, of interest to note that the discrimination factors in milk lipid synthesis were similar to those found in adipose tissues (14) because both mainly consist of triglycerides. Thus, *trans* 20:1, but not *trans* 22:1, appears to be utilized with the same efficiency as *trans* 18:1 for synthesis of triglycerides, while *trans* 18:1 is preferred to *trans* 20:1 in synthesis of phospholipids (14).

The results of Expt. 2 clearly demonstrate and confirm previous results in pigs (25), namely, that the level of linoleic acid in the milk is a linear function of dietary linoleic acid content. In agreement with previous results in rats (11), the content of *trans* fatty acids in the diets had no consistent effect on the linoleic fatty acid levels in the milk.

The tendency in the data was that the level of long chain PUFA (*i.e.*, fatty acids containing 20 carbon atoms or more) in the milk lipids was somewhat depressed when the sows received diets with high contents of *trans* fatty acids. The results agree with our previous findings in tissue lipids of sows and their offsprings (14,15), but are contrary to the results of previous studies on milk lipids in rats (11). It has been hypothesized that a depression of the activities of the Δ6 (26,27) and Δ5 (28,29) desaturation enzymes by *trans* fatty acids should reduce the rate of synthesis of higher homologues of the n-6 series of fatty acids, including 20:4n-6, from linoleic acid. However, a later study (30) did not confirm these findings.

The 22:1 monoenes, present in appreciable levels in PHFO and HFO diets and the 22:0 of HFO, were only found at low levels in milk lipids; and 24:1 was not detected. Thus, as has previously been found in tissue lipids (14,15), these fatty acids are discriminated against in the synthesis of milk lipids. Since this was the case with *cis* as well as with *trans* isomers, the reason is evidently related to chain length.

The different experimental fats used in this study varied substantially in fatty acid composition. Although some characteristics of the dietary fatty acid profiles were reflected in the milk lipids, the fatty acid composition of the milk lipids in the various groups was remarkably similar, and this is in line with previous studies in humans (21). The most consistent effect of the hydrogenated fats containing *trans*, regardless of control fat, was a reduction in the level of saturated fatty acids and a compensating increase in the level of *cis* and *trans* monoenoic fatty acids.

In conclusion, the results show that dietary *trans* 18:1 and *trans* 20:1 from PHFO and PHSBO are incorporated to a similar degree into milk lipids. The content of *trans* monoenes as a percentage of total *cis* plus *trans* monoenes

was reduced by some 40% from diet to milk. Only low levels of dietary *cis* and *trans* 22:1 were found in milk lipids from sows fed PHFO.

The fatty acid composition of the milk lipids remained remarkably constant despite a wide range in dietary fatty acid composition, though the feeding of fats containing *trans* fatty acids did lead to a reduction in the level of saturated and an increase in the level of *cis* and *trans* monoenoic fatty acids. The overall conclusion was that the effects of dietary fats containing *trans* fatty acids on the fat content and fatty acid composition of colostrum and milk in sows were moderate to minor.

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Dietary Fat Composition Influences Fatty Acid Composition of Milk Fat Globule Membrane in Lactating Cows[1]

D.L. Palmquist* and **F.L. Schanbacher**
Department of Dairy Science, Ohio Agricultural Research and Development Center, The Ohio State University, Wooster, Ohio 44691

Milk fat globule membranes are derived directly from the apical plasma membrane of mammary epithelial cells. To evaluate the effect of dietary fat on mammary membranes, we determined the fatty acid composition of the milk fat globule membrane in lactating dairy cows fed diets supplemented with fats of different fatty acid composition, or infused intravenously with soy oil emulsion. A preliminary survey, using an abbreviated preparation procedure (membranes isolated at 48,000 × *g-max* for 15 min), yielded about 45% of the total membrane fatty acids that could be recovered by centrifuging at the same speed for 120 min, and showed that changes in fatty acid composition of membranes reflected dietary fatty acids to some extent. Dietary palmitic acid increased the content of 16:0 in the membranes. A high corn diet increased ruminal formation of *t*18:1, and its level increased to 12% of membrane fatty acids. Infusion of soy oil emulsion increased 18:2 membrane content, and decreased the levels of 18:1 and 20:4. All treatments decreased the ratio of unsaturated/saturated fatty acids as compared to controls, whereas the ratio of polyunsaturated/saturated fatty acids was increased by feeding a high corn diet or by infusing soy oil. The ratio of 18:2/c18:1 increased from 0.31 to 1.0 after infusing soy oil for 4 days. The fatty acids of membranes isolated upon 120-min centrifugation were slightly more saturated. The differences were not sufficiently large, however, to affect overall results significantly.
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The milk fat globule membranes (MFGM) surround the fat droplets secreted by lactating mammary cells. The membranes are derived from the apical portion of the cell, surrounding and enveloping the fat globules as they are extruded from the cells. Eventually the membranes are pinched off during the apocrine secretion process (1,2). Membrane composition and cellular function has been shown to be influenced by dietary fat composition (3) in Ehrlich ascites cells (4), rat aorta (5), rat liver (6), rat hepatocyte culture (7), and rat adipocytes (8). There is little information on the effects of dietary fat on membranes of ruminant animals; however, milk fat composition is influenced by dietary fat under normal feeding conditions (9,10), and it is not unreasonable to speculate that tissue membranes may be influenced as well. Smith *et al.* (11) reported that feeding a mixture of sunflower and soybean seeds protected from ruminal biohydrogenation increased the proportion of linoleic acid in both core and membrane lipids of the milk fat globule, whereas Anderson (12) found that coconut oil similarly protected did not change fatty acid composition of milk fat globule membrane phospholipids.

In this study, we surveyed the effects of various types of fats, or diets known to influence lipid metabolism in lactating cows, on the fatty acid composition of milk fat globule membranes. Because MFGM are derived from intracellular membranes (1,2), it is likely that changes in fatty acid composition of MFGM represent similar changes in intracellular membrane composition. A preliminary study examined effects of several different diets; results were confirmed in a second experiment.

MATERIALS AND METHODS

Animals and diets. Five cows in mid-lactation (15–35 kg milk/day) were fed standard diets containing (dry matter basis) 40–50% concentrate mix, with the balance from roughages (alfalfa hay and corn silage) in a 5 × 5 Latin square design. Concentrate mixes contained no added fat or 10% added yellow grease, tallow, saturated fatty acids or calcium soap of palm fatty acids (Table 1). Additionally, two cows were fed a diet of whole shelled corn and ground, pelleted alfalfa (75:25, w/w), which is known to induce milk fat depression and to alter milk fat composition (13), and two cows were maintained on a standard diet and infused intravenously with 1900 mL/d (380 g oil) of a commercial soy emulsion (Intralipid, Baxter Healthcare Corp., Deerfield, IL). All cows except those infused with Intralipid were fed their respective diets for at least 3 wk prior to sampling milk.

Yellow grease is primarily restaurant cooking fat recycled by renderers, and tallow comes from renderers. The saturated fatty acid supplement is primarily palmitic, stearic, and oleic acids (Energy Booster 100, Milk Specialties Co., Dundee, IL); the calcium soap (Megalac, Church and Dwight Co., Inc., Princeton, NJ) is prepared from palm fatty acid distillate. Intralipid is a commercial soybean emulsion manufactured for intravenous nutritional support for humans.

Milk fat globule membrane. Milk samples for determination of milk fat globule fatty acid composition were obtained on one day from two cows on each treatment. Milk from the p.m. milking was prepared for harvesting milk fat globules essentially according to Patton and Huston (14). Fresh milk (175 mL, containing 5% sucrose) was put into each of six 250-mL polyethylene bottles and overlayed with 75 mL of phosphate-buffered saline (0.14 M NaCl in 0.01 M phosphate, pH 7.3). The milk was centrifuged at 1500 × *g* for 20 min at room temperature, and the bottles were held at 4°C overnight. The fat layer was harvested, sealed under N_2, and frozen until churning. The fat was thawed and churned while on ice by working with a spatula to release the milk fat globule membrane. This approach, rather than using a blender, was taken to limit exposure to air. In the preliminary survey, membranes

[1]Ohio Agricultural Research and Development Center. Manuscript No. 190-90.

*To whom correspondence should be addressed.

Abbreviations: CV, coefficient of variation; GLC, gas-liquid chromatography; MFGM, milk fat globule membrane; P/S, polyunsaturated fatty acids/saturated fatty acids + *t*18:1; U/S, unsaturated fatty acids − *t*18:1/saturated fatty acids + *t*18:1.

TABLE 1

Fatty Acid Content and Composition of the Major Fatty Acids in Diet Components and Intralipid

Treatment	Fatty acid, mg/g	Weight %								
		14:0	16:0	16:1	18:0	*t*18:1	*c*18:1	18:2	18:3	IV[a]
Control[b]	20	5.4	21.3	—	3.2	—	21.5	40.1	3.4	91
Yellow grease[c]	721	0.9	15.9	1.7	8.2	9.1	42.9	18.8	1.0	85
Tallow[c]	810	2.9	24.2	2.9	21.4	4.1	38.6	2.0	0.4	45
Saturated fatty acid[c,d]	990	2.2	44.1	0.9	34.7	2.9	9.4	0.9	—	14
Calcium soap of palm FA distillate[c,e]	838	1.5	50.1	—	4.2	—	34.1	7.8	0.3	45
High grain[b]	35	3.3	25.7	—	2.4	—	16.3	44.2	9.2	119
Intralipid[c,f]	190	—	11.1	—	4.2	—	20.2	55.8	6.4	136

[a]Calculated iodine value: 100% 18:1 = 90.

[b]Fatty acid (FA) content of total diet.

[c]Fatty acid content of fat supplement.

[d]Energy Booster 100, Milk Specialties Co., Dundee, IL.

[e]Megalac, Church and Dwight Co., Inc., Princeton, NJ.

[f]20% IV Fat Emulsion, Baxter Healthcare Corp., Deerfield, IL.

were precipitated by centrifuging for 15 min at 48,000 × *g-max* at 4°C, resuspended, washed once in phosphate-buffered saline, and recentrifuged. The washed pellet was extracted with hexane/isopropanol/HCl (60:40:1, v/v/v) as described by Radin (15), and the material in the hexane layer was methylated (16). Fatty acid analysis of feeds, MFGM, and milk fat taken from the cream before churning were as described by Sukhija and Palmquist (16). In a second study, milk was obtained on three different days from a single cow fed a standard diet, and duplicate sets of membranes were prepared by churning as above. After harvesting the pellet from the 15-min centrifugation (48,000 × *g-max*), the supernatant was recentrifuged for 2 hr at 48,000 × *g-max* to obtain a second pellet. Membrane fatty acids were analyzed as above, and additionally, were quantitated by gas-liquid chromatography (GLC), using nonadecanoic acid (19:0, Nu-Chek Prep, Elysian, MN) as internal standard (16).

Data from the first study show fatty acid composition of MFGM averaged from two animals on each treatment; no statistical manipulations were performed. In the second study, data were subjected to Least Squares Analysis of Variance (17) to determine precision of analysis, repeatability of daily sampling, and similarity of membrane composition from different isolation procedures.

RESULTS

Table 1 shows fatty acid composition of the control diet and various fat supplements. The small amount of fat in the control diet is typically highly unsaturated (65% unsaturated 18-carbon fatty acids). The fatty acid composition of the supplements was intentionally different; yellow grease provided low 16- and high unsaturated 18-carbon fatty acids; tallow provided more 16- and less unsaturated 18-carbon fatty acids. Saturated fatty acids and calcium soaps of palm fatty acid distillate were high in 16:0 and have little or no effect on rumen function. The high grain diet provided high 18:2 (from corn lipids) and is known to change ruminal biohydrogenation activity. Intralipid infusion provided a high amount of 18:2 directly to the mammary gland (18).

Data in Table 2 show daily intakes of fatty acid, milk yield, and milk fat percent to indicate the magnitude of daily fat metabolism of the lactating cows. Yield differences are primarily due to differences in lactation stage and are not intended to suggest differences among treatments in ability to support lactation.

Average fatty acid composition of milk fat obtained from cows on the different treatments is in Table 3. Dietary fat supplements decreased fatty acids of 4- to 14-carbon chain length, whereas content of 16- and 18-carbon fatty acids increased or decreased in accordance with the amount of these consumed. The content of *t*18:1 was increased 8-fold and 15-fold by feeding the yellow grease and the high corn diet, respectively. Infusing Intralipid changed 4- to 14-carbon fatty acids very little, and increased 18:2 4-fold, primarily by replacing 16:0, 18:0, and 18:1.

The fatty acid composition of MFGM is given in Table 4. No short-chain fatty acids were detected. The content of 14:0 varied from 4 to 10%; there was little variation in content of 16:0 (26 to 30%), except for the saturated fatty acid and calcium soap supplements, which increased 16:0 to 33 and 37%, respectively. Patterns of 16:1, 18:0, and *t*18:1 of MFGM followed those of milk fat. The proportion of *c*18:1 in MFGM from cows fed control or high fat diets was 27 to 33%, whereas *c*18:1 was less than 20% in high grain and Intralipid infusion treatments. Conversely, 18:2 was higher in the latter two treatments than control or fat-fed treatments. Arachidonic acid (20:4) was observed in all MFGM preparations except those from Intralipid infusion. Ratios of unsaturated/saturated fatty acids in MFGM were lower than those of controls for all treatments; the same was true for ratios of polyunsaturated/saturated fatty acids except for the high grain diet and Intralipid infusion. Calculated iodine values were highest for the latter two treatments.

In Table 5, data are shown from sampling of one cow on three different days, with sequential centrifugation of duplicate samples for 15 and 120 min. Coefficients of variation (CV, n=6) for determination of the proportion of individual fatty acids ranged from 0.6 to 103% of the mean. Only CV values of 16:0 (15.0%) and 18:2 (103%) exceeded 10%; inspection of data showed that variation was caused by a single sample. There was no difference ($P>0.05$) in fatty acid composition among days; however,

TABLE 2

Total Fatty Acid Consumption, Milk Yield and Milk Composition of Cows Fed Experimental Diets

Item	Diet						
	Control	Yellow grease	Tallow	Sat. FA	Ca soap	High grain	Intralipid
Fatty acid intake, g/d[a]	400	1400	1060	1460	1400	820	780
Milk yield, kg/d	35	22	14	32	26	16	31
Milk fat, %	3.26	2.60	3.52	2.77	3.42	1.80	4.08
Milk protein, %	3.22	3.15	2.81	3.03	3.00	3.60	3.30

[a]Basal diet plus supplement.

TABLE 3

Average Composition (wt% of Methyl Esters) of the Principal Fatty Acids of Milk Fat from Cows with Different Fat Treatments

Treatment	Fatty acid														
	4:0	6:0	8:0	10:0	12:0	14:0	14:1	15:0	16:0	16:1	17:0	18:0	*t*18:1	*c*18:1	18:2
Control	2.40	2.28	1.58	4.06	5.10	13.34	0.85	1.45	37.21	2.14	0	9.05	0.70	17.24	2.91
Yellow grease	1.61	2.11	0.79	1.20	1.62	7.51	0	1.03	27.92	1.90	0.36	14.16	5.95	31.60	2.16
Tallow	1.52	2.02	0.73	1.46	1.78	7.78	1.01	1.03	30.20	2.68	0.90	11.67	1.67	25.14	1.44
Saturated fatty acids	1.91	1.90	0.88	1.96	2.42	9.50	1.42	1.02	35.88	1.69	0.90	8.86	2.40	23.70	2.52
Ca soap, palm fatty acid distillate	1.75	1.68	0.68	1.54	1.89	7.88	0.94	0.79	37.26	1.64	0.42	9.04	1.86	27.16	3.05
High grain	0.60	0.88	0.51	1.61	2.94	9.28	3.24	2.58	25.82	3.62	0.76	3.38	11.06	16.76	7.60
Intralipid infusion	1.68	1.70	1.42	4.44	5.96	14.60	1.31	1.01	32.04	1.50	0	6.06	1.50	13.12	12.48

TABLE 4

Average Composition (wt% of Methyl Esters) of the Principal Fatty Acids of Milk Fat Globule Membrane from Cows with Different Fat Treatments (n=2/Treatment)

Treatment	Fatty acid										
	14:0	16:0	16:1	18:0	*t*18:1	*c*18:1	18:2	20:4	U/S[a]	P/S[b]	IV[c]
Control	8.76	29.22	2.36	11.67	0	30.23	9.42	1.10	0.87	0.21	48
Yellow grease	4.68	26.46	0.73	20.38	3.89	33.24	5.42	0.39	0.72	0.10	45
Tallow	5.40	28.60	1.70	15.50	0.95	33.68	5.36	1.28	0.83	0.13	47
Saturated fatty acids	6.25	33.10	0.75	14.58	0	30.86	9.80	0.52	0.78	0.19	48
Ca soap, palm fatty acids	5.33	37.38	1.04	14.30	0	27.72	6.91	1.10	0.64	0.14	42
High grain	6.90	26.45	3.90	6.32	12.02	18.02	12.13	1.11	0.68	0.26	56
Intralipid infusion	10.35	30.79	0	10.78	0	18.95	19.32	0	0.74	0.37	52

[a] $\frac{16:1 + c18:1 + 18:2 + 20:4}{14:0 + 16:0 + 18:0 + t18:1}$ [b] $\frac{18:2 + 20:4}{14:0 + 16:0 + 18:0 + t18:1}$ [c]Calculated iodine value: 100% 18:1 = 90.

several fatty acids differed significantly between 15- and 120-min isolations. These changes caused higher saturation in the 120-min pellet. The 120-min pellet contained more unidentified 14 and 16, more 18:0, *t*18:1, 22:0, and 24:0, and less 16:0, *c*18:1, 18:3, and 20:4 than the 15-min pellet.

Because individual samples in this study varied in mass, a weighted mean composition was computed as the sum of mass and weight percent of each fatty acid, and is shown in Table 5. This value is representative of the composition if the MFGM had not been separated by centrifuging sequentially for 15 and 120 min.

DISCUSSION

Ruminant diets are typically low in fat (2% in this basal diet), and the fatty acid profile of the basal diet reflects the unsaturated nature of corn oil and leaf lipids (Table 1). However, these unsaturated fatty acids are 80–90% biohydrogenated by rumen microorganisms; thus, little unsaturated fatty acid is absorbed (19). The amount of fat supplemented was within the range recommended for commercial practice (20). Yellow grease and tallow are metabolized extensively in the rumen, with conversion of C_{18} unsaturates primarily to 18:0 and *t*18:1n-7. The

TABLE 5

Composition (wt% of Methyl Esters) of Fatty Acids in Milk Fat Globule Membrane Isolated by Centrifuging at 48,000 × *g-max* for 15 or 120 min[a]

FA	Mean	SEM[b]	Day			Time		P			Weighted mean[c]
			1	2	3	15	120	Day	Time	DXT	
12:0	2.47	0.019	2.79	2.28	2.35	2.64	2.31	0.063	0.065	0.21	2.51
14:0	7.31	0.158	7.05	7.62	7.26	7.34	7.27	0.58	0.87	0.24	7.42
U14[d]	0.89	0.0081	1.06	0.70	0.92	0.76	1.03	0.062	0.028	0.20	0.92
15:0	1.10	0.0023	1.08	1.14	1.07	1.06	1.13	0.58	0.22	0.38	1.12
16:0	22.64	0.980	21.64	23.95	22.35	21.63	23.65	0.27	0.10	0.36	22.99
U16	2.76	0.0726	3.23	2.26	2.78	2.21	3.30	0.087	0.009	0.17	2.84
16:1	1.02	0.0018	0.93	1.02	1.10	1.18	0.84	0.061	0.001	0.71	0.99
18:0	12.94	0.219	13.69	12.96	12.17	11.92	13.96	0.12	0.007	0.30	13.19
*t*18:1	2.70	0.0550	2.92	2.54	2.62	2.43	2.96	0.53	0.077	0.15	2.73
*c*18:1	21.99	0.588	20.38	22.10	23.51	24.36	19.64	0.056	0.002	0.82	21.51
U18:1	3.11	0.0497	3.25	2.90	3.17	3.03	3.18	0.50	0.56	0.035	3.13
18:2	10.42	3.10	10.76	8.95	11.54	10.72	10.11	0.55	0.75	0.50	9.37
18:3	0.92	0.0162	0.86	0.91	0.98	1.18	0.66	0.77	0.010	0.83	0.87
20:3	0.88	0.0031	0.94	0.89	0.80	0.93	0.82	0.20	0.13	0.13	0.87
22:0	1.11	0.0027	1.17	1.03	1.14	0.92	1.31	0.19	0.001	0.082	1.11
20:4	0.71	0.0019	0.71	0.71	0.71	0.80	0.63	1.00	0.012	0.21	0.69
24:0	0.98	0.0023	1.04	0.89	1.03	0.82	1.15	0.11	0.001	0.047	0.98
Total, mg[e]	45.0	9.15	51.4	50.4	33.2	19.9	25.1	0.35	0.36	0.28	—

[a]Means of duplicate samples run on three different days.

[b]SEM = standard error of the mean. n=6.

[c]Weighted by summing product of weight percent of each fatty acid and mass of each sample.

[d]Unidentified fatty acid of presumed 14-carbon chain length.

[e]Total weight of fatty acids (sum of 15- and 120-min preparations) in membrane of a single churned sample.

calcium salt of palm fatty acid distillate is moderately (about 50%) biohydrogenated in the rumen (19). Hydrogenated fatty acids undergo little change in the rumen. After ruminal lipolysis, linoleic acid of corn oil is converted extensively to *t*18:1n-7 (21); however, if ruminal pH is low (<6), lipolysis is limited (22), and 18:2n-6 escapes ruminal biohydrogenation and is absorbed intact.

Supplementing a basal diet with fat caused increased mammary uptake of long-chain fatty acids and reduced output of fatty acids with 4–15 carbons (Table 3). Lower short-chain fatty acid output is due to feedback inhibition of *de novo* synthesis in the mammary gland (23). The content of fatty acids with 16 and 18 carbons reflected their content in the diet. The most dramatic effects on long-chain fatty acid composition are seen with feeding a high corn-low roughage diet, or infusing soybean oil intravenously. Both milk fats had very low 18:0 content. Milk fat from cows fed high corn had very low short-chain fatty acid content, also by inhibition of *de novo* synthesis (14) and a high content of *t*18:1, reflecting abnormal ruminal biohydrogenation patterns (22–24). The very high 18:2 content indicated that a large amount of this fatty acid escaped ruminal metabolism (22). Even though *c*18:1 was not increased, 16:1 was increased, suggesting conversion of 16:0 to 16:1 by Δ9 desaturase activity. Activity of the desaturase would be expected to increase because it is stimulated by insulin (25), and insulin also increases in cows fed high corn diets (13). The large amount of 18:2 in Intralipid was transferred directly to milk fat. The milk fat composition from cows infused with Intralipid is unusual in that the composition of short-chain fatty acids was changed very little from control. There seems to be a nearly 1:1 substitution of 18:2 for 16:0 + 18:0 + 18:1. This pattern is not typical of that observed with feeding of protected polyunsaturated fats (26).

The content of fatty acids with 4–12 carbons in MFGM in the preliminary study was nil or low (data not shown), indicating that the membrane fatty acids were not contaminated with milk fatty acids. The content of 14:0 was significant (Table 4). Palmitic and 18:1 were present in highest amounts, approximately 30% each, with variations reflecting chain length of diet fat source. For example, diets with commercial saturated fatty acid or calcium soap supplements contributed to higher 16:0, whereas yellow grease and tallow induced higher 18:1 levels. The sum of *t*18:1 and *c*18:1 was 30% and 18:2 was highest, except for Intralipid infusion, with the high corn diet. Intralipid infusion for only 4 days lowered the level of *c*18:1 and increased the level of 18:2 compared to control or fat-supplemented treatments. Interestingly, 20:4 was not detected in MFGM when Intralipid was infused. Plasma contents of 18:2 and 20:4 were inversely related in lambs fed a diet high in protected polyunsaturated fatty acids (27) and in sheep maintained for long periods on intravenous glucose infusions (28). Spector *et al.* (29) discussed in detail the decreased incorporation of 20:4 into cellular phospholipids in the presence of high 18:2 in cultured human umbilical vein endothelial cells. They suggested that the endothelial cell has a limited capacity to convert 18:2 to 20:4, probably by low activity of Δ6 desaturase. Further, 18:2 apparently competitively inhibits incorporation of 20:4 into *sn*-2 of phospholipids, and the effect was expressed fully within 24 hr. Clandinin *et al.* (30) found similar 16:0 and 18:0, lower 18:2, and much higher 18:3 and 20:4 in phospholipids of rat mucosal plasma membrane than we found in MFGM.

The unsaturated/saturated (U/S) index (Table 4) was computed with *t*18:1 included as a saturated fatty acid because its contribution to membrane structure would be much more similar to 18:0 than to 18:1. It is interesting that no treatment, including Intralipid infusion, induced membrane composition with a higher unsaturation index than that of the control. Tallow caused little change, whereas the index was lower for yellow grease, calcium soap, and high corn. The low index for high corn is unexpected, considering the high 18:2 content; however, the high *t*18:1 and low *c*18:1 content lowered the ratio. Alternatively, the calculated iodine values were higher than the control with high grain feeding and Intralipid infusion, and were lowest when calcium soap was fed, because *t*18:1 is included in the calculation of iodine value.

Since our initial procedure for precipitating MFGM was not standard, a confirming study evaluated yield and composition of membranes by obtaining a pellet first at 48,000 × *g-max* for 15 min, followed by recentrifugation of the supernatant for 120 min at 48,000 × *g-max* (31). We repeated the separation on duplicate samples of milk from the same cow on three different days in order to document precision of determining membrane fatty acid composition. The data in Table 5 document that precision of estimating fatty acids was good (CV < 10%), with the exceptions of 16:0 and 18:2, in which variation was due to a single sample. The analysis showed no difference by day of sampling; however, fatty acids from membranes separated by centrifuging 15 min were less saturated (less unidentified 14 and 16, 18:0, 22:0, and 24:0; more 16:1, *c*18:1, 18:3, and 20:4), than those obtained by recentrifuging the supernatant for 120 min. Quantitation showed that the distribution of total fatty acid was approximately 45:55 (w/w) in the 15- and 120-min pellets, respectively. Calculated weighted mean fatty acid composition of the total membrane, obtained by summing the product of proportion and mass of fatty acid in each fraction (Table 5, last column), was not different from the statistical mean (Table 5, first column). Further, differences between the weighted mean and the composition of membranes obtained by 15-min centrifugation were not sufficient to invalidate conclusions drawn from data in Table 4.

Patton and Keenan (2) discussed the possibility that the large amount of neutral glyceride in MFGM may be caused by association of high-melting glycerides from milk fat with the inner membrane surface. They noted that high-melting glycerides were lower in membranes prepared at 37°C from uncooled milk. Since our membranes were prepared at 0-4°C, contamination by high-melting glycerides cannot be discounted. If such were to occur, buoyancy of the glyceride should cause these membranes to precipitate more slowly, and perhaps to contain a greater proportion of saturated fatty acids. That we indeed observed higher saturation in the 120-min pellet is consistent with contamination by high-melting glycerides. Though measurable and consistent, the higher saturation had a relatively small effect on overall fatty acid composition of membranes, and did not change or confound conclusions drawn about treatment effects on membrane composition. Finally, it would be expected that any methodological artifacts would be expressed in all treatments, resulting in small effects on differences among treatments.

We have no documentation as to whether the observed differences in membrane fatty acid composition are related to differences in mammary function. With exception of Intralipid infusion, all cows were fed the experimental diets for a minimum of 3 wk. It is interesting that dramatic changes in membrane fatty acid composition were observed after only four days of Intralipid infusion, which is consistent with the high turnover of cell membranes associated with milk fat secretion (32).

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Tocopherol Contents of Lipoproteins from Frozen Plasma Separated by Affinity Chromatography

Beverly A. Clevidence[a,*] and **Rachel Ballard-Barbash**[b]

[a]Lipid Nutrition Laboratory, Beltsville Human Nutrition Research Center, ARS-USDA, Beltsville, Maryland 20705 and [b]Division of Cancer Prevention and Control, National Cancer Institute, NIH, DHHS, Bethesda, Maryland 20892

We investigated whether freezing and storage of plasma altered α-tocopherol levels of whole plasma or the lipoprotein fractions derived from such plasma. Plasma from 24 men, at each of two collection periods, was frozen at −20°C for six weeks, then high-density lipoproteins (HDL) were separated from low- plus very low-density lipoproteins (LDL-VLDL) by heparin affinity chromatography. Whole plasma and the lipoprotein fractions were analyzed for α-tocopherol content and compared to counterparts from fresh plasma. Freezing and storage did not reduce α-tocopherol levels of plasma or the lipoprotein fractions. α-Tocopherol values from fresh and frozen plasma were highly correlated for both plasma (period 1, r = 0.94; period 2, r = 0.93) and the LDL-VLDL fractions (periods 1 and 2, r = 0.97). Percent distribution of α-tocopherol between the two lipoprotein fractions was comparable for lipoproteins derived from fresh and frozen plasma. Under the storage conditions used in this study, plasma can be frozen for at least six weeks prior to lipoprotein fractionation with no detectable detrimental effects on α-tocopherol content of either plasma or lipoproteins.

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α-Tocopherol is a major lipophilic antioxidant that is transported in plasma by lipoproteins. Low-density lipoproteins (LDL) and high-density lipoproteins (HDL) are the major transport vehicles for α-tocopherol in human plasma (1–3), and the amount of tocopherol carried by these lipoproteins is strongly influenced by their lipid concentrations (4). The amount of tocopherol carried by very low-density lipoproteins (VLDL) from fasting, normolipemic subjects is a minor portion of the total (1,2).

α-Tocopherol has been measured less frequently on lipoprotein fractions than on whole plasma, but recently there has been considerable interest in structural and biochemical properties of lipoproteins as they relate to lipoprotein tocopherol levels (1,2,5–8). This interest in measuring tocopherol levels of lipoproteins suggests that reliable and convenient methods for determining tocopherol levels of human lipoproteins would be useful. We recently reported that commercially available affinity columns allow quick separation of the two major α-tocopherol-containing lipoproteins from human plasma (9). Using this separation method in combination with high-performance liquid chromatography (HPLC), analysis of lipoprotein tocopherol can be carried out the day of blood collection with complete recovery of α-tocopherol in lipoprotein fractions.

*To whom correspondence should be addressed at USDA, ARS, Lipid Nutrition Laboratory, Bldg. 308, Rm. 115, BARC-East, 10300 Baltimore Avenue, Beltsville, MD 20705-2350.

Abbreviations: apoB, apolipoprotein B; HDL, high-density lipoproteins; HPLC, high-performance liquid chromatography; LDL, low-density lipoproteins; VLDL, very low-density lipoproteins.

Yet, practical considerations often require delayed sample analysis bringing into question the suitability and effect of storage conditions. While α-tocopherol appears to be stable for over a year in frozen plasma (10) or serum (11), little is known about how freezing and storage affect subsequent analysis of tocopherol from lipoprotein fractions. It is generally recommended that plasma be held in the liquid state prior to lipoprotein analysis, and that determinations be carried out promptly. However, if samples are to be held more than a few days, then freezing is recommended, in part, to prevent oxidation of unsaturated lipids (12). If lipoproteins rapidly decompose during freezing and storage of plasma, as generally (13,14) but not universally (15–17) believed, then α-tocopherol levels of lipoprotein fractions could be greatly reduced. It is also possible that redistribution of tocopherols among lipoproteins occurs during freezing and thawing, thus affecting lipoprotein tocopherol levels without altering tocopherol levels of whole plasma.

The objective of this study was to determine whether freezing and storage of plasma would alter α-tocopherol contents of lipoproteins and thus compromise results obtained following separation of the lipoproteins by affinity chromatography.

MATERIALS AND METHODS

Twenty-four subjects, 24 to 57 years of age, were chosen at random from 40 nonsmoking men participating in a controlled diet study (18). For that study, applicants with plasma α-tocopherol levels of less than 6 μg/mL or greater than 12 μg/mL were not accepted as subjects. Study protocols were approved by the Institutional Review boards of the Georgetown University School of Medicine, Washington, D.C., and the National Cancer Institute, DHHS, Bethesda, Maryland.

During two ten-week periods, men took 15 soft gelatin capsules each day with each capsule providing 1 g of fat and 1 IU of all-*rac*-α-tocopherol (0.74 d-α-tocopherol equivalents) as a preservative. During period 1, fat in the capsules was a blend of stripped lard, hormone-free beef tallow and corn oil (48:40:12, w/w/w). During period 2, the capsules contained a concentrate of refined anchovy oil (ROPUFA®, Hoffmann-La Roche, Inc., Nutley, NJ) that provided 6.5 g per day of n-3 fatty acids (eicosapentaenoic plus docosahexaenoic acids). For both periods, fat from the diet, including capsules, provided 40% of energy. The minimum intake of α-tocopherol from diet and capsules was estimated to be 18 d-α-tocopherol equivalents per day. Menus excluded vitamin E-enriched foods, such as fortified breakfast cereals, and the use of vitamin supplements was not allowed. Plasma cholesterol and triglyceride levels of subjects averaged (mean ± SEM) 173 ± 7.0 and 87 ± 6.9 mg/dL, respectively, for period 1 and 169 ± 6.7 and 60 ± 5.1 mg/dL for period 2.

Blood was drawn during the tenth week of each period after an overnight (≥12 hr) fast. Blood samples, collected

in vacuum tubes containing potassium EDTA (1.5 mg/mL final concentration), were cooled on ice and plasma promptly separated from red blood cells by low-speed centrifugation (1600 × *g* for 20 min) at 4°C. Plasma samples were protected from light at all stages of processing and analysis.

Plasma lipoproteins were fractionated as described previously (9) using heparin affinity columns (LDL Direct, Isolab Inc., Akron, OH). Whole plasma (50 μL) and eluates (200 μL) containing HDL or LDL plus VLDL (LDL-VLDL) were mixed with 1 to 2 mL of 100% methanol and centrifuged at 1600 × *g* for 10 min. Fifty μL of the methanolic extracts were assayed for α- and γ-tocopherol by HPLC using fluorescence detection (19). Each plasma sample and its corresponding lipoprotein fractions were chromatographed, in duplicate, in the order: plasma, HDL, LDL-VLDL.

Fresh samples were processed and analyzed within 10 hr of collection. Replicate plasma samples were frozen at −20°C immediately after separation from red blood cells. Samples were frozen in 0.5-mL aliquots in tightly sealed plastic vials, but no further precautions were taken against oxidation of stored samples. Following six weeks of storage, samples were thawed at room temperature, then fractionated and analyzed under the same conditions as the fresh counterparts. To assure analytical consistency across time, α-tocopherol standards were validated prior to analysis of each storage-period combination using reference standards of reconstituted serum (National Institute of Standards and Technology, Gaithersburg, MD).

An electroimmunoassay (20) for apolipoprotein B (apoB) was used to determine whether freezing and storage of plasma inhibited the binding of apoB-containing lipoproteins to heparin columns. Column eluates from a subset of eight plasma samples were diluted in electrophoresis buffer to a final concentration of 1:12 (v/v) and, for HDL only, to a final concentration of 1:6 (v/v). Fractions were analyzed, in duplicate, in 1.5% agarose gels containing 1.2% antisera to human apoB, raised in rabbits (Behring Diagnostics, La Jolla, CA). Buffers and conditions for electrophoresis have been described (9).

Data were analyzed using the SAS system (SAS Institute, Cary, NC). Correlation coefficients were determined by the Pearson product-moment method, and the paired t-test was used to compare fresh and frozen/stored samples for plasma and lipoprotein tocopherol content (21).

RESULTS

Plasma α- and γ-tocopherol levels. Frozen/stored plasma was not different from fresh plasma when compared for α- and γ-tocopherol contents (Table 1). For period 1, plasma α-tocopherol levels were 10.5 ± 0.5 mg/dL (mean ± SEM) for both fresh and frozen/stored plasma. For period 2, plasma α-tocopherol levels were 9.4 ± 0.4 and 9.5 ± 0.4 μg/mL for fresh and frozen/stored plasma, respectively. Mean levels of γ-tocopherol for fresh and frozen/stored plasma were identical for both period 1 (1.5 ± 0.1 μg/mL) and period 2 (1.4 ± 0.1 μg/mL).

Fresh and frozen/stored plasma samples were strongly correlated (Fig. 1) for both plasma α-tocopherol (period 1: $r = 0.94$, $P<0.001$; period 2: $r = 0.93$, $P<0.001$) and plasma α-tocopherol (period 1: $r = 0.93$, $P<0.001$; period 2: $r = 0.94$, $P<0.001$).

α-Tocopherol content of lipoprotein fractions. α-Tocopherol levels of lipoprotein fractions were not reduced as a function of freezing and storage of plasma (Table 1). However, the period 1 value for LDL-VLDL from fresh plasma was 9% lower than its frozen/stored counterpart (6.1 *vs* 6.7 μg/mL). This difference was not observed for LDL-VLDL at period 2 or for HDL at either period.

Correlations were strong between LDL-VLDL levels of α-tocopherol from fresh and frozen/stored plasma samples (Fig. 2 $r = 0.97$, $P<0.001$ for both periods). Correlations were weaker for HDL α-tocopherol levels of fresh and frozen/stored plasma (period 1: $r = 0.73$, $P<0.001$; period

TABLE 1

Tocopherol Levels in Plasma, LDL-VLDL and HDL. Effect of Freezing Plasma Prior to Lipoprotein Fractionation by Affinity Chromatography[a]

	Fresh	Frozen/stored[b]	Paired t-test	
			Dif ± SE[c]	P[d]
	μg/mL			
Period 1				
α-Tocopherol				
Plasma	10.5 ± 0.53	10.5 ± 0.55	<0.1 ± 0.19	NS
LDL-VLDL	6.1 ± 0.44	6.7 ± 0.49	−0.5 ± 0.13	<0.001
HDL	3.8 ± 0.13	3.7 ± 0.15	0.1 ± 0.11	NS
γ-Tocopherol				
Plasma	1.5 ± 0.12	1.5 ± 0.12	<−0.1 ± 0.05	NS
Period 2				
α-Tocopherol				
Plasma	9.4 ± 0.42	9.5 ± 0.38	−0.1 ± 0.16	NS
LDL-VLDL	6.0 ± 0.38	6.0 ± 0.39	<−0.1 ± 0.10	NS
HDL	3.6 ± 0.12	3.6 ± 0.17	<0.1 ± 0.10	NS
γ-Tocopherol				
Plasma	1.4 ± 0.12	1.4 ± 0.12	<−0.1 ± 0.04	NS

[a]Mean ± SEM (N = 24).

[b]Samples stored at −20°C for six weeks prior to lipoprotein fractionation and tocopherol analysis.

[c]Mean difference and standard error (SE) of the difference.

[d]Probability determined by paired t-test, P>0.10 = nonsignificant (NS).

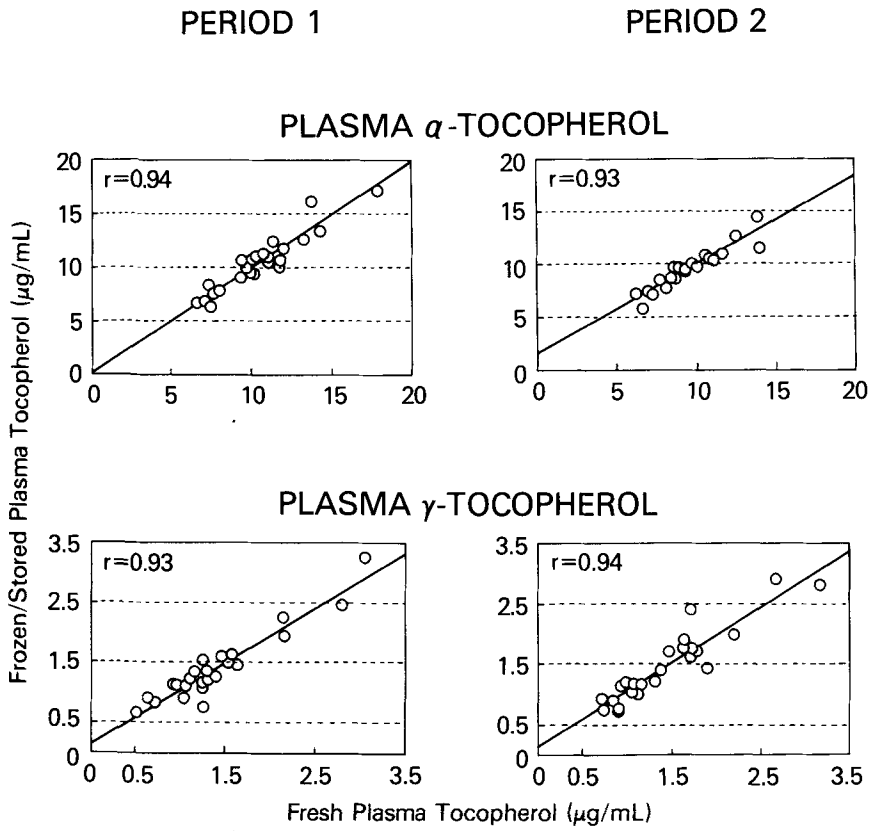


FIG. 1. Correlations of α- and γ-tocopherol levels in frozen/stored *vs* fresh plasma for two blood collection periods.

2: r = 0.81, P<0.001), due largely to the narrow range of HDL α-tocopherol values.

Distribution of α-tocopherol between LDL-VLDL and HDL. Distribution of α-tocopherol between the LDL-VLDL and the HDL fractions is shown in Table 2. With the exception of the LDL-VLDL fraction for period 1, the percentage of tocopherol carried by lipoprotein fractions did not differ for fresh and frozen/stored samples. The higher percentage distribution of α-tocopherol observed in period 1 for LDL-VLDL from frozen/stored plasma (62 *vs* 57%) was probably a function of the improved recovery (99 *vs* 95%) associated with the frozen/stored samples, as discussed below, rather than a direct effect of freezing and storage.

ApoB in column fractions. ApoB was confined exclusively to the LDL-VLDL fraction from each of the eight frozen/stored plasma samples analyzed.

DISCUSSION

As anticipated, plasma α- and γ-tocopherol were stable under the storage conditions of this study. Craft *et al.* (10) reported that α-tocopherol in heparinized plasma was stable for at least 15 months at −20°C and that purging plasma samples with nitrogen gas prior to storage had no detectable beneficial effects. Driskell *et al.* (11), using a single-donor pool of human serum, found that α-tocopherol was stable for 16 months when stored at −20°C. Furthermore, α-tocopherol levels were unaffected by 17 cycles of freezing and thawing. In the present study, all plasma samples contained EDTA as an added precaution against oxidative degradation of lipoprotein components (12) including loss of α-tocopherol (7).

The suitability of frozen plasma for determination of lipoprotein tocopherol levels appears to depend more on the stability of the lipoproteins than the stability of tocopherol. Several studies have been conducted to investigate the influence of freezing and storage of plasma on stability of lipoproteins as judged by recovery of lipoprotein cholesterol following ultracentrifugation. Two reports (13,14) concluded that lipoproteins are rapidly degraded by freezing, but others (16,17) concluded that decomposition of lipoproteins in frozen plasma was negligible for at least several months.

Frozen plasma has certain advantages over fresh plasma for lipoprotein fractionation and α-tocopherol analysis. Use of frozen replicate samples provides the opportunity to repeat chromatography and analysis of samples when recovery of α-tocopherol in lipoprotein fractions is incomplete. Storage also provides the convenience of pacing sample analysis. This can be important when large numbers of samples are to be analyzed; or, as frequently

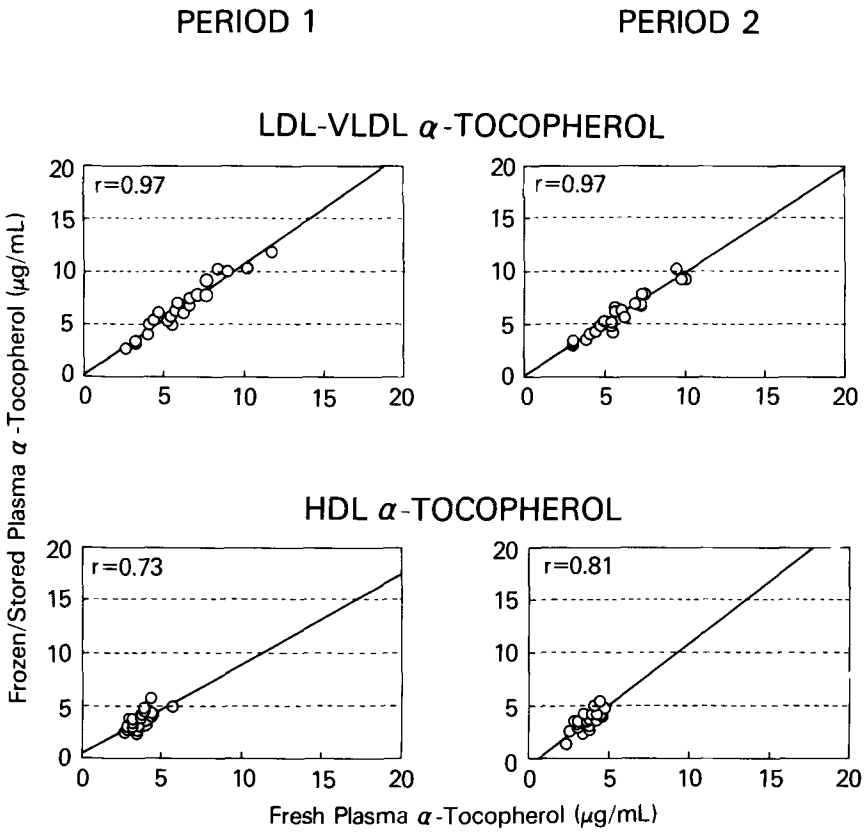


FIG. 2. Correlations of α-tocopherol levels in lipoprotein fractions separated by affinity chromatography from frozen/stored *vs* fresh plasma for two blood collection periods.

TABLE 2

Percent of Whole Plasma α-Tocopherol Recovered in LDL-VLDL and HDL Fractions Separated by Affinity Chromatography Using Fresh or Frozen/Stored Plasma[a]

	Fresh	Frozen/ stored[b]	Paired t-test	
			DIF ± SE[c]	P[d]
	Percent			
Period 1				
LDL-VLDL	57 ± 1.8	62 ± 2.0	−5 ± 1.3	<0.001
HDL	38 ± 1.7	37 ± 2.1	1 ± 1.0	NS
Recovery	95 ± 1.2	99 ± 1.2	−4 ± 1.7	0.019
Period 2				
LDL-VLDL	63 ± 1.9	62 ± 2.1	<1 ± 1.3	NS
HDL	40 ± 1.6	38 ± 2.1	1 ± 1.1	NS
Recovery	103 ± 1.2	100 ± 0.9	2 ± 1.8	NS

[a]Mean ± SEM (N = 24).

[b]Samples stored at −20°C for six weeks prior to lipoprotein fractionation and tocopherol analysis.

[c]Mean difference and standard error (SE) of the difference.

[d]Probability determined by paired t-test, P>0.10 = nonsignificant (NS).

required, when lipids, proteins, or other components of column fractions are to be concurrently measured. Use of frozen plasma also provides the opportunity to analyze, in one run, plasma samples collected at different times from a single donor.

In spite of such advantages, most investigators have chosen to use fresh plasma, and thus avoid the possibility that physical forces involved in freezing and thawing could alter lipoprotein integrity. In the present study, plasma that had been frozen at −20°C for six weeks was examined for evidence of lipoprotein decomposition. Ineffective binding of apoB to heparin affinity columns and loss of LDL tocopherol were not evident as might be expected if LDL particles had been substantially altered.

Freezing and storage of plasma did not appear to disrupt the binding of apoB-containing lipoproteins to affinity columns. If the affinity of apoB for the heparin ligand had been lost, then apoB should have been found in the unbound (HDL) fraction. Yet, no apoB was detected in that fraction for the eight plasma samples analyzed.

Furthermore, α-tocopherol content of lipoproteins was not decreased due to fractionating plasma that had been subjected to a single freeze-thaw cycle. Although a difference in the α-tocopherol content of LDL-VLDL was observed for period 1, the lower α-tocopherol value was from the LDL-VLDL fraction that was produced from fresh plasma. It is likely that this lower value was the result of an unexplained 5% lower recovery of LDL-VLDL from fresh plasma during period 1. That conclusion is supported by the α-tocopherol values for LDL-VLDL during period 2. There, the difference between mean α-tocopherol values for fresh and frozen/stored LDL-VLDL was negligible (-0.01 ± 0.10, $P = 0.900$). This illustrates the importance of complete recovery for accurate determination of lipoprotein tocopherol levels. Our 95% recovery from period 1, fresh plasma, was associated with a 9% decrease in the mean value for LDL-VLDL α-tocopherol as compared to frozen plasma where recovery averaged 99%. Yet, recoveries as high as 95% are generally not achieved using traditional methods of lipoprotein separation such as ultracentrifugation and gel filtration (1,2).

Because our observations were limited to a single storage temperature and to a relatively brief period of storage, they cannot be generalized to all conditions of low-temperature storage. Additionally, it is not known whether the relatively low lipid levels of the plasma samples used in this study influenced the outcome. In hypertriglyceridemic subjects VLDL can carry an unusually large proportion of α-tocopherol (3), and decay of VLDL and chylomicrons appears to be more pronounced in plasma samples with relatively high concentrations of these lipoproteins (15). Since fasting, normolipemic subjects carry only a minor proportion of α-tocopherol in VLDL (1-3), α-tocopherol contents of the LDL-VLDL fractions from this study predominately reflect α-tocopherol contents of LDL.

Measuring α-tocopherol content of LDL may prove useful for assessing the degree of protection this lipophilic antioxidant provides against certain oxidative processes. Recent studies have implicated oxidatively modified LDL as a promoter of atherosclerosis (22,23). *In vitro*, modified LDL are chemotactic for human monocytes (22) and promote lipid accumulation in macrophages (5,24). They are also toxic to cells in culture (25-27). Yet, it has been clearly demonstrated that oxidative modification of LDL, *in vitro*, is effectively inhibited by α-tocopherol (7,26,27). If these processes are operative *in vivo*, then the α-tocopherol carried by LDL could play a significant role in protecting this lipoprotein from oxidative modification.

We have previously described a rapid method for determining α-tocopherol levels of plasma lipoproteins. The present study expands the versatility of that method by demonstrating that plasma can be stored at −20°C for at least six weeks prior to lipoprotein fractionation by affinity chromatography without loss of α-tocopherol from either LDL-VLDL or HDL fractions. This simplified and convenient method for assessing α-tocopherol content of LDL may have practical application for assessing susceptibility to oxidative stresses.

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Diet-Induced Type IV-Like Hyperlipidemia and Increased Body Weight Are Associated with Cholesterol Gallstones in Hamsters[1]

K.C. Hayes*, Pramod Khosla and **Andrzej Pronczuk**
Foster Biomedical Research Laboratory, Brandeis University, Waltham, Massachusetts 02254

Male Syrian hamsters (60–70 g) were fed purified diets containing 5% fat (American Fat Blend) and 15% fiber with or without 0.3% cholesterol (0.86 mg/kcal), for 12 weeks. Hamsters fed the cholesterol-supplemented challenge diet revealed a major increase in plasma triglyceride between 9 and 12 weeks, whereas plasma cholesterol (which reflected body weight dynamics) increased threefold up to nine weeks and plateaued (342 ± 22 *vs.* 122 ± 5 mg/dL). The greatest increases in cholesterol occurred in the very low density lipoprotein (VLDL) and high density lipoprotein (HDL_2) fractions. Gallstone incidence was similar (69% *vs.* 78%) for cholesterol-supplemented *vs.* control hamsters, but the type of stones differed. Of the cholesterol-supplemented hamsters with gallstones, 45% had cholesterol stones and 55% had pigment stones. Only pigment stones were seen in control hamsters. Hamsters with cholesterol stones were 25% heavier and transported most cholesterol in VLDL (33 ± 5%), approximately double that in VLDL of cholesterol-supplemented hamsters with no stones (19 ± 3%) or cholesterol-supplemented hamsters with pigment stones (21 ± 3%). Hamsters with pigment stones or no stones (regardless of diet fed) transported the majority of their cholesterol in HDL_2 (44%), whereas this figure was only 27% in hamsters that developed cholesterol stones. Thus pigment stones develop routinely in hamsters fed casein-based purified diets. Adding dietary cholesterol resulted in cholesterol gallstones only in those hamsters that gained the most weight and whose terminal VLDL/HDL cholesterol ratio exceeded 1.0, not unlike the lipoprotein profile of obese humans who develop cholesterol gallstones.
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Syrian hamsters have been used extensively as an animal model for the study of gallstones because they are one of the few species in which gallstones can be readily induced by dietary manipulation (1–4). Their gallbladder bile seems to contain the requisite mucus, and biliary stasis with "sludging" appears to enhance their gallstone formation once lithogenic bile develops (5,6). Although both pigment stones and cholesterol stones occur (3–6), the dietary factors and the mechanisms responsible for these two types of stones are unresolved.

Diets with defined composition, as opposed to crude diet formulations or laboratory chow diets, allow study and identification of the specific dietary factor(s) causing lithogenesis. Recent work from this (5,6) and other investigators (3,4) describe purified diets that induce either pigment (3,5) or cholesterol gallstones (4,6).

Although it is well appreciated that cholesterol gallstones in humans are associated with abnormal cholesterol metabolism (7) and elevated plasma triglycerides (8), and that cholesterol metabolism itself is linked to the metabolism of plasma lipoproteins, minimal information exists concerning the relationship between lipoprotein metabolism and gallstone development in any species (9–12). In a recent study (6) we identified an association between an elevated very low density lipoprotein (VLDL) cholesterol pool, bile lithogenecity and cholesterol gallstones in Charles River (Lakeview, MA) hamsters fed butter fat and cholesterol.

To follow the time-course of the changes in circulating lipoproteins and gallstone development (including the type of gallstones induced), we have fed hamsters purified diets differing solely in their cholesterol content. The Sasco strain of male golden Syrian hamsters was studied because a recent report suggested that this hamster was especially sensitive to cholesterol gallstone induction (4).

MATERIALS AND METHODS

One hundred and ten male golden Syrian hamsters (*Mesocricetus auratus*) were obtained from Sasco Inc. (Omaha, NE; Sasco, golden Syrian outbred hamster derived from a closed colony from the University of Nebraska Medical Center, Eppley Cancer Institute) at a starting weight of 60–70 g. Hamsters were housed 4 per cage in a controlled environment with a 12-hr light-dark cycle (lights on at 1800 hr). All hamsters were fed a purified, cholesterol-free control diet with 5% fat (w/w). After 5 days of feeding the control diet, and just prior to feeding of the cholesterol-supplemented challenge diet (designated t=0 weeks), plasma lipids and lipoproteins were evaluated in 16 hamsters selected at random. Of the 16 hamsters, 7 were sacrificed for gallstone evaluation (no visible lesions or gallstones were observed). The data from these 16 animals constitutes the basal (t=0 week) values. The hamsters were then randomly divided into two groups. Group 1 (n=25) was continued on the control diet, whereas Group 2 (n=78) was fed the control diet supplemented with 0.3% (w/w) cholesterol (0.86 mg/kcal) for the remainder of the study. Hamsters had access to fresh food and water (provided daily) *ad libitum*. The composition of the diets (Table 1) reveals a polyunsaturated to saturated fatty acid ratio (P/S) of 0.54. The experimental design and procedures were approved by the Brandeis University Animal Use Committee.

In addition to the analysis at t=0 weeks, lipoprotein changes were also assessed at t=2, 5, 9 and 12 weeks in the cholesterol-supplemented hamsters. For this purpose, 16 hamsters were randomly selected from the pool of 78 animals. Individual hamsters were fasted overnight in wire-bottomed cages prior to being anesthetized lightly

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*To whom correspondence should be addressed.

Abbreviations: ANOVA, analysis of variance; EDTA, ethylenediaminetetraacetic acid; HDL, high density lipoprotein; HPLC, high-performance liquid chromatography; IDL, intermediate density lipoprotein; LDL, low density lipoprotein; PLSD, protected least significant difference; P/S, ratio of polyunsaturated to saturated fatty acids; VLDL, very low density lipoprotein.

TABLE 1

Composition of Purified Diets Fed to Hamsters[a]

	Control diet (cholesterol-free)	Challenge diet (cholesterol-supplemented)
	g/100g	
Casein	20	20
Glucose	18.9	18.9
Corn starch	35	34.7
Fat[b]	5	5
Cellulose	10	10
Wheat bran	5	5
Mineral mix[c]	4.6	4.6
Vitamin mix[d]	1.2	1.2
Choline chloride	0.3	0.3
Cholesterol	—	0.3

[a]Diets were fed as gel blocks, prepared by withholding 60 g corn starch (per Kg mix) from the formulation and premixing it with 800 mL of simmering water to form a gel to which the remaining ingredients were added.

[b]American fat blend (2.6 butter, 1.6 canola oil, 0.8 corn oil) had a P/S ratio of 0.54 and a P/M/S ratio of 1.0:1.0:1.9 (P, polyunsaturated; M, monounsaturated; S, saturated).

[c]Ausman-Hayes Salt Mix (BioServ Inc., Frenchtown, NJ).

[d]Hayes-Cathcart Vitamin Mix; the composition of the vitamin mix has been detailed previously (5).

for blood sample collection (2-3 mL) by cardiac puncture, into ethylenediaminetetraacetic acid (EDTA)-wetted syringes using a 25-gauge needle. (Unless sacrificed for gallstone evaluation, all hamsters recovered from the blood sampling). Lipoproteins were also analyzed at t=12 weeks in 16 animals from the control group. To allow for gallstone evaluation (at t=12 weeks), all 16 hamsters fed the cholesterol-supplemented diet (and 9 of the 16 animals fed the control diet) were exsanguinated by cardiac puncture. Gallbladders from the 9 control and 16 test hamsters were opened under an Olympus M100 dissecting microscope and all gallstones present were examined under regular and polarized light. Spherical white cholesterol gallstones (0.1-1.2 mm) and dark green-to-black pleomorphic pigment gallstones (<0.1-0.6 mm) were identified. Upon drying, representative gallstones of both cholesterol and pigment varieties were analyzed by high-performance liquid chromatography (HPLC) for their cholesterol content (13). The cholesterol content of cholesterol gallstones was routinely over 90%, whereas this figure was less than 2% for pigment gallstones. A detailed description of the two types of gallstones has been reported elsewhere (6).

Sodium azide (10%, w/v) was added to the plasma samples (10 μL/mL plasma). Throughout the study, lipoproteins were isolated from plasma pooled from 3-4 hamsters, with the exception of the analysis at t=12 weeks for the cholesterol-supplemented animals when lipoproteins were isolated from the plasma of individual hamsters. In all cases lipoproteins were isolated using stock density solutions of d=1.006 g/mL and d=1.38 g/mL by sequential ultracentrifugation (14) in Quick seal tubes®™ (Beckman, Palo Alto, CA) using a Ti 70.1 rotor at 12°C. Stock density solutions contained EDTA, gentamycin sulfate, sodium azide (15) and benzamidine (16). Five lipoprotein fractions were isolated, VLDL (d<1.006 g/mL), intermediate density lipoprotein (IDL) (1.006<d<1.019 g/mL), low density lipoprotein (LDL) (1.019< d<1.055 g/mL), HDL_2 (1.055<d<1.125 g/mL) and HDL_3 (1.125<d<1.21 g/mL). These density cuts for hamsters were determined in an initial study in which lipoproteins were isolated by discontinuous density-gradient ultracentrifugation (17) (data not shown). With the exception of VLDL and IDL, all lipoprotein fractions were dialyzed against 0.15M NaCl/1 mM EDTA, pH 7.4, at 4°C, for 24-36 hr. Lipoprotein cholesterol and triglyceride content were measured by enzymatic methods. Lipoprotein protein was measured using a modification (18) of the procedure of Lowry *et al.* (19). The sum of the cholesterol measured in each lipoprotein fraction was divided by the total plasma cholesterol to determine the percent recovery (20). The percent recovery for the lipoprotein isolations carried out at 12 weeks (n=21; 5 controls and 16 experimental) was 81% ± 2%. The final concentration of cholesterol, triglyceride and protein in each lipoprotein fraction was calculated by applying the same percent recovery.

To assess whether gallstone formation might be associated with differences in cholesterol esterification and/or changes in essential fatty acid availability, VLDL free cholesterol and individual cholesteryl esters were determined by HPLC (13). Cholesterol (free and esterified) was extracted by a double-solvent extraction procedure (isopropanol/0.75 M NaOH, 33:17, v/v and n-octane). The individual cholesteryl esters were separated on a Waters Radial-Pak Resolve™ C18 column by isocratic elution with acetonitrile/isopropanol (45:55, v/v) at 2 mL/min with a Beckman 110B solvent delivery module. The absorbance of the eluate was measured at 210 nm using a Waters model 480 LC spectrophotometer (Waters Associates, Wilmington, MA).

Plasma and lipoprotein cholesterol and triglyceride concentrations were determined enzymatically using kits (#336 and #352, respectively) obtained from Sigma Diagnostics (St. Louis, MO). Statistical analyses were carried out on a Macintosh Plus®™ computer (Apple Computer Inc., Cupertino, CA) using the Statview 512®™ software package (Brain Power, Inc., Calabasca, CA). Statistical differences between mean values for the control and cholesterol-supplemented dietary groups was assessed using Student's unpaired *t*-test. For subgroup analysis of the cholesterol-supplemented hamsters, the various parameters related to the gallstone response were analyzed by a one-factorial analysis of variance (ANOVA), and the Fisher protected least significant difference (PLSD) was used to test for statistical significance.

RESULTS

Time course of the changes in body weights, plasma lipids and lipoproteins. The time course of the changes in body weights and plasma lipid concentrations in hamsters fed the cholesterol-supplemented challenge diet over the 12 weeks of the study is shown in Figure 1. The final body weights represented a two-fold increase (p<0.001) in comparison to the body weights observed at the start of the study (68 ± 5 g). The plasma lipid values reflected body weight dynamics in that plasma cholesterol increased three-fold up to nine weeks (p<0.001), and then plateaued. By contrast, the plasma triglyceride concentration

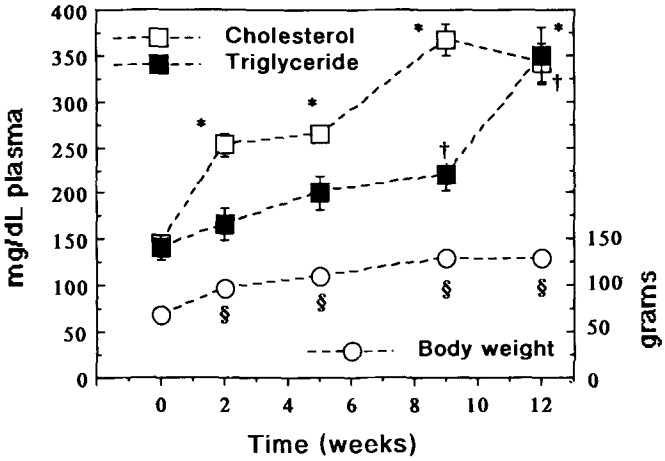


FIG. 1. Time-course of the change in body weight, plasma cholesterol and plasma triglyceride concentrations in hamsters fed the cholesterol-supplemented diet. Each point is the mean ± SEM of 16 hamsters. The data was analyzed with a one-way ANOVA followed by Fisher's PLSD to test for statistical significance ($p<0.05$). Symbols indicate values that were significantly different from the zero-time value.

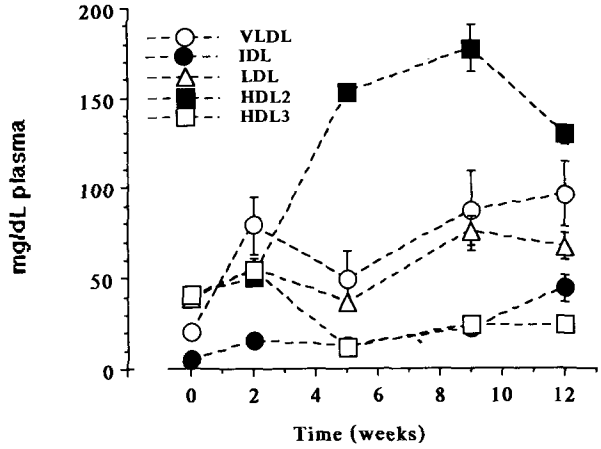


FIG. 2. Time-course of the change in lipoprotein cholesterol concentrations in hamsters fed the cholesterol-supplemented diet. Values are means ± SEM of four isolations using the plasma pooled from 3–5 animals, with the exception of the analysis at 12 weeks, which is the mean ± SEM of 16 individual hamsters. The data was analyzed with a one-way ANOVA followed by Fisher's PLSD to test for statistical significance ($p<0.05$). For VLDL and IDL, all values were significantly higher than the zero-time values. For HDL_2 the 5, 9 and 12 week values were significantly higher than the zero-time values. For LDL, the 9 and 12 week values were significantly higher than the zero-time values.

increased throughout the study, but significant increases were noted at 9 and 12 weeks ($p<0.001$).

The time-course for changes in lipoprotein cholesterol concentrations in the cholesterol-supplemented hamsters over the 12 weeks of the study is depicted in Figure 2. Fluctuating cholesterol concentrations in most of the lipoprotein fractions were apparent during the first 5 weeks, with the greatest relative increase in VLDL and HDL_2 cholesterol. After 5 weeks the cholesterol increased in all the lipoprotein fractions except HDL_3, but the most striking increase in mass occurred in HDL_2 until 9 weeks. By 12 weeks, HDL_2 cholesterol had decreased while only VLDL and IDL cholesterol revealed further increases.

Terminal weights and plasma lipid responses. Body weights, liver weights and plasma lipid levels after 12 weeks of feeding the two diets are presented in Table 2. Although body weights were comparable in the two dietary groups (125 ± 4 *vs.* 129 ± 4 g), the liver weights (as a percentage of the body weight) were significantly higher in hamsters fed the cholesterol-supplemented challenge diet ($p<0.001$). The terminal plasma cholesterol (361 ± 28 *vs.* 122 ± 5 mg/dL; $p<0.001$) and plasma triglyceride concentrations (405 ± 63 *vs.* 141 ± 13 mg/dL; $p<0.001$) were almost threefold higher in the challenge group. The final plasma cholesterol concentration in the hamsters fed the control diet was modestly, but significantly, lower than the value obtained at the start of the study (122 ± 5 *vs.* 144 ± 5 mg/dL; $p<0.003$), whereas the concentration of plasma triglyceride was similar (142 ± 12 *vs.* 141 ± 13 mg/dL).

The 12-week lipoprotein data revealed a greater lipoprotein mass in hamsters fed the challenge diet (Table 3). For both dietary groups the HDL_2 fraction transported the greatest percentage of cholesterol. The hypercholesterolemia in the experimental group was characterized by a four-fold increase in VLDL cholesterol, a seven-fold increase in IDL cholesterol, a three-fold increase in LDL cholesterol and a two-fold increase in HDL_2 cholesterol.

TABLE 2

Body Weights, Liver Weights and Plasma Lipid Concentrations in Hamsters Fed the Control and Challenge Diets for 12 Weeks[a]

	Control diet (cholesterol-free)	Challenge diet (cholesterol supplemented)
Body weight (g)	125 ± 4	129 ± 4
Liver weight (% body weight)	3.5 ± 0.5	5.6 ± 0.5
Plasma cholesterol (mg/dL)	122 ± 5	361 ± 28
Plasma triglyceride (mg/dL)	141 ± 13	405 ± 63

[a]Of the 16 hamsters fed the control diet, 9 hamsters were sacrificed for gallstone evaluation. Therefore, with the exception of the liver weights for the hamsters fed the control diet (n=9), all values are the means ± SEM of 16 animals. All parameters were significantly different between groups as determined by Student's unpaired *t*-test ($p<0.001$) with the exception of body weights.

These changes in the terminal cholesterol distribution represented a relative decrease in HDL_2 cholesterol, but relative and absolute increases in VLDL cholesterol as compared to control hamsters. The hypertriglyceridemia in the supplemented group was reflected principally in the VLDL fraction. In terms of cholesterol distribution, cholesterol-feeding almost doubled the cholesterol load in the VLDL/IDL pool (38% *vs.* 22%) while decreasing the cholesterol in HDL by 30% and leaving the LDL pool unaltered.

Gallstone incidence. No gallstones were detected in hamsters fed the cholesterol-supplemented challenge diet over the first five weeks of the study (Table 4). At nine weeks, three of five hamsters killed had early pigment stone formation only (represented by soft, mucus-rich clusters of greenish, sawdust-like material which was typically amorphic and less than 0.1 mm long). By 12

TABLE 3

Lipoprotein Lipid and Protein Concentrations in Hamsters Fed the Control or Challenge Diets for 12 Weeks[a]

	Control diet (cholesterol-free)	Challenge diet (cholesterol-supplemented)
VLDL (d < 1.006 g/mL)		
Cholesterol	22 ± 1 (17.4)[b]	96 ± 18[c] (26.2)
Triglyceride	85 ± 6	302 ± 61[d]
Protein	16 ± 1	22 ± 5
IDL (1.006 < d < 1.019 g/mL)		
Cholesterol	6 ± 0.1 (4.6)	44 ± 7[c] (12.4)
Triglyceride	13 ± 0.2	38 ± 3[d]
Protein	5 ± 0.2	16 ± 2[c]
LDL (1.019 < d < 1.055 g/mL)		
Cholesterol	23 ± 0.9 (18.3)	67 ± 7[e] (18.4)
Triglyceride	10 ± 0.3	24 ± 1[d]
Protein	14 ± 0.3	44 ± 5[e]
HDL_2 (1.055 < d < 1.125 g/mL)		
Cholesterol	57 ± 2 (45.9)	129 ± 5[e] (36.2)
Triglyceride	9 ± 0.6	30 ± 2[e]
Protein	114 ± 6	257 ± 9[e]
HDL_3 (1.125 < d < 1.21 g/mL)		
Cholesterol	17 ± 0.9 (13.8)	24 ± 2 (6.7)
Triglyceride	3 ± 0.3	11 ± 1[c]
Protein	50 ± 5	77 ± 4[c]
VLDL-C/HDL_2-C	0.4 ± 0.04	0.8 ± 0.2

[a]mg/dL Plasma. Values are the means ± SEM; n=5 for the control group and n=16 for the experimental group. For the control group lipoproteins were isolated from plasma pooled from 3-4 hamsters, whereas for the experimental group, lipoproteins were isolated from the plasma of individual animals.

[b]Values in parentheses represent percent distribution.

[c,d,e]Statistical significance between mean values for the two dietary groups was calculated using Student's unpaired *t*-test. p Values: [c]p<0.02; [d]p<0.05; [e]p<0.001.

TABLE 4

Gallstone Incidence in Hamsters Fed the Control and Challenge Diets

Time (weeks)	Criterion	Control diet (cholesterol-free)	Challenge diet (cholesterol supplemented)
0	Gallstone incidence[a]	0/7 [0%]	
2	Gallstone incidence		0/16 [0%]
5	Gallstone incidence		0/11 [0%]
9	Gallstone incidence		3/5 [60%][b]
12	Gallstone incidence	7/9 [78%]	11/16 [69%]
	Cholesterol stones[c]	0/7 [0%]	5/11 [45%]
	Pigment stones[c]	7/7 [100%]	6/11 [55%]
	Gallstone severity[d]		
	Cholesterol stones	0	5
	Pigment stones	3.6 ± 0.5	3.3 ± 1.5

[a]The number of hamsters with gallstones/number of hamsters examined.

[b]Only early pigment stone formation observed. Additional hamsters were not killed.

[c]The number of hamsters with cholesterol or pigment stones/number of hamsters with gallstones.

[d]Both cholesterol and pigment stones were scored microscopically on a scale of 0-5 to characterize relative size and number.

TABLE 5

Body Weights, Liver Weights, Plasma Lipid and Lipoprotein Concentrations in Subgroups of Hamsters Fed the Challenge Diet for 12 Weeks (Based on Gallstone Response)[a]

		No stones	Cholesterol stones	Pigment stones
Number of hamsters		5	5	6
Body weight (g)		120 ± 8[b]	149 ± 4[b,c]	121 ± 4[c]
Liver weight (% body wt.)		4.6 ± 1.0[d,e]	6.6 ± 1.5[d]	6.7 ± 1.0[e]
Plasma cholesterol (mg/dL)		319 ± 35	421 ± 70	346 ± 34
Plasma triglyceride (mg/dL)		338 ± 64	562 ± 173	329 ± 54
VLDL[f]	Cholesterol	64 ± 17[g] (19.8)[h]	152 ± 46[g,i] (35.3)	77 ± 19[i] (21.4)
	Triglyceride	231 ± 60	462 ± 166	228 ± 55
	Protein	25 ± 6	49 ± 13	27 ± 6
IDL	Cholesterol	39 ± 9 (13.2)	60 ± 15 (14.1)	36 ± 10 (10.5)
	Triglyceride	36 ± 4	43 ± 6	35 ± 2
	Protein	14 ± 3	21 ± 5	14 ± 4
LDL	Cholesterol	53 ± 8 (16.3)	78 ± 18 (18.4)	69 ± 4 (20.3)
	Triglyceride	23 ± 3	25 ± 3	23 ± 1
	Protein	43 ± 10	49 ± 8	41 ± 5
HDL_2	Cholesterol	136 ± 4[j] (42.4)	110 ± 6[j,k] (27.0)	139 ± 7[k] (40.6)
	Triglyceride	34 ± 3	22 ± 4	33 ± 5
	Protein	260 ± 17[l]	224 ± 12[l,m]	281 ± 12[m]
HDL_3	Cholesterol	26 ± 2 (8.1)	22 ± 5 (5.2)	25 ± 2 (7.1)
	Triglyceride	14 ± 2	10 ± 1	11 ± 1
	Protein	75 ± 7	74 ± 9	82 ± 6
VLDL-C / HDL_2-C		0.47 ± 0.12[n]	1.43 ± 0.45[n,o]	0.53 ± 0.11[o]

[a] Mean ± SEM.

[f] mg/dL Plasma.

[h] Values in parentheses represent the percent distribution of cholesterol among the lipoprotein fractions. Data from the 16 hamsters fed the Challenge diet (cholesterol-supplemented) are expressed in terms of the gallstone response. The data was analyzed by a one factorial ANOVA followed by Fishers PLSD test for significance between the subgroups. Mean values, sharing a common superscript are significantly different.

p Values: [b,c] $p<0.02$; [j,k] $p<0.03$; [d,e,n,o] $p<0.05$; [g,i,l,m] $0.05<p<0.10$.

weeks, the incidence of gallstones in the two dietary groups was comparable (78% *vs.* 69%). When the control hamsters were sacrificed, the livers in the first nine hamsters were dark red-brown and smaller than test hamster livers, and since none of these had cholesterol crystals in their bile and the only gallstones present were pigment stones, the other seven animals in this group were not killed. By contrast, hamsters fed the cholesterol-supplemented challenge diet were found to have enlarged, pale-yellow livers. Among the 69% with gallstones, either cholesterol stones (45%) or pigment stones (55% incidence) were observed. Both number and size of pigment stones in the control dietary group were similar to those in the cholesterol-supplemented group.

Lipoprotein distribution in relation to gallstone response. Since the cholesterol-supplemented group revealed three responses in terms of gallstones (no stones, cholesterol stones, or pigment stones), the data from this dietary group was re-analyzed according to gallstone subgroup (Table 5). Although all three subgroups experience elevated total plasma cholesterol and triglyceride concentrations by comparison to controls, the cholesterol-stone formers tended to have higher lipid values than the other two subgroups, and their lipoprotein profile was characterized by 20% less HDL_2 cholesterol ($p<0.03$). The HDL protein tended to be lower as well ($0.05<p<0.10$). Their VLDL cholesterol, however, tended to be higher ($0.05<p<0.10$). The combined effect of the lipoprotein differences resulted in the ratio of VLDL to HDL_2 cholesterol being essentially three-fold higher ($p<0.05$) in hamsters with cholesterol stones as compared to cholesterol-supplemented hamsters having no stones or pigment stones. The percentage of plasma cholesterol in the VLDL fraction of hamsters with cholesterol stones (35%) was almost double that of the other two subgroups. All other lipoprotein parameters between the three subgroups of hamsters were similar. Furthermore, no differences were noted in any of the measured lipoprotein parameters between hamsters with no stones and those with pigment stones. In essence, the two types of gallstones were associated with distinct VLDL and HDL_2 cholesterol distributions such that pigment stone bearers transported the majority of their lipoprotein cholesterol in the HDL_2 fraction (41%), whereas hamsters bearing cholesterol gallstones transported the majority of cholesterol in the VLDL fraction (35%).

A striking finding was that the mean body weight of cholesterol-supplemented hamsters developing cholesterol stones was 25% greater ($p<0.02$) than either of the other two subgroups having no stones or pigment stones.

VLDL cholesterol. The two-fold increase in total VLDL cholesterol observed in the subgroup of hamsters with

TABLE 6

VLDL Cholesteryl Ester Distribution in Hamsters Fed the Challenge Diet for 12 Weeks[a]

Criterion	No stones	Cholesterol stones	Pigment stones
	mg/dL plasma		
Total VLDL cholesterol	64 ± 17[b]	152 ± 46[b,c]	77 ± 19[c]
VLDL free cholesterol	15 ± 3 (23.5)[d]	44 ± 12 (28.7)	24 ± 7 (31.6)
Cholesteryl esters	49 ± 11[e] (76.5)	108 ± 20[e,f] (71.2)	53 ± 7[f] (68.4)
Arachidonate (20:4)	1.1 ± 0.3 (4.0)	2.1 ± 0.4 (2.0)	1.3 ± 0.2 (2.6)
Linoleate (18:2)	11.1 ± 2.7[g] (22.8)	27.3 ± 5.7[g,h] (25.1)	11.3 ± 1.8[h] (21.5)
Palmitate (16:0)	9.1 ± 1.8 (18.7)	18.6 ± 3.3 (17.1)	10.0 ± 2.2 (19.0)
Oleate (18:1)	27.7 ± 6.3[i] (56.4)	60.0 ± 11.3[i,j] (55.2)	30.4 ± 3.6[j] (57.9)

[a]Values are the mean ± SEM for five hamsters with no stones, five with cholesterol stones and six with pigment stones. Mean values sharing a common superscript differ significantly between groups using a one factorial ANOVA.

[d]Parentheses represents percent distribution of the total cholesterol fraction or the fatty acid cholesteryl esters, respectively.

p values: [e,f] $p<0.05$; [b,c,g–j] $0.05<p<0.10$.

cholesterol stones was reflected in the mass of cholesterol esters (Table 6), although the relative distribution between free and esterified cholesterol was similar for the three subgroups. Furthermore, the cholesteryl ester fatty acid profile was similar for the three subgroups with no difference in the 18:1/18:2 ratio.

DISCUSSION

The objective of this investigation was to define the progressive impact of dietary cholesterol on hamster plasma lipoproteins and to determine the relationship of the lipoprotein changes to the induction of cholesterol gallstones observed previously (6). In addition, we wanted to establish the time-frame of the associated lipoprotein alterations. Upon termination of our previous study we noted an elevated VLDL/HDL cholesterol ratio in hamsters with cholesterol gallstones, but the diet in that study was more severe, containing 5% butter, more glucose, and more cholesterol (0.4% or 1.14 mg/kcal). Evidence of mild essential fatty acid depletion also was deduced from plasma fatty acid profiles. Our previous hamsters (Charles River) were allegedly more resistant to gallstones than the Sasco strain used in the present experiment (4), yet with the previous cholesterol challenge diet and hamster strain we found a much higher overall incidence of cholesterol gallstones (93% *vs.* 45%) over the same experimental period (6).

In the present study an American Fat Blend (P/S ratio 0.54) that provided 2.6% dietary energy as 18:2 was fed to improve essential fatty acid status, and dietary cholesterol was reduced by 25%. Furthermore, the sequential changes in plasma lipids, including the lipoprotein profile, were measured during gallstone induction to document the time-course in lipoprotein alterations. This identified HDL_2 as the predominant lipoprotein in the normal hamster. Feeding a 0.86 mg/kcal cholesterol supplement (0.3%, w/w) initiated a rapid rise in VLDL and HDL_2 cholesterol, but the latter pool of cholesterol then declined somewhat by 12 weeks, while VLDL and IDL cholesterol continued to increase. This distribution of cholesterol was most pronounced in the subset of hamsters that developed cholesterol gallstones, a process that appeared to occur between 9 and 12 weeks in our study. By contrast, the hamsters fed the same diet that managed to resist the VLDL-cholesterol increase and maintain their HDL_2, remained free of cholesterol gallstones and were either free of all gallstones or developed only pigment stones. The animals that developed cholesterol stones also gained 25% more body weight than controls fed no cholesterol or the other two subgroups (no stones, pigment stones) also fed cholesterol. Essential fatty acid status did not appear compromised in any group. In fact, the cholesterol-stone formers had the highest percentage of 18:2 among their cholesteryl esters.

At least three implications can be inferred from these data. First, cholesterol gallstone induction in response to cholesterol feeding in hamsters appeared to be associated with an expansion of the VLDL cholesterol pool and relative decrease in HDL_2, the principal lipoprotein cholesterol pool found in normal hamsters. Previously in hamsters fed a comparable diet with a similar shift in their lipoprotein profile (6), we found that the gallbladder bile was markedly supersaturated with cholesterol (lithogenic index of 1.72 with bile acids, phospholipids and cholesterol of 79, 13 and 8 mol%, respectively) and that the liver was enlarged and cholesterol laden (2 mg *vs.* > 20 mg cholesterol/g liver) as it was herein. In the present study the plasma cholesterol (and triglyceride) concentrations tended to be higher in hamsters with cholesterol stones than in the two other subgroups that were fed cholesterol without developing cholesterol stones, supporting our previous observation that increased cholesterol transport was associated with cholesterol gallstone induction (6). However, both the plasma cholesterol and triglyceride values in all three cholesterol-fed subgroups were higher than the control values. This suggests that 0.3% cholesterol (0.86 mg/kcal) approximates the minimal threshold for initiating a cholesterol "overload", causing approximately one-third of the hamsters to exceed their ability to metabolize the increased rate of cholesterol transport. Similar to the situation in gallstone patients where

triglyceridemia could not be separated from decreased HDL levels (7), we are not able to ascertain why HDL_2 should protect against, or VLDL-C should be associated with, cholesterol supersaturation of bile in hamsters. One possibility might be that VLDL-C (as VLDL remnants) has more direct access to the biliary cholesterol pool or that HDL_2 cholesterol has preferential access to bile acid synthesis, since we previously noted an increase in biliary cholesterol (8 *vs.* 2 mol%) and a decrease in the relative concentration of bile acids (79 *vs.* 90 mol%) in hamsters with cholesterol-gallstones under similar circumstances (6). It is interesting that Syrian hamsters, as a species, like women whose gender seems to predispose to gallstones (21), have a predominant HDL_2 profile.

The second point relates to the greater body weight of hamsters with cholesterol gallstones. It is well accepted that obesity is the single most important risk factor for cholesterol stones in humans (7,8,21,22). Obese persons typically experience increased cholesterol transport rates and a lipoprotein profile (expanded VLDL and LDL pools) reflective of hypercholesterolemia and hypertriglyceridemia (7,23,24). Even in non-obese women with increased gallstone incidence, the factor most associated with gallstones was excessive caloric intake (22). If the propensity for increased body weight in hamsters bearing cholesterol gallstones eventually proves to be a reflection of increased energy flux through lipoproteins with its attendant cholesterol transport, it would suggest that at least one mechanism of lithogenesis in cholesterol-fed hamsters and humans may be comparable, *i.e.*, increased caloric consumption resulting in elevated cholesterol transport and increased flux and catabolism of VLDL cholesterol through the liver. If bile acid production is compromised, or the direct transport of free cholesterol into bile is enhanced, or phospholipid synthesis is curtailed in any way, lithogenic bile may ensue.

Although the adipose stores were not measured in these hamsters, subsequent experiments have revealed that weight increments above 100 g body weight in male hamsters are highly correlated (r=0.88) with the size of the adipose depots (unpublished observations). Thus, it would appear that the extra body weight in the cholesterol-stone formers represented fat and that this subgroup had assumed certain characteristics of the moderately obese, type-IV hyperlipidemic human characterized by elevated plasma cholesterol and triglyceride concentrations (7,8,23-26). It will be important to determine whether the propensity to gain weight by the subgroup of susceptible hamsters represents excessive food intake (behavioral) or whether this subpopulation is metabolically more energy efficient resulting in an aberrant shift in cholesterol transport and disposition (genetic).

Finally, as we noted previously (6), susceptibility and induction of pigment gallstones would appear to be independent of changes in cholesterolemia or the lipoprotein profile, as the incidence of pigment stones and the degree of pigment stone involvement (number, size, etc.) was similar in control and cholesterol-fed hamsters.

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Metabolism of Meadowfoam Oil Fatty Acids in Mice[1]

E.A. Emken[a,*], R.O. Adlof[a] and S. Abraham[b]

[a]Food Quality and Safety Research, National Center for Agricultural Utilization Research, ARS, U.S. Department of Agriculture, Peoria, Illinois 61604, and [b]Children's Hospital Medical Center, Oakland, California 94609

Meadowfoam oil is unusual because over 95% of the fatty acids are 20- and 22-carbon aliphatic acids with *cis* double bonds located principally at the 5- and/or 13-position. Since little information is available on the metabolism of the 5*c*-20:1 and 5*c*,13*c*-22:2 fatty acids, an exploratory study in mice was conducted to investigate the metabolism of purified samples of the free fatty acids isolated from meadowfoam oil, and to determine the effect of meadowfoam oil on weight gain and tissue lipid composition. Mice fed diets containing 5% by wt of the purified 5*c*-20:1 or 5*c*,13*c*-22:2 for 6 days exhibited no apparent physiological problems. Total liver lipids from mice fed the purified fatty acid diets contained mean values of 2.0% 5*c*-20:1 and 2.1% 5*c*,13*c*-22:2; total heart lipids contained 1.7% 5*c*-20:1 and 10.7% 5*c*,13*c*-22:2. Liver total phospholipids from mice fed a 5% meadowfoam oil diet for 19 wk contained 1.4% 5*c*-20:1 and 1.9% 5*c*,13*c*-22:2. There was no evidence of desaturation, elongation or retroconversion. Weight gain for mice fed the meadowfoam oil diet for 19 wk was similar to mice fed corn oil, and was higher than for mice fed hydrogenated cottonseed oil. Considering the high 5*c*-20:1 and 5*c*,13*c*-22:2 content of the diets, the percentages of these fatty acids in mouse tissue lipids from both the short- and long-term studies were low. Weight gain was surprisingly good since the meadowfoam oil diet was essential fatty acid-deficient. Results of this initial investigation suggest that the 5*c*-20:1 and 5*c*,13*c*-22:2 fatty acids were utilized primarily for energy. In the short-term study, these fatty acids did not produce toxic effects or cause metabolic problems.

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The fatty acid composition of *Limnanthes douglasii* or meadowfoam oil (MFO) was characterized in 1960 (1). The composition of the oil is unusual because over 95% of the fatty acids are 20- and 22-carbon aliphatic acids with *cis* double bonds located principally at the 5- and/or 13-position. This unique composition and the high oxidative stability of the oil has resulted in its use in cosmetics, surfactants, lubricants and waxes (2,3). No data have been reported on the metabolism of the 5*c*-20:1 and 5*c*,13*c*-22:2 fatty acids which comprise over 80% of the oil.

The health concerns caused by the physiological and metabolic data reported for rats fed high erucic acid (13*c*-22:1) rapeseed oil (4) suggest that the metabolism and physiological properties of the structurally related 5*c*,13*c*-22:2 should be evaluated before meadowfoam oil is used in food products. The structure of 5*c*-20:1 suggests the possibility that it could compromise the metabolism of arachidonic acid (5,8,11,14-20:4) or eicosapentaenoic acid (5,8,11,14,17-20:5).

The purpose of this initial evaluation of the metabolism of meadowfoam oil was to determine if the 5*c*-20:1 and 5*c*,13*c*-22:2 fatty acids (i) accumulate or influence the fatty acid composition of mouse tissue lipids, (ii) influence weight gain, (iii) alter lipogenesis, or (iv) are desaturated or elongated to other structures.

EXPERIMENTAL PROCEDURES

Materials. Alkali refined and bleached meadowfoam oil was supplied by Richard Purdy Inc. (Ukiah, CA). Hydrogenated cottonseed oil was supplied from Wilfey Food Co. (Oakland, CA). Corn oil was a gift from Best Foods (Union, NJ). Samples of 5*c*-20:1 and 5*c*,13*c*-22:2 acids were obtained by transesterification of meadowfoam oil and saponification of the methyl esters that were isolated by silver resin chromatography (5). The enriched 5*c*-20:1 fraction contained 82% 5*c*-20:1, 4% 5*c*-22:1, 11% 13*c*-22:1, and about 1% each of 9*c*-18:1, 20:0 and 22:0. The 5*c*,13*c*-22:2 fraction contained 98% 5*c*,13*c*-22:2, 0.5% 18:2, and 1.5% 20:2. The purity of the oleic acid (9*c*-18:1) and linoleic acid (9*c*,12*c*-18:2) was 99% (Nu-Chek Prep. Inc., Elysian, MN).

Diets. The experimental diets were prepared by starting with a fat-free dry base mixture. This mixture contained by weight, 60% glucose, 20% vitamin-free casein, 5% cellulose, 3.5% salt mix (AIN mineral mixture 76), 1% vitamin mixture (Vitamin Diet Fortification Mixture), 0.3% methionine, 0.2% choline and 0.01% butylated hydroxytoluene. NBC Biochemicals (Cleveland, OH) was the source of all diet components except for glucose and butylated hydroxytoluene. The final diets were formulated by adding 5% by weight of the pure fatty acid plus 5% additional glucose or 10% by weight of the meadowfoam oil, corn oil or hydrogenated cottonseed oil to the dry mixture. The mice were provided free access to the diets, and

TABLE 1

Fatty Acid Composition of Dietary Fats

Fatty acid	Meadowfoam oil (%)	Corn oil (%)	Hydrogenated cottonseed oil (%)
14:0	0.5	0.0	2.0
16:0	0.2	13.0	27.0
18:0	0.2	2.0	64.0
18:1	1.8	28.0	5.0
9*c*,12*c*-18:2	0.8	55.0	0.0
5*c*-20:1	59.6		
5*c*-22:1	3.7		
13*c*-22:1	11.0		
5*c*,13*c*-22:2	18.9		
Other	3.3	2.0	2.0

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

*To whom correspondence should be addressed at National Center for Agricultural Utilization Research, 1815 N. University Street, Peoria, IL 61604.

Abbreviations: CO, corn oil; EFA, essential fatty acid; FAME fatty acid methyl ester; FAS, fatty acid synthetase; GC, gas chromatography; HCTO, hydrogenated cottonseed oil; MFO, Meadowfoam oil.

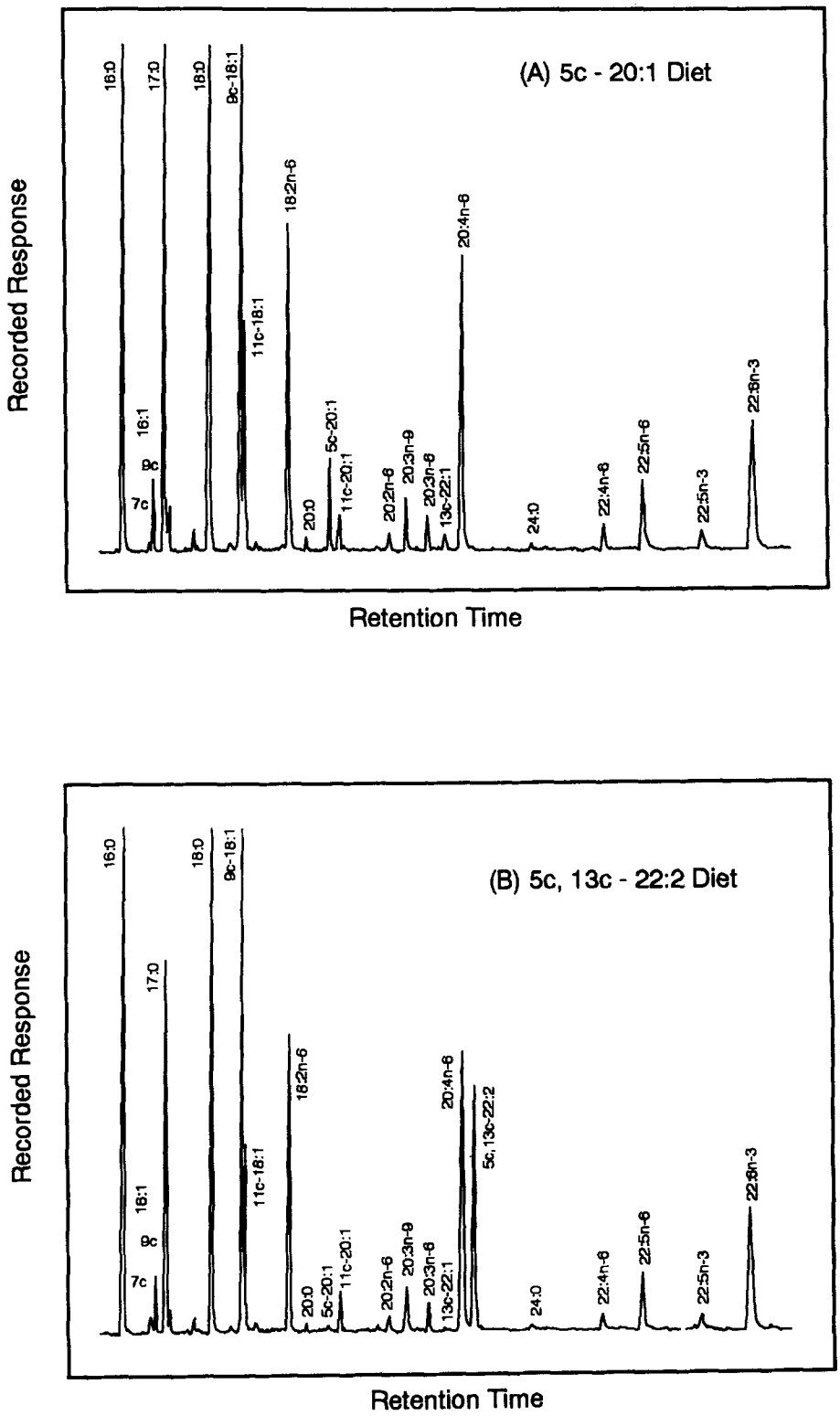


FIG. 1. Separation of fatty esters from mouse heart total lipids by capillary gas chromatography. A) Mice fed purified 5c-20:1 acid diet. B) Mice fed purified 5c,13c-22:2 acid diet. GC conditions: SP2560 column; temperature program 175° for 15 min; 1.5°/min increase to 220°; final hold for 10 min. Detector temperature, 240°. Injector temperature, 235°.

the portion of diet that was not consumed was discarded. The diet was replaced daily.

Meadowfoam oil study. Diets containing 10 wt% of meadowfoam oil (MFO), hydrogenated cottonseed oil (HCTO) or corn oil were fed to groups of 11–16 young adult BALB/c female mice (initial wt, *ca.* 20 g) obtained from the breeding colony at The Children's Hospital Medical Center, Oakland, CA. The fatty acid composition of the oils are summarized in Table 1. The mice were housed in groups of 3–5 per cage and the diets were fed *ad libitum*. Temperature was maintained at 22° with a 12 hr light/12 hr dark cycle. Weight gain was determined, and the mice were sacrificed after 19 wk. Organs were removed, placed in ice-cold sucrose solution and homogenized. Portions of the tissue homogenate were removed for fatty acid analysis. Liver mitochondria and microsomes were isolated by centrifugation of the liver homogenates as previously described (6). Free fatty acids were obtained by saponification with aqueous/ethanolic potassium hydroxide (7). Methyl esters were prepared either by refluxing the free fatty acid extracts with sulfuric acid/methanol or by treatment with diazomethane (8).

Purified fatty acid study. Diets containing 5 wt% of either 9*c*-18:1, 9*c*,12*c*-18:2, 5*c*-20:1 or 5*c*,13*c*-22:2 as the free fatty acid were fed to groups of 4 BALB/c weanling female mice (age 3–4 wk; wt 10–12 g). Free fatty acid was used to facilitate absorption. After 6 days, 500 microcuries of tritiated water was injected intraperitoneally and the mice were sacrificed after 15 min. The procedures used to analyze the organ lipids were the same as described for the meadowfoam oil study.

Portions of the homogenized tissues were analyzed also for tritiated fatty acids to determine fatty acid synthetase activity as described previously (9,10).

Analytical methods. Fatty acid synthetase activity was determined as described elsewhere (9,10). Extraction, methyl ester preparation, and determination of fatty acid composition by gas chromatography (GC) have been described (10).

Liver, heart, and small intestine lipid extracts from the purified fatty acid study were analyzed by both capillary and packed column GC. Mammary gland, kidney, lung, and brain lipid extracts from the purified fatty acid study and liver samples from the meadowfoam oil study were analyzed by packed column GC only (6 ft × 1/4 in DEGS column). The chromatograms shown in Figure 1 for heart lipid methyl esters illustrate the separation obtained with the 100-m SP2560 coated fused silica capillary column. The 5*c*-20:1 and 5*c*,13*c*-22:2 esters were completely resolved from other fatty acid methyl esters present in these mouse tissue lipids.

Statistical methods. A SAS statistical program was utilized (11). A paired t-test was used to analyze the differences between mean values. Linear regression analysis of 0–9 wk and 10–19 wk data was used to evaluate differences in weight gain.

RESULTS

Weight gain. The mean body weights and regression slopes for 0–9 wk and 10–19 wk are plotted in Figure 2 for mice fed corn oil (CO), hydrogenated cottonseed oil (HCTO) and meadowfoam oil (MFO) diets.

Rate of weight gain for each mouse was estimated by linear regression analysis. Individual weight gains were used to calculate regression slopes. For mice fed the CO diet, a small weight gain occurred during the first 9 wk (regression slope = 0.12 g/wk/mouse, $p = 0.07$ for a t-test of null hypothesis (H_0)). No weight gain occurred during the last 10 wk (slope = 0.04, $p = 0.38$). Overall weight gain was nearly zero (slope = −0.02, $p = 0.78$). For mice fed the MFO diet, weight gain occurred during the first 9 wk (slope = 0.15, $p < 0.01$) and then remained stable for the last 10 wk (slope = −0.01, $p = 0.19$). The mice fed the HCTO diet gained weight during the first 9 wk (slope = 0.18, $p < 0.01$) and lost weight during the last 10 wk (slope = −0.32, $p < 0.01$). Comparison of mean weight gains during the first 9 wk by analysis of variance indicated no significant difference due to diet (all $p > 0.25$). For the last 10 wk of the study, weight gains were similar ($p = 0.99$) for the mice fed the CO and MFO diets. Mean weight gains for mice fed CO and MFO diets were significantly different ($p < 0.01$) from mean weight gains for mice fed the HCTO diet.

Weight loss for the HCTO-fed mice is probably related to the linoleic acid deficiency of these diets. Although the weight loss for the MFO-fed mice was not significantly different from that of the CO-fed mice, they did develop a scruffy and sometimes scaly appearance that is typical of essential fatty acid deficiency (12).

Liver lipids. The fatty acid composition of liver total lipids from mice fed the purified fatty acid diets is listed in Table 2. The differences in the total liver lipid fatty acid composition for the mice fed the purified 9*c*-18:1, 5*c*-20:1 and 5*c*,13*c*-22:2 diets were relatively small. The most notable effect of the 5*c*-20:1 and 5*c*,13*c*-22:2 diets compared to the 18:1 diet was a 25% to 50% depression of the 18:2n-6, 20:4n-6 and 22:6n-3 percentages. A smaller decrease in 18:0 (10–30%) and a 10–30% increase in 16:0 and 16:1 also occurred. Samples from the MFO-acid-fed mice contained only 1.99% 5*c*-20:1 and 2.13% 5*c*,13*c*-22:2, which is less than the reduction in the total percentages for 20:4n-6 and 22:6n-3. These data suggest the 5*c*-20:1 and 5*c*,13*c*-22:2 acids did not merely replace other 20- and 22-carbon fatty acids, but that they also influenced the metabolism of other fatty acids. In particular, when 5*c*,13*c*-22:2 was fed, the percentage of oleic acid increased 8% (48.5% *vs* 40.6%) compared to that in mice fed the oleic acid diet, but the 5*c*-20:1 diet did not alter the percentage of oleic acid in the liver lipids. Both the 5*c*-20:1 and 5*c*,13*c*-22:2 diets substantially reduced 20:4n-6 levels. Fatty acids were not detected that could be associated with chain elongation, desaturation or retroconversion of the 5*c*-20:1 or 5*c*,13*c*-22:2 structures.

The liver mitochondrial and microsomal lipids from mice fed MFO for 19 wk contained 3.6% and 2.0% of 5*c*-20:1 and 5*c*,13*c*-22:2, respectively (Table 2). When compared to data from three mice fed the CO diet, the values for 18:2n-6 were lower in the samples from the MFO-fed mice. The difference is likely due to the low 18:2n-6 content of the MFO diet. The percentage of 20:4n-6 was reduced in the MFO-fed mice compared to that in corn oil-fed mice, but the percentage of 22:6n-3 was similar in samples from both the CO and MFO diets. The MFO diet was essential fatty acid (EFA)-deficient and produced the expected increase in 20:3n-9 as did the HCTO diet and the pure 18:1 diet (12). Since mouse liver mitochondrial and microsomal lipid fractions contain about 90%

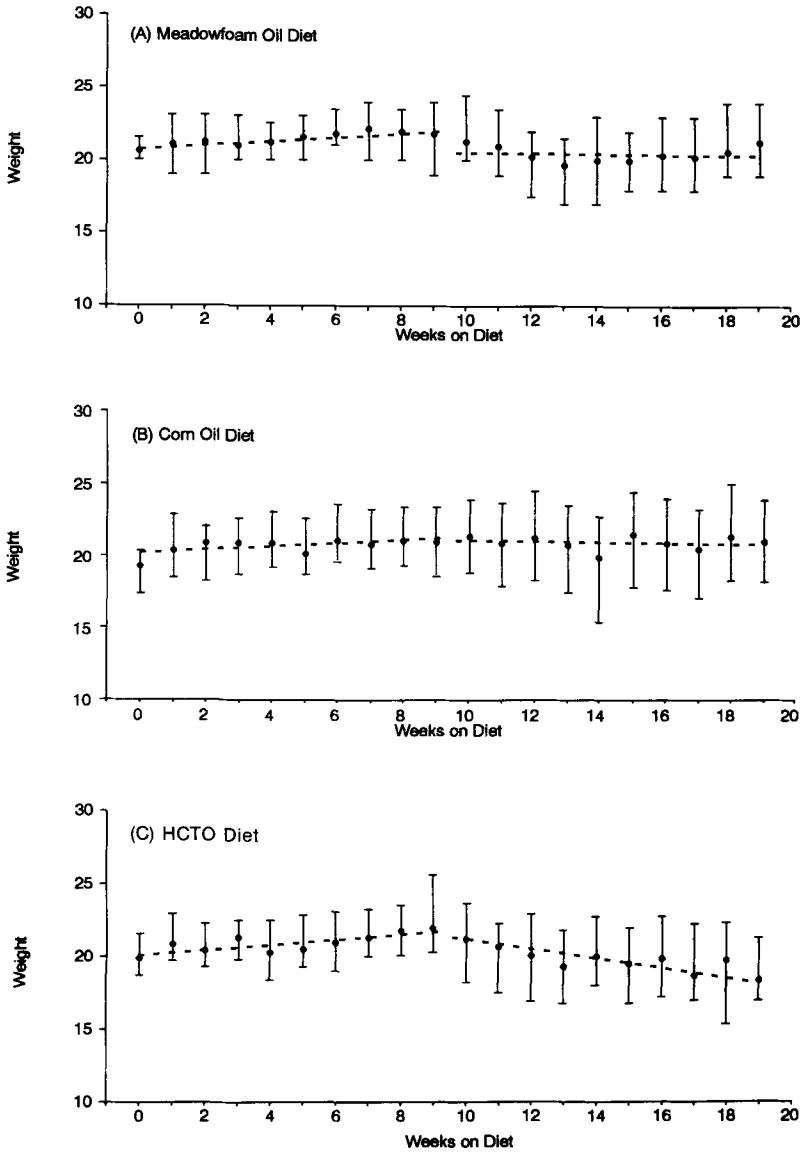


FIG. 2. Weights of mice fed triglyceride diets for 19 wk. A) Meadowfoam oil diet (n=10). B) Corn oil diet (n=10). C) Hydrogenated cottonseed oil diet (n=15). Symbols represent mean values and bars indicate range of body weights. Dashed lines represent linear regression analysis of 0–9- and 10–19-wk data.

phospholipids (13), the fatty acid data reflect the total liver phospholipid fatty acid composition.

Heart lipids. The fatty acid composition of the heart total lipids from mice fed the purified fatty acids is listed in Table 3. The influence of the purified 9*c*-18:1, 5*c*-20:1 and 5*c*,13*c*-22:2 diets on the total heart lipid fatty acid composition was different than that on the liver. The heart lipids contained 2.2% 5*c*-20:1 (mice fed 5*c*-20:1) and 8.4% 5*c*,13*c*-22:2 (mice fed 5*c*,13*c*-22:2). The percentage of 5*c*-20:1 in the liver and heart lipids were similar, but the percentage of 5*c*,13*c*-22:2 was nearly 5 times higher in heart lipids than in liver lipids. There was no evidence for fatty acids with structures that would be indicative of chain elongation, desaturation or retroconversion of 5*c*-20:1 or 5*c*,13*c*-22:2. An interesting effect of the 5*c*-20:1 diet was a 20% increase in 20:4n-6 compared to the data for 9*c*-18:1-fed mice. For the 5*c*,13*c*-22:2 *vs* 9*c*-18:1 diets, there was a 15% decrease in the 22:6n-3 percentages.

Other tissue lipids. The 5*c*-20:1 and 5*c*,13*c*-22:2 percentages in kidney, intestine, lung, brain and mammary tissues are compared to heart and liver total lipids in Table 4. These data were from mice fed the purified 5*c*-20:1 and 5*c*,13*c*-22:2 diets. The percentages for 5*c*-20:1 and 5*c*,13*c*-22:2 were the highest in intestine and mammary tissue. Brain tissue contained only traces of 5*c*-20:1 and 5*c*,13*c*-22:2. In all tissues, the percentage of 5*c*,13*c*-22:2 was significantly higher than that of 5*c*-20:1.

Fatty acid synthetase activity. The influence of dietary 5*c*-20:1 and 5*c*,13*c*-22:2 on fatty acid synthetase (FAS)

TABLE 2

Fatty Acid Composition (%) of Total Liver Lipids from Mice Used in Purified Fatty Acid Study and Mitochondria Plus Microsomes from Mice Used in Long-Term Study

	Purified fatty acid diets (n=4)								Long-term diets (n=4)		
	9*c*-18:1		9*c*,12*c*-18:2		5*c*-20:1		5*c*,13*c*-22:2		Meadowfoam oil		Corn oil
Fatty acid	Mean	SD	Mean	SD	Mean	SD	Mean	SD	Mean	SD	Mean[a]
14:0	0.3	0.1	0.3	0.1	0.3	0.1	0.5	0.1	0.1	0.0	0.2
16:0	16.9	1.2	18.1	1.6	20.2	0.7	18.9	0.6	22.5	0.3	19.8
7*c*&9*c*-16:1	4.0	0.2	3.3	0.3	5.3	0.7	5.6	0.4	3.8	0.2	1.8
18:0	7.2	0.1	7.9	1.3	6.7	0.9	4.9	0.4	10.0	1.0	12.9
9*c*&11*c*-18:1	41.5	1.7	20.8	2.3	42.0	5.5	49.3	0.7	30.0	3.9	14.3
18:2n-6	6.2	0.5	27.1	2.3	4.4	0.6	3.7	0.1	4.0	0.9	17.5
5*c*-20:1	0.0		0.0		1.4	0.1	0.0		3.7	0.7	0.0
11*c*-20:1	1.4	0.1	0.6	0.0	1.4	0.3	1.9	0.5	0.4	0.1	0.3
20:2n-6	0.2	0.1	0.9	0.2	0.0		0.0		0.0	0.0	0.4
20:3n-9	2.9	0.3	0.0		4.5	0.8	2.9	0.2	6.6	0.3	0.0
20:3n-6	1.6	0.1	1.5	0.3	1.1	0.3	0.8	0.1	1.0	0.3	1.6
20:4n-6	11.2	1.1	12.8	2.6	8.1	1.1	5.3	0.1	6.9	1.3	19.5
20:5n-3	0.3	0.0	0.2	0.0	0.1	0.0	0.0		0.5	0.2	0.1
5*c*,13*c*-22:2	0.0		0.0		0.0		2.2	0.5	2.0	0.0	0.0
22:4n-6	0.2	0.0	0.9	0.4	0.2	0.0	0.5	0.1	0.0		0.6
22:5n-6	1.6	0.5	2.7	0.6	1.2	0.3	1.2	0.3	0.7	0.0	2.9
22:5n-3	0.3	0.1	0.1	0.1	0.0		0.9		0.0	0.0	0.3
22:6n-3	4.1	0.3	2.8	0.3	3.2	0.8	2.3	0.1	7.7	0.0	7.5
μg FAME[b]/ mg liver	51.0	3.7	60.6	7.0	49.5	12.2	79.6	9.7			

[a]Single-pooled samples of mitochondria plus microsomes isolated from mouse livers.
[b]Fatty acid methyl esters.

TABLE 3

Fatty Acid Composition (%) of Total Heart Lipids from Mice Used in Purified Fatty Acid Study

	Purified fatty acid diets (n=4)							
	9*c*-18:1		9*c*,12*c*-18:2		5*c*-20:1		5*c*,13*c*-22:2	
Fatty acid	Mean	SD	Mean	SD	Mean	SD	Mean	SD
14:0	0.8	0.31	0.3	0.05	0.02	0.08	0.2	0.08
16:0	14.4	1.92	13.4	1.15	14.3	0.04	12.4	0.06
7*c*&9*c*-16:1	2.3	0.27	1.5	0.55	2.0	0.04	1.8	0.05
18:0	14.9	0.98	16.4	0.11	15.7	0.52	15.1	0.25
9*c*&11*c*-18:1	22.7	1.51	9.4	1.15	17.5	0.23	19.5	1.47
18:2n-6	8.2	0.14	20.3	1.26	8.3	0.30	7.4	0.09
20:0	0.1	0.02	0.2	0.02	0.2	0.01	0.2	0.01
5*c*-20:1	0.8		0.0		2.2	0.05	0.1	0.01
11*c*-20:1	0.8	0.08	0.2	0.02	0.7	0.04	1.3	0.09
20:2n-6	0.8	0.03	1.1	0.05	0.7	0.08	0.8	0.41
20:3n-9	1.2	0.06	0.0		2.0	0.03	1.4	0.26
20:3n-6	1.1	0.25	0.8	0.01	1.3	0.01	1.2	0.25
20:4n-6	11.4	0.87	12.8	1.52	13.8	0.25	11.4	0.05
20:5n-3	0.3	0.05	0.2	0.02	0.2	0.01	0.2	0.08
13*c*-22:1	0.0		0.0		0.3	0.01	0.0	
5*c*,13*c*-22:2	0.0		0.0		0.9		8.6	0.42
22:4n-6	1.5	0.19	2.7	0.52	1.6	0.02	1.4	0.29
22:5n-6	4.6	0.35	7.9	0.68	4.6	0.83	3.9	0.36
22:5n-3	1.5	0.25	1.3	0.35	1.6	0.21	1.5	0.42
22:6n-3	13.4	1.97	11.5	1.57	13.2	0.15	11.7	1.15
μg FAME/ mg tissue	29.80	2.76	25.80	3.00	27.20	5.40	27.40	3.40

TABLE 4

Comparison of 5*c*-20:1 and 5*c*,13*c*-22:2 Percentages in Mouse Tissue Lipids (n=4)[a]

	5*c*-20:1		5*c*,13*c*-22:2		
Tissue	Mean	SD	Mean	SD	p Value[b]
Liver	1.4	0.13	1.9	0.52	<0.03
Heart	2.2	0.05	8.4	0.42	<0.04
Intestine	7.7	0.09	10.0	1.40	<0.15
Mammary	9.2	0.12	13.2	0.58	<0.15
Lung	1.0	0.03	5.3	0.91	<0.05
Kidney	2.9	0.05	6.2	1.52	<0.05

[a]5*c*-20:1 and 5*c*,13*c*-22:2 were not detected in brain.
[b]p value for comparison of 5*c*-20:1 to 5*c*,13*c*-22:2 in tissues calculated by paired t-test.

activity in mouse tissue is compared to the effect of 9*c*-18:1 and 9*c*,12*c*-18:2 in Table 5. Fatty acid synthesis in livers from mice fed the purified 5*c*-20:1 and 5*c*,13*c*-22:2 diets were similar and was intermediate between that in livers of mice fed 9*c*-18:1 and 9*c*,12*c*-18:2. Both of the fatty acids from MFO significantly depressed FAS activity compared to 9*c*-18:1. The FAS activity data for livers from mice fed 5*c*-20:1 and 5*c*,13*c*-22:2 were higher than for 18:2n-6-fed mice, but the data were not significantly different at $p < 0.05$.

TABLE 5

Effect of Dietary 5c-20:1 and 5c,13c-22:2 on *in vivo* Fatty Acid Synthesis in Mouse Tissues[a] (n=4)

	Liver			Kidney			Small intestine		
Diet fed	Tritium[b]	SD	p Value[c]	Tritium	SD	p Value	Tritium	SD	p Value
9c-18:1	10.1	1.94		1.88	0.45		1.56	0.40	
18:2n-6	4.9	1.43	<0.01	1.15	0.17	<0.04	0.87	0.37	<0.05
5c-20:1	7.5	1.61	<0.02	0.80	0.16	<0.04	1.07	0.40	<0.07
5c,13c-22:2	7.8	2.64	<0.02	1.12	0.35	<0.12	0.77	0.13	<0.05

[a]Fatty acid synthetase activity in mammary gland, brain, heart and lung was low and data for mice fed different diets were not significantly different.

[b]Micromoles of tritiated water converted to fatty acids per gram wet tissue per 15 min.

[c]p Values for comparison of 9c-18:1 diet to other diets calculated by paired t-test.

DISCUSSION

Weight gain for MFO-fed mice was no different than for CO-fed mice. The EFA-deficient nature of the MFO diet was reflected in the general scruffy appearance of the mice and the high triene-to-tetraene ratio (20:3n-9/20:4n-6 = 1.26) in the liver samples.

In the 19-wk study, absorption of the MFO long-chain acids is believed to have been good to produce weight gains that were comparable to the CO and HCTO diets during the first 9 wk of the study (Fig. 2). Total diet consumed per day was similar for each group which indicates the MFO-fed mice did not need to compensate for poor fat absorption by increasing carbohydrate intake. For the short-term pure acid study, the 9–13% levels of 5c-20:1 and 5c,13c-22:2 in the mammary gland (an adipose-like tissue) and intestinal cells indicate absorption of both acids was similar (Table 4). The acids rather than triglycerides were used in the purified fatty acid study to avoid potential problems due to poor absorption of the monoacid triglyceride.

Excessive accumulation of MFO acids in livers was not observed in either the short-term purified acid or the long-term MFO feeding studies. The levels of 5c-20:1 and 5c,13c-22:2 in liver lipids were about 2%. This percentage was lower than expected considering the high amount of these acids in the diets and the percentages in the intestine and mammary tissue. The data suggest that a substantial portion of MFO acids is efficiently oxidized to meet energy needs. The lower percentages of the MFO acids in the liver compared to the mammary gland and intestinal cells may also be a result of discrimination against the MFO acids by the phospholipid acyltransferases. Although 5c,13c-22:2 did not accumulate, livers from mice fed 5c,13c-22:2 contained substantially more total lipid. This difference in liver lipid concentration may indicate a possible metabolic problem associated with 5c,13c-22:2, but not with 5c-20:1. The lack of detectable levels of desaturation or elongation products indicates that 5c-20:1 and 5c,13c-22:2 are not rapidly desaturated or elongated. The 11% 13c-22:1 impurity in the 5c-20:1 diet was not detected in the liver lipids.

In total heart lipids, the percentage of 5c, 13c-22:2 was more than 6 times the percentage of 5c-20:1, and the 5c-20:1-to-13c-22:1 ratio was the same as in the fed diet. These data suggest that 5c,13c-22:2 has a greater tendency to accumulate in heart lipids than either 5c20:1 or 13c-22:1. The reason for accumulation of 5c,13c-22:2 is unknown, but it may be related to the same mechanism that induces accumulation of 13c-22:1 in heart lipids from rapeseed oil-fed rats (4).

Both 5c-20:1 and 5c,13c-22:2 produced similar effects on fatty acid synthesis. The values in Table 5 for these fatty acids were between the fatty acid synthetase depressed values for 18:2 and the non-depressed values for 18:1. Data suggest that the 5c bond position has some influence on the regulation of fatty acid synthesis but is 50% less effective as a fatty acid synthetase inhibitor than the 9c,12c methylene-interrupted structure.

The results show that MFO acids are absorbed, incorporated into tissue lipids, used as an energy source, do not accumulate excessively in liver microsomal and mitochondrial phospholipids, and are not converted to other fatty acid structures. Toxic effects were not observed and the gross appearance of the organs was normal. However, the presence of some, more subtle physiological effects cannot be ruled-out. Our initial data on the metabolism of MFO acids are encouraging and suggest that the use of small amounts of refined MFO as a food additive may become acceptable in the future.

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Prostaglandin Synthesis and Fatty Acid Composition of Phospholipids and Triglycerides in Skeletal Muscle of Chicks Fed Combinations of Flaxseed Oil and Animal Tallow

J.M. Olomu and **V.E. Baracos***
Department of Animal Science, University of Alberta, Edmonton, Alberta, Canada T6G 2P5

Chicks were fed isocaloric and isonitrogenous diets containing 6% (w/w) added fat consisting of various proportions of animal tallow and flaxseed oil (FSO). No differences among treatments were seen in growth rate, muscular deposition of protein and lipids nor in the muscle phospholipid (PL) and triglyceride (TG) contents. Prostaglandin (PG)E_2 synthesis in isolated skeletal muscle was depressed significantly by feeding FSO or by treatment with naproxen (6-methoxy-α-methyl-2-napthaleneacetic acid), an inhibitor of PG synthesis. The changes associated with diet may be related to differences in the fatty acid composition of muscle lipids. Levels of saturated fatty acids in muscle PL and TG were relatively insensitive to dietary treatments. Monounsaturated fatty acid levels were significantly lower in the FSO-fed groups. FSO diets caused significant depression in muscle PL 20:4ω6 and almost completely inhibited 22:5ω6 incorporation. FSO diets decreased ratios of ω6/ω3 fatty acids and increased the unsaturation index of muscle PL. Muscles of chicks fed FSO showed increased levels of 18:3ω3, and of its derivatives 20:4ω3 and 22:5ω3. These results suggest that FSO inhibits PG synthesis and modifies the fatty acids of PL and TG of chick muscle. These changes may have implications for PG-dependent and/or membrane-dependent processes in muscle metabolism.
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It has been suggested that in skeletal muscle, prostaglandins (PG) are involved in the regulation of protein and amino acid metabolism (1–3), differentiation (4), and the local control of blood flow in active and reactive hyperemia (5). It is known that incubated skeletal muscle *in vitro*, as well as muscle cells in tissue culture produce PG and other metabolites of arachidonic acid, including PGE_2, $PGF_{2\alpha}$, PGI_2 and thromboxane A_2 (1). However, the physiological, pathological and nutritional factors affecting the synthesis of these metabolites in skeletal muscles have not yet been fully characterized.

A number of studies with laboratory rodents (6–8) have shown that PG synthesis in several cell types is depressed by the addition of long-chain ω3 fatty acids (mainly 20:5ω3 and 22:6ω3) in the form of fish oil to the diet. Similar results have been obtained with rats fed flaxseed (FSO) oil (9,10) which contains 18:3ω3. It would appear that the inhibitory effects of the fish oils on PG synthesis are related to characteristic changes in the fatty acid composition of tissue phospholipids (6,7,11–13). In particular, ω3 fatty acids are thought to i) competitively inhibit the synthesis of arachidonic acid (20:4ω6), the immediate precursor of PG (10–12); ii) act as an alternate substrate for PG synthesis (20:5ω3), leading to synthesis of the less potent 3-series PG; iii) activate PG catabolism (6); and iv) competitively inhibit the PG synthetase complex (20:5ω3) (11).

Dietary modification of the fatty acid composition of platelets, liver, heart, kidney, plasma and dermis has been well described (7,8,10,13). By contrast, information on the influence of dietary oils on the fatty acid composition and metabolism of skeletal muscle is limited. A number of earlier studies (*e.g.*, reference 14 and references therein) have shown that fatty acids from dietary fish oil are incorporated into muscle lipids. In these studies no distinction was made between fatty acid incorporation into phospholipids and triglycerides. Whether the effect of feeding long-chain ω3 fatty acids of marine origin can be duplicated by the feeding of 18:3ω3 found in the oils of terrestrial plants, such as flaxseed is not clear. It would depend upon the availability of 18:3ω3 for desaturation and elongation, and would presumably be species-dependent. Cunnane *et al.* (15), for example, have shown increased levels of 20:5ω3 in porcine skeletal muscle after feeding flaxseed, but longer chain derivatives such as 22:5ω3 and 22:6ω3 were not detected.

The objective of our study was to determine to which extent dietary ω3 fatty acids can be incorporated into chicken skeletal muscle triglycerides and phospholipids and how fatty acid incorporation relates to FSO feeding and tissue PG synthesis. The effects are compared with those caused by naproxen, a direct inhibitor of PG synthesis (16).

MATERIALS AND METHODS

Animals and diets. Broiler chicks of the Hubbard strain, vaccinated for Marek's disease, were obtained on the day of hatching from the Cooperative Hatchery (Edmonton, Canada). The chicks were housed in thermostatically controlled, electrically heated batteries (33–35°C) with raised mesh floors and stainless steel waterers, feeders and partitions. Birds were maintained under continuous light. Feed and water were provided for *ad libitum* consumption. Experiments were done according to the guidelines of the Canadian Council on Animal Care.

Two experiments were conducted. Birds were placed on treatments on the day of hatching. At the start of each experiment, birds were weighed individually and divided into groups of 12 (Expt. 1) or 24 birds (Expt. 2) such that the average body weight and weight range were similar

*To whom correspondence should be addressed.

Abbreviations: AT, animal tallow; EDC, extensor digitorum communis; EMS, error mean square; FSO, flaxseed oil; MUFA, monounsaturated fatty acid(s); PG, prostaglandin; PL, phospholipid(s); PUFA, polyunsaturated fatty acid(s); SAT, saturated fatty acid(s); TG, triglyceride(s).

TABLE 1

Composition of Experimental Diets (%)

Ingredients	Diets			
	1	2	3	4
Ground corn	53.75	53.75	53.75	53.75
Soybean meal	34.60	34.60	34.60	34.60
Limestone	1.50	1.50	1.50	1.50
Dicalcium phosphate[a]	1.60	1.60	1.60	1.60
NaCl	0.35	0.35	0.35	0.35
Vit-Min premix[b]	2.00	2.00	2.00	2.00
DL-methionine	0.20	0.20	0.20	0.20
Animal tallow[c]	6.00	4.50	3.00	1.50
Flaxseed oil	—	1.50	3.00	4.50
Calculated composition				
Crude protein (%)	22.00	22.00	22.00	22.00
ME (Kcal/Kg)	3164	3164	3164	3164
Met + cys (%)	0.86	0.86	0.86	0.86
Lysine (%)	1.20	1.20	1.20	1.20
Calcium (%)	1.00	1.00	1.00	1.00
Available P (%)	0.46	0.46	0.46	0.46

[a]18% calcium, 21% phosphorus.

[b]Vit-Min premix supplied per Kg of diet the following: Vitamin A, 400 IU; Vitamin D_3, 600 ICU; Vitamin E, 10 IU; menadione, 1 mg; riboflavin, 5 mg; calcium pantothenate, 10 mg; niacin, 20 mg; choline chloride, 100 mg; folic acid, 1 mg; Vitamin B_{12}, 10 μg; Mn, 108 mg; Zn, 72 mg.

[c]Ethoxyquin, at 0.25% of the diet, was added to the dietary fats before mixing, as an antioxidant.

for each group. Three such groups were placed on each treatment. Equal numbers of male and female chicks were used per group in Expt. 1, while male chicks only were used in Expt. 2.

The diets used were isonitrogenous (22% crude protein) and isocaloric (3164 Kcal/Kg diet) with similar amounts of calcium and phosphorus (Table 1). The diets were based on ground corn and soybean meal. The diets contained 6% added fat, consisting of 100% animal tallow (AT); 75% AT/25% flaxseed oil (FSO); 50% AT/50% FSO; and 25% AT/75% FSO (Table 1). A further experimental treatment included the administration of naproxen (6-methoxy-α-methyl-2-napthaleneacetic acid), an inhibitor of PG synthesis (16). This agent was administered in the drinking water (76 mg/L), to birds fed Diet 1, as in previous studies (2,3). Fresh flaxseed oil (cold-pressed) stored in light- and air-tight bottles was obtained from Omega Nutrition Inc. (Vancouver, Canada). Animal tallow rendered from slaughterhouse waste at 118°C was obtained from Northern Alberta Processors (Edmonton, Canada).

Diets were prepared weekly and stored at −4°C. Table 2 shows the fatty acid contents of the oils and diets studied. Diets were fed for 7 days (Expt. 1) or 21 days (Expt. 2). In Expt. 1, diets 1 to 4 (Table 1) were tested while in Expt. 2, diet 3 was not included in the tests. Records of body weight and feed consumption were kept on a weekly basis. On day 7 (Expt. 1,2) and 21 (Expt. 2), four birds were taken from each replicate and killed by cervical dislocation. One extensor digitorum communis (EDC) muscle from the wing and sartorius muscles from both thighs were dissected immediately after killing. Sartorius muscles were weighed individually and then pooled by replicate for lipid extraction (see below).

Analytical. The EDC muscle is a thin, long muscle suited to incubation studies where oxygen and nutrients are provided by superfusion (17). Freshly dissected EDC muscles were mounted at their resting length *in situ* on stainless steel supports (17) and immediately incubated in 3 mL Krebs Ringer bicarbonate medium (NaCl 119 mM; KCl 4.8 mM; $CaCl_2$ 1.0 mM; $NaHCO_3$ 25 mM; $MgSO_4$ 1.25 mM; NaH_2PO_4 1.24 mM; HEPES 2.0 mM, pH 7.4) containing 15 mM glucose and insulin (0.01 U/mL) as previously described (17). Media were continuously gassed with O_2/CO_2 (19:1, v/v) and the temperature was maintained at 35°C in a shaking water bath. Muscles were preincubated for 30 min, then transferred to fresh media and incubated for 3 hr. PGE_2 production was estimated by radioimmunoassay of the incubation medium (2,3). The antibody used showed 100% cross-reactivity with PGE_1, but no cross-reactivity with $PGF_{2\alpha}$. Since skeletal muscle does not appear to synthesize PGE_1 (18), this cross-reactivity does not interfere with the assay of PGE_2.

Total lipid was extracted from sartorius muscles and feed samples by mechanical homogenization with 20 vol of chloroform/methanol (2:1, v/v). The crude lipid extracts were washed with 0.2 vol of 0.88% NaCl, and then with 0.65 vol of chloroform/methanol/water (3:48:47, v/v/v) as described by Folch *et al.* (19). The lipid extract was evaporated to dryness at 60°C under N_2 for determination of total lipid content. Total sartorius lipids were separated by two-dimensional thin-layer chromatography on a 20 × 20 cm Redi/Pit Silica Gel GF (Fisher Scientific, Ottawa, Canada). Benzene/diethyl ether/ethanol/acetic acid (50:40:2:0.2, by vol) was the solvent in the first dimension (15–20 min) to separate the phospholipids from the monoglycerides. Hexane/diethyl ether/formic acid (80:20:2, v/v/v) was the solvent used in the second dimension (30–40 min) to completely separate cholesteryl esters and triglycerides. The fractions were visualized under ultraviolet light after spraying with 0.1% 2,4-dichloro-

TABLE 2

Fatty Acid Composition of Animal Tallow, Flaxseed Oil and Lipids From Diets Fed[a]

	Total Fatty acids (%)					
Fatty acid	Animal tallow	Flaxseed oil	Diet 1	Diet 2	Diet 3	Diet 4
14:0	1.9	0.1	1.0	0.8	0.5	0.4
15:0	ND	ND	0.1	0.1	0.1	0.1
16:0	20.8	6.0	17.3	14.8	12.5	11.0
16:1	2.3	0.3	1.6	1.2	1.0	0.6
17:0	0.7	ND	0.6	0.4	0.3	0.2
18:0	11.6	4.0	10.3	8.2	7.3	5.4
18:1(*trans*)	5.4	ND	4.7	3.3	2.3	1.2
18:1ω9	43.7	21.5	35.7	32.4	29.3	26.7
18:1ω7	3.1	0.9	2.1	1.6	1.4	1.1
18:2ω6	7.4	15.6	23.1	25.3	25.5	25.8
18:3ω3	1.5	51.0	2.0	10.7	18.6	26.5
20:0	0.8	0.3	0.6	0.5	0.5	0.4
20:1ω9	0.8	0.3	0.7	0.5	0.5	0.4
22:0	ND	ND	0.2	0.2	0.2	0.2
Σ SAT	35.8	10.4	30.2	25.0	21.4	17.7
Σ MUFA	55.3	23.0	44.8	39.0	34.5	30.0
Σ PUFA	8.9	66.6	25.1	36.0	44.1	52.3
Σ ω6	7.4	15.6	23.1	25.3	25.5	25.8
Σ ω3	1.5	51.0	2.0	10.7	18.6	26.5

[a]SAT, saturated; MUFA, monounsaturated; PUFA, polyunsaturated fatty acids; ND, not detectable. Total lipid was extracted from feed samples after mixing of the diets. No fatty acids longer than 22 carbons were detected in the feed or fat samples. Results are means of duplicate analyses.

fluorescein in methanol and identified by comparison with standards. The phospholipids and triglyceride bands were scraped off the plate directly into methylating tubes. Nonadecanoic acid (19:0) was added as internal standard and the tubes were tightly closed with Teflon-lined screw caps. Prior analysis of sartorius muscle showed that it did not contain nonadecanoic acid.

The lipid extracts were then converted to fatty acid methyl esters using a mixture of boron trifluoride (in 10% methanol), hexane and methanol (35:20:45, v/v/v). The resulting fatty acid methyl esters were separated and quantified by automated gas-liquid chromatography (Varian model 3400 equipped with autosampler and flame ionization detector, Varian Associates, Palo Alto, CA), using a 30-m fused silica capillary column (International Scientific, Folsom, CA). Helium was used as the carrier gas at a flow rate of 3.0 mL/min. The column temperature was increased from 60°C to 230°C at a rate of 30°C/min up to 160°C and then at 5°C/min up to 230°C. Conditions were chosen to separate fatty acids ranging from 12 to 24 carbons in chain length. Fatty acids were identified by comparison of retention times with those of standard fatty acid methyl esters (Sigma Chemical Co., St. Louis, MO). The results are expressed as percent distribution of fatty acid methyl esters. The amount of phospholipid and triglyceride was determined from the percent fatty acid contents, using the procedures and factors earlier proposed by Christie *et al.* (20).

Muscle protein content was determined by the Kjeldahl method (21), or using a modified Bradford method (22) in the case of EDC muscles, which were too small for Kjeldahl analysis. Bovine serum albumin served as the protein standard in the Bradford method.

Data obtained were tested by one-way and two-way analysis of variance, and differences between means were determined using Student Neuman-Keuls multiple range test. Planned comparisons were carried out using procedures of orthogonal contrast, to determine effects of duration of feeding, FSO addition to the diets, and FSO levels. Fatty acid data are presented as least squares means and the error mean square presented as the error term. The criterion for significance was a probability of 0.05.

RESULTS

Feed consumption, and body and muscle weights. Feed intake averaged 35.8 ± 1.5 (standard deviation) g/bird/day, and liveweight gain averaged 528 ± 17.8 g/bird over the 21-day study period. These variables and the wet weights of the sartorius (1.5 ± 0.1 g) and EDC (57.1 ± 3.5 mg) muscles on day 21 were not significantly affected by experimental treatments (n = 12; data not shown).

Muscle protein and lipid deposition. There were no effects of treatment on the total amount of protein and lipid deposited in the muscles studied nor on the phospholipid (PL) and triglyceride (TG) contents of the sartorius muscle (Table 3). The proportion of PL and TG in the muscle was age-dependent, irrespective of the treatment, the PL fraction being higher and the TG fraction lower at 3 wk of age ($p<0.05$).

PGE_2 synthesis. After 7 days of feeding FSO, PGE_2 synthesis in the EDC muscle was significantly depressed (Fig. 1); this inhibition was not evident at 2 or 4 days after initiating treatments (data not shown). The inhibition was proportional to the FSO content of the diet. The continuous administration of naproxen also inhibited PGE_2 production and its effect was quantitatively

TABLE 3

Sartorius Muscle Protein and Lipid Content[a]

	Diet			
	1	2	4	1 + Naproxen
Wet weight (g)	1.6 ± 0.2	1.5 ± 0.2	1.6 ± 0.2	1.4 ± 0.1
Protein (mg/muscle)	308 ± 43	294 ± 40	293 ± 53	275 ± 25
Lipid (mg/muscle)	81.1 ± 1.8^{bc}	87.6 ± 9.4^{bc}	92.9 ± 8.2^{c}	70.1 ± 5.5^{b}
Lipid classes (%)				
7 Days				
Phospholipid	24.6 ± 1.8	28.1 ± 3.5	27.3 ± 3.3	32.3 ± 1.7
Triglyceride	67.8 ± 3.6	66.2 ± 3.0	65.4 ± 3.0	61.2 ± 1.4
21 Days				
Phospholipid	35.9 ± 3.0	37.4 ± 3.2	36.8 ± 0.4	36.9 ± 0.1
Triglyceride	57.6 ± 1.6	55.9 ± 2.8	58.9 ± 3.3	58.0 ± 3.3

[a]Values in the same row with common superscripts are not different ($p<0.05$). Values are mean ± SEM. Values are for chicks fed from hatch to 3 wk except where indicated otherwise.

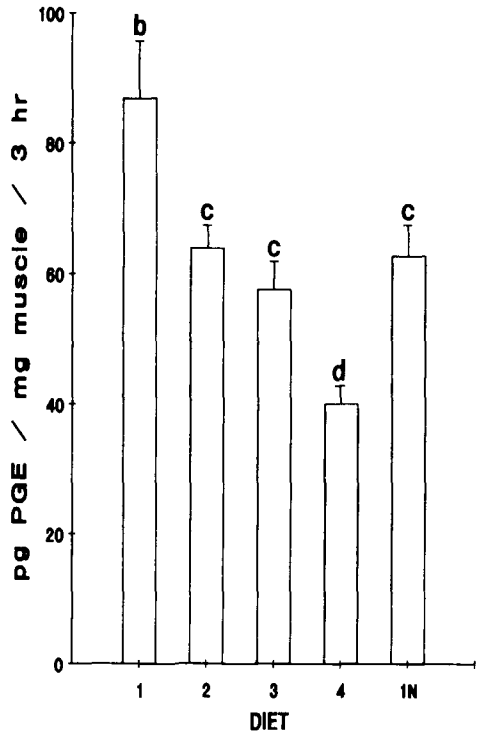


FIG. 1. PGE_2 synthesis in extensor digitorum communis muscle. Broiler chicks were fed diets described in Table 1 for a period of 7 days. One group of birds on Diet 1 received naproxen (N), an inhibitor of prostaglandin synthesis, in the drinking water (76 mg/L). At the end of the period of study birds were killed and the extensor digitorum communis muscles were incubated for the determination of PGE_2 synthesis. Values with different superscripts are different ($p<0.05$; n = 12). Values are mean ± SEM.

similar to that of the lower levels of FSO feeding. There was no further reduction in muscle PGE_2 synthesis when FSO was fed, or naproxen administered for longer periods up to 21 days.

Fatty acid composition of sartorius muscles. In order to simplify presentation of the results, only data obtained on day 21 are shown (Tables 4 and 5). Since initial statistical analysis revealed no effects of naproxen on the level of any fatty acid in PL or TG, these data were pooled with data from other birds fed diet 1.

Phospholipids. In spite of substantial differences in the levels of dietary saturated fatty acids, there were few differences seen in the levels of saturated fatty acids between groups (Table 4). Levels of several individual and total saturated fatty acids declined over the study period. Only 18:0 was significantly affected by diet, and was increased in FSO-fed chicks. Total monounsaturated fatty acids (MUFA) content decreased with increasing dietary FSO level. All of the MUFA, with the exception of 16:1ω7 were significantly decreased by the addition of FSO to the diets, and for 16:1ω7 and the three 18:1 fatty acids, these changes were progressive with continued feeding from 7 to 21 days.

There were marked alterations in the individual and total polyunsaturated fatty acid (PUFA) contents of sartorius PL when FSO was consumed. Only 18:2ω6 was not affected by diet. For many PUFA, these alterations were significantly related to the level of FSO fed, and/or progressive with time. Consumption of FSO reduced levels of 20:2ω6 and 20:3ω6. Arachidonic acid (20:4ω6) was significantly reduced within one week by the 4.5% FSO diet while feeding a 1.5% FSO diet even for 3 wk did not reduce 20:4ω6. The level of 22:4ω6 was depressed by FSO feeding and little or no 22:5ω6 was detected in birds consuming FSO. Total ω3 fatty acids increased as much as four-fold on the FSO diets, while total ω3 fatty acids decreased 40% in PL of birds fed only animal tallow. Levels of 18:3ω3 did not increase further after the first week, while levels of 20:3ω3, 20:5ω3 and 22:5ω3 continued to rise. Levels of 22:6ω3 were increased after 3 wk of feeding. As a result of these changes in PUFA levels, chicks fed FSO showed a marked increase in the index of unsaturation (sum of the product of the percentage unsaturated fatty acid and number of double bonds).

Triglycerides. Although the level of total saturated fatty acids was similar in TG and PL, levels of MUFA were higher and levels of PUFA were lower in the TG fraction (Table 5). In contrast to the PL fraction, FSO lowered levels of all saturated fatty acids except 20:0 in TG. These changes were progressive, and were related to the FSO level of the diet. Total and individual MUFA decreased with increased dietary FSO, and only 16:1ω7 was not affected by FSO. As in PL, 18:2ω6 was the principal PUFA

TABLE 4

Percent Fatty Acid Composition of Sartorius Muscle Phospholipid[a]

Fatty acids	Diet			Main Effects			
	1	2	4	Day	FSO	Level	EMS
Saturated (SAT)							
14:0	0.06[b]	0.30[b]	ND	NS	NS	NS	0.029
16:0	14.8[b]	14.8[b]	14.7[b]	NS	NS	NS	0.801
17:0	0.32[c]	0.58[b]	0.28[c]	**	NS	NS	0.012
18:0	17.8[c]	18.3[bc]	18.5[bc]	**	*	NS	0.465
20:0	0.08	0.21	ND	**	NS	NS	0.010
22:0	0.04	0.19	ND	NS	NS	NS	0.021
Σ SAT	33.1	34.4	33.4	**	NS	NS	1.732
Monounsaturated (MUFA)							
16:1ω9	0.16	0.06	ND	NS	NS	NS	0.014
16:1ω7	0.72	0.48	0.52	**	*	NS	0.023
18:1(*trans*)	1.32[b]	0.80[c]	0.61[c]	**	**	*	0.033
18:1ω9	18.6[b]	15.5[c]	15.5[c]	**	**	NS	1.198
18:1ω7	3.92[b]	3.34[c]	2.86[d]	**	**	**	0.024
20:1ω9	0.41	0.27	0.21	NS	**	NS	0.008
Σ MUFA	25.2[b]	20.5[cd]	19.7[d]	NS	**	*	1.647
Polyunsaturated (PUFA)							
18:2ω6	21.1	20.4	21.0	**	NS	NS	0.900
20:2ω6	1.04[b]	0.65[c]	0.57[c]	**	**	**	0.002
20:3ω6	1.09[b]	0.82[c]	0.84[c]	**	**	NS	0.002
20:4ω6	7.96[b]	7.21[b]	5.21[c]	**	**	*	0.811
22:4ω6	1.13[b]	0.68[c]	0.43[c]	**	**	**	0.028
22:5ω6	0.49[b]	0.14[b]	ND[c]	**	**	NS	0.037
Σ ω6	32.8[b]	29.9[bc]	28.1[c]	NS	**	NS	2.241
18:3ω3	0.31[d]	0.95[c]	2.00[b]	NS	**	**	0.012
20:3ω3	0.11[c]	0.15[c]	0.42[b]	*	**	**	0.017
20:5ω3	0.50[d]	1.53[c]	2.91[b]	**	**	**	0.013
22:5ω3	0.88[d]	2.50[c]	3.58[b]	**	**	**	0.034
22:6ω3	1.29[c]	3.72[b]	3.47[b]	NS	**	NS	0.357
Σ ω3	3.09[d]	8.84[c]	12.4[b]	**	**	**	0.715
Σ PUFA	35.9	38.8	40.5	NS	**	NS	3.970
Unsaturation index	127[c]	143[b]	149[b]	NS	**	NS	53.45
Others	5.86	6.30	6.43	**	NS	NS	0.366

[a]Values in the same row with common superscripts are not different ($p<0.05$; n=6, diet 1; n=3, diets 2,4). Significance of main effects, *, $p\leq0.05$; **, $p<0.01$, NS, $p>0.05$. FSO, flaxseed oil; EMS, error mean square; ND, not detectable; NS, not significant.

found in muscle TG, and comprised >85% of total PUFA in chicks fed AT only. Levels of 18:2ω6 acid were increased by FSO feeding. The levels of other ω6 fatty acids including 20:2ω6 and 20:3ω6 were reduced by FSO feeding, while levels of 20:4ω6 were low and not affected by diet. Neither 22:4ω6 or 22:5ω6 was present in TG. When FSO was fed, 18:3ω3 was deposited in the sartorius muscle TG, and its level increased progressively, and with the level of FSO fed, to as much as 48% of total PUFA. Levels of other ω3 fatty acids including 18:4ω3, 20:3ω3, 20:5ω3, 22:5ω3 and 22:6ω3 were low in TG compared with PL, but were increased by feeding FSO. As a result of these changes in PUFA, the unsaturation index of muscle TG was increased.

DISCUSSION

Muscle prostaglandin synthesis. The results of the present study indicate that PG synthesis by skeletal muscle in the rapidly growing broiler chick may be altered by dietary means on a rapid time scale. Dietary FSO decreased PG synthesis as much, or more, than an orally administered inhibitor of PG synthesis. At the concentration used here, naproxen has been shown to alter skeletal muscle protein metabolism in different pathological states (1-3). It remains to be clarified whether this would also be the case when PG synthesis is modified by dietary means. A depression in PG synthesis by feeding ω3 fatty acids is in agreement with data obtained on other tissues with other species of animals (6-8,10). Several factors may have contributed to the decrease in muscle PGE_2 production in chicks fed FSO. The depressed level of 20:4ω6, the precursor of PG of the 2 series, and enhanced levels of ω3 fatty acids of sartorius phospholipid, may help to explain the depression of PGE_2 synthesis by FSO. It has been proposed (7,10-12) that increased levels of ω3 fatty acids competitively inhibit the synthesis of 20:4ω6. The enhanced level of 20:5ω3 in the FSO groups may also have competitively inhibited the PG synthetase complex (10,23,24). Naproxen did not alter the fatty acid composition of muscle; this inhibitor's action is at the level of fatty acid cyclooxygenase (16).

TABLE 5

Percent Fatty Acid Composition of Sartorius Muscle Triglyceride[a]

Fatty acids	Diet			Main Effects			
	1	2	4	Day	FSO	Level	EMS
Saturated (SAT)							
14:0	1.04^b	0.92^c	0.56^d	**	**	**	0.002
15:0	0.14^b	0.19^b	0.07^c	*	*	**	0.002
16:0	21.8^b	19.6^b	18.0^c	**	**	**	1.831
17:0	0.39^b	0.35^b	0.19^c	**	**	**	0.003
18:0	7.01^b	6.89^b	4.90^c	*	**	**	0.351
20:0	0.19	0.12	0.13	NS	NS	NS	0.000
Σ SAT	30.6^b	28.1^b	23.9^c	**	**	**	2.375
Monounsaturated (MUFA)							
16:1ω9	0.60^b	0.54^c	0.48^c	**	**	*	0.001
16:1ω7	4.99	4.74	4.33	NS	NS	NS	0.527
18:1(*trans*)	2.91^b	2.68^b	0.74^c	**	**	**	0.213
18:1ω9	39.6^b	34.8^b	32.8^c	NS	**	**	3.442
18:1ω7	2.69^b	2.22^c	1.63^d	**	**	**	0.021
20:1ω9	0.50^b	0.41^c	0.30^d	**	**	**	0.001
Σ MUFA	51.2^b	45.4^b	40.2^c	NS	**	**	4.643
Polyunsaturated (PUFA)							
18:2ω6	14.3^c	15.8^b	16.3^b	**	**	NS	1.329
20:2ω6	0.20	0.12	0.11	NS	*	NS	0.003
20:3ω6	0.15	ND	0.11	**	**	**	0.001
20:4ω6	0.17^c	0.27^b	0.17^b	**	NS	NS	0.002
Σ ω6	14.8^c	16.2^b	16.7^b	**	**	NS	1.420
18:3ω3	1.28^d	8.03^c	16.8^b	**	**	**	2.225
18:4ω3	0.27^c	0.40^b	0.37^b	**	**	NS	0.002
20:3ω3	0.03	0.05	0.13	NS	**	*	0.002
20:5ω3	0.04^c	0.52^b	0.43^b	**	**	NS	0.009
22:5ω3	0.04^c	0.14^c	0.27^b	**	**	*	0.004
22:6ω3	0.04^c	0.13^c	0.31^b	**	**	*	0.007
Σ ω3	1.69^d	9.26^c	18.3^b	**	**	**	2.443
Σ PUFA	16.5^d	26.5^c	35.1^b	**	**	**	7.027
Unsaturation index	87.0^d	113^c	132^b	**	**	**	47.24
Others	1.78^b	1.53^b	0.87^c	**	**	**	0.072

[a]Values in the same row with common superscripts are not different ($p<0.05$; n=6, diet 1; n=3, diets 2,4). Significance of main effects, *, $p<0.05$; **, $p<0.01$, NS, $p>0.05$. FSO, flaxseed oil; EMS, error mean square; ND, not detectable; NS, not significant.

Fatty acid incorporation into skeletal muscle lipids. The levels of total lipid, PL and TG in skeletal muscle were not affected by composition of the diets fed in this study. PL are the main constituents of cell membranes and are relatively resistant to quantitative changes from dietary lipids (25,26). However, consistent with some results obtained on chicks (27), PL increased and TG decreased, with age in this study.

Fatty acid composition of muscle phospholipids. The fatty acid composition of PL was not strictly a reflection of that of the dietary lipids. Although levels of saturated fatty acids in the diets ranged from 18 to 30% of total fatty acids, these had little influence on the total saturated fatty acid incorporation into sartorius muscle PL. Levels of PUFA in the diets ranged from 25.5 to 52.6% of total fatty acids, yet levels of total PUFA in muscle PL varied little. Although total muscle MUFA decreased with decrease in dietary MUFA, differences in the PL MUFA were also small compared to differences in dietary total MUFA. Finally, although the ratios of saturated to unsaturated fatty acids were constant at 0.5–0.6 for all groups, the index of unsaturation was increased by FSO feeding. These observations support the hypothesis that a homeostatic mechanism prevents membranes from significant compositional changes in PL, thus preserving a relatively constant state of membrane fluidity (28). The lower level of 18:1 in the FSO groups may be due to its lower level in the FSO diets and/or a reduced rate of desaturation of endogenous 18:0 due to increased total substrate (18:2ω6, 18:3ω3) competing for desaturation.

The depressed level of 20:4ω6 in PL of the FSO groups in this study is consistent with that reported for microsomal fractions of white and dark meat of FSO-fed chickens (29). This may have been due to competition between 18:3ω3 and 18:2ω6 for Δ6 desaturation, thus limiting the synthesis of 20:4ω6 from 18:2ω6 (11,27). Our results also revealed that FSO diets reduced the deposition of 22:4ω6 and that virtually no 22:5ω6 was found after extended feeding. FSO decreased levels of ω6 fatty acids in a dose- and time-dependent fashion, and it seems likely that even greater modifications could be produced with longer feeding periods or higher levels of FSO in the

diet. Diet 2 contained 11% 18:3ω3 and did not affect PL levels of 20:4ω6 at any stage. However, the deposition of 22:4ω6 and 22:5ω6 was significantly reduced in chicks fed this diet for 3 wk. Diet 4 provided 26% 18:3ω3 and caused a reduction of 20:4ω6 within one week of feeding, but required a longer feeding period to significantly depress levels of 22:4ω6 and 22:5ω6. Studies with rats (25) showed that 18:3ω3 at levels less than 15% of dietary fat did not inhibit the conversion of 18:2ω6 to 20:4ω6 but the subsequent conversion to the C_{22} PUFA was reduced, as seen here.

Although the muscle total PL PUFA was similar for all the dietary groups, there was a dose- and time-dependent change in the ratios of ω6/ω3 and 20:4ω6/20:5ω3 with FSO feeding. The present results thus show that ω3 PUFA of the diet is incorporated into chick muscle PL. The accumulation of ω3 fatty acids in the FSO-fed chicks is not surprising since 18:3ω3, the predominant fatty acid in FSO, serves as the precursor in the synthesis of longer chain ω3 fatty acids by Δ6 and Δ5 desaturases (11,30). Other reports have not been consistent in demonstrating long-chain ω3 fatty acids in skeletal muscle. For example, 20:5ω3 was detected in rat (12) and pig (15) muscle, but not in muscle of chicks (29) and rats (31). These discrepancies may be due to the use of total muscle lipids in these studies, where the low content of this fatty acid and its derivatives in TG would tend to mask changes in PL.

Fatty acid composition of muscle triglycerides. In the TG fraction, as the level of FSO in the diet increased, total saturated fatty acids and MUFA decreased, while total PUFA increased, in direct relationship to their levels in the diets. There would appear to be a much greater incorporation of dietary fatty acids into TG than PL; for example the maximum level of 18:3ω3 in TG reached 16.8%, while in PL this fatty acid reached 2.0%. Longer-chain derivatives of the dietary fatty acids were less abundant in the TG than in the PL, also tending to reflect storage of fatty acids in the TG fraction rather than metabolism.

These studies demonstrate the inhibitory effect of 18:3ω3 from FSO on PGE_2 synthesis in the chick. Chicks metabolize 18:3ω3 to longer chain ω3 fatty acids; this in turn enriches the tissue lipids with ω3 and alters the pattern of fatty acid incorporation. Although the composition of PL was generally less influenced than that of TG, it is apparent that both fractions contain dietary fatty acids as well as elongated and desaturated derivatives. The altered fatty acid composition may have implications for muscle metabolism. For example, in related studies it has been shown that the incorporation of ω3 fatty acids into muscle lipids, by feeding fish oil, increases the responsiveness of glucose transport to insulin (32). The importance of fatty acid composition for other membrane-associated functions of muscle including substrate transport, excitation/contraction coupling, and lysosomal sequestration, and for PG-dependent processes (1–3) remains to be characterized.

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Distribution Among Tissues of Intravenously Administered Sucrose Octaoleate

F.H. Mattson[1] and **R.J. Jandacek***
Miami Valley Laboratories, Procter & Gamble Company, Cincinnati, Ohio 45239-8707

The distribution of [^{14}C]oleate label in rat tissues in the 6 hr after intravenous administration of sucrose octa-[^{14}C]oleate (7.5 mg; SuO_8) was compared with that observed after administration of [^{14}C]triolein. The [^{14}C]-oleate label, whether injected as triolein emulsion, or as chylomicrons obtained from donor animals, rapidly cleared from the serum; only 10% or less remained in the serum 15 min after injection. Labeled SuO_8 disappeared less rapidly from the serum; about one-third of the dose was present after 15 min, and after 120 min 14% remained. In the liver, there was an initial greater accumulation of fatty acid label when an emulsion of either triolein or SuO_8 was given rather than the chylomicrons. The octa-ester continued to accumulate in the liver throughout the 6 hr of study, and 78% of the initial dose was present at that time. By contrast, although one-third of the triolein, as of SuO_8, was found in the liver shortly after injection, levels subsequently decreased; at 6 hr, 12% of the label remained associated with that organ. A small portion, up to 8% of the acid label, whether administered as chylomicrons or as a triolein emulsion, was found in the epididymal fat pads. Smaller amounts, usually 1% or less, of the [^{14}C]oleate label were found in fat pads following the injection of labeled SuO_8. In a separate study, the levels of acid label in the liver and spleen were monitored for 21 days following the intravenous administration of [^{14}C]SuO_8. There was an initial accumulation of approximately half of the injected lipid label in the liver and one-quarter in the spleen. By day 21, the level in the liver had decreased to one-third of that administered, while the level in spleen remained at one-quarter.
***Lipids 26,* 750–753 (1991).**

Olestra (sucrose polyester) is the now common name for the mixture of hexa-, hepta-, and octaesters that results from esterification of sucrose with long-chain fatty acids derived from edible fats. Olestra has the physical, culinary, and organoleptic properties of customary dietary fat of similar fatty acid composition. However, the biological behavior of olestra differs markedly. Unlike triglyceride, olestra is not hydrolyzed by the lipolytic enzymes of the pancreas (1). Hence, olestra is not absorbed, but rather is excreted unchanged in the feces (2,3). Olestra has also been reported to reduce the absorption of dietary cholesterol (4), presumably through entrapment of intestinal cholesterol in the non-absorbable oil phase (5), which in turn results in a decrease of plasma total cholesterol and low-density lipoprotein cholesterol (6,7). Olestra's nonabsorbability and its physical similarity with common fats have suggested its utility as a noncaloric, partial replacement for dietary fat.

Although olestra is not absorbed, it was nevertheless, of interest to determine its distribution in body tissues following intravenous (i.v.) injection. Rats were therefore injected with an emulsion of [^{14}C]oleate-labeled sucrose octaoleate (SuO_8), one of the major components of olestra. The levels of labeled lipid in serum, liver, and adipose tissue were then monitored over 6 hr, and over 21 days in liver and spleen. The fate of [^{14}C]oleate-labeled chylomicrons and emulsified [^{14}C]triolein was followed over 6 hr after i.v. administration.

MATERIALS AND METHODS

Animals. Male Sprague-Dawley rats (Charles River Laboratories, Portage, MI) were used in all studies. The rats were housed in individual stainless steel cages in a room maintained at 24 ± 3°C and at 30–70% relative humidity, using a twelve-hour light/dark cycle. The animals received rat chow (Ralston-Purina, St. Louis, MO) and tap water *ad libitum,* unless noted otherwise.

Materials. [^{14}C]Oleic acid used was homogeneous as judged by thin-layer chromatography (TLC). Triolein was prepared by base-catalyzed esterification of glycerol. Following purification by Florisil column chromatography, the isolated triolein was shown by TLC to have a purity of greater than 99%. SuO_8 was synthesized similarly; the octaester fraction from the Florisil column was better than 99% pure.

A portion of the labeled triolein was administered *per os* to rats fitted with a thoracic duct cannula (3). The lymph of these animals was collected and the chylomicrons isolated (8). The isolated chylomicrons were taken up in 0.85% NaCl to a lipid concentration of 15 mg/mL.

Emulsions of triolein and SuO_8 were prepared by the method of Rodbell and Scow (9) at a lipid concentration of 125 mg/mL. On the day of injection, 1 mL of lipid emulsion was added dropwise, while vortexing, to 7.5 mL of fresh serum obtained from rats that had been fasted for 24 hr. The final concentration of labeled lipids was 15 mg/mL. The specific activities of these preparations were: SuO_8, 6.9 μCi/g; triolein, 2.7 μCi/g; and chylomicron lipids, 1.1 μCi/g.

Experimental protocol. In the first study, the early fate of intravenously administered SuO_8 was determined. A control group received a similar emulsion of oleate-labeled triolein. The effect of the emulsifying system was examined by injecting rats with chylomicrons that were harvested from the lymph of rats fed [^{14}C]oleate. Based on the results obtained, the duration of a subsequent study was extended to encompass 21 days, but observation was limited to liver and spleen. Emulsified triolein or chylomicrons were not administered in the long-term study.

Prior to treatment, rats were fasted overnight. However, in order to minimize catabolism of the injected lipids, the "water" bottles during this period supplied 20% glucose and 0.45% NaCl (w/v). Two hours prior to i.v. injection, the rats were given 5 mL of the glucose-saline solution by stomach tube (10). Immediately prior to use, the lipid preparation was passed through a 1.2-μm Millipore filter and 0.5 mL of the filtrate was injected into the tail vein.

[1] Present address: Department of Medicine, University of California, San Diego, California 92093.

*To whom correspondence should be addressed.

The exact weight of administered emulsion was determined by weighing the syringe before and after injection. Each animal received 7.5 ± 0.2 mg of labeled lipid. With each injection, an aliquot of the material administered was taken for radiochemical assay. The animals were returned to their cages and offered water and Purina Laboratory Chow (Ralston Purina).

At the time points indicated, animals were killed by guillotine. In the initial study, the blood was collected from the carcass, allowed to clot, and the serum, which was recovered by centrifugation, was weighed. The liver was flushed *in situ* with 0.85% NaCl until pallid. Tissues were removed, rinsed, blotted dry, weighed, and stored at −4°C. To verify the success of the i.v. injection, the entire tail was severed and assayed for labeled lipid.

Tissue analysis. Serum, homogenized tissues, and the emulsions administered were extracted with $CHCl_3$/MeOH (2:1, v/v). The chloroform phase was washed with phosphate buffer, dried with Na_2SO_4, and evaporated to dryness. The lipid extract was assayed for ^{14}C label by liquid scintillation counting.

Serum was assumed to constitute 3.5% of body weight. Values for liver and spleen were obtained by analysis of the entire organ. Values given for epididymal fat pads are representative of total label accumulation, but do not indicate the absolute amounts of label in total adipose tissue.

After i.v. administration, the tails of some animals contained more than 5% of the material administered indicating faulty injection; values from these animals were not considered. In the animals used, remaining label at the injection site was typically only 1 to 2 percent of the dose injected.

Statistical methods. To compare the fit of a piecewise (two pool) linear regression model with a single (one pool) model, a piecewise mode was fitted to the log-transformed blood serum data for each treatment, assuming a common point of intersection (at 60 min for the two segments of the regression lines) (11). The fit of the piecewise model was then compared with the single linear trend model for each treatment (12). Once we had verified the piecewise model as providing the best fit, we checked the point of intersection, by estimating separately for each treatment the point of intersection of the two trend lines (13). To make comparisons between the three treatments, the average of the three estimates for the point of intersection was used to fit a single model with dummy variables to accommodate a piecewise linear regression for each treatment. F-tests of the appropriate linear combinations of the regression coefficients were used to test hypotheses corresponding to linear combinations of the parameters of the three piecewise regression lines. For each linear regression, the correlation coefficient (R) was calculated (14).

A single model (one pool), with dummy variables to accommodate a simple linear regression for each treatment, was fitted to the data for each tissue, *i.e.*, liver and fat pads without log transformation. F-tests of the appropriate regression coefficients were used to test hypotheses corresponding to the parameters of the three regression lines.

RESULTS

The percentage recoveries in serum of the intravenously administered ^{14}C-labeled lipids are given in Figure 1.

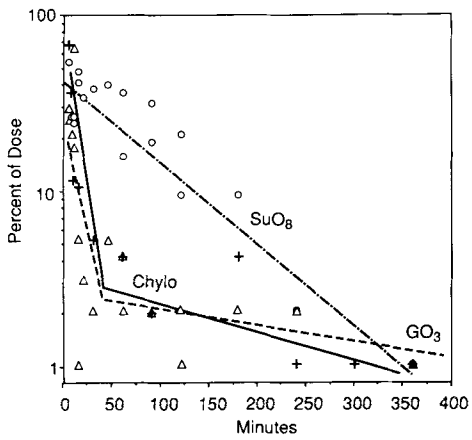


FIG. 1. Percent of ^{14}C administered in serum lipids after i.v. injection into rats at (t = 0) of sucrose octa-[^{14}C]oleate (SuO_8, O, —·—) ^{14}C-labeled triolein (GO_3, Δ, ——) or chylomicrons (Chylo, +, ——).

TABLE 1

Equations Describing the Two Pools for Chylomicrons and Remaining Serum Levels[a]

	Log % of dose	R
Pool 1		
Chyl	1.727 − 0.0382t[b]	0.79
GO_3	1.330 − 0.277t	0.58
Pool 2		
Chyl	0.520 − 0.00158t	0.62
GO_3	0.393 − 0.00084t	0.59
Time of breakpoint of pool 1 and 2:		
Chyl	33.0 min	
GO_3	34.9 min	

[a]Rate of removal from the serum was calculated from the data in Figure 1.

[b]t, time in minutes.

Statistical analyses showed the disappearance of the administered chylomicrons (Chyl) and triolein emulsion (GO_3) to fit a two-pool model. The rates of removal calculated from the data in these plots of the initial study are given in Table 1. The rates of removal for the chylomicrons and for the triolein emulsion from the pools do not differ statistically ($p > 0.1$). The pattern seen following the administration of the sucrose ester (SuO_8) was quite different, with a one-pool model sufficiently describing the data. The rate of removal of the material, log % of dose = 1.63 − 0.00451t (t = min, R = 0.94), was slower than that observed for pool 1 of the chylomicrons and triolein in serum (Table 1). Whereas only 10% or less of the label from the administered chylomicrons or triolein was present in the serum 15 min after injection, the same level was still found two hours following SuO_8 injection.

The portions of ^{14}C-labeled lipids found in the liver are shown in Figure 2. The three preparations each exhibited different patterns. Shortly after administration, approximately 12% of the label that was given as chylomicrons was present in liver. Subsequently the level, as percent of dose, decreased at a linear rate, 12.12 − 0.020t (R =

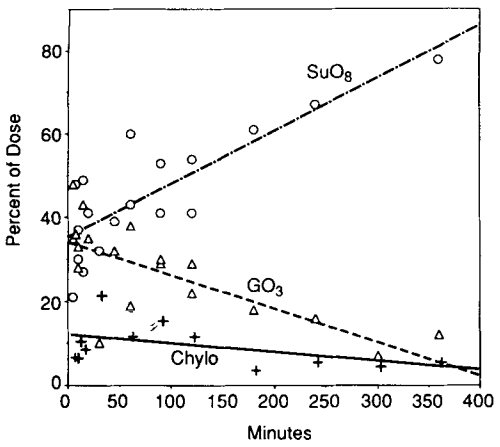


FIG. 2. Percent of ^{14}C administered found in the livers of rats after i.v. injection (at t = 0) of sucrose octa-[^{14}C]oleate (SuO_8, O, —·—), ^{14}C-labeled triolein (GO_3, Δ, ——) or chylomicrons (Chylo, +, —).

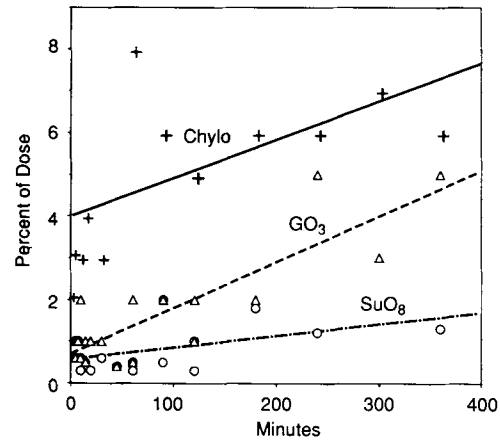


FIG. 3. Percent of ^{14}C administered in the epididymal fat pads of rats after i.v. injection (at t = 0) of sucrose octa-[^{14}C]oleate (SuO_8, O, —·—), ^{14}C-labeled triolein (GO_3, Δ, ——) or chylomicrons (Chylo, +, —).

0.48). When the labeled lipid was administered as an emulsion, whether as GO_3 or SuO_8, approximately 35% of the dose rapidly appeared in liver. The level supplied as GO_3 subsequently decreased; the rate as percent of dose was 34.44 − 0.078t (R = 0.3). When the label was administered as SuO_8, the level in the liver increased throughout the study. The rate as percent of dose was 35.31 + 0.126t (R = 0.82). Six hours after intravenous injection, 80% of the label given as SuO_8 was found in the liver, whereas that organ contained only about 10% of the label from the other lipids. The rates of change for the labeled lipid supplied by the three sources, chylomicrons, GO_3, or SuO_8, differed significantly from each other ($p < 0.05$).

Throughout the course of the experiment, less than 2% of the labeled lipid was found in the epididymal fat pads following administration of SuO_8 (Fig. 3). When the labeled fatty acid was given as triolein emulsion (GO_3), a somewhat higher level was found in the fat pads. An even larger amount, up to 8%, was found following the injection of chylomicrons (chyl). The accumulation of labeled

TABLE 2

Percentage of Sucrose Octa-[^{14}C]oleate Found in Liver and Spleen of Rats at Various Time Points After Intravenous Administration[a]

	Days after injection						
Tissue	1/4	1	2	3	7	14	21
Liver	60	51	53	58	35	49	34
Spleen	22	22	29	24	31	15	26
Liver and spleen	82	73	82	82	66	64	60
Number of animals	5	3	3	4	1	1	2

[a]At zero time each animal received i.v. (tail vein) 7.5 mg of sucrose octa-[^{14}C]oleate. Subsequently, the animals had access to Purina Laboratory Chow.

fatty acid from the three sources as a function of time as percentage of dose was: chyl, 3.90 + 0.00948t (R = 0.62); GO_3, 0.704 + 0.011t (R = 0.855); SuO_8, 0.558 + 0.0030t (R = 0.517). The rates in the GO_3 and chyl equations did not differ significantly ($p = 0.66$), but both differed from the rate of SuO_8 ($p < 0.05$). The intercepts of the GO_3 and SuO_8 equations differed from that of the chyl equation ($p < 0.0001$), but not from each other ($p = 0.70$).

In a subsequent study that was of longer duration, the tissue levels of ^{14}C-labeled lipids upon intravenous injection of the SuO_8 emulsion were followed. During the first three days following SuO_8 administration, serum, lung, and epididymal fat pads each contained less than 1% of the dose (data not shown). As shown in Table 2, initially 60% of the ^{14}C label that was administered as SuO_8 was found in liver lipids. Another 22% was recovered in the spleen. Since the mass of the spleen is typically 3 to 5% of that of the liver, the affinity of SuO_8 for spleen was greater than that for liver, when expressed as per unit mass of tissue. Over the course of the study there was a decrease in label in the liver; label in spleen was essentially unchanged. Even 21 days after injection, 60% of the dose was still present in both liver and spleen.

DISCUSSION

Following ingestion, triglycerides are partially hydrolyzed (1), and the products of digestion are absorbed (3). By contrast, SuO_8 is not hydrolyzed (1) and is not absorbed (1,3). The fate of the two lipids also differs when they are administered intravenously. Triglycerides are rapidly removed from the blood stream and distributed to various tissues and organs where they undergo a sequence of catabolic conversion. By contrast, SuO_8 disappears from the blood stream more slowly. A major portion of SuO_8 is taken up by the liver and spleen where it is retained tenaciously.

A concern in this study was that the emulsifying system used may affect the metabolic fate of SuO_8 as it affects in part the fate of triolein (15). Therefore, triolein was administered in the same emulsifying system as was SuO_8 and the redistribution was compared with that of chylomicron lipids. The disappearance of lipids from the chylomicron and triolein emulsions (Fig. 1) followed a two-pool pattern with similar rates (Table 1). The half-life of both in pool 1 was approximately 10 min, similar to values reported by others (16–18). Removal of the triolein

emulsion from serum was similar to that of chylomicrons. Since the same emulsifying system was used for SuO_8, the markedly different pattern of removal of SuO_8 from serum does not appear to be attributable to the emulsifying system employed, but rather appears to be a consequence of differing metabolic pathways.

The greater accumulation in the liver and subsequent slower release of triolein administered as emulsion, rather than as chylomicrons, has been observed by others (19-21), and this effect was attributed to the emulsified fat in the serum being taken up by the reticulo-endothelial system of the liver. In the present study (Fig. 2), as in those of others, the fatty acids from triolein initially accumulated in the respective organ and subsequently were slowly released. After several hours, the level in the liver approximated that seen following administration of the chylomicrons. The emulsified sucrose octaoleate, like the triolein emulsion, was rapidly taken up by the liver, here too probably by the reticulo-endothelial system. However, unlike the triolein, the labeled lipid in the liver subsequently increased rather than decreased. Three days following the injection of sucrose octaoleate, about 80% of the dose was recovered in liver and spleen (Table 2). By day 21, the amount of SuO_8 in liver and spleen had decreased, but only to 60% of the dose that was administered. This decrease could be due to redistribution to other tissues, secretion into the bile, or catabolism. The accompanying paper by Jandacek and Holcombe (22) shows that a major route of removal from the liver is by way of the bile with subsequent egestion in the feces. Whether other pathways are operative as well remains to be determined.

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The Excretion and Characterization of Intravenously Administered Olestra

R.J. Jandacek* and **B.N. Holcombe**
Miami Valley Laboratories, The Procter and Gamble Company, Cincinnati, Ohio 45239-8707

Although sucrose octaoleate that is consumed is neither digested or absorbed, following intravenous injection it is found mainly in the liver. Olestra is a mixture of the hexa-, hepta-, and octaesters of sucrose. To follow the metabolic fate of intravenously administered [^{14}C]sucrose-labeled olestra, we measured its urinary elimination, and the rate of excretion of ^{14}C in the feces, and characterized the ^{14}C-labeled material that appeared in bile. The fecal excretion for days 4-14 after dosing was found to be first order with the half-life of the injected olestra being 5.0 ± 0.5 days. The ^{14}C recovered in the bile was soluble in chloroform. Two-dimensional thin-layer chromatography autoradiograms of the biliary lipid showed the pattern of the biliary ^{14}C to be essentially the same as that of the dosed olestra. Biliary excretion and subsequent fecal egestion of essentially unhydrolyzed sucrose esters is the principal route for the removal of intravenously administered olestra. Only traces of ^{14}C were found in the urine.
Lipids 26, 754–758 (1991).

Olestra, the mixture of the hexa, hepta, and octa long-chain fatty acid esters of sucrose, is not digested (1) and not absorbed (2). Investigation of the fate of the material in tissues other than the gastrointestinal tract is possible only following intravenous administration. Such a study, described in the preceding paper (3), showed that one component of olestra, sucrose octaoleate, is principally taken up and retained by the liver and spleen. To further investigate the fate of intravenously administered sucrose polyesters, we have utilized ^{14}C-labeled olestra to determine the rate of excretion and the identity of the excreted labeled material.

In three studies [^{14}C]sucrose-labeled olestra was administered intravenously to rats. We measured organ uptake and the rate of fecal excretion of ^{14}C (Studies 1 and 2), measured the elimination of urine (Study 2), and characterized the labeled material in excreted bile (Study 3).

MATERIALS AND METHODS

Animals. Young, adult male Sprague-Dawley rats (Charles River Laboratories, Portage, MI) were utilized in all studies. Body weights ranged from 163–172 g in Study 1, 110–125 g in Study 2, and 180–200 g in Study 3. Housing and feeding were the same as described previously (3). The schedule for assay of tissues and excreta is given in Table 1.

Materials. [^{14}C]Olestra was synthesized by base-catalyzed reaction of methyl oleate and [^{14}C] sucrose (uniformly labeled). The specific activity was 0.6 μCi/mg in Study 1. This level of activity proved to be sufficient for the tissue and fecal assays used in this study. The components of this material were resolved by one-dimensional thin-layer chromatography (TLC) silica gel GF, heptane/toluene/methanol, 100:94:6, v/v/v). Its composition was found to be 65% octaester and 34% combined hexa- and heptaester, based on a radio-scan of the plate. The R_f of the octaester was 0.88–1.0; the R_f values of the hexa- and heptaesters, consisting of isomer mixtures, ranged from 0.28–0.87. The distribution of mass determined by charring a plate after TLC development was consistent with the radiochemical scan.

To increase the assay sensitivity for the urinary excretion and biliary characterization required in Studies 2 and 3, olestra of higher specific activity, 8.9 μCi/mg, was obtained. The ester distribution of this olestra sample was 60% octaester and 39% combined hepta- and hexaester, as determined by the TLC method described above. The material used in Studies 2 and 3 was also analyzed by two-dimensional TLC [north, hexane/diethyl ether/acetic acid (190:10:2, v/v/v); east, same solvents (140:60:2); north, same solvents (190:10:2, v/v/v)]. An autoradiogram of the two-dimensional TLC plate was made by placing X-ray film in contact with the plate after development. The film was developed after 30 days of dark storage of plate and film. The autoradiogram was consistent with the two-dimensional olestra pattern observed on a charred TLC plate. The two-dimensional TLC procedure and autoradiography were also used in the analysis of biliary lipids by visual comparison of position and intensity of exposed areas.

Dosing regimen. After an overnight fast, the animals were anesthetized with Nembutal (0.1 mL/100 g body weight, intraperitoneal), and an incision made in a hind

TABLE 1

Design and Assay Schedule of the Three Studies of Intravenous [^{14}C]Olestra Administration to Rats

Study	Design	Assay schedule
1	14-Day measurement of fecal excretion and tissue distribution of ^{14}C.	5 Animals—necropsy and tissues at 24 hr. 4 Animals—necropsy and tissues at 7 days. 4 Animals—fecal collection days 1–14; necropsy and tissues at day 14.
2	35-Day study of excretion and tissue accumulation.	4 Animals fecal and urine samples, days 1–4, 11–15. Necropsy and tissues at day 35.
3	5-Day study of biliary excretions.	Bile collection from 3 animals for 5 days.

*To whom correspondence should be addressed at The Procter and Gamble Miami Valley Laboratories, P.O. Box 398707, Cincinnati, OH 45239-8707.

Abbreviations: TLC, thin-layer chromatography.

limb to expose the saphenous vein. The preparation of the dosing emulsion was according to the procedure of Rodbell and Scow (4) as described in the preceding paper (3). The emulsion was injected into the vein and the incision closed with a suture. The syringe was weighed before and after dosing to determine the mass of injected emulsion. A typical dose was 0.6 g of emulsion, and the amount of olestra administered was approximately 800 μg in Study 1. The availability of higher specific activity material allowed a reduction of dose to 450 μg in Study 2, and 300 μg in Study 3.

Collection of excreta, bile, and tissues. In Studies 1 and 2, a screen was attached beneath the cage of each animal and 24-hr collections of feces were made daily throughout Study 1, and collectively for days 1–5 and 11–15 of Study 2. Samples were stored frozen until analyzed. In Study 2, urine from the animals was collected in iced containers during the fecal collection periods.

In Study 3, after injection of the emulsion and while the animals were still anesthetized, their bile ducts were cannulated. The animals were placed in restraining cages. Bile was collected daily in containers in ice baths and then frozen until analyzed. The animals were provided chow pellets and water. At the times shown in the Table 1, the animals were killed by carbon dioxide inhalation. Livers, spleens, kidneys, lungs, and epididymal fat pads were removed and frozen until analyzed. In Study 3, the absence of bile in the cannula in one animal prevented biliary analysis, although the livers from all four animals were analyzed. Fecal samples from Study 1 were mixed with enough distilled water and then ground so as to form a pasty suspension. Samples were combusted and the trapped carbon dioxide assayed for radioactivity.

Multiple aliquots confirmed the homogeneity of the mixture. In Study 2, fecal lipids were extracted with chloroform/methanol (2:1, v/v) and aliquots of the washed chloroform were assayed for radioactivity. Radioactivity in urine was measured by direct scintillation counting of an aliquot of urine in a gelled scintillation solution.

Analysis of excreta, tissues, and bile. All tissues were homogenized and extracted with chloroform/methanol (2:1, v/v), washed with a solution of KH_2PO_4 and then dried as described in the preceding paper (3).

To determine total radioactivity, aliquots of the bile were counted directly. Another portion of the bile was extracted with chloroform/methanol (2:1, v/v) which was then washed with a solution of KH_2PO_4 (2%, wt). The chloroform and aqueous phases were each assayed for radioactivity. Following evaporation of a portion of the chloroform, the lipid residue was analyzed by two-dimensional TLC as described above. Unlabeled olestra (35 μg) was spotted on the plate at the origin directly over the bile extract before development to provide a mass that could be visualized by charring for comparison with the distribution of radioactivity on the plate.

RESULTS

Distribution of olestra in tissues. The distribution of ^{14}C in the tissues taken in Study 1 is shown in Table 2. As reported in the previous study (3), most of the radioactivity was found in the liver. The radioactivity in the spleen accounted for less than 2.5% of the dose, a fraction that is much less than was previously observed. A large decrease in the radioactivity in the livers was evident between days 1 and 7. The levels on days 7 and 14 (Study 1) and day 35 (Study 3) did not differ appreciably from each other. The radioactivity in spleen did not change with time. Less than 1% of the administered material was found in the other tissues that were analyzed. The recovery of radioactivity from all of the tissues that were examined was approximately 35% at days 7 and 14.

The concentrations of olestra in the spleen and liver were similar in this study. At 24 hr after dosing, the concentration in the spleen was 4.1 ± 0.5% of dose/g of tissue compared with 6.9 ± 0.4% of dose/g of tissue for the liver. Thus the accumulation of the olestra in the liver reflects the mass of the organ, typically 20–30 times that of the spleen, rather than a greater affinity for the liver.

Excretion. The recovery of ^{14}C in feces, urine, and bile is summarized in Table 3. A significant portion was found in bile and feces from intact animals (Studies 1 and 2); only trace amounts were in the urine (Study 2). Less than 0.1% of the activity was recovered in the feces of the bile duct-cannulated rats in Study 3.

Extraction of bile with chloroform/methanol (2:1, v/v) resulted in recovery of essentially all of the label in the chloroform phase; only trace amounts were in the aqueous phase. This extract was separated by two-dimensional TLC. Autoradiograms of this material (Fig. 1b) and of the injected olestra (Fig. 1a) were similar. The majority of the activity in the bile was associated with octa-, hepta-, and hexaesters.

TABLE 2

Distribution of Carbon-14[a] in Tissues of Rats Injected Intravenously with [^{14}C]Sucrose-Labeled Olestra[b]

	Time after dosing[c]				
Tissue	24 hr (n=5)	5 Days (n=4)	7 Days (n=4)	14 Days (n=4)	35 Days (n=4)
Liver	68.6 (2.1)	64.7 ± 3.3	27.9 (7.7)	32.7 (2.9)	33.3 (6.8)
Spleen	2.2 (0.2)		1.1 (0.4)	1.6 (0.3)	2.4 (0.7)
Kidney	0.5 (0.2)		0.4 (0.1)	0.5 (0.2)	0.1 (0.02)
Lung	0.2 (0.03)		0.2 (0.02)	0.1 (0.02)	
Fat pad	0.2 (0.1)		0.2 (0.1)	0.2 (0.1)	0.03 (0.01)

[a]Percent of dose, mean (SEM).

[b]A mixture of the hexa, hepta, and octa long-chain fatty acid esters of sucrose.

[c]Data at 35 days are from Study 2; at 5 days, from animals with bile-duct cannulation in Study 3; other data from Study 1.

TABLE 3

Recovery[a] at Indicated Times of ^{14}C in Bile, Urine, and Feces of Animals Following Intravenous Injection with [^{14}C]Sucrose-Labeled Olestra

Study	Day	Feces	Urine	Bile
1 (n=4)	1	1.5 (0.8)		
	2	2.1 (0.4)		
	3	4.7 (0.6)		
	4	5.0 (0.2)		
	5	4.2 (0.2)		
	6	3.8 (0.2)		
	7	3.1 (0.3)		
	8	2.8 (0.2)		
	9	2.5 (0.1)		
	10	2.3 (0.2)		
	11	1.8 (0.2)		
	12	1.5 (0.1)		
	13	1.7 (0.2)		
	14	1.0 (0.1)		
2 (n=4)	1-5	21.6 (5.2)	0.2 (0.03)	
	11-15	8.0 (2.4)	0.12 (0.02)	
3 (n=3)	1	0.02 (0.004)		0.9 (0.06)
	2	0.05 (0.01)		4.6 (1.2)
	3	0.05 (0.01)		6.3 (1.9)
	4	0.04 (0.01)		4.1 (1.3)
	5	0.03 (0.01)		1.2 (0.6)

[a]Percent of dose, mean (SEM).

DISCUSSION

The sucrose esters administered intravenously were found primarily in the liver at levels similar to those seen in the preceding study (3). Accumulation in the spleen was also evident, but to a lesser extent than previously seen (3). Even some five weeks after administration, a considerable portion was still present in those organs. The possible metabolic fate of the remainder of the intravenously administered olestra includes hydrolysis, distribution to other tissues, and excretion into bile and feces. If hydrolysis had occurred, the resulting free sucrose would have been excreted in urine. Since no more than a trace of ^{14}C was found in the urine, this pathway was not significant. The presence of only trace amounts of ^{14}C in tissues other than the liver and spleen indicates that general distribution to tissues is insignificant. The presence of appreciable levels of ^{14}C in bile and feces shows this to be the major pathway for removal. After discharge into the intestinal lumen, olestra administered intravenously, like that consumed in the diet, is not digested or absorbed, but is evacuated as a component of the feces.

From the data in Study 1 the rate constant of fecal excretion was calculated for each rat for days 4-14 from the least squares determination of the linear equations for the logarithm of the daily excretion *vs.* time. The rate constant was found to be 0.14 ± 0.01 days^{-1}. This value corresponds to a half life of 5.0 ± 0.5 days for the injected olestra. The amounts of injected ^{14}C found in the livers of the rats at 14 days (32.7%, Table 1), 21 days (34%, ref. 3), and 35 days (33.3%, Table 1) suggest a slower rate of removal. It is possible that the rate calculated from the fecal data for the initial 14 days reflects a pool that turns over rapidly, whereas a pool that turns over more slowly is indicated by the liver values.

The feces of unoperated animals from Studies 1 and 2 contained appreciable levels of ^{14}C, whereas the feces of the animals fitted with bile duct cannulas had quite low levels of activity. This low fecal activity conforms with the appreciable activity that was present in the bile of these animals.

A comparison of the TLC autoradiograms of the excreted biliary radioactivity (Fig. 1b) with the autoradiogram of the injected olestra (Fig. 1a) shows that olestra is not significantly metabolized or hydrolyzed prior to excretion in bile. Essentially all of the excreted radioactivity was found in the areas of the TLC plate corresponding to the octaester (area A), and the several isomers of the hepta- and hexaesters (area B) of olestra. The radioactivity of the administered material found in area C, whose identity is not known, did not appear in the bile. Traces of radioactivity in the bile outside of areas A and B might have resulted from a small amount of hydrolysis to more polar sucrose esters, but it is clear that the principal components of olestra, as administered, account for essentially all of the excreted radioactivity. Apparently, olestra can be suspended by bile acids and phospholipids and then excreted into the intestine. Presumably the olestra in bile was suspended in the biliary aqueous system as an emulsion rather than in solution, since its size would exclude it from the mixed micelles of the components of bile. The bile was normal in appearance, as might be predicted from the very low concentration of olestra. Since the sucrose esters excreted in the bile are similar in composition to olestra (Fig. 1), which is not absorbed, this excreted material, like dietary olestra, passes out in the feces.

The deposition of olestra in the liver seen in these studies is consistent with that reported in the accom-

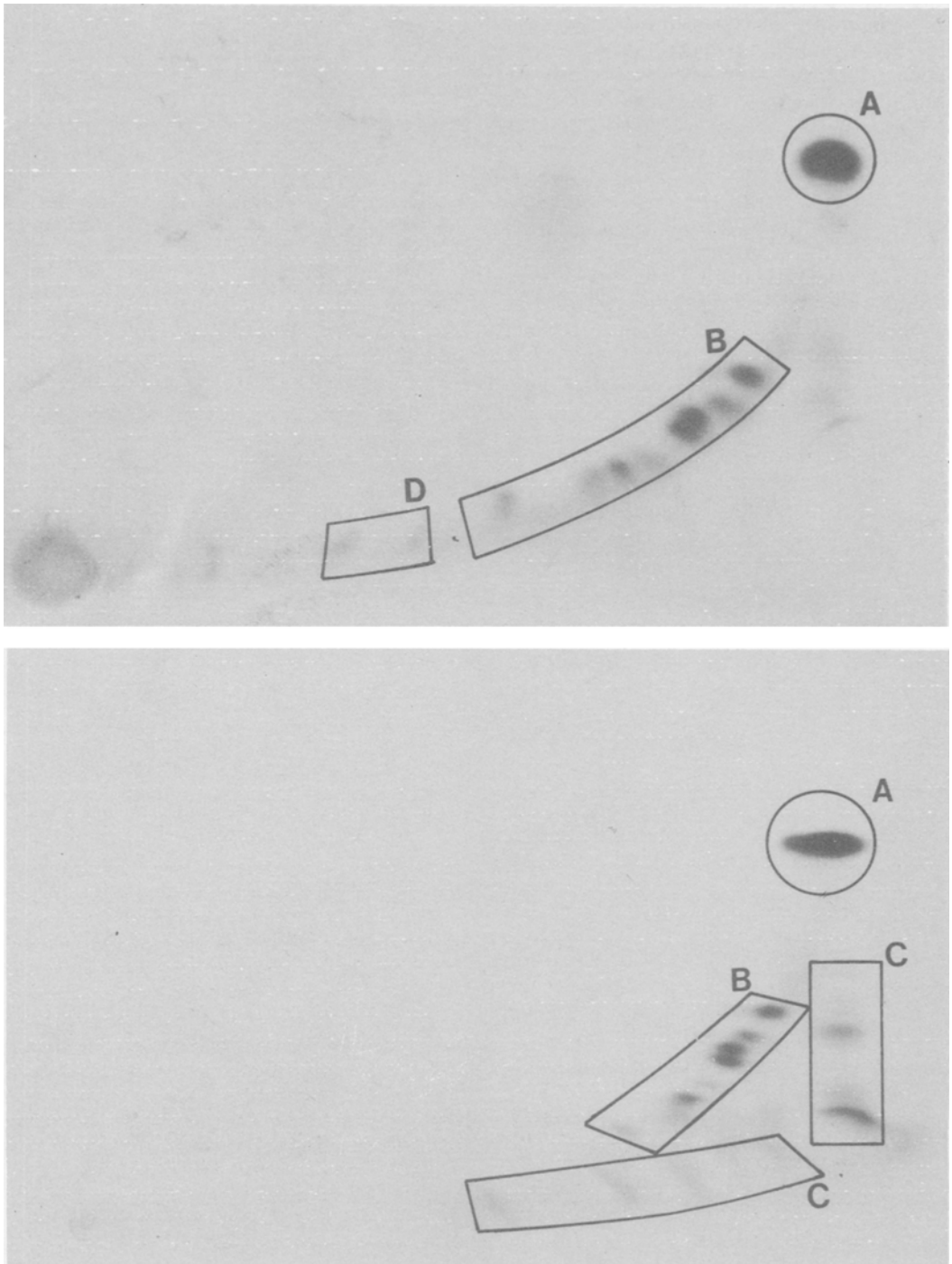


FIG. 1. Top (1a). Two-dimensional TLC autoradiogram of the [^{14}C]olestra used in Studies 2 and 3. A denotes the octaester; B, hepta- and hexaesters; C, unknown species. Bottom (1b). Two-dimensional TLC autoradiogram of the chloroform-soluble ^{14}C extracted from rat bile in Study 3. A and B are as in 1a; traces of radioactivity in area D may reflect lower esters.

panying paper (3). The fraction of the injected dose found in the spleen, however, is approximately 10% of that previously found. This smaller accumulation in contrast with that seen in the previous study (3) suggests that the accumulation in the spleen was dependent on the dose of injected fat. Thus, the high level found in the spleen in the previous paper (3) resulted when 7.5 mg of sucrose octaoleate was injected, whereas the 300–800 μg of olestra administered in this study resulted in only a small accumulation in that organ. Since the olestra used in this study comprised more than 60% sucrose octaester, it seems unlikely that the different levels of accumulation in the spleen resulted from the compositional differences of the olestra and sucrose octaoleate.

Thus far it has not been possible to demonstrate the absorption of any olestra by rats (2) or humans (5). Since it is impossible to establish zero absorption of any material, the conclusion from each of those studies was necessarily that within the limits of the sensitivity of the methods that were used, absorption could not be demonstrated. The results obtained in this and the preceding paper partially resolve the fate of sucrose polyesters, even if trace amounts were absorbed. Such material would be rapidly removed from the circulation, primarily by the liver and secondarily by the spleen—probably by the reticulo-endothelial system. Subsequently, the sucrose ester would be slowly released and excreted in the bile. In the intestinal tract the biliary excreted sucrose ester, like that taken orally, would be egested in the feces.

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Interaction Between Dietary Proteins and Lipids in the Regulation of Serum and Liver Lipids in the Rabbit. Effect of Fish Protein[1]

N. Bergeron, Y. Deshaies[2], C. Lavigne and H. Jacques*
Département de Nutrition Humaine et de Consommation, Faculté des Sciences de L'Agriculture et de L'Alimentation, Université Laval, Québec G1K 7P4, Canada

Purified diets varying in dietary protein, namely casein (CA), soy protein (SP), fish protein (FP), and lipid origin (corn oil (CN), coconut oil (CO)) were fed to rabbits to evaluate the effects of protein and fat source, as well as protein-lipid interactions, on serum total, lipoprotein and hepatic lipid levels. Dietary proteins and lipids exerted a separate effect on serum total cholesterol (C), very low-density lipoprotein cholesterol (VLDL-C), and low-density lipoprotein cholesterol to high density lipoprotein cholesterol (LDL-C/HDL-C) ratio. Hence, CA increased serum cholesterol compared to SP, while coconut oil enhanced serum and VLDL-C, and decreased LDL-C/HDL-C compared to corn oil. Dietary proteins interacted with dietary lipids to modulate HDL-C levels. Thus, FP maintained a high level of HDL-C regardless of lipid origin, compared to CA and SP whose HDL-C levels were decreased by corn oil, compared to coconut oil. A dietary protein-lipid interaction was also observed in the regulation of liver cholesterol levels. Coconut oil, compared to corn oil, decreased liver cholesterol in rabbits fed FP, whereas hepatic cholesterol concentration was unaltered by dietary lipid source in CA- and SP-fed rabbits. These results demonstrate that dietary proteins act synergistically with dietary lipids to regulate cholesterol metabolism in the rabbit.
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The hypercholesterolemic effect of casein compared to soy protein, irrespective of the high saturated or polyunsaturated nature of the fats included in the purified diets of rabbits, is well established (1,2). Nevertheless, the origin of dietary lipids has been shown to affect the magnitude of the cholesterolemic response of rabbits to dietary proteins (3). Butter, a fat rich in saturated fatty acids, has been reported to intensify casein-induced hypercholesterolemia compared to corn oil, a fat rich in polyunsaturated fatty acids (2).

The effect of fish protein as well as the modulatory role exerted by saturated and unsaturated dietary lipids included in a fish protein diet on cholesterol metabolism are more variable and still poorly understood. While fish protein exerts a cholesterolemic action similar to that of casein when fed to the rabbit as part of a low-fat (1%) corn oil diet (2), fish protein is hypocholesterolemic compared to casein, when included in a high-fat (14%) coconut oil diet (4). In a corn oil-coconut oil diet with an intermediate lipid content (5%), fish protein exerts a cholesterolemic action intermediary to that of casein and soy protein (5). Meanwhile, regardless of the lipid content of the purified diet, fish protein, compared to casein (4,5) and soy protein (5), has consistently been shown to increase high-density lipoprotein cholesterol (HDL-C) levels in the rabbit. The variation in serum cholesterol levels induced by fish protein when included in purified diets which differ in their lipid content and origin suggests that dietary proteins and lipids may act synergistically to regulate cholesterol metabolism in the rabbit.

Numerous studies have been conducted to determine the individual effects of various dietary proteins (1,2) or lipids (6) on serum and lipoprotein cholesterol levels. However, studies that have been statistically designed to isolate the dietary protein effects from the dietary lipid effects in rabbits (3,7) and other animal models (8-10) are scarce. It is therefore not possible at present to identify with certainty which specific nutrients are responsible for serum total and lipoprotein cholesterol changes subsequent to feeding purified diets. Inasmuch as dietary proteins and lipids are normally consumed as part of a mixed diet and are thus liable to exert an overall effect on cholesterol metabolism, the present study was designed by means of a 3 × 2 factorial model, to isolate the dietary protein effect from the dietary lipid effect, and to detect the interaction between dietary proteins and lipids simultaneously present in purified diets on cholesterol metabolism in the rabbit. For this purpose, rabbits were fed casein, soy protein and fish protein in purified diets differing in their lipid sources. The latter consisted of either corn oil, rich in n-6 polyunsaturated linoleic acid (18:2n-6), or coconut oil, rich in saturated lauric acid (12:0), two dietary lipid sources known to have contrasting effects on lipid and lipoprotein metabolism (6).

MATERIALS AND METHODS

Experimental animals. Upon arrival, 53 male New Zealand white rabbits were housed individually in stainless steel cages with mesh floors, suspended over sawdust in a room maintained under constant conditions of temperature (20-24°C), lighting (12-hr cycle) and humidity (45-55%). Following a 5-day adaptation to the animal quarters where animals were maintained on a non-purified diet (NPD) (Performance blend no. 5301, Ralston Purina, Québec, Canada), rabbits were divided into 6 experimental groups balanced for initial body weight (1400 g), and gradually adapted to their experimental rations by feeding mixtures of NPD and purified diets over a 5-day period. For the duration of the 30-day study, fresh food and water were provided once daily on an *ad libitum* basis. Food

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[2]Département de Physiologie, Faculté de Médecine, Université Laval, Québec, Canada G1K7P4.

*To whom correspondence should be addressed at the Département de Nutrition Humaine et de Consommation, Pavillon Paul-Comtois, Université Laval, Québec, Canada G1K 7P4.

Abbreviations: CA-CN, casein-corn oil; CA-CO, casein-coconut oil; C, cholesterol; FP-CN, fish protein-corn oil; FP-CO, fish protein-coconut oil; GWGE, gross weight gain efficiency; HDL, high-density lipoproteins; LDL, low-density lipoproteins; LPL, lipoprotein lipase; NPD, non-purified diet; PUFA, polyunsaturated fatty acids; SFA, saturated fatty acids; SP-CN, soy protein-corn oil; SP-CO, soy protein-coconut oil; TG, triglycerides; VLDL, very low-density lipoproteins.

intake was measured daily while growth of animals was monitored twice a week.

Purified diets. In the present study high fat (11%), cholesterol-free, powdered purified diets, assigned to rabbits in a 3 × 2 factorial arrangement, consisted of either casein-corn oil (CA-CN), casein-coconut oil (CA-CO); soy protein-corn oil (SP-CN), soy protein-coconut oil (SP-CO); fish protein-corn oil (FP-CN) and fish protein-coconut oil (FP-CO). The composition of the corn oil and coconut oil based purified diets is detailed in Table 1. The coconut oil diets were supplemented with 1% corn oil in order to meet the essential fatty acid requirements of the rabbit (11). The nutrient content of the vitamin and mineral mix has been described in a previous study (5). Casein and soy protein were purchased from ICN Biomedicals (Cleveland, OH). Fish protein, prepared in our laboratory, was obtained by lyophilization of frozen cod fillets which were then delipidated in an industrial Soxhlet-type apparatus during 12-hr cycles, using a mixture of ethyl acetate/hexane (1:1, v/v) in order to extract both neutral and polar lipids. The delipidation procedure was done with the collaboration of the Food Research and Development Centre (St-Hyacinthe, Québec, Canada). Residual lipid content of casein (0.02%), soy protein (0.4%) and fish protein (0.2%) was verified with a Goldfish Lipid Extractor (model 35001, Labconco Corporation, Kansas City, MO). The protein content (N × 6.25) of casein (88.5%), soy protein (87.4%) and fish protein (92.0%) was assayed by the Kjeldahl method using a Kjell Foss autoanalyzer (Model 16210, Foss Co., Hillerod, Denmark). The level of protein in the purified diets was then adjusted to 20% on an isonitrogenous basis, at the expense of cornstarch. As confirmed by bomb calorimetry, the CA-CN (4.71 kcal/g), CA-CO (4.64 kcal/g), SP-CN (4.65 kcal/g), SP-CO (4.56 kcal/g), FP-CN (4.59 kcal/g) and FP-CO (4.43 kcal/g) purified diets were isocaloric providing an average of 4.60 kcal/g of feed. The American Institute of Nutrition (AIN) mineral mix, cornstarch, and cellulose (Alphacel) were purchased from ICN Biomedicals. Coconut oil was obtained from Sigma Chemical Company (St. Louis, MO) and the vitamin mix was purchased from Teklad test diets (Madison, WI). Corn oil was bought from a local food market.

Serum, lipoprotein and hepatic lipid analyses. At the end of the 30-day experimental period, 18-hr fasted rabbits were submitted to a light ketamine-xylazine anesthesia (0.4 mL/kg body weight) before extracting blood by cardiac puncture. Blood samples were allowed to clot at room temperature, followed by low-speed centrifugation to isolate serum. Livers were also removed, weighed, frozen in liquid nitrogen, and stored at −70°C until subsequent chloroform/methanol (2:1, v/v) extraction of hepatic lipids by the method of Folch *et al.* (12). All 4-mL aliquots of serum were stained with Sudan black dye (13) to later visualize the lipoprotein bands, and were submitted to a 25-hr discontinuous gradient ultracentrifugation (14) using a SW-41 swinging bucket rotor in a Beckman ultracentrifuge, model L8 M70 (Palo Alto, CA). Very low-density lipoproteins (VLDL; d< 1.006 g/mL), low-density lipoproteins (LDL; 1.006 < d < 1.063 g/mL) and high-density lipoproteins (HDL; 1.063 < d < 1.21 g/mL) thus separated were collected by aspiration and frozen at −70°C until determination of their cholesterol and triglyceride (TG) contents.

Serum, liver and lipoprotein cholesterol and TG concentrations were determined enzymatically using the CHOD-PAP and triglycerides without free glycerol kits supplied by Boehringer Mannheim (Laval, Québec, Canada) according to the methods described by Siedel *et al.* (15) and Kohlmeier (16), respectively.

Statistical analyses. Data were subjected to an analysis of variance (ANOVA) according to a 3 × 2 factorial arrangement (17) in a randomized block design using the statistical analysis system (SAS) general linear model (GLM) procedure allowing for missing cells in factorial models (18), in order to determine the main protein and lipid effects, and to identify any interaction among dietary protein and lipid sources (P × L) at a probability level inferior to 0.05. Overall dietary protein and lipid effects, as well as protein-lipid interactions detected by ANOVA, were then submitted to least square mean (LSmean) multiple comparisons (18) to identify the statistical differences (P<0.05) among the groups. All data were log transformed (18) prior to statistical analysis in order to make comparisons among homogeneous means. Values in tables and figures are however expressed as the original means ± SEM.

TABLE 1

Composition of the Purified Diets[a]

Ingredients	Corn oil diets	Coconut oil diets
	(g/100 g feed)	
Protein	20	20
Cornstarch	51	51
Corn oil	11	1
Coconut oil	—	10
Cellulose	12	12
Vitamin mix	1	1
Mineral mix	5	5

[a]Composition of the vitamin and mineral mix has been described previously (5).

RESULTS

Food intake, weight gain and gross weight gain efficiency (GWGE) (g of body weight gained/g of food ingested daily) are presented in Table 2. Although the CA-CO rabbits had a significantly (P<0.05) lower food intake than the animals of the other groups, weight gain and GWGE were similar in all dietary groups. These results indicate that all experimental rations supported normal and linear growth throughout the study period and were thus nutritionally adequate and equivalent.

Mean serum total cholesterol, VLDL-C, LDL-C and LDL-C/HDL-C are listed in Table 3. The overall analyses of variance performed on these data are reported in the top half of Table 4, while the multiple comparisons are shown on the bottom half of this table. As indicated by the P values lower than 0.05, both dietary proteins and lipids separately and significantly affected serum total cholesterol, VLDL-C, and the LDL-C/HDL-C ratio. Hence feeding FP, as well as CA, significantly (P<0.05) increased serum total cholesterol compared to SP. By comparison, VLDL-C concentrations were significantly (P<0.05) decreased by FP, in contrast to the CA and SP groups

TABLE 2

Food Intake, Weight Gain and Gross Weight Gain Efficiency (GWGE) of Rabbits Fed the Purified Diets[a]

Dietary group	n[b]	Food intake (g/day)	Weight gain (g/day)	GWGE
CA-CN	9	55.2 ± 3.5[c]	14.8 ± 1.4[c]	0.26 ± 0.01[c]
CA-CO	10	45.0 ± 3.2[d]	10.9 ± 2.2[c]	0.23 ± 0.04[c]
SP-CN	8	57.6 ± 2.5[c]	14.0 ± 1.6[c]	0.24 ± 0.03[c]
SP-CO	10	55.5 ± 3.8[c]	11.6 ± 2.3[c]	0.19 ± 0.04[c]
FP-CN	6	62.1 ± 5.0[c]	18.0 ± 2.5[c]	0.28 ± 0.03[c]
FP-CO	10	55.5 ± 2.4[c]	16.7 ± 1.7[c]	0.28 ± 0.02[c]

[a]Means ± SEM. Values in a given column bearing different letters are significantly different (P<0.05). CA, casein; SP, soy protein; FP, fish protein; CN, corn oil; CO, coconut oil.

[b]Number of rabbits per group.

TABLE 3

Mean Serum Total and Lipoprotein Cholesterol Levels in Rabbits Fed the Purified Diets[a]

Dietary group	Cholesterol (mg/dL)			
	Total	VLDL	LDL	LDL-C/HDL-C
CA-CN	70 ± 10	4 ± 1	28 ± 7	1.5 ± 0.6
CA-CO	99 ± 10	10 ± 1	33 ± 7	0.8 ± 0.2
SP-CN	37 ± 2	4 ± 1	15 ± 2	1.6 ± 0.5
SP-CO	71 ± 9	8 ± 3	25 ± 8	1.0 ± 0.4
FP-CN	79 ± 14	3 ± 1	23 ± 3	0.5 ± 0.1
FP-CO	81 ± 5	2 ± 1	20 ± 3	0.5 ± 0.1

[a]Refer to Table 4 for two-way analysis of variance performed on these data. Means ± SEM. CA, casein; SP, soy protein; FP, fish protein; CN, corn oil; CO, coconut oil.

whose VLDL-C levels were similar. The lipid effect observed translated into a significant (P<0.05) enhancement in serum total cholesterol and VLDL-C in the CA and SP groups fed coconut oil, in relation to corn oil. Interestingly, this rise in serum and VLDL-C consequent to coconut oil compared to corn oil feeding was not observed in the FP group (Table 3), indicating that dietary proteins tended to act synergistically with dietary lipids to regulate serum total cholesterol (P<0.1) and VLDL-C (P<0.08) levels.

Although LDL-C levels were not affected by the type of dietary protein or lipid, the LDL-C/HDL-C ratio was significantly influenced by diet composition. Thus, FP caused a significant (P<0.05) decrease in LDL-C/HDL-C ratio compared to CA and SP, which induced similar LDL/HDL ratios. Moreover, feeding corn oil, in contrast to coconut oil, significantly (P<0.05) increased the LDL-C/HDL-C ratio in the CA and SP groups. This lipid effect was again not experienced by the FP-fed rabbits.

The significant (P<0.05) interactions illustrated in Figures 1 and 2 indicate that proteins and lipids exerted synergistic, as opposed to distinct, effects on HDL and hepatic cholesterol levels, respectively. Although corn oil, compared to coconut oil, significantly (P<0.05) decreased HDL-C in the rabbits fed CA-CN and SP-CN diets, it did not have this effect with FP; HDL-C levels remained high and unchanged in both the FP-CN- and FP-CO-fed rabbits (Fig. 1). Concomitantly, feeding the FP-CO and

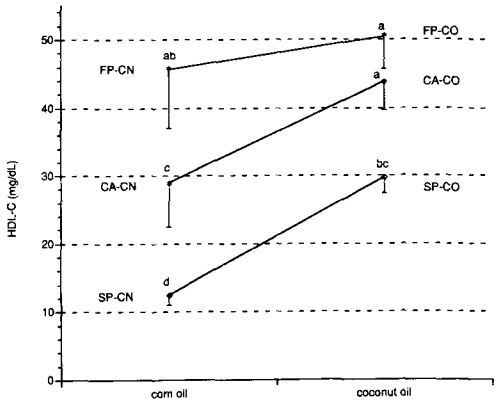


FIG. 1. Protein-lipid interaction on HDL-C levels. Values are expressed as mean ± SEM. Groups bearing different letters are significantly different at P < 0.05.

TABLE 4

Overall Analysis of Variance of the Dietary Protein and Lipid Effects on Serum Total, Hepatic and Lipoprotein Cholesterol Levels

Source of variation	P values					
	Total-C	VLDL-C	LDL-C	HDL-C	LDL-C/HDL-C	liver-C
Protein	0.0005	0.0001	0.1	0.0001	0.03	0.001
Lipid	0.0002	0.02	0.4	0.0001	0.04	0.04
P × L	0.1	0.08	0.6	**0.04**	0.5	**0.03**
Comparisons[a]						
Protein	CA > SP FP = CA FP > SP	CA = SP FP < CA FP < SP	CA = SP FP = CA FP = SP	**Figure 1**	CA = SP FP < CA FP < SP	**Figure 2**
Lipid	CO > CN	CO > CN	CO = CN		CO < CN	

[a]The symbols designate: =, no significant difference among the groups at P<0.05; >, significantly higher than the group to which it is compared at P<0.05. CA, casein; SP, soy protein; FP, fish protein; CN, corn oil; CO, coconut oil.

FP-CN diets resulted in a significant ($P<0.05$) rise in HDL-C, compared to the respective counterparts fed the SP-CO, SP-CN, and CA-CN diets. However, when included in coconut oil-based diets FP-CO and CA-CO exerted similar effects on HDL-C levels.

Similarly, while hepatic cholesterol levels (Fig. 2) induced by CA and SP were unaffected by the origin of dietary lipids, feeding coconut oil, compared to corn oil, resulted in a significant ($P<0.05$) decrease in liver cholesterol in the FP rabbits. The P × L interaction on hepatic cholesterol may thus be ascribed to FP. Nevertheless, when included in a corn oil diet, the liver cholesterol level of the FP-CN rabbits was intermediary to, and not significantly different from that of the CA-CN and SP-CN groups. By comparison, when incorporated into a coconut oil diet, FP-CO and SP-CO caused a significant ($P<0.05$) reduction in liver cholesterol, compared to CA-CO. A significant ($P<0.05$) increase in hepatic cholesterol concentration was observed in the rabbits fed CA, in contrast to SP, irrespective of the saturated or n-6 polyunsaturated origin of dietary lipids.

Mean serum, hepatic and lipoprotein TG levels are presented in Table 5. The overall ANOVA, along with the multiple comparisons are summarized in Table 6. Although no significant differences were observed in serum total TG levels, which remained low in all dietary groups, dietary proteins significantly ($P<0.05$) affected VLDL-TG and HDL-TG, whereas dietary lipids significantly ($P<0.05$) influenced the liver and HDL-TG concentration. Interestingly, in parallel to its cholesterol lowering effect in

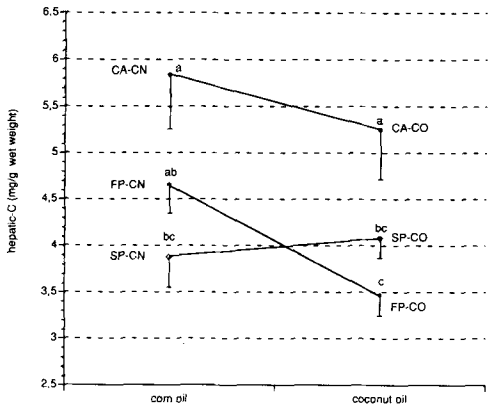


FIG. 2. Protein-lipid interaction on hepatic cholesterol levels. Values are expressed as mean ± SEM. Groups bearing different letters are significantly different at $P < 0.05$.

TABLE 5

Mean Serum Total, Hepatic and Lipoprotein Triglyceride Levels in Rabbits Fed the Purified Diets[a]

Dietary group	TG (mg/dL)				Liver TG (mg/g wet weight)
	Total	VLDL	LDL	HDL	
CA-CN	25 ± 2	7 ± 1	7 ± 1	9 ± 2	6 ± 1
CA-CO	32 ± 3	14 ± 2	6 ± 1	11 ± 1	13 ± 2
SP-CN	33 ± 5	14 ± 3	9 ± 1	8 ± 1	7 ± 2
SP-CO	39 ± 8	17 ± 5	10 ± 3	11 ± 1	14 ± 3
FP-CN	23 ± 3	6 ± 1	6 ± 1	11 ± 2	4 ± 1
FP-CO	26 ± 2	6 ± 1	7 ± 1	13 ± 1	8 ± 2

[a]Refer to Table 6 for two-way analysis of variance performed on these data. Means ± SEM. CA, casein; SP, soy protein; FP, fish protein; CN, corn oil; CO, coconut oil.

TABLE 6

Overall Analysis of Variance of the Dietary Protein and Lipid Effects on Serum Total, Hepatic and Lipoprotein Triglyceride Levels

Source of variation	P values				
	Total-TG	VLDL-TG	LDL-TG	HDL-TG	liver-TG
Protein	0.1	0.0007	0.5	0.02	0.1
Lipid	0.1	0.07	0.4	0.007	0.0007
P × L	0.8	0.2	0.9	0.6	0.7
Comparisons[a]					
Protein	CA = SP FP = CA FP = SP	CA = SP FP < CA FP < SP	CA = SP FP = CA FP = SP	CA = SP FP > CA FP > SP	CA = SP FP = CA FP = SP
Lipid	CO = CN	CO = CN	CO = CN	CO > CN	CO > CN

[a]The symbols designate: =, no significant difference among the groups at $P<0.05$; >, significantly higher than the group to which it is compared at $P<0.05$. CA, casein; SP, soy protein; FP, fish protein; CN, corn oil; CO, coconut oil.

VLDL, FP significantly ($P<0.05$) decreased VLDL-TG and concomitantly, significantly ($P<0.05$) raised HDL-TG, in relation to the CA and SP groups. No significant difference was observed in serum, lipoprotein and liver TG levels of the CA- and SP-fed rabbits. The significant lipid effect on HDL and hepatic TG was translated by a significant ($P<0.05$) coconut oil-induced rise in TG, compared to corn oil. VLDL-TG were however unaffected by dietary lipids. Likewise, dietary proteins did not interact with dietary lipids to regulate serum total, lipoprotein and hepatic TG levels in the rabbit.

DISCUSSION

Results from the current investigation confirm the recognized hypercholesterolemic effect of casein in relation to soy protein (1). Moreover, in agreement with previous trials (2,5), FP was shown to induce serum cholesterol levels similar to those of the CA-fed rabbits (2,5) and greater to those of the SP-fed rabbits (2). Likewise the cholesterol-raising effect of coconut oil, compared to corn oil, observed in CA and SP groups is consistent with the established hypercholesterolemic influence of saturated in contrast to polyunsaturated fatty acids (PUFA) (6,19). However, this lipid effect was not expressed in the FP groups where feeding coconut oil (FP-CO), compared to corn oil (FP-CN), failed to further enhance serum cholesterol levels. In this sense, although dietary proteins and lipids were shown to exert a separate effect on serum cholesterol in the CA- and SP-fed rabbits, as also demonstrated by Sanchez *et al.* (7), the present results suggest that dietary proteins tended to act synergistically with the lipid component of the purified diet to modulate serum cholesterol levels in rabbits fed fish protein.

Consistent with prior observations, FP, despite its similarity to CA with respect to serum total cholesterol levels (5), was shown to significantly increase HDL-C, rendering this lipoprotein the predominant serum cholesterol carrier (4,5). High HDL-C levels observed in rabbits fed both FP-CN and FP-CO diets demonstrate that dietary proteins and lipids acted synergistically and suggest that the FP effect may override the lipid effect in the regulation of HDL-C levels in the rabbit. Likewise, dietary proteins tended ($P<0.08$) to interact with dietary lipids to down-regulate VLDL-C levels, in contrast to CA and SP. Absence of significant differences in VLDL-C between CA and SP groups could be expected since changes in VLDL-C concentrations have been reported to occur only when serum cholesterolemia exceeds 600 mg/dL (20).

Since a hypercholesterolemic state has been shown to be conditional to LDL-C increase in the rabbit (20), failure to observe changes in LDL-C levels in the present study may be due to serum cholesterol levels which remained below 100 mg/dL in all dietary groups. The cornstarch content of the purified diets may be responsible for the low serum, and thus unaltered LDL cholesterol levels observed in the present investigation. Cornstarch diets, as opposed to molasses-dextrose purified diets, have recently been shown to attenuate serum total cholesterol and LDL-C response to casein feeding (21). Despite the similar LDL-C levels observed, feeding FP resulted in an average 2.5-fold decrease in LDL-C/HDL-C ratio compared to CA and SP. Furthermore, the increase in LDL-C/HDL-C ratio in CA and SP rabbits fed corn oil, as compared to coconut oil, is consistent with the established HDL-C lowering action of n-6 PUFA against saturated fatty acids (SFA) (6, 10). It is however noteworthy that FP again seemed to counteract the lipid effect to maintain low LDL-C/HDL-C ratios. These findings are in agreement with those of previous studies where FP, by raising HDL-C (4,5), has constantly been reported to decrease LDL-C/HDL-C (5) as well as aortic atherosclerosis (4) compared to CA. The present results suggest that whereas CA and SP may generate a beneficial lipoprotein profile depending on the dietary lipid source to which they are combined, FP, by increasing HDL-C, may consistently be efficient in reverse cholesterol transport in the rabbit, whether combined to saturated or polyunsaturated dietary fats.

Absence of protein-induced changes in serum TG levels is in agreement with data reported by Bauer (22). However, coconut oil, in contrast to corn oil intake has already been associated with an increase in serum TG, and a decrease in liver TG, in casein-fed rabbits (23). Since TG metabolism is also known to be influenced by dietary carbohydrate origin (21,24), discrepancies may be due to the 40% sucrose content of the purified diets used by Kritchevsky *et al.* (23), while the current diets provided 51% cornstarch. Indeed, coconut oil supplemented to a non-purified diet (NPD) containing complex carbohydrates has been shown to result in an efficient removal of TG-rich particles and unaltered TG levels in the rabbit (19).

The assembly of HDL particles is known to be closely related to VLDL metabolism (25). HDL lipid and protein constituents have been shown to result from the lipoprotein lipase (LPL)-dependent lipolysis of VLDL (25). Increased catabolism of VLDL, activated by LPL, in turn enhances HDL formation. Hence, decreased VLDL-C coupled to the concomitant rise in HDL-C consequent to FP feeding suggests that the FP-induced rise in HDL-C may result from increased VLDL catabolism either through the promoted activity of LPL, or consequent to a more efficient catabolism due to changes in VLDL composition. The similar low VLDL-TG and high HDL-TG relationship observed in FP groups provides further evidence that the FP-induced increase in HDL may depend on the activity of LPL. In CA and SP rabbits, the increase in cholesterol and TG content in both VLDL and HDL upon feeding coconut oil, as opposed to corn oil, is in agreement with the enhanced production rate of apo A-I and HDL particles demonstrated by Quig and Zilversmit (19) in coconut oil-fed rabbits.

The differential influence of dietary proteins on cholesterol metabolism has been suggested to originate from a hepatic cholesterol saturation threshold (4.64 mg liver cholesterol/g wet weight) (26) and suppressed liver receptor-mediated uptake of circulating lipoproteins (27) causing an increase in serum cholesterol in CA- as opposed to SP-fed rabbits. These findings are consistent with the current data where a direct relationship was observed between liver and serum cholesterol levels which both rose in rabbits fed CA, while they both remained low in the SP groups. Conversely, while serum cholesterolemia induced by FP and CA were similar, FP decreased liver cholesterol in the coconut oil-fed rabbits, in contrast to corn oil-fed animals, to a level below that observed in CA rabbits. These results suggest that FP acted synergistically with coconut oil to decrease cholesterolemia below the liver saturation threshold and thus prevent a serum

cholesterol rise in rabbits fed the FP-CO compared to FP-CN diets.

Although CA and SP, as well as coconut oil and corn oil, are established potent regulators of cholesterol metabolism, the present investigation has additionally shown that these nutrients act separately, as opposed to synergistically, to modulate serum, lipoprotein and liver lipid distribution in the rabbit. However, dietary proteins were shown to interact with dietary lipids to regulate liver cholesterol concentration, and maintain high HDL-C levels in FP-fed rabbits. Combining FP to other dietary lipids varying in degree of unsaturation would, however, be required to confirm the overriding effect of FP on cholesterol metabolism in the rabbit. Nevertheless, the HDL-C rise, combined with the VLDL-C decrease, currently observed in FP-fed rabbits suggests that the formation of HDL particles may be regulated by the extent of VLDL catabolism. These results emphasize the need to further investigate the activity of lipoprotein lipase as well as apoprotein C-II-induced LPL activation of TG-rich particle catabolism consequent to fish protein feeding.

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METHOD

A Simple Assay for Lipid Hydroperoxides Based on Triphenylphosphine Oxidation and High-Performance Liquid Chromatography

Takashi Nakamura* and **Hiroyuki Maeda**
Laboratory of Fisheries Technology, Faculty of Agriculture, Kyushu University, Fukuoka 812, Japan

A simple and sensitive method was developed to measure total hydroperoxides. After the reduction of hydroperoxides with triphenylphosphine (TP) in cyclohexane at 30°C, the amount of triphenylphosphine oxide (TPO) produced is determined by reverse-phase or normal-phase high-performance liquid chromatography (HPLC) in combination with an ultraviolet (UV)-detector by measuring absorption at 220 or 260 nm. TPO was shown to be generated stoichiometrically by reduction of known amounts of either cumene hydroperoxide or methyl 13-hydroperoxyoctadecadienoate. For various lipids at low levels of oxidation, the peroxide values determined by this method were in good agreement with those obtained by conventional iodometry. The detection limit of TPO in HPLC using absorption at 220 nm was less than 10 pmol. Consequently, total hydroperoxides in lipids at levels corresponding to less than a peroxide value of 1 can be estimated by the TP method on a 10-mg sample.
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Lipid peroxidation in biological systems has been implicated in the pathogenesis of various degenerative diseases (1–3). Hence, there is considerable interest in the development of methods to determine very small quantities of hydroperoxides, the primary products of lipid oxidation. Among these methods, the most popular is the thiobarbituric acid (TBA) test (4–6). This test is based on the acid-catalyzed decomposition of lipid peroxides to form malondialdehyde, and its subsequent reaction with TBA to form a red chromogen. The TBA test is neither specific for hydroperoxides, nor is it positive for all lipid hydroperoxides. Spectrophotometric methods based on the oxidation of iodide ions (7–9), ferrous ions (10), sesamol dimer (11) and methylene blue (12) by hydroperoxides are also available. Nanomolar levels of hydroperoxides can be determined by these methods; however, sensitivity problems exist and the procedures remain tedious.

In recent years, methods based on high-performance liquid chromatography (HPLC) have been developed for measuring hydroperoxides. Yamada *et al.* (13) quantitatively determined hydroperoxy phospholipids in rat liver by a combination of reverse-phase HPLC and an electrochemical detector. Miyazawa *et al.* (14,15) and Yamamoto *et al.* (16) developed chemiluminescence HPLC systems for the detection of lipid hydroperoxides in biological samples at the picomolar levels. Akasaka *et al.* (17–19) utilized 1-naphthyldiphenylphosphine, 9-anthryldiphenylphosphine and diphenyl-1-pyrenylphosphine for the postcolumn fluorometric HPLC assay of lipid peroxides. These HPLC methods are excellent and can detect picomolar levels of resolved lipid hydroperoxides. However, specialized HPLC/equipment and/or tedious procedures can be involved, and poor reactivity of the latently fluorescent phosphines with lipid hydroperoxides can be encountered.

Triphenylphosphine (TP), which has long been used for the structural analysis of lipid hydroperoxides (20–22), reduces hydroperoxides quantitatively to the corresponding hydroxy compounds, while being converted to triphenylphosphine oxide (TPO). Consequently, peroxide content of lipids can be estimated by quantitatively measuring the TPO produced or the TP consumed in the reaction. Bernard and Wong (23) and Akasaka *et al.* (17) assayed the residual TP after reducing the hydroperoxides with a modest excess of TP, using gas-liquid chromatography and reverse-phase HPLC, respectively. However, at low lipid oxidation levels, the determination of trace amounts of TP consumed based on the measurements of unreacted TP can lead to substantial errors.

This paper describes a first attempt to determine total hydroperoxides in lipid samples by direct measurement of the TPO produced using reverse-phase or normal-phase HPLC and ultraviolet (UV)-detection.

MATERIALS AND METHODS

Materials. Linoleic acid (purity 99%), cumene hydroperoxide, and soybean lipoxygenase (type 1-S) were purchased from Sigma Chemical Co. (St. Louis, MO). TP (purity 98%) and TPO (purity 98%) were obtained from Kishida Chemical Co. (Osaka, Japan) and Aldrich (Milwaukee, WI), respectively. TP was purified by chromatography on a silicic acid column, using cyclohexane/benzene (85:15, v/v), to remove traces of TPO which are present as impurity in the reagent. Methyl 13-hydroperoxyoctadecadienoate (Me-13-HPO) was prepared enzymatically from linoleic acid using lipoxygenase, followed by methylation with diazomethane (24,25). The product was purified by normal-phase HPLC (μ-Porasil 7.8 i.d. × 300 mm, Waters, Milford, MA; n-hexane/2-propanol, 99:1, v/v). The purity of cumene hydroperoxide as estimated by iodometry (26) was 86.1%. Other materials such as methyl linolenate (purity 70%, Tokyo Kasei Co., Tokyo, Japan), olive oil, safflower oil, linseed oil and squid liver oil were obtained from commercial suppliers. Sardine muscle lipids were extracted from frozen sardines (*Sardinops melanosticta*) by the method of Folch *et al.* (27).

TP reaction. TP (400 nmol) in 100 μL of cyclohexane was added to lipid hydroperoxides, or oxidized lipids, in 100 μL

*To whom correspondence should be addressed at Laboratory of Fisheries Technology, Faculty of Agriculture, Kyushu University, 6-10-1, Hakozaki, Fukuoka 812, Japan.

Abbreviations: HPLC, high-performance liquid chromatography; Me-13-HPO, methyl 13-hydroperoxyoctadecadienoate; POV, peroxide value(s); TBA, thiobarbituric acid; TP, triphenylphosphine; TPO, triphenylphosphine oxide; UV, ultraviolet.

of cyclohexane in a screw-capped reaction tube. The mixture was shaken at 30°C in the dark. After the prescribed reaction period, usually 30 min, 5 to 10 μL of the mixture was analyzed by HPLC. TP reagent was freshly prepared just before use.

HPLC. Using a Hitachi HPLC system with a UV-detector, Model 4000, and a data processor, Model D-2500 (Hitachi Ltd., Tokyo, Japan), TPO and TP were separated and assayed by reverse-phase HPLC on a column of Inertsil ODS-2 (5 μm, 4.6 i.d. × 250 mm, Gasukuro Kogyo Inc., Tokyo, Japan) using CH_3CN/H_2O (90:10, 70:30, v/v, etc.) as eluent. For normal-phase HPLC, a Nova-pak Silica column (3.9 i.d. × 150 mm, Waters) and n-hexane/2-propanol (98:2, v/v) at a flow rate of 1.5 mL/min were used.

Other methods. Peroxide values (POV) were also determined iodometrically by the standard method of the Japanese Oil Chemists' Society (26). The reaction of TP with hydroperoxides was monitored by thin-layer chromatography on Silicagel 60 plates (E. Merck, Darmstadt, Germany) using n-hexane/diethyl ether/acetic acid (60:40:1, v/v/v) as developing solvent.

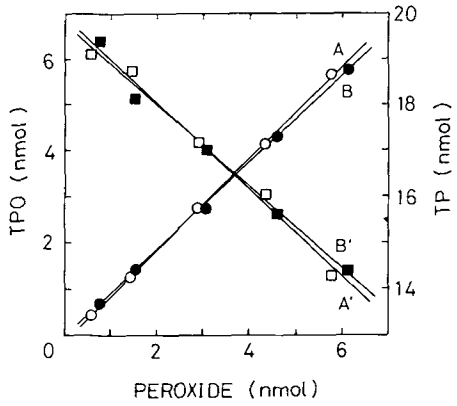


FIG. 2. Correlation between hydroperoxides used and TPO produced or TP remaining. A known amount of cumene hydroperoxide or Me-13-HPO was reduced with TP (400 nmol) at 30°C for 30 min as described in Materials and Methods. The amounts of TPO (—○— cumene hydroperoxide, —●— Me-13-HPO) and TP (—□— cumene hydroperoxide, —■— Me-13-HPO) were measured by reverse-phase HPLC using CH_3CN/H_2O (90:10, v/v) as eluent for Me-13-HPO and CH_3CN/H_2O (60:40, v/v) changing to CH_3CN at 7 min for cumene hydroperoxide.

RESULTS AND DISCUSSION

Reaction of TP with hydroperoxides. The time course of the reaction of TP with hydroperoxides was studied. TP (400 nmol) in 100 μL of cyclohexane was added to 50 μg (153 nmol) of Me-13-HPO in 100 μL of cyclohexane. The mixture was shaken at 30°C in the dark. After the prescribed reaction period, 5μL of the mixture was analyzed by reverse-phase HPLC and by measuring absorption at 260 nm. As shown in Figure 1, the reaction was complete within a short time after addition of the TP solution. The production of TPO correlated well with the molar quantity of Me-13-HPO added. No traces of Me-13-HPO were detected by thin-layer chromatography, even after a 10-min reaction.

Figure 2 shows the correlation between the amounts of hydroperoxides used and the amounts of TPO produced or TP remaining after completion of the reaction. For both the reduction of cumene hydroperoxide and Me-13-HPO, the amount of TPO produced correlated well with the amount of hydroperoxide present (correlation coefficient = 0.999 for both hydroperoxides). These results confirm that, for practical purposes, the hydroperoxides were completely reduced within 30 min, and that TP was stoichiometrically oxidized to TPO in relation to the amount of hydroperoxides present. Less than 10 pmol of TPO was detectable by HPLC based on absorption at 220 nm.

Estimation of POV in lipid samples. Peroxide content of frozen sardines stored at −20°C for a month was measured after reduction of the extracted lipids with TP and subsequent normal-phase HPLC. Figure 3 shows

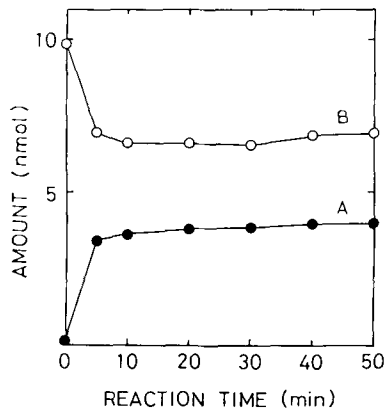


FIG. 1. Time course of the reaction of TP with hydroperoxides in cyclohexane. Me-13-HPO (153 nmol) was reduced with TP (400 nmol) in 200 μL of cyclohexane, as described in Materials and Methods. After the reaction, 5 μL of the mixture was analyzed by reverse-phase HPLC using CH_3CN/H_2O (90:10, v/v) as eluent at a flow rate of 1.5 mL/min. TPO produced (—●—) and TP remaining (—○—) were determined based on absorption at 260 nm.

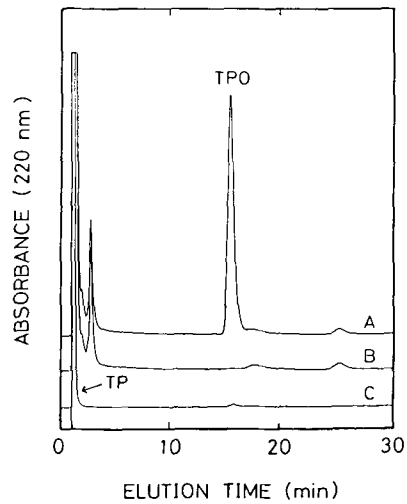


FIG. 3. HPLC of TPO produced by the lipids of sardine muscle. Lipids from sardine muscle were reduced at 30°C for 30 min as described in Materials and Methods, and analyzed by normal-phase HPLC based on absorption at 220 nm. (A): TP reaction mixture of the extracted lipids, (B): the extracted lipids. (C): TP reagent incubated under the same conditions as (A). With an injection of 483 μg of lipid, 2.08 nmol of TPO (equivalent to POV 8.61) was determined.

the elution pattern of the TPO generated; 2.08 nmol of TPO was detected by injection of 483 μg of the lipids. The POV was calculated to be 8.61.

The POV of various other oxidized lipids were estimated by the TP method and by conventional iodometric assay. Figure 4 shows the good agreement between the results obtained for various oils by the two methods. For the TP method, 10 mg of lipid was sufficient for the assay. Figure 5 shows changes in POV with oxidation time during autoxidation of methyl linolenate, as measured by the TP method and by iodometry. For low oxidation levels of the ester, POV obtained by the two methods showed good agreement. However, as autoxidation progressed, the values obtained by the TP method were lower than those obtained by iodometry. By the TP method, a maximum was reached at 68 hr. The discrepancy may be the result of differences between the reducing ability of KI in the acidic medium of $CHCl_3$/acetic acid and of TP in cyclohexane against the peroxidized products of methyl linolenate, including monocyclic and bicyclic endoperoxides and polymers.

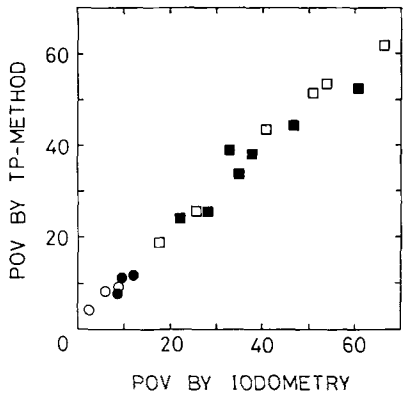


FIG. 4. Relationship between POV obtained by the TP method and by the iodometric method. Olive Oil (●), safflower oil (○), linseed oil (■) and squid liver oil (□) were oxidized in an incubator set at 40°C, and POV was estimated. The conditions for HPLC were the same as in Figure 1.

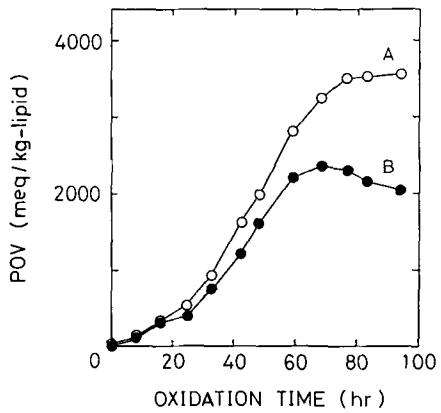


FIG. 5. Changes in POV with oxidation time during autoxidation of methyl linolenate. Methyl linolenate was oxidized at 40°C in the dark with stirring. POV was estimated by the iodometric method (—○—) and by the TP method (—●—) using reverse-phase HPLC. The conditions of HPLC were the same as in Figure 1, except that CH_3CN/H_2O (70:30, v/v) was used as eluent.

TPO in the HPLC solvents used in our experiment has absorption maxima at 220 and 265 nm. Hence, at either wave length, the TPO generated can satisfactorily be determined by HPLC. However, since lipids from biological sources can contain various substances with absorption at the UV-region, care should be taken to exclude peaks that could overlap with TPO. Generally, for lipids from biological sources which contain polar lipids, such as phospholipids, normal-phase HPLC can be used (Fig. 3). However, control experiments are essential to establish background values for HPLC analysis. By our method, the detection limit of TPO in HPLC based on absorption at 220 nm was less than 10 pmol. Consequently, total hydroperoxides in lipids corresponding to oxidation levels of less than POV 1 can be estimated by the TP method using a 10-mg sample of lipid.

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High-Performance Liquid Chromatographic Separation of Enantiomeric Alkyl Glycerol Ethers[1]

Thomas A. Foglia* and **Kazuhito Maeda**[2]
U.S.D.A., ARS, Eastern Regional Research Center, Philadelphia, Pennsylvania 19118

The resolution of a series of racemic 1-alkyl-3-trityl-*rac*-glycerols and 1-alkyl-2-benzyl-*rac*-glycerols into their enantiomers by high-performance liquid chromatography (HPLC) was investigated. Of the racemic glycerol derivatives studied, only three (1,2-*O*-isopropylidene-*rac*-glycerol, 1,2-*O*-isopropylidene-3-benzyl-*rac*-glycerol and 1-hexadecyl-2-benzyl-*rac*-glycerol) could be resolved into their enantiomers. The compounds were resolved on a silica (10 μm) column coated with cellulose tribenzoate and eluted isocratically with hexane/isopropanol as mobile phase. The effects of mobile phase composition, column temperature and flow rate on the resolution of enantiomeric pairs were investigated. Also, the effect of substituents, *i.e.*, trityl, benzyl, hydroxyl or alkyl, on the resolution of the alkyl glycerol ethers by HPLC was evaluated. *Lipids 26*, 769–773 (1991).

1-*O*-Alkyl-2-acetyl-*sn*-glycero-3-phosphocholine (commonly referred to as platelet activating factor, PAF) and its analogs have been attracting considerable attention because of their role in initiating or mediating various biological processes (1). It also has been shown that there are distinct differences in the biological activity between the enantiomers of PAF and of its analogs (2,3). In order to prepare pure PAF enantiomers, several total synthetic methods, employing optically active starting materials, have been developed (3–6). However, such stereospecific syntheses usually require numerous steps, and overall yields are relatively low. Moreover, in some cases synthetic intermediates are labile and racemize quite readily. Thus, convenient methods for the preparation of pure enantiomeric ether lipids are urgently needed.

High-performance liquid chromatography (HPLC) technology for the chiral separation of enantiomers has undergone rapid development in recent years. Several types of chiral stationary phases (CSP) for HPLC separation are now available (7). Some of the more useful CSP are the cellulose derivatives on which various compounds have been separated successfully into pure enantiomers without prior derivatization (8–12). Moreover, even large scale chiral separations by HPLC appear feasible, since cellulose derivatized CSP columns are reported to have good stability and durability. Therefore, we considered that in preparing enantiomeric ether lipids, it may be easier to separate selected racemic intermediates into their enantiomers by HPLC, rather than to synthesize them by multistep stereospecific procedures. The enantiomeric resolution of diradylglycerols by HPLC has been reported previously (13) on other chiral liquid phases. However, such resolutions are accomplished after prior conversion of the diacylglycerols into urethane or naphthyl urea diastereomeric pairs.

In the present study, we investigated the use of HPLC for the chiral separation of glycerol ethers without derivatization. Several of the compounds are intermediates of PAF synthesis and of other biologically active ether lipids. Here we describe the enantiomer separation of some glycerol ethers by HPLC using a cellulose derivatized CSP column.

MATERIALS AND METHODS

Materials. Racemic glycerol acetonide 1 was obtained from Aldrich Chemical Co. (Milwaukee, WI). All other glycerol ether derivatives (compounds 2–12) (Fig. 1) were synthesized in our laboratory. 1-Bromohexadecane, benzyl chloride, trityl chloride, methyl iodide, and all catalysts used were obtained from Aldrich Chemical Co. n-Hexane and isopropanol used for HPLC separations were obtained from American Burdick & Jackson (Muskegon, MI).

High-performance liquid chromatography. The HPLC instrument used in this study was an HP 1090 Liquid Chromatograph (Hewlett-Packard, Avondale, PA) that included an ultraviolet detector. A Waters Differential Refractometer Model R 401 detector (Waters Associates, Milford, MA) was employed when necessary. The CSP HPLC column used was a 4.6 mm ID × 25 cm stainless steel type prepacked with 10 μm silica coated with cellulose tribenzoate (Bakerbond Chiralcel OB, J.T. Baker, Phillipsburg, NJ). An analytical silica column (Zorbax SIL, 4.6 mm ID × 25 cm, 5–6 μm, DuPont, Wilmington, DE) was used for determining purity of the glycerol ethers prepared. The integrator/recorder was a Chromatopac C-R3A System (Shimadzu, Columbia, MD). The samples were eluted isocratically with n-hexane/isopropanol varying in composition from 90:10 to 99:1 (v/v), at flow rates of 0.25 to 1 mL/min. Samples were dissolved in mobile phase (1% w/v; injection volumes for the chiral HPLC column were 20 μL). Column temperature was 25 ∼ 27°C, unless stated otherwise.

Syntheses. The glycerol ether derivatives used in this study were prepared using the procedures illustrated in Figure 1.

1-*O*-Hexadecyl-2,3-*O*-isopropylidene-*rac* glycerol 2 was synthesized by alkylation of 1 with 1-bromohexadecane in the presence of sodium hydride in dimethylformamide (4). 1-*O*-Hexadecyl-*rac*-glycerol 3 was obtained by hydrolysis of 2 with conc. HCl in methanol solution. Tritylation at the 3-position of 3 with trityl chloride in triethylamine gave 1-*O*-hexadecyl-3-*O*-trityl-*rac*-glycerol 4 (4). Benzylation at the 2-position of 4 with benzyl chloride in the presence of sodium hydride gave 1-*O*-hexadecyl-2-*O*-benzyl-3-*O*-trityl-*rac*-glycerol 5 (5). 1-*O*-Hexadecyl-2-*O*-benzyl-*rac*-

[1]Presented in part at the AOCS 80th Annual Meeting, Cincinnati, Ohio, May 1989. Mention of brand or firm names does not constitute an endorsement by the U.S. Department of Agriculture over others of a similar nature not mentioned.

*To whom correspondence should be addressed at U.S. Department of Agriculture, ARS, Eastern Regional Research Center, 600 East Mermaid Lane, Philadelphia, PA 19118.

[2]Present address: Nippon Oil & Fats Co., Amagasaki, Japan.

Abbreviations: α, Selectivity; CSP, chiral stationary phase; HPLC, high-performance liquid chromatography; IR, infrared; κ′, capacity factor; PAF, platelet activating factor; Rs, resolution.

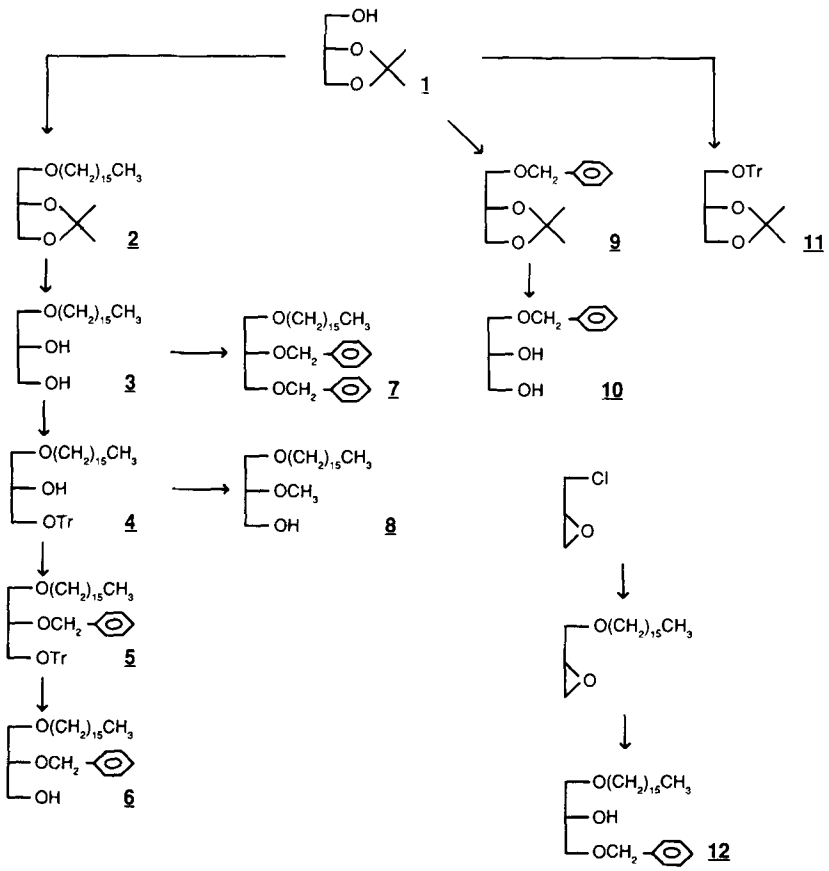


FIG. 1. Preparation of glycerol ethers.

glycerol **6** was prepared by detritylation of **5** with *p*-toluenesulfonic acid in methanol (6).

1-*O*-Hexadecyl-2-*O*-methyl-*rac*-glycerol **8** was obtained from **4** by methylation with methyl iodide in the presence of sodium hydride in dimethylformamide followed by detritylation (5). Benzylation of **3** gave 1-*O*-hexadecyl-2, 3-*O*-dibenzyl-*rac*-glycerol **7** (6).

1-*O*-Benzyl-2,3-*O*-isopropylidene-*rac*-glycerol **9** was prepared from **1** by benzylation; hydrolysis of **9** with conc. HCl in methanol at 0°C gave 1-*O*-benzyl-*rac* glycerol **10** (5). 1-*O*-Trityl-2,3-*O*-isopropylidene-*rac*-glycerol **11** was obtained by tritylation of **1** (5).

1-*O*-Hexadecyl-3-*O*-benzyl-*rac*-glycerol **12** was prepared from epichlorohydrin and 1-hexadecanol in the presence of sodium hydride in dimethylformamide, followed by selective reaction at the 3-position with benzyl alcohol in the presence of sodium hydride in dimethylformamide (6).

All glycerol ether derivatives were identified by infrared (IR) and ^{13}C nuclear magnetic resonance (NMR) spectroscopy, and purity was confirmed by HPLC with the analytical silica column (Table 1).

RESULTS AND DISCUSSION

Enantiomer separation by CSP column. Various chiral compounds have been reported to be separated into their enantiomers on the cellulose tribenzoate CSP column (Chiralcel OB). However, only three of the glycerol derivatives (**1**, **6** and **9**) which we prepared were separated into their enantiomers on the column used (Fig. 2).

Table 2 gives the chromatographic data for the enantiomeric separation of compounds **1**, **6** and **9**. Although elution times with lower polarity mobile phases were longer, better enantiomer separations were obtained. Racemic glycerol acetonide **1** was separated into its enantiomers, as previously reported (14). Good resolution (Rs 1.24) was obtained with n-hexane/isopropanol (99:1, v/v). However, **1** may not be a good intermediate for enantiomer separation since the enantiomers tend to racemize in the process. 1-*O*-Benzyl-2,3-*O*-isopropylidene-*rac*-glycerol **9** was separated into its enantiomers. However, resolution (Rs) was less than 0.8 under optimal conditions. 1-*O*-Hexadecyl-2-*O*-benzyl-*rac*-glycerol **6** was separable into its enantiomers although it has a long alkyl chain at the 1-position of the glycerol backbone. A desirable baseline resolution (Rs 1.47) was obtained with n-hexane/isopropanol (99:1, v/v) as mobile phase. The observed order of elution, R enantiomers before S enantiomers, shown in Figure 2, is in agreement with work previously reported for compound **1** (14). Moreover, the order of elution for compounds **1** and **9** was confirmed by the direct preparation of the R enantiomer of **1** and the S enantiomer of **9**.

We also checked the effect of mobile phase flow rate as well as column temperature on the enantiomer separation of **6** (Table 3). Column selectivites (α) were similar at all flow rates studied. However, enantiomer resolution (Rs)

TABLE 1

Characterization of Glycerol Derivatives 1–12

Glycerol derivative	IR (cm^{-1})	Purity (%)[c] HPLC	Retention time (min)
1	3580, 3460 (OH, 1380, 1370 (gem. dimethyl) 1210, 1160, 1050 (C-O-C)	100[d]	11.0[g]
2	1380, 1370 (gem. dimethyl)[a] 1110, 1050 (C-O-C)	100[e]	8.6[f]
3	3580, 3460 (OH), 1120 (C-O-C)[b]	100[d]	10.8[g]
4	3580 (OH) 3100 ~ 3000, 1495 (arom)[a] 1220, 1120, 1095 (C-O-C)	99[e]	12.0[f]
5	3100 ~ 3000, 1495 (arom)[a] 1220, 1120, 1095 (C-O-C)	95[e]	8.3[f]
6	3580 (OH) 3100 ~ 3000, 1495 (arom)[a] 1220, 1120, 1095 (C-O-C)	100[a]	13.2[f]
7	3100 ~ 3000, 1495 (arom)[a] 1180, 1100 (C-O-C)	83[a]	8.5[f]
8	3580 (OH) 1120, 1040 (C-O-C)[a]	100[d]	7.9[g]
9	3100 ~ 3000, 1495 (arom)[a] 1380, 1370 (gem. dimethyl) 1220, 1160, 1090 (C-O-C)	94[d]	9.1[f]
10	3400 (OH) 3200 ~ 3000, 1495 (arom)[a]	100[d]	18.1[g]
11	3100 ~ 3000, 1600, 1495 (arom) 1380, 1370 (gem. dimethyl) 1220, 1160, 1080 (C-O-C)	100[d]	8.3[f]
12	3580 (OH), 3200 ~ 3000, 1495 (arom)[a] 1120, 1095 (C-O-C)	94[e]	8.4[g]

[a]In CCl_4.

[b]In $CHCl_3$.

[c]Peak area (%) of HPLC with analytical silica column. Column: Zorbax Sil 4.6 mm ID × 25 cm, 5–6 μm (DuPont).

[d]Mobile phase, n-hexane/isopropanol (90:10, v/v).

[e]Mobile phase, n-hexane/isopropanol (90:2, v/v).

[f]Flow rate, 0.5 mL/min.

[g]Flow rate, 1.0 mL/min.

TABLE 2

Enantiomer Separation of Glycerol Derivatives 1, 6 and 9

Glycerol derivative		Mobile phase (n-hexane/isopropanol) 99:1	99:2	95:5	Enantiomer elution order
1	κ_1'[c]	4.00	2.33	1.21	R,S
	α[d]	1.21	1.21	1.15	
	Rs[e]	1.24	1.04	0.57	
6[a]	κ_1'	1.00	0.52	0.30	R,S
	α	1.46	1.34	1.21	
	Rs	1.47	0.95	0.54	
9[b]	κ_1'	3.37	2.77	2.26	R,S
	α	1.21	1.14	1.08	
	Rs	0.75	0.48	0.42	

[a]Flow rate of mobile phase, 0.25 mL/min; column temperature, 25°C.

[b]Flow rate of mobile phase, 0.5 mL/min; column temperature, 25°C.

[c]Capacity factor of the first eluted enantiomer.

[d]Column selectivity: $\alpha = \kappa_1'/\kappa_1'$, the ratio of the capacity factors.

[e]Resolution: $RS = 2\,(t_2\text{-}t_2)/(wt_1 + wt_2)$; t, retention time; wt, peak width.

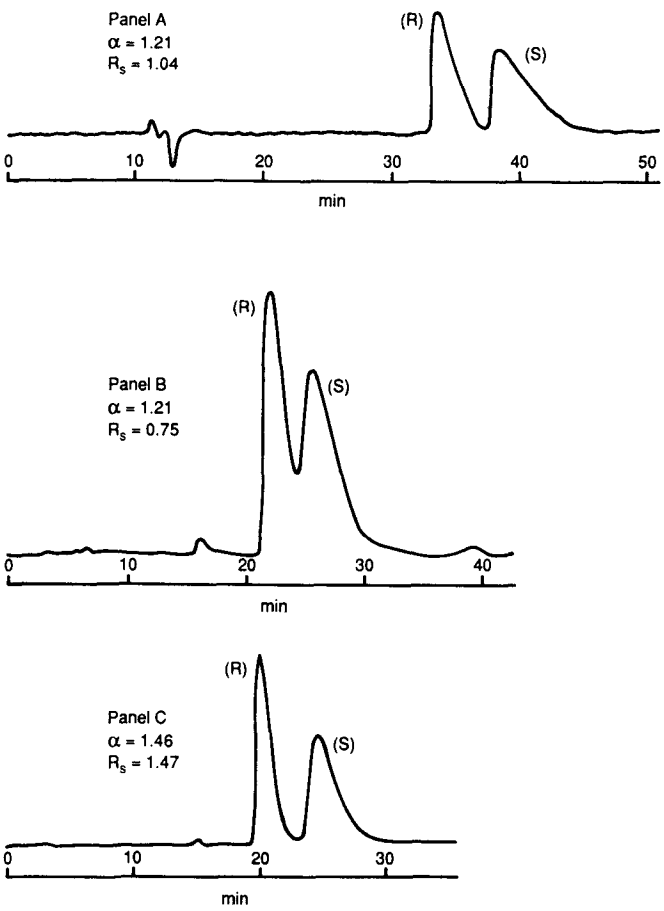


FIG. 2. Enantiomer separation of glycerol derivatives 1, 6 and 9 on Chiralcel OB column (4.6 × 250 mm). Panel A: compound 1, mobile phase, n-hexane/isopropanol (98:2, v/v); flow rate, 0.25 mL/min; RI detector. Panel B: compound 9; mobile phase n-hexane/isopropanol (99:1, v/v); flow rate, 0.5 mL/min; UV detector (254 nm). Panel C: compound 6; mobile phase, n-hexane/isopropanol (99:1, v/v) flow rate, 0.25 mL/min; UV detector (254 nm).

decreased at higher flow rates, presumably because of decreased column efficiency. No appreciable difference in Rs was observed between 25°C and 32°C. However, resolution decreased at 40°C. This may be attributed to a decrease in column efficiency at the higher column temperature. As a result of the above experiments, we concluded that in order to obtain good enantiomer separation, this particular CSP column should be used with a lower polarity solvent, at relatively low flow rate, and at, or near, room temperature (20–30°C).

Structure of glycerol ethers and separation of enantiomers. In the present study only three of the glycerol derivatives, **1**, **6** and **9** were resolved into their enantiomers. Judging from the structure of compounds **1**, **6** and **9**, the combination of a hydroxy group, a benzyl group and an isopropylidene group seems to provide chiral recognition by CSP. The structures probably interact with CSP through hydrogen bonding and π–π interaction that are also governed by steric effects. Furthermore, more than two of the three groups appear to be required for successful enantiomer separation. This seems plausible because multiple site interactions between the racemates

TABLE 3

Dependence of Enantiomer Separation of (6) on Flow Rate and Column Temperature

		α	R_s
Flow rate[a]	0.25 mL/min	1.42	1.32
Mobile phase	0.5 mL/min	1.41	1.08
	1 mL/min	1.41	0.86
Column temperature[b]	25°C	1.46	1.48
	32°C	1.39	1.51
	40°C	1.32	1.17

[a]Mobile phase, n-hexane/isopropanol (99:1, v/v); column temperature, 25°C.

[b]Mobile phase, n-hexane/isopropanol (99:1, v/v); flow rate of mobile phase, 0.25 mL/min.

and CSP would be expected to be required for good chiral recognition (14). If two of the functional groups occur in the structure, they are not necessarily effective as recognition sites, as compound **10** and **12** were not separated into enantiomers. These results support the idea that for chiral recognition to be effective, factors other than the mere presence of chemical recognition sites (11) of the analyte play a role. It appears that the morphology of cellulose tribenzoate on the silica gel, for example, is also important. Moreover, it has been reported that electron-donating substituents on the aromatic ring of a racemate (15) or the cellulose tribenzoate CSP (11) tend to improve enantiomer separations. Such substituents seem to work by changing the electron density of the aromatic ring which relates to the π-π interaction of the analyte and CSP. Accordingly, the enantiomer separation of **6** and **9** might be improved by attaching an electron-donating unit to the benzyl group.

In conclusion, of the glycerol derivatives studied, compound **6** has the best potential for preparative enantiomer separation by HPLC because of the good column selectivity (α) and resolution (Rs) obtained with this compound (Table 2). The S-isomer of **6** would be the intermediate of choice for the stereoselective synthesis of PAF.

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COMMUNICATION

The Effects of Tricaine Methanesulfonate (MS-222) on Plasma Nonesterified Fatty Acids in Rainbow Trout, *Oncorhynchus mykiss*

A.J. Harrington, K.A. Russell, T.D. Singer and **J.S. Ballantyne***
Department of Zoology, University of Guelph, Guelph, Ontario, K1A 2W1 Canada

The effects of tricaine methanesulfonate (MS-222), a commonly used fish anesthetic, on plasma nonesterified fatty acid levels (NEFA) were examined in rainbow trout, *Oncorhynchus mykiss*. Total NEFA levels declined with increasing duration of exposure to MS-222. Most of the decline in total NEFA was due to decreases in saturated fatty acids (14:0, 16:0 and 18:0). The fatty acid displaying the most rapid response to exposure to MS-222 was 20:5n-3. The lower plasma NEFA levels in anesthetized fish may be explained by depressed lipolysis in the presence of the anesthetic.
***Lipids* 26, 774–775 (1991).**

The anesthetic, tricaine methanesulfonate (MS-222), is commonly used to reduce stress in fish for blood sampling and other experimental procedures. As indicated by a recent study (1), the anesthetics themselves may induce stress. Levels of a variety of blood parameters during anesthesia with MS-222 have been investigated. These include glucose and/or lactate (2–6), blood amino acid levels (6), blood hormone levels (1,3), blood cholesterol and urea (3), as well as blood electrolytes (3,7). There have been no studies of the effects of MS-222 on pathways of lipid catabolism. Studies of the effects of similar anesthetics in mammals indicate that inhibition of lipolysis occurs (8,9). Since plasma nonesterified fatty acids are the most metabolically labile form of plasma lipid in teleost fish, some understanding of the effects of MS-222 on plasma fatty acids would be useful in interpreting a variety of experimental manipulations of fish involving anesthesia with MS-222.

MATERIALS AND METHODS

Experimental animals. Rainbow trout (*Oncorhynchus mykiss, Salmo gairdneri*) with an average weight of 150–200 g were obtained from a local supplier and maintained in freshwater at 10 ± 1.0°C for three weeks prior to use. Animals were fed *ad libitum* a commercial salmonid diet (Martin Feed Mills, Ltd., Elmira, Ontario, Canada). The fatty acid composition of the diet (wt%) was 14:0, 5.9%; 14:1, 0.3%; 16:0, 12.6%; 16:1n-7, 6.3%; 18:0, 1.4%; 18:1n-9 , 13.1%; 18:2n-6, 11.2%; 18:3n-3, 1.3%; 18:4n-3, 1.4%; 20:1 isomers, 14.2%; 20:2n-6, 0.1%; 20:3n-6, 0.04%; 20:4n-6, 0.2%; 20:3n-3, 0.1%; 20:4n-3, 0.3%; 20:5n-3, 5.0%; 22:1 isomers, 20.4%; 22:5n-3, 0.6%; 22:6n-3, 4.6%; 24:0, 0.8%; 24:1, 0.3%.

Treatment of animals. Blood was drawn into unheparinized syringes from the caudal vein before killing the fish. The control group (no anesthetic) was removed and bled as quickly as possible, with an average sampling time of 30 sec. Another group of fish was exposed to MS-222 at a concentration of 100 mg/L until equilibrium was lost, a process which took approximately 30 sec. The final group of fish was exposed to the same concentration of MS-222 until cessation of respiration, taking approximately 5 min.

Determination of plasma nonesterified fatty acids. Blood was immediately centrifuged for 5 min at 28,000 × *g* in a refrigerated centrifuge (BHG Hermle Z320K, Gosheim, Germany), after which the supernatant was removed and kept at −20°C until use. Nonesterified fatty acids (NEFA) were determined as described previously (10). This method is essentially the same as that first described by Lepage and Roy (11) using acid catalyzed methylation (methanol/acetyl chloride). The assay allows quantitative identification of NEFA from small amounts of plasma. Methyl esters were redissolved in 50 μL CS_2. Two μL of CS_2 containing methyl esters was injected into the gas chromatograph (Hewlett-Packard, HP5890 series II, Hewlett-Packard, Mississauga, Ontario, Canada) fitted with a flame ionization detector. Fatty acid methyl esters were analyzed on a DB-225 megabore fused silica column (Chromatographic Specialties Inc., Brockville, Ontario, Canada). The column temperature was initially 60°C, then was increased to 200°C over 3 min, and kept at 200°C for the remaining 22 min. Fatty acid methyl esters were identified by comparison of their retention times with those of known standards. Absolute amounts of fatty acids were calculated with the aid of an internal standard, heptadecanoic acid (17:0).

Statistical analysis. Differences between the levels of individual and total NEFA between treatment groups were tested with a one-way analysis of variance (ANOVA) and those parameters with significant differences were further analyzed with Duncan's New Multiple-Range test ($P<0.05$ was considered significant) according to Steele and Torrie (12). Log transformation of data was used to meet the assumption of parametric tests.

RESULTS AND DISCUSSION

Plasma NEFA levels declined with increasing duration of exposure to MS-222 (Table 1). The longest duration of anesthesia (to the point of cessation of respiration) resulted in a significantly lower plasma fatty acid level (total fatty acids were almost 50% lower than unanesthetized controls). Most of this decline was due to decreases in the levels of saturated fatty acids (14:0, 16:0 and 18:0). Significant but quantitatively smaller decreases occurred in 20:1 and 20:5n-3. The fatty acid displaying the most rapid response to exposure to MS-222 was 20:5n-3.

*To whom correspondence should be addressed.

Abbreviations: ANOVA, analysis of variance; MS-222, tricaine methanesulfonate; NEFA, nonesterified fatty acids; pO_2, partial pressure of oxygen.

TABLE 1

Effects of MS-222 on Blood Nonesterified Fatty Acid Content of Rainbow Trout[a]

Fatty acid	Concentration (nmol/mL)		
	Control	Anesthesia to loss of equilibrium	Anesthesia to cessation of respiration
14:0	123.76 ± 29.02^{b}	62.66 ± 11.13^{bc}	35.58 ± 8.04^{c}
16:0	345.13 ± 47.29^{b}	240.07 ± 46.81^{bc}	188.45 ± 10.42^{c}
16:1n-7	101.56 ± 16.79^{b}	82.92 ± 18.89^{b}	71.04 ± 5.25^{b}
18:0	74.96 ± 10.58^{b}	49.29 ± 12.05^{bc}	27.28 ± 2.16^{c}
18:1n-9	233.36 ± 32.84^{b}	212.72 ± 57.27^{b}	161.68 ± 13.44^{b}
18:2n-6	55.73 ± 12.87^{b}	73.58 ± 12.21^{b}	63.12 ± 1.76^{b}
20:1	136.85 ± 21.34^{b}	81.04 ± 19.64^{bc}	51.99 ± 4.83^{c}
20:4n-6	34.73 ± 20.07^{b}	17.15 ± 3.66^{b}	17.00 ± 4.39^{b}
20:5n-3	84.72 ± 8.59^{b}	53.62 ± 9.11^{c}	54.65 ± 3.61^{c}
22:1	94.28 ± 10.95^{b}	60.94 ± 15.85^{b}	33.43 ± 8.13^{b}
22:6n-3	237.94 ± 40.32^{b}	149.18 ± 10.85^{b}	165.55 ± 12.26^{b}
Total	1523.04 ± 177.12^{b}	1083.17 ± 206.19^{bc}	869.75 ± 38.86^{c}

[a]Absolute amounts of individual nonesterified fatty acids in plasma of rainbow trout, *Oncorhynchus mykiss*, subjected to 100 mg/L of MS-222. Values are presented as means ± SE for five determinations. Conditions were as described in Materials and Methods. For each fatty acid, values with a common superscript are not significantly different.

Significantly lower levels of this fatty acid were observed after about 30 sec (anesthesia to the point of loss of equilibrium).

The decreased plasma NEFA observed in fish exposed to MS-222 may be due either to elevated oxidation of fatty acids or a decrease in lipolysis. It has been found that stress causes an increased mobilization of lipids from the liver of fish (13). Stress, such as that associated with blood sampling of unanesthetized fish would therefore cause an increase in NEFA levels in the blood. Further studies are required to establish true "resting" plasma NEFA levels. Regardless of the origin of the high levels of plasma NEFA in unanesthetized fish, the reduction in plasma NEFA levels with increasing level of anesthetization may indicate a reduction in lipolysis rather than increased levels of utilization of fatty acids. It has been shown that blood partial pressure of oxygen (pO_2) in rainbow trout declines with MS-222 anesthesia due to impaired respiration (4). Cessation of oxygen delivery to fat catabolizing tissues would decrease fatty acid oxidation sparing blood NEFA elevating plasma NEFA. An antilipolytic effect of MS-222 may be responsible for the reduced plasma NEFA levels.

Other anesthetics including prilocaine (9), dibucaine and piperocaine (8) have been shown to reduce lipolysis in mammals. Tricaine methanesulfonate (MS-222) may have a similar effect in fish. The present study suggests that caution must be exercised in interpreting studies of hormonal effects on the metabolism of fish when MS-222 is used as an anesthetic. MS-222-induced depression of lipolysis would obscure hormonal effects on blood NEFA levels.

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ARTICLE

Adenosine Triphosphate (ATP) and Other Nucleotides Stimulate the Hydrolysis of Phosphatidylethanolamine in Intact Fibroblasts

Zoltan Kiss* and **Karan S. Crilly**
The Hormel Institute, University of Minnesota, Austin, Minnesota 55912

Addition of adenosine triphosphate (ATP) to [^{14}C]ethanolamine-prelabeled NIH 3T3 fibroblasts resulted in rapid formation of [^{14}C]ethanolamine from the prelabeled cellular phosphatidylethanolamine (PtdEtn) pool. After 2-min exposure, 10 μM ATP had near maximal effects on PtdEtn hydrolysis. Several other nucleotides, including UTP, ITP, and the stable ATP analog adenosine 5′-*O*-(3-thiotriphosphate) (ATPγS), also had stimulatory effects on PtdEtn hydrolysis with a potency comparable to that observed with ATP. The same nucleotides which acted on PtdEtn hydrolysis also had similar stimulatory effects on the hydrolysis of phosphatidylcholine (PtdCho) in [^{14}C]choline-labeled cells. In isolated membranes, Mg^{2+} greatly enhanced the stimulatory effects of ATP and ATPγS, but not of other nucleotides, on the hydrolysis of PtdEtn and PtdCho. Results indicate that in isolated membranes, both ATP and ATPγS stimulate phospholipid hydrolysis by two different mechanisms, but in intact cells only one of these mechanisms appears to be responsive to externally added nucleotides.
Lipids 26, 777–780 (1991).

External adenosine triphosphate (ATP) has several purinergic receptor-mediated effects in mammalian cells (reviewed in ref. 1). Most relevant to the present studies, ATP was found to stimulate both phospholipase C-mediated degradation of phosphatidylinositol 4,5-bisphosphate (2–11), and phospholipase D-mediated hydrolysis of phosphatidylcholine (PtdCho) (12,13).

Recently, we showed that in membranes isolated from NIH 3T3 fibroblasts, ATP also stimulates phospholipase D-mediated hydrolysis of phosphatidylethanolamine (PtdEtn) (14). However, the site and the mechanism of this ATP action remained unknown. Here we show that in intact fibroblasts, externally added ATP and several other nucleotides can stimulate the hydrolysis of PtdEtn, suggesting the role of purinergic receptors in the mediation of the ATP effect. In addition, in membranes, ATP and adenosine 5′-*O*-(3-thiotriphosphate) (ATPγS), but not other nucleotides, also stimulate PtdEtn hydrolysis by a second mechanism which appears to be unrelated to the effects observed in intact cells.

EXPERIMENTAL PROCEDURES

Materials. Dowex-50W (H^+ form) and all nucleotides were purchased from Sigma Chemical Company (St. Louis, MO). [2-^{14}C]Ethanolamine (60 mCi/mmol), [*methyl*-^{14}C]-choline chloride (50 mCi/mmol) and [γ-^{32}P]ATP (3000 ci/mmol) were from Amersham Corporation (Arlington Heights, IL). Tissue culture reagents were purchased from Gibco (Grand Island, NY), and fetal calf serum was purchased from Flow Laboratories (McLean, VA).

Cell culture. NIH 3T3 cells were cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal calf serum, penicillin/streptomycin (50 units/mL and 50 μg/mL, respectively), and glutamine (2 mM). For all experiments, the cells were used during the exponential phase of growth.

Measurement of phospholipid hydrolysis in cells prelabeled with ^{14}C-labeled precursors. NIH 3T3 cells were grown in humidified CO_2/air (5/95%) at 37°C and were used between passages 16–42. Cells were incubated with [2-^{14}C]ethanolamine (0.25 μCi/mL) or [*methyl*-^{14}C]-choline chloride (0.30 μCi/mL) for 48 hr, washed with fresh medium, and incubated in fresh DMEM for 3 hr. This last step reduced the amount of unincorporated ^{14}C-labeled precursors from 5,000–6,000 dpm/10^6 cells to 600–1,000 dpm/10^6 cells. Cells were harvested by gentle scraping from the dish and counted hemocytometrically. The harvested cells, usually pooled from 3–6 dishes (150 cm^2) were again washed with fresh medium. At this step, 95–98% of cells were viable, determined by the trypan blue dye exclusion assay. Aliquots of cell suspension (0.7–0.9 × 10^6 cells/mL) were incubated in a final volume of 0.3 mL at 37°C for 2–20 min. The incubation medium contained 2 mM unlabeled ethanolamine or choline, as appropriate, to suppress phosphorylation and reesterification of newly formed ^{14}C-labeled ethanolamine or choline as described earlier (15,16). Incubations were terminated by the addition of 4 mL of a mixture of chloroform/methanol (1:1, v/v). Phase separation was initiated by the addition of 3 mL of water. Fractionation of choline and ethanolamine metabolites was performed on Dowex-50W(H^+)-packed columns (Bio-Rad Econo columns—Richmond, CA; 1 mL bed volume) as described by Cook and Wakelam (17) for the choline derivatives with minor modifications. The initial flow-through (5 mL) along with a following 3- or 5-mL wash (water) contained glycerophosphoethanolamine or glycerophosphocholine, respectively. Ethanolamine phosphate and choline phosphate were eluted by 15 and 20 mL of water, respectively. Finally, ethanolamine and choline were eluted with 8 and 20 mL of 1 M HCl, respectively. Appropriate ^{14}C-labeled standards were prepared as described earlier (18). For the determination of the elution profiles of standards, 1-mL fractions were collected. The metabolites of [^{14}C]ethanolamine and [^{14}C]choline were further identified by thin-layer chromatography (TLC) (Silica Gel G), using the solvent system of 0.5% aqueous NaCl/methanol/concentrated aqueous NH_3 (50:50:5, v/v/v) (15). Phospholipids were separated as described earlier (19). For the preparation of crude membrane fractions, [^{14}C]ethanolamine-labeled cells were collected by scraping and disrupted by Dounce homogenization (A pestle, 100 strokes) in ice-cold 20 mM-

*To whom correspondence should be addressed.

Abbreviations: ATPγS, adenosine 5′[β,γ-*O*-(3-thiotriphosphate); AdoPP[NH]P, adenosine 5′[β,γ-imido)triphosphate; AdoPP[CH_2]P, adenosine 5′[β,γ-methylene]triphosphate; DMEM, Dulbecco's modified Eagle's medium; GTPγS, guanosine-5′-*O*-(3-thiotriphosphate); PtdCho, phosphatidylcholine; PtdEtn, phosphatidylethanolamine; TLC, thin-layer chromatography.

Tris/HCl, pH 7.5. After centrifuging the homogenates at 1,000 × *g* for 5 min, the resulting supernatants were further centrifuged at 16,000 × *g* for 20 min at 4°C. Membranes were incubated in the presence of nucleotides as described earlier (14). For the determination of degradation of ATP, intact cells or membranes were incubated in the presence of [γ-^{32}P]ATP (10^6 cpm; 0.01–1.0 mM) for 0–10 min. Reactions were stopped, and ATP was separated on polyethyleneimine cellulose F plates (20).

Statistical methods. The Student's t-test was used. P values of < 0.05 were considered significant.

RESULTS AND DISCUSSION

Addition of 10 μM ATP to [^{14}C]ethanolamine-labeled NIH 3T3 cells for 2 min significantly stimulated the formation of [^{14}C]ethanolamine from the prelabeled PtdEtn pool; 1 mM ATP had a similar effect (Fig. 1). However, between 2–10 min of incubation, 1 mM ATP, but not 10 μM ATP, had an additional stimulatory effect on [^{14}C]PtdEtn hydrolysis (Fig. 1). These data suggested that the effect of 10 μM ATP on PtdEtn hydrolysis was transient, partly because of its rapid degradation. Indeed, we found that about 90% of 10 μM ATP was degraded by cells within 2 min. During the same period, only about 15% of 1 mM ATP was degraded.

After labeling the cells with [^{14}C]ethanolamine for 48 hr, ^{14}C activity in PtdCho was only 2.2% of that found in PtdEtn. Even though, assuming that ethanolamine and choline comigrate on the Dowex column, degradation of PtdCho still could contribute to the above results. However, under the conditions used, there was less than 10% overlapping between the ethanolamine and choline peaks judged from the elution profiles of standards (21). In addition, the exclusive formation of [^{14}C]ethanolamine in ATP-treated cells (Fig. 1) was confirmed using the TLC method for its identification as described in Methods.

We still should note that accumulation of [^{14}C]ethanolamine both in the absence and presence of ATP occurred exponentially as a function of time (Fig. 1). This phenomenon was probably due to the delayed stimulatory action of a serum factor, because the formation of [^{14}C]ethanolamine was linear in the absence of serum (data not shown). The following experiments with intact cells were performed in the presence of serum, because it did not affect interpretation of data and results were more reproducible.

ATPγS is more resistant to hydrolysis than ATP; therefore, it is often used to elicit ATP-like biological effects. As shown in Figure 2, after 10 min incubation 1–10 μM concentrations of ATPγS appeared to be more potent than ATP in stimulating the formation of [^{14}C]ethanolamine. When the incubation time was shorter (2 min), these nucleotides were nearly equipotent stimulators of PtdEtn hydrolysis (data not shown). Taken together, the data indicate that a low (~10 μM) concentration of external ATP is capable of significantly, although transiently, stimulating PtdEtn hydrolysis in intact NIH 3T3 cells.

If the effect of ATP on PtdEtn hydrolysis is mediated by a purinergic receptor, then one would expect a similar action by other nucleotides as well, because the nucleotide specificity of purinergic receptors is relatively broad (1). Indeed, as shown in Table 1, ATPγS, ITP and UTP each stimulated the hydrolysis of PtdEtn at 100 μM, although with significant differences in potency being detected at

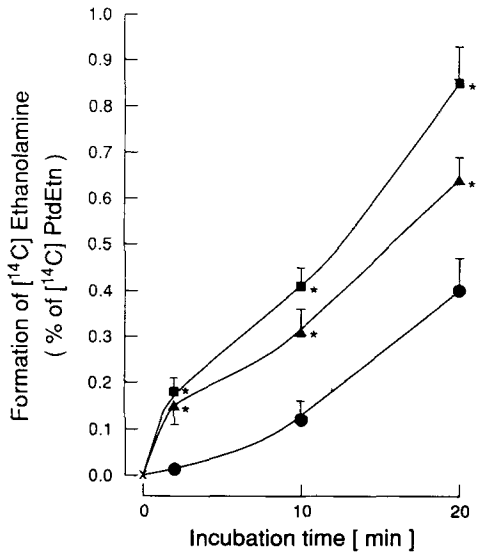


FIG. 1. Time-dependent effects of ATP on the hydrolysis of [^{14}C]-PtdEtn in NIH 3T3 cells. Cells were prelabeled with [^{14}C]-ethanolamine for 48 hr, followed by incubation of suspended cells for 2–20 min in the absence (●) or presence of 10 μM (▲) or 1000 μM ATP (■), as described in the Methods section. [^{14}C]Ethanolamine was separated by ion-exchange chromatography. The mean ^{14}C content of PtdEtn was 1.55×10^6 dpm/10^6 cells. Data are the means ± SEM of two independent experiments each performed in triplicate. Significance: $p < 0.05$ (*). Similar results were obtained in two additional experiments.

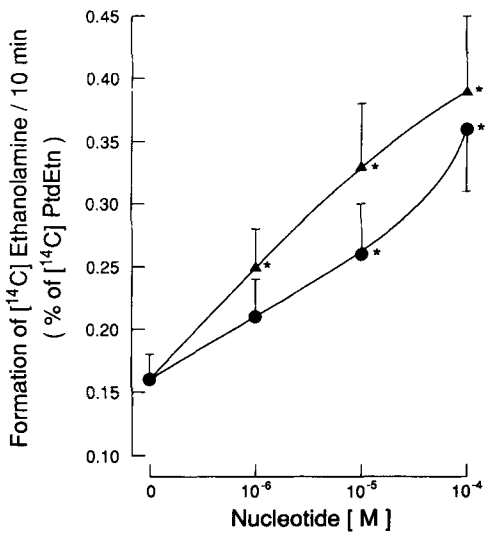


FIG. 2. Comparison of the effects of ATP and ATPγS on the hydrolysis of [^{14}C]PtdEtn in NIH 3T3 cells. Cells were prelabeled with [^{14}C]ethanolamine for 48 hr, followed by incubation of suspended cells for 10 min in the presence of varying concentrations of ATP (●) or ATPγS (▲), as described in the Methods section. The mean ^{14}C content of PtdEtn was 1.48×10^6 dpm/10^6 cells. Data are the mean ± SEM of two independent experiments each performed in triplicate. Significance: $p < 0.05$ (*). Qualitatively similar results were obtained in a third experiment.

TABLE 1

Effects of Nucleotides on Phospholipid Hydrolysis in Prelabeled NIH 3T3 Cells[a]

Nucleotide (100 μM)	Formation of [^{14}C]ethanolamine	Formation of [^{14}C]choline
	% of respective [^{14}C]phospholipid	
None	0.16 ± 0.02	0.07 ± 0.04
ATPγS	0.37 ± 0.04*	0.20 ± 0.03*
ATP	0.31 ± 0.04*	0.28 ± 0.03*
ITP	0.27 ± 0.04*	0.19 ± 0.06*
UTP	0.26 ± 0.03*	0.25 ± 0.05*
ADP	0.25 ± 0.06	0.21 ± 0.04*
AdoPP[NH]P	0.22 ± 0.06	0.11 ± 0.04
AdoPP[CH_2]P	0.20 ± 0.05	0.09 ± 0.05

[a]Cells prelabeled with [^{14}C]ethanolamine or [^{14}C]choline for 48 hr were incubated for 10 min. [^{14}C]Ethanolamine and [^{14}C]choline were separated by ion-exchange chromatography. The mean ^{14}C content of PtdEtn and PtdCho was 1.26 × 10^6 and 1.43 × 10^6 dpm/10^6 cells, respectively. Data are the mean ± SEM of two independent experiments each performed in quadruplicate.

*Significance: $p < 0.05$, according to Student's t-test. Qualitatively similar results were obtained in a third experiment.

this concentration. On the other hand, ADP, adenosine 5′[β,γ-imido)triphosphate (AdoPP[NH]P) and adenosine 5′[β,γ-methylene]triphosphate (AdoPP[CH_2]P) had only small, statistically insignificant effects on PtdEtn hydrolysis (Table 1). The same nucleotides which stimulated PtdEtn hydrolysis also stimulated the hydrolysis of PtdCho, except that in this latter case ADP also had a statistically significant effect (Table 1).

At present, we cannot exclude the possibility that at lower concentrations, the above nucleotides might exhibit greater differences in their effects on PtdEtn hydrolysis. Unfortunately, at low nucleotide concentrations the formation of [^{14}C]ethanolamine is not sufficiently stimulated over a relatively high background radioactivity (600–1,000 dpm/10^6 cells) to permit unequivocal evaluation of concentration-dependent effects. In addition, it is difficult to take into account how the rate of nucleotide hydrolysis (which differs for various nucleotides) would affect the effect of a given nucleotide on phospholipid hydrolysis. Despite these uncertainties, we can conclude that in intact fibroblasts, several phosphorylating (ATP, ATPγS) and nonphosphorylating nucleotides (UTP, ITP) can stimulate the hydrolysis of both PtdEtn and PtdCho. It also seems clear that, in these cells, nucleotide-stimulated phospholipase D similarly utilizes PtdEtn and PtdCho as substrates.

In order to investigate the mechanism of ATP action in more detail, we also compared the effects of nucleotides in plasma membrane-enriched membrane fractions isolated from [^{14}C]ethanolamine-prelabeled fibroblasts. Since most biological actions of extracellular ATP involve GTP and are affected by Mg^{2+} (1–13), we determined how these agents modified the actions of nucleotides on PtdEtn hydrolysis. As shown in Table 2, each nucleotide which enhanced phospholipid hydrolysis in intact cells also stimulated PtdEtn hydrolysis in membranes. However, while in the presence of Mg^{2+}, GTP potentiated the effect of each nucleotide, Mg^{2+} alone significantly potentiated only the effects of ATP and ATPγS on PtdEtn hydrolysis (Table 2).

It is of additional interest to note that the effects of ATP and the other nucleotides were not affected by 2 mM ethyleneglycol *bis*(2-aminoethyl)*N,N′*-tetraacetic acid used as calcium chelator, or by 10–500 μM calcium. In addition, the nucleotide effect did not require the presence of phospholipid bases (ethanolamine, choline, or L-serine). Thus, it appears that a base-exchange mechanism (22) did not mediate the effects of nucleotides on phospholipid hydrolysis. On the other hand, it was previously shown that in isolated membranes ATP stimulates the formation of phosphatidic acid from ^{32}P-labeled PtdEtn (14). Collectively, these data suggest that the effects of each

TABLE 2

Magnesium- and Guanine Nucleotide-Dependent Effects of ATP on the Hydrolysis of PtdEtn in Membranes from NIH 3T3 Cells[a]

Nucleotide	Formation of [^{14}C]ethanolamine: No $MgCl_2$, No GTPγS	No $MgCl_2$, + GTPγS	+ $MgCl_2$, No GTPγS	+ $MgCl_2$, + GTPγS
	% of [^{14}C]PtdEtn			
None	0.14 ± 0.02	0.19 ± 0.03	0.18 ± 0.03	0.23 ± 0.04
ATP	0.27 ± 0.02*	0.33 ± 0.06*	1.08 ± 0.06**	1.66 ± 0.08**
ATPγS	0.34 ± 0.04*	0.39 ± 0.07*	1.05 ± 0.09**	1.39 ± 0.05**
UTP	0.22 ± 0.06	0.29 ± 0.03*	0.32 ± 0.04*	0.47 ± 0.06*
ITP	0.20 ± 0.05	0.36 ± 0.05*	0.26 ± 0.03	0.42 ± 0.04*
AdoPP[NH]P	0.15 ± 0.04	0.20 ± 0.05	0.23 ± 0.05	0.36 ± 0.06
AdoPP[CH_2]P	0.13 ± 0.01	0.19 ± 0.02	0.20 ± 0.05	0.30 ± 0.03

[a]Cells were prelabeled with [^{14}C]ethanolamine for 48 hr, and crude membrane fractions were isolated and then incubated (74 or 89 μg protein/sample) with $MgCl_2$ (2 mM) and/or guanosine 5′-*O*-(3-thiotriphosphate) (GTPγS) (10 μM) in the absence or presence of 250 μM nucleotides for 15 min. The mean ^{14}C content of PtdEtn was 913,000 dpm/100 μg protein. Data are the mean ± SEM of two independent experiments each performed in quadruplicate.

*Significance = $p < 0.05$; ** $p < 0.01$, according to Student's t-test.

stimulatory nucleotide on PtdEtn hydrolysis involve both a phospholipase D enzyme and a GTP-binding protein, while Mg^{2+} specifically potentiates the effects of ATP and ATPγS.

In view of the Mg^{2+}-dependent effects of ATP and ATPγS, we considered the possibility that the full actions of these nucleotides involved a protein kinase activity. However, in membranes, 1-(5-isoquinolinesulfonyl)-2-methylpiperazine (300 μM) and staurosporine (5 μM), potent inhibitors of protein kinase C and other protein kinases (23–26) failed to inhibit the stimulatory effects of 1 mM ATP on the hydrolysis of PtdEtn and PtdCho (data not shown). In NIH 3T3 cells, the protein kinase C activator 12-*O*-tetradecanoylphorbol-13-acetate (27) stimulates phospholipase D-mediated hydrolysis of both PtdEtn and PtdCho (15,18). However, addition of this compound (100 nM) to membranes in the absence or presence of ATP (0.1–1 mM) failed to significantly stimulate the hydrolysis of these phospholipids (data not shown). Thus, experimental data collected so far indicate that the stimulatory effect of ATP on phospholipid hydrolysis is not mediated by protein kinase C.

In conclusion, our data demonstrate that in NIH 3T3 fibroblasts, ATP and several other nucleotides rapidly, but only transiently, stimulate the degradation of both PtdEtn and PtdCho. These results together with our earlier observations (14) strongly suggest the involvement of a phospholipase D. However, the effect of external ATP observed in intact cells appears to represent only a portion of the previously characterized Mg^{2+}-dependent ATP effects observed in isolated membranes (14). Further work is required to clarify the mechanism and judge the importance of the Mg^{2+}-dependent and -independent effects of ATP on phospholipid metabolism.

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Hypothyroidism and Thyroxin Substitution Affect the n-3 Fatty Acid Composition of Rat Liver Mitochondria

D. Raederstorff*[,a], C.A. Meier[1,b], U. Moser[a] and P. Walter[b]

[a]F. Hoffmann-La Roche Ltd., Department of Vitamin and Nutrition Research, CH-4002 Basel and [b]Department of Biochemistry, University of Basel, CH-4051 Basel, Switzerland

The effects of hypothyroidism and of daily treatment for up to 21 days with thyroxin (T4, 0.5 μg/100 g body weight) on the fatty acid composition of total lipid, phosphatidylethanolamine, and phosphatidylcholine of rat liver mitochondria were studied. The fatty acid compositions of hypothyroid and euthyroid (control) rats of similar age were compared. The n-6 and n-3 polyunsaturated fatty acids (PUFA) were affected differently by the hypothyroid state. The levels of linoleic (18:2n-6), γ-linolenic (18:3n-6) and dihomo-γ-linolenic acids (20:3n-6) were higher in hypothyroid rats than in controls, while the level of arachidonic acid (20:4n-6) was lower, which suggests an impairment of the elongase and desaturase activities. The n-3 polyunsaturated fatty acids, eicosapentaenoic (EPA, 20:5n-3) and docosapentaenoic (22:5n-3) acids, were higher in hypothyroid rats, whereas the linolenic acid (18:3n-3) content remained constant. The level of docosahexaenoic acid (DHA, 22:6n-3) was dramatically decreased in hypothyroid rats, while the levels of C_{22} n-6 fatty acids were unchanged. The differences were probably due to the competition between n-3 and n-6 PUFA for desaturases, elongases and acyltransferases. When hypothyroid rats were treated with thyroxin, the changes induced by hypothyroidism in the proportions of n-6 fatty acids were rapidly reversed, while the changes in the n-3 fatty acids were only partially reversed. After 21 days of thyroxin treatments, the DHA content was only half as high in hypothyroid rats than in euthyroid rats. These results suggest that the conversion of 18:2n-6 to 20:4n-6 is suppressed in the hypothyroid state which favors the transformation of 18:3n-3 to 20:5n-3. The marked decrease in DHA content indicates an impairment of the enzymes involved in the DHA metabolism, possibly the n-3 Δ4 desaturase or the acyltransferases. The increased levels of EPA and 22:5n-3 is consistent with the inhibition of the n-3 pathway at the Δ4 desaturase step. Observed modifications in the fatty acid composition may significantly alter eicosanoid synthesis and membrane functions in hypothyroidism.
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Decreases observed in the body basal metabolic rates in hypothyroidism have been linked to decreases in mitochondrial respiration rates (1). Thyroid hormones regulate both lipogenesis and fatty acid oxidation (2). Changes which may occur in the fatty acid composition of liver and heart mitochondrial phospholipids reflect the thyroid status of experimental animals. Thyroid conditions influence cell membrane lipids (3–7) which, in turn, may modulate membrane enzymes, transporters and receptors (2,8,9), such as L-glycerol-3-phosphate dehydrogenase (10), pyruvate translocator (11), and cation permeability (12). Hoch (2) proposed that synthesis and desaturation of fatty acids regulate mitochondrial oxidation *via* altered membrane fatty acyl compositions. Numerous studies in rats have indicated a role for thyroid hormones in the regulation of hepatic fatty acid synthesis (13–21). In addition, elevated levels of linoleic acid (18:2n-6) and decreased levels of arachidonic acid (AA, 20:4n-6) have been found in the hypothyroid state (2,3,8,14–17). However, only few data are available concerning the n-3 fatty acids derived from α-linolenic acid (18:3n-3) and the possible metabolic interactions between n-6 and n-3 fatty acids in different thyroid states. The Δ6 and Δ5 desaturases, which are involved in the conversion of 18:2n-6 into 20:4n-6, were found to be decreased in hypothyroid rats compared to normal rats when n-6 fatty acids were used as substrates (22). These desaturases are also involved in the conversion of 18:3n-3 into eicosapentaenoic acid (EPA, 20:5n-3), but no data are available using n-3 fatty acids as substrates. Recently, a number of reports have indicated that n-3 fatty acids may have beneficial effects in various chronic diseases, such as atherosclerosis, heart disease and cancer, as well as in certain inflammatory diseases (23–27). These reports have stimulated interest in the metabolism of n-3 fatty acids and the interaction between n-3 and n-6 fatty acids. The n-3 and n-6 fatty acid families have antagonistic metabolic, physiological and pathological functions (Fig. 1) (28,29). Therefore, we studied the possible influence of hypothyroidism and replacement therapy with physiological doses of thyroxin (T4) on the n-3 and n-6 fatty acid composition in liver mitochondria.

MATERIALS AND METHODS

Chemicals. L-3,3′,5,5′-Tetraiodothyronine (thyroxin, T4) and solvents of analytical grade were purchased from Fluka (Buchs, Switzerland). 6-Propyl-2-thiouracil and fatty acid standards were obtained from Sigma Chemical Co. (St. Louis, MO) and Nu-Chek Prep, Inc. (Elysian, MN), respectively. Sucrose was purchased from Serva (Heidelberg, Germany) and all other reagents were obtained from Merck (Darmstadt, Germany).

Animal treatment. Thirty male Wistar rats, each weighing between 100 and 120 g, received an iodine depleted diet (Altromin No. 1042 from Altromin AG, Lage, Germany) for 30 days. Five control rats (euthyroid, Group F) were given 1 ppm potassium iodide in the drinking water and, after 30 days, 50 μL of a 0.2 mM NaOH solution orally used as vehicle for thyroxin in the experimental groups for 19 days. The remaining 25 animals were

*To whom correspondence should be addressed at F. Hoffmann-La Roche Ltd., VFRB, B. 60/317A, CH-4002 Basel, Switzerland.

[1]Present address: National Institutes of Health, NIDDK, MCNEB, Bethesda, MD 20892.

Abbreviations: AA, arachidonic acid, 20:4n-6; DHA, docosahexaenoic acid, 22:6n-3; DPA, docosapentaenoic acid, 22:5n-3; EFA, essential fatty acid; EPA, eicosapentaenoic acid, 20:5n-3; FAME, fatty acid methyl ester; PC, phosphatidylcholine; PE, phosphatidylethanolamine; PUFA, polyunsaturated fatty acid(s); SEM, standard error of the mean; T3, 3,3′,5-triiodothyronine; T4, 3,3′,5,5′-tetraiodothyronine, thyroxin; UI, unsaturation index.

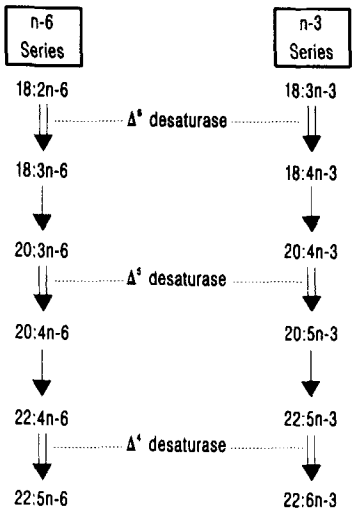


FIG. 1. Pathways for the biosynthesis of the n-6 and n-3 series of polyunsaturated fatty acids. The steps are catalyzed by either an elongation (single arrow) or a desaturation (double arrow) enzyme.

rendered hypothyroid by adding 1% $KClO_4$ to the drinking water for the 30 days. 6-Propyl-2-thiouracil (0.1%) was added to the diet for the first six days of this period. After 30 days, the hypothyroid rats were randomly assigned to five groups, each containing five animals. Group A (hypothyroid rats) received orally 50 μL of a 0.2 mM NaOH solution used as vehicle for thyroxin for 11 days. The other hypothyroid animals were orally given T4 (0.5 μg/100 g body weight) on a daily basis as sodium salt dissolved in a final concentration of 0.2 mM NaOH for various durations. Groups B, C, D and E received T4 orally for 2, 5, 9 and 21 days, respectively. The fatty acid weight percent composition of the experimental diet was: 10.4% 16:0 (palmitic acid), 3.7% 18:0 (stearic acid), 22.9% 18:1n-9 (oleic acid), 1.5% 18:1n-7 (vaccenic acid), 54.8% 18:2n-6 (linoleic acid), and 6.3% 18:3n-3 (α-linolenic acid). Heart blood was collected from the rats under short anesthesia, and the liver was quickly removed and placed into ice-cold saline buffer. The animals were sacrificed after the experimental periods.

Hormone measurement. Total L-3,3',5-triiodothyronine (T3) and free L-thyroxin (T4) levels were measured in serum using the Rapignost assay from Behring Werke (Marburg, Germany).

Preparation of mitochondria. Liver mitochondria from rats were isolated in 0.25 M mannitol/0.07 M sucrose/2 mM Hepes, pH 7.4, by the method of Johnson and Lardy (30).

Lipid analysis. Lipids were extracted from the homogenates by the method of Bligh and Dyer (31). The individual phospholipids were isolated from the lipid extracts by thin-layer chromatography on Silica Gel G 60 (Merck 5714), using the solvent chloroform/isopropanol/ethyl acetate/methanol/0.25% aqueous KCl (30:25:18:9:5, by vol). The plates were sprayed with berberine, and the bands were made visible under ultraviolet light (366 nm). The adsorbent bands corresponding to the phosphatidylethanolamine (PE) and phosphatidylcholine (PC) fractions were immediately scraped from the plates, and the lipids in the presence of the adsorbent were subjected to methanolysis by reaction with methanolic hydrogen chloride (10%) at 95°C for 25 min. Heptadecanoic acid was added as internal standard. An aliquot of the total lipid fraction was transesterified in the same way to obtain the total fatty acid composition. The fatty acid methyl esters were analyzed using a Hewlett-Packard HP 5890A gas chromatograph (Hewlett-Packard, Palo Alto, CA) equipped with a flame ionization detector. All samples were analyzed on a 50 m × 0.25 mm ID fused silica column coated with OV1. The column was temperature programmed from 55°C to 177°C at 10°C/min, then to 218°C at 1°C/min, and finally to 270°C at 4°C/min, and held at this temperature for 25 min. Peaks were identified by comparison with known standards. Quantitative data were derived from the integrator (HP 3393A) using a standard mixture of fatty acid methyl esters (FAME) to establish the response factors for the components identified. The internal standard (heptadecanoic acid) was used to convert the peak areas into μg of FAME in the samples. The results were then converted into mole percentages of the identified FAME, which represent more than 98% of the integrated area.

Statistical analysis. All results are expressed as means with their standard errors of the mean (SEM). The statistical significance of differences between means was determined by Student's *t*-test, with comparison made to the euthyroid controls (Group F).

RESULTS

Animal characteristics. The hypothyroid rats cease to grow after 10 days, and their body weight remained constant over the test period at about 170 g. The euthyroid animals grew steadily; after 30 days they weighed 74% more than the hypothyroid rats. Thyroxin treatment for 9 days and 21 days increased the weight of hypothyroid animals by 26% and 46%, respectively.

Total T3 and free T4 levels in rat serum are given in Table 1. The free T4 level increased sharply in hypothyroid rats after two days of thyroxin treatment, but leveled off at 10 pmol/L after nine days, remaining five times lower than in euthyroid animals. T3 level also increased rapidly reaching the level of euthyroid rats after two and five days of treatment, but then fell at about half the level of euthyroid animals. Neither total T3 nor free T4 in serum

TABLE 1

Total Triiodothyroxin (T3) and Free Thyroxin (T4) Levels in Rat Serum[a]

Group[b]	Total T3 (nmol/L)	Free T4 (pmol/L)
GR.A	0.14 ± 0.04	0.4 ± 0.10
GR.B	0.82 ± 0.03	5.5 ± 0.06
GR.C	0.91 ± 0.07	8.7 ± 1.30
GR.D	0.51 ± 0.05	10.1 ± 0.80
GR.E	0.46 ± 0.09	10.2 ± 1.50
GR.F	1.06 ± 0.12	49.2 ± 2.30

[a]Results are given as means ± SEM (n=5).

[b]GR.A, hypothyroid; GR.B, hypothyroid, T4 for 2 days; GR.C, hypothyroid, T4 for 5 days; GR.D, hypothyroid, T4 for 10 days; GR.E, hypothyroid, T4 for 21 days; GR.F, euthyroid, control.

TABLE 2

Relative Percent of Total Fatty Acids Composition in Total Lipids of Liver Mitochondria[a]

Fatty acids	Group A hypothyroid (n=5)[b]	Group B ± 2 day T4 (n=5)	Group C + 5 day T4 (n=5)	Group D + 9 day T4 (n=4)	Group E + 21 day T4 (n=5)	Group F euthyroid (n=5)
16:0	21.84 ± 0.63	20.83 ± 0.21	21.68 ± 0.46	23.35 ± 0.36[c]	23.42 ± 0.40[c]	20.47 ± 0.29
16:1n-7	9.07 ± 1.15[d]	11.93 ± 0.64[c]	9.46 ± 0.85[d]	9.55 ± 1.51[d]	9.02 ± 0.41[c]	4.83 ± 0.42
18:0	11.57 ± 0.45	10.92 ± 0.44	11.30 ± 0.69	12.10 ± 1.14	11.75 ± 0.81	12.54 ± 0.59
18:1n-9	8.24 ± 0.32[c]	7.51 ± 0.37[d]	6.23 ± 0.38	6.42 ± 0.36	4.67 ± 0.23[d]	5.90 ± 0.26
18:1n-7	2.91 ± 0.10[c]	3.83 ± 0.15[c]	4.07 ± 0.08[c]	4.04 ± 0.26[c]	4.81 ± 0.23[d]	7.06 ± 0.36
18:2n-6	18.97 ± 1.27	15.44 ± 1.26	14.95 ± 1.15	14.54 ± 1.20	14.91 ± 0.75	16.03 ± 0.66
18:3n-3	0.28 ± 0.03	0.40 ± 0.07	0.42 ± 0.04	0.32 ± 0.03	0.36 ± 0.03	0.38 ± 0.04
18:3n-6	2.52 ± 0.06[c]	2.45 ± 0.39[c]	1.54 ± 0.11[c]	0.99 ± 0.10[c]	0.77 ± 0.09[c]	0.18 ± 0.02
20:3n-9	0.59 ± 0.04[c]	0.69 ± 0.09[d]	0.42 ± 0.10	0.30 ± 0.06	0.38 ± 0.02[e]	0.28 ± 0.03
20:3n-6	1.53 ± 0.06	1.57 ± 0.10	1.14 ± 0.07	1.21 ± 0.17	1.53 ± 0.08	1.28 ± 0.10
20:4n-6	15.19 ± 0.37[c]	16.77 ± 0.37[c]	20.02 ± 0.80	19.16 ± 0.61	19.32 ± 0.63	20.35 ± 0.49
20:5n-3	1.58 ± 0.07[c]	1.75 ± 0.10[c]	1.29 ± 0.12[c]	0.99 ± 0.16[e]	1.26 ± 0.14[d]	0.59 ± 0.06
22:4n-6	0.20 ± 0.02	0.22 ± 0.01	0.19 ± 0.01	0.21 ± 0.02	0.20 ± 0.02	0.19 ± 0.01
22:5n-6	0.57 ± 0.02	0.66 ± 0.03[e]	0.70 ± 0.07[d]	0.54 ± 0.02	0.56 ± 0.05	0.53 ± 0.04
22:5n-3	1.25 ± 0.04[c]	1.35 ± 0.11[d]	1.78 ± 0.11[c]	1.71 ± 0.15[c]	1.59 ± 0.07[c]	0.73 ± 0.07
22:6n-3	2.13 ± 0.03[c]	2.34 ± 0.13	3.41 ± 0.37[c]	3.28 ± 0.14[c]	3.76 ± 0.43[c]	7.18 ± 0.22
Saturated	34.59 ± 0.38	32.84 ± 0.33	34.09 ± 0.58	36.51 ± 1.21	36.54 ± 0.72	34.03 ± 0.84
Monoenes	20.30 ± 1.44	23.36 ± 0.72[c]	19.86 ± 1.14	20.08 ± 2.07	18.63 ± 0.70	17.96 ± 0.50
Polyenes	44.90 ± 1.46	43.73 ± 1.00[e]	45.98 ± 1.41	43.34 ± 1.86	44.76 ± 1.03	47.95 ± 1.01
UI[f]	165 ± 2.04[c]	171 ± 1.20[c]	181 ± 4.33	171 ± 3.70[d]	175 ± 4.00[e]	191 ± 2.56
n-6/n-3	7.59 ± 0.34[c]	6.53 ± 0.34[c]	5.71 ± 0.28[d]	5.87 ± 0.10[c]	5.47 ± 0.23[d]	4.41 ± 0.17

[a]Values expressed as mole percent of total fatty acids are means ± SEM. Several small components are not shown. Statistical significant differences were calculated by Student's *t*-test.

[b]Number in parenthesis represents the number of samples analyzed.

[e]$p<0.05$, [d]$p<0.01$, [c]$p<0.001$, Significance with respect to euthyroid rats.

[f]UI, unsaturation index is the sum of the percentages of individual unsaturated fatty acids × number of double bonds/100.

could be normalized in this experiment after 21 days of thyroxin treatment.

Mitochondrial fatty acid modification. The relative fatty acid composition of total lipid, PE and PC in rat liver mitochondria is presented in Tables 2, 3 and 4. The fatty acid composition of total lipids reflects the total phospholipid content because phospholipids represent more than 85% of the liver mitochondrial lipids (32). Moreover, the two major phospholipids analyzed comprise 75% of the total phospholipids (33). The level of total saturated fatty acids in liver mitochondria was only slightly higher in hypothyroid rats than in controls. Palmitic (16:0) and stearic (18:0) acids, the major saturated fatty acids in mitochondrial lipids, were not significantly changed in total lipids. Palmitic acid was only increased in PC of hypothyroid rats. Thyroxin replacement significantly increased the proportion of 16:0 in total lipids, PC and PE. The proportion of monounsaturated fatty acids was also influenced by the thyroid state of the rats. The mitochondria from hypothyroid animals had a higher relative content of oleic (18:1n-9) and palmitoleic (16:1n-7) acids and a lower content of vaccenic acid (18:1n-7) in total lipids, PC and PE as compared to the mitochondria from controls. The level of total n-6 PUFA remained unchanged in total lipids and PE, and was slightly decreased (by 13%) in PC of hypothyroid animals. The relative contents of 18:2n-6, 18:3n-6 and 20:3n-6 were higher in the different lipid fractions of mitochondria of hypothyroid animals, while the AA content was significantly lower. T4 administration rapidly reversed the changes in n-6 PUFA in PE and total lipids. The total n-3 PUFA of total lipids (8.9%), PC (8.4%) and PE (15.2%) decreased in hypothyroid rats to 5.2%, 4.9% and 7.5%, respectively. This was due to the considerable decrease of DHA from 7.2%, 6.5%, 12.6% to 2.1%, 1.7%, 3.0% in those fractions. The precursors of DHA, *i.e.*, EPA and 22:5n-3, were significantly increased in the mitochondrial lipids of hypothyroid rats compared to euthyroid animals. The decrease in DHA was not reversed by T4 treatment.

DISCUSSION

The results show that hypothyroidism affects the phospholipid fatty acid composition of rat liver mitochondria. Moreover, thyroid hormone treatment partially reversed the changes in the phospholipid fatty acid patterns. The altered fatty acid composition in hypothyroid rats was not due to starvation and essential fatty acid deficiency because the ratio of 20:3n-9/20:4n-6 was about 10 times lower than the generally accepted normal limit of 0.4 (34). Emphasis was given to changes in the n-6 and n-3 fatty acids that are essential for humans (35,36). The proportions of n-3 and n-6 fatty acids were affected differently. The observed decrease in AA and the increase in 18:2n-6 in hypothyroid rat liver lipids confirm the previously reported alterations in AA metabolism (14–17). Microsomal Δ6 and Δ5 desaturases have been reported to be below normal in hypothyroid animals (22); these low activities may be correlated with the fatty acid composition of lipids in liver microsomes, mitochondria and nuclei (14–16,22). In the hypothyroid state, the level of 18:2n-6 may increase as a consequence of lower Δ6 desaturase

TABLE 3

Relative Percent of Total Fatty Acids in Phosphatidylcholine of Liver Mitochondria[a]

Fatty acids	Group A hypothyroid (n=5)[b]	Group B ± 2 day T4 (n=5)	Group C + 5 day T4 (n=5)	Group D + 9 day T4 (n=4)	Group E + 21 day T4 (n=5)	Group F euthyroid (n=5)
16:0	26.90 ± 0.48[c]	25.37 ± 1.04	27.04 ± 0.47[c]	28.06 ± 0.71[c]	28.06 ± 0.90[c]	22.92 ± 0.73
16:1n-7	6.36 ± 0.68[c]	6.98 ± 0.81[c]	4.69 ± 0.42[d]	4.63 ± 0.43[d]	7.19 ± 0.62[e]	3.28 ± 0.27
18:0	12.72 ± 0.44	13.58 ± 0.62	13.36 ± 1.64	16.19 ± 0.62[c]	10.98 ± 0.58	11.90 ± 0.84
18:1n-9	9.71 ± 0.77[e]	8.73 ± 0.36[e]	6.38 ± 0.35[c]	6.34 ± 0.18[c]	5.47 ± 0.39	4.86 ± 0.24
18:1n-7	2.35 ± 0.16[e]	3.50 ± 0.22[e]	3.91 ± 0.15[e]	3.88 ± 0.34[c]	4.44 ± 0.25[c]	6.21 ± 0.40
18:2n-6	14.91 ± 0.86[c]	13.12 ± 0.84[d]	10.16 ± 0.46	10.99 ± 1.01	12.25 ± 0.64	11.27 ± 0.21
18:3n-3	0.38 ± 0.03	0.48 ± 0.03	0.45 ± 0.01	0.41 ± 0.03	0.37 ± 0.02	0.37 ± 0.06
18:3n-6	3.89 ± 0.07[e]	3.87 ± 0.57[e]	2.27 ± 0.28[e]	1.75 ± 0.37[c]	1.41 ± 0.18[c]	0.64 ± 0.05
20:3n-9	0.74 ± 0.06[d]	0.96 ± 0.15[d]	0.65 ± 0.15	0.62 ± 0.12	0.60 ± 0.05	0.43 ± 0.07
20:3n-6	1.72 ± 0.10	1.88 ± 0.16[d]	1.23 ± 0.14	1.60 ± 0.20	1.83 ± 0.07[d]	1.49 ± 0.12
20:4n-6	13.18 ± 0.36[e]	15.10 ± 1.39[e]	22.77 ± 1.75	18.85 ± 1.13[e]	19.21 ± 1.47[c]	25.99 ± 0.71
20:5n-3	1.91 ± 0.11[e]	2.07 ± 0.15[e]	1.34 ± 0.19[d]	1.23 ± 0.14	1.50 ± 0.13[d]	0.84 ± 0.10
22:4n-6	0.17 ± 0.02[d]	0.19 ± 0.01[d]	0.18 ± 0.01[e]	0.21 ± 0.02	0.20 ± 0.01[d]	0.24 ± 0.01
22:5n-6	0.64 ± 0.06	0.57 ± 0.04	0.64 ± 0.01[d]	0.52 ± 0.06	0.52 ± 0.05	0.54 ± 0.04
22:5n-3	0.88 ± 0.06[d]	0.96 ± 0.14	1.32 ± 0.11[e]	1.00 ± 0.10[d]	1.11 ± 0.08[e]	0.66 ± 0.04
22:6n-3	1.71 ± 0.06[e]	1.76 ± 0.21[e]	2.59 ± 0.29[e]	2.08 ± 0.09[e]	2.97 ± 0.42[e]	6.56 ± 0.38
Saturated	41.17 ± 0.67[c]	39.65 ± 1.21[d]	41.24 ± 1.61[d]	45.65 ± 0.21[e]	40.67 ± 0.88[c]	36.17 ± 0.78
Monoenes	18.52 ± 1.13[d]	19.21 ± 0.62[e]	15.00 ± 0.64	14.85 ± 0.76	17.14 ± 1.08	14.48 ± 0.61
Polyenes	40.25 ± 0.80[e]	41.08 ± 1.78[c]	43.70 ± 1.91[d]	39.45 ± 0.93[e]	42.12 ± 1.24[c]	49.28 ± 0.91
UI[f]	150 ± 1.86[e]	157 ± 6.78[e]	173 ± 7.37[d]	153 ± 3.44[e]	166 ± 6.28[c]	201 ± 4.25
n-6/n-3	7.32 ± 0.42[e]	6.93 ± 0.48[c]	6.68 ± 0.22[e]	7.39 ± 0.21[e]	6.19 ± 0.41[d]	4.87 ± 0.14

[a] Values expressed as mole percent of total fatty acids are means ± SEM. Several small components are not shown. Statistical significant differences were calculated by Student's *t*-test.

[b] Number in parenthesis represents the number of samples analyzed.

[d] $p<0.05$, [c] $p<0.01$, [e] $p<0.001$, Significance with respect to euthyroid rats.

[f] UI, unsaturation index is the sum of the percentages of individual unsaturated fatty acids × number of double bonds/100.

TABLE 4

Relative Percent of Total Fatty Acids in Phosphatidylethanolamine of Liver Mitochondria[a]

Fatty acids	Group A hypothyroid (n=5)[b]	Group B ± 2 day T4 (n=5)	Group C + 5 day T4 (n=5)	Group D + 9 day T4 (n=4)	Group E + 21 day T4 (n=5)	Group F euthyroid (n=5)
16:0	22.60 ± 1.77	23.69 ± 0.82	24.19 ± 0.62[c]	25.98 ± 0.27[d]	25.27 ± 1.11[c]	21.49 ± 0.54
16:1n-7	2.92 ± 0.50[e]	4.19 ± 0.77[c]	2.88 ± 0.35[d]	2.57 ± 0.29[d]	2.85 ± 0.22[d]	1.23 ± 0.03
18:0	21.87 ± 1.31	19.17 ± 0.96	18.19 ± 0.96	20.06 ± 0.71	17.83 ± 0.57	18.46 ± 1.24
18:1n-9	6.69 ± 0.37[d]	4.97 ± 0.51[e]	3.10 ± 0.13	3.47 ± 1.04	2.68 ± 0.08	2.96 ± 0.19
18:1n-7	2.03 ± 0.15[d]	3.01 ± 0.17[e]	3.13 ± 0.10[e]	3.01 ± 0.30[c]	3.73 ± 0.22	4.83 ± 0.44
18:2n-6	7.52 ± 0.66[d]	5.52 ± 0.65[c]	4.65 ± 0.63	3.87 ± 0.58	4.13 ± 0.37	3.32 ± 0.18
18:3n-3	0.63 ± 0.07	0.72 ± 0.06	0.79 ± 0.09	0.55 ± 0.05	0.67 ± 0.02	0.66 ± 0.09
18:3n-6	1.34 ± 0.04[d]	1.30 ± 0.28[d]	0.85 ± 0.11[c]	0.49 ± 0.06	0.49 ± 0.08	0.48 ± 0.06
20:3n-9	0.67 ± 0.05[d]	0.62 ± 0.04[d]	0.43 ± 0.08	0.39 ± 0.04[c]	0.40 ± 0.03[c]	0.26 ± 0.03
20:3n-6	1.02 ± 0.02[d]	0.87 ± 0.06[e]	0.65 ± 0.03	0.75 ± 0.08	0.80 ± 0.05[e]	0.57 ± 0.03
20:4n-6	23.78 ± 0.57[e]	26.48 ± 0.76	29.10 ± 0.52	28.31 ± 0.75	29.04 ± 0.42	28.78 ± 1.15
20:5n-3	1.93 ± 0.16[d]	2.12 ± 0.10[d]	1.82 ± 0.16[d]	1.41 ± 0.15[e]	1.54 ± 0.19[e]	0.72 ± 0.07
22:4n-6	0.33 ± 0.02	0.29 ± 0.01	0.33 ± 0.01	0.33 ± 0.04	0.35 ± 0.02	0.34 ± 0.03
22:5n-6	0.81 ± 0.05	0.87 ± 0.03	0.98 ± 0.10	0.78 ± 0.01	0.82 ± 0.08	0.82 ± 0.06
22:5n-3	1.99 ± 0.09[d]	2.03 ± 0.24[c]	2.72 ± 0.18[d]	2.36 ± 0.32[e]	2.55 ± 0.11[d]	1.24 ± 0.10
22:6n-3	3.01 ± 0.02[d]	3.31 ± 0.25[d]	5.15 ± 0.56[d]	4.52 ± 0.22[d]	5.88 ± 0.79[d]	12.59 ± 1.09
Saturated	45.27 ± 0.42[c]	43.65 ± 0.40	43.34 ± 1.01	47.13 ± 0.73[c]	44.11 ± 0.94	40.92 ± 1.92
Monoenes	11.64 ± 0.79[c]	12.17 ± 0.55[e]	9.10 ± 0.38	9.04 ± 1.09	9.29 ± 0.41	9.11 ± 0.46
Polyenes	43.03 ± 1.03[c]	44.13 ± 0.73[c]	47.49 ± 1.25	43.77 ± 1.69	46.68 ± 0.90	49.91 ± 2.01
UI[f]	176 ± 2.87[e]	186 ± 3.15[e]	203 ± 4.73	188 ± 5.30[c]	202 ± 5.80	228 ± 9.87
n-6/n-3	4.71 ± 0.18[d]	4.47 ± 0.30[d]	3.57 ± 0.20[d]	3.97 ± 0.08[d]	3.45 ± 0.23[e]	2.30 ± 0.10

[a] Values expressed as mole percent of total fatty acids are means ± SEM. Several small components are not shown. Statistical significant differences were calculated by Student's *t*-test.

[b] Number in parenthesis represents the number of samples analyzed.

[c] $p<0.05$, [e] $p<0.01$, [d] $p<0.001$, Significance with respect to euthyroid rats.

[f] UI, unsaturation index is the sum of the percentages of individual unsaturated fatty acids × number of double bonds/100.

activity. The 2.8- and 6-fold increase in the 18:3n-6 content of PC and PE and the relatively small changes in the level of 20:3n-6 seem to be related to a decrease in the activity of the fatty acid elongase in hypothyroid animals. Previous investigators reported a decrease in microsomal, but not mitochondrial, fatty acid chain elongation activity in hypothyroid rats (37,38). The increase in the level of 16:1n-7 and the concomitant decrease in the 18:1n-7 level in hypothyroid animals suggest that fatty acid chain elongation is inhibited. Finally, the lower AA level in liver lipids may be a consequence of the inhibition of Δ5 desaturase. The AA content decreased less in PE than in PC, as reported by Hoch *et al.* (15). Moreover, the changes in fatty acid composition after T4 treatment were more completely reversed in PE.

T4 treatment for five days normalized the ratio of 20:4n-6/18:2n-6 which represents the overall metabolic conversion of 18:2-6 into 20:4-6 including Δ6 desaturase, elongase and Δ5 desaturase (Fig. 2). Within five days of T4 treatment, the fatty acid composition was close to normal, suggesting that the changes were due to thyroxin deficiency. In addition, T4 treatment increased the 16:0 content of mitochondrial lipids. Thyroid hormones have been shown to increase the activity and amount of fatty acid synthetase (20,39).

The decreased ratio of 20:4n-6/18:2n-6 in hypothyroid rats may have been partially compensated for by an increase in the ratio of 20:5n-3/18:3n-3. This increase in the ratio of 20:5n-3/18:3n-3 is consistent with a higher rate of overall conversion of 18:3n-3 into 20:5n-3 (Fig. 3). T4 treatment of hypothyroid rats partially reversed the ratio of 20:5n-3/18:3n-3 (Fig. 3), confirming a thyroxin deficiency effect. The n-6 and n-3 PUFAs are thought to be metabolized by the same enzyme sequence, but a competitive inhibition occurs between the two series at each step in the normal metabolism of these PUFA (40,41). Therefore, if hypothyroidism suppresses Δ5 and Δ6 desaturases, the competition between the n-3 and n-6 fatty acids may have favored the synthesis of long-chain n-3 PUFA up to 22:5n-3. The ratio of 22:6n-3/22:5n-3 was lower in hypothyroid rats than in controls consistent with an inhibition of the Δ4 desaturase of n-3 fatty acids. Previously, Hoch *et al.* (15) have documented a less marked decrease in the level of 22:6n-3 in mitochondrial lipids of hypothyroid rats. The diet fed and the strain of rat used may explain the differences in the relative changes of the fatty acid composition compared to our study. In thyroidectomized dogs the serum level of 20:5n-3 increased and the one of 22:6n-3 decreased (42). T4 replacement failed to correct the decreased level of DHA; the ratio of 22:6n-3/22:5n-3 was only slightly increased (Fig. 4). In fact, the DPA level was even further increased in hypothyroid rats after thyroxin treatment, suggesting that the Δ4 desaturase activity may be partially blocked, resulting in an accumulation of the substrate level. However, factor others than desaturation and elongation activities may influence the fatty acid composition of hypothyroid rats, such as fatty acid synthesis, esterification and oxidation, cell membrane degradation and transport of fatty acids from one site to another. Recently, Rosenthal *et al.* (43) suggested that the ratio of C_{22}/C_{20} PUFA in cells was regulated by the relative rate of retroconversion and chain length elongation, both of which may be affected by thyroid hormone levels. In addition, the n-3 PUFA also compete with 20:n-6 for acylation in the tissue phospholipids (29,40). Higher doses of thyroxin or a longer treatment period or injection may be necessary to correct the activity of the enzymes of the DHA metabolism affected by hypothyroidism. The lower PUFA content and UI in hypothyroid rats compared to controls are mainly due to a redistribution of fatty acids in favor of less unsaturated fatty acids, and to a marked decrease in the proportion

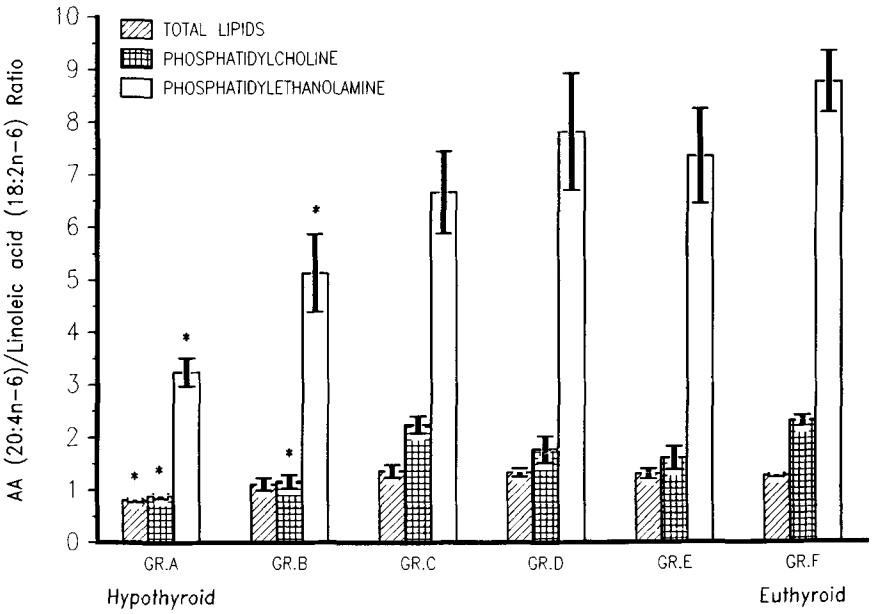


FIG. 2. Changes in the fatty acid ratio of 20:4n-6/18:2n-6 in mitochondrial lipids of hypothyroid rats given thyroxin for 2, 5, 9 and 21 days. Each bar represents the mean ± SEM (n≥4). Bars with * are significantly different from euthyroid rats ($p<0.01$).

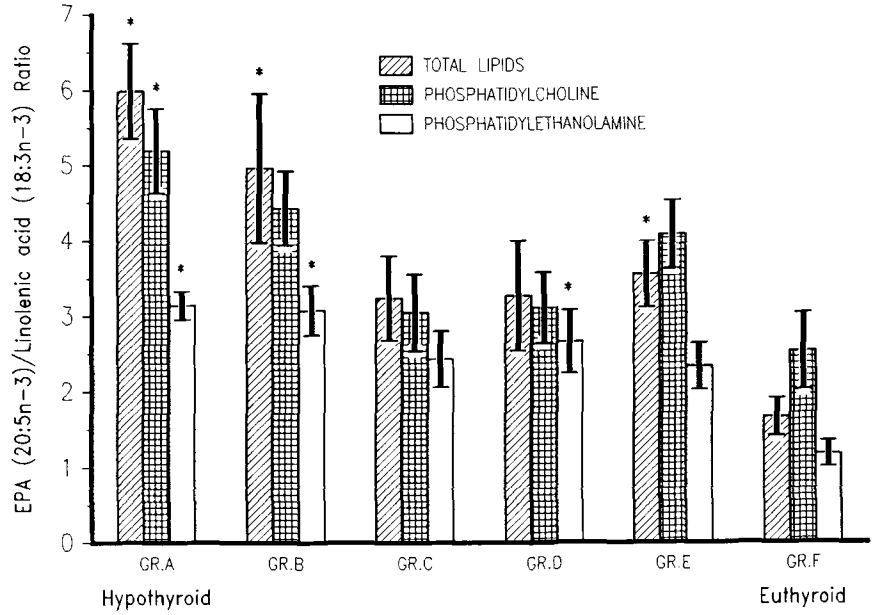


FIG. 3. Changes in the fatty acid ratio of 20:5n-3/18:3n-3 in mitochondrial lipids of hypothyroid rats given thyroxin for 2, 5, 9 and 21 days. Each bar represents the mean ± SEM (n≥4). Bars with * are significantly different from euthyroid rats ($p<0.01$).

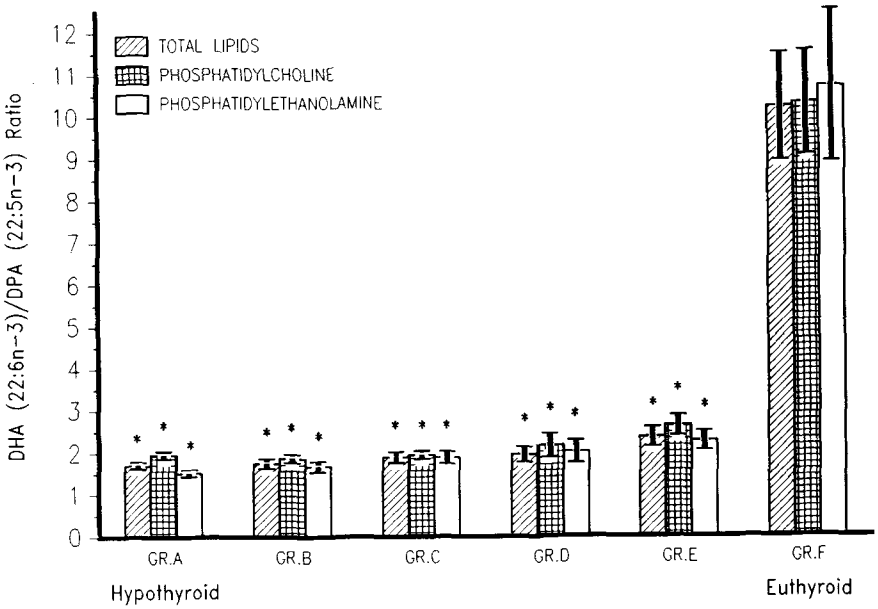


FIG. 4. Changes in fatty acid ratio of 22:6n-3/22:5n-3 in mitochondrial lipids of hypothyroid rats given thyroxin for 2, 5, 9 and 21 days. Each bar represents the mean ± SEM (n≥4). Bars with * are significantly different from euthyroid rats ($p<0.01$).

of DHA, which was not corrected by T4 replacement. The modification of the balance between n-3 and n-6 fatty acids in tissues may have significant biological implications because eicosanoids derived from the two PUFA series exert different biological activities (44–46).

Our study using hypothyroid rats and thyroid hormone replacement suggests that the biosynthesis of n-6 and n-3 PUFA is altered by thyroid hormones. However, each series seems to be affected at different steps in the biosynthesis, leading to the accumulation of different products or intermediates of desaturation and chain elongation. The decrease of the ratio of 20:4n-6/18:2n-6 confirms the previously reported impairment of the Δ5 and Δ6 desaturases by hypothyroidism, whereby the competition between n-3 and n-6 fatty acids favors the conversion of 18:3n-3 to 22:5n-3. In addition, the considerable decrease

in the ratio of 22:6n-3/22:5n-3 may be the consequence of a reduced n-3 Δ4 desaturase or of other enzymes involved in the DHA metabolism. The biosynthesis of n-6 fatty acids is inhibited at an earlier step up to the 20:4n-6 formation. T4 treatment rapidly reverses the changes in n-6 PUFA of hypothyroid rats, while changes in n-3 fatty acid levels are only slowly normalized.

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Incorporation of n-3 Fatty Acids into WB-F344 Cell Phospholipids Inhibits Gap Junctional Intercellular Communication

Clare M. Hasler[a,1], **James E. Trosko**[b] and **Maurice R. Bennink**[a,*]
[a]Departments of Food Science and Human Nutrition and [b]Pediatrics and Human Development, Michigan State University, East Lansing, Michigan 48824

In this investigation, we demonstrate that rat liver epithelial (WB-F344) cells grown in medium supplemented with n-3 fatty acids (FA) results in the inhibition of gap junctional intercellular communication (GJIC). Cells incubated for 48 hr in medium containing 50 μM α-linolenate (18:3n-3) resulted in a 60% inhibition of GJIC, compared to control cells, while treatment with γ-linolenate (18:3n-6) had no effect. Supplementation with octadecatetraenoate (18:4n-3), eicosapentaenoate (20:5n-3), and docosahexaenoate (22:6n-3), inhibited GJIC by 42%, 28%, and 18%, respectively. Incubation with each of the n-3 FA markedly increased the total n-3 FA content of cellular phospholipids (PL). Growing cells in medium containing 50 μM arachidonate (20:4n-6) plus 50 μM 18:3n-3 partially attenuated the inhibition of GJIC induced by 18:3n-3. The mechanism by which n-3 FA inhibit GJIC remains to be determined.
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Previous work conducted in our laboratory (1) demonstrated that in rat liver epithelial (WB-F344) cells, the n-3 fatty acid (FA) α-linolenate (18:3n-3) was a potent inhibitor of gap junctional intercellular communication (GJIC). GJIC is a fundamental biological process thought to be critical in the maintenance of cellular homeostasis, and in the regulation of cell proliferation and differentiation (2,3). Saturated FA (laurate, 12:0; palmitate, 16:0; stearate, 18:0), unsaturated FA from the n-6 family (linoleate, 18:2n-6; arachidonate, 20:4n-6), and oleate (18:1n-9), a monounsaturated FA from the n-9 family, did not modulate GJIC. The inhibition of GJIC induced by 18:3n-3 was not due to alterations in membrane fluidity, production of intracellular free radicals, or activation of protein kinase C. We hypothesized that 18:3n-3 inhibits GJIC through alterations in the FA composition of cellular phospholipids (PL).

In the present study, we have continued our investigation into the influence of n-3 FA on GJIC by examining several metabolic desaturation/elongation products of 18:3n-3 namely octadecatetraenoate (18:4n-3), eicosapentaenoate (20:5n-3), and docosahexaenoate (22:6n-3). The results of this study demonstrate that all n-3 FA tested in our cell system inhibit GJIC. The inhibition of GJIC may be related to an elevated n-3/n-6 FA ratio subsequent to an increase in the total n-3 FA content of cell PL.

MATERIALS AND METHODS

Cell culture. WB-F344 cells (passages 10–30) were obtained from Drs. J. W. Grisham and M. S. Tsao of the University of North Carolina, Chapel Hill, NC (4). The medium consisted of a modified Eagles's medium containing Earle's balanced salt solution with a 50% increase of vitamins and essential amino acids except glutamine, a 100% increase of non-essential amino acids (Gibco Laboratories, Grand Island, NY), 1 mM sodium pyruvate, 5.5 mM glucose, 14.3 mM sodium chloride, 11.9 mM sodium bicarbonate (pH 7.3), and 50 μg/mL gentamicin (Boehringer Mannheim Biochemicals, Indianapolis, IN). The culture medium (designated D medium) was supplemented with 3% fetal bovine serum (Gibco Laboratories). Cells were grown at 37°C in a humidified incubator containing 5% CO_2/95% air. For propagation, cells were subcultured weekly by trypsinization and resuspended in fresh D medium to yield a split ratio of 1:10.

Fatty acid supplementation. γ-Linolenate (18:3n-6), 18:3n-3, 18:4n-3, 20:4n-6, 20:5n-3, and 22:6n-3 (Sigma Chemical Co., St. Louis, MO) were added to D medium as complexes with FA-free bovine serum albumin in an approximate molar ratio of 4:1 according to the method of McGee (5). Briefly, FA were dissolved in a warm, rapidly stirring solution of 20 mM sodium hydroxide, to yield a 10 mM stock FA solution. The appropriate quantity of bovine serum albumin was added to yield a final bovine serum albumin concentration of 2.5 mM. The pH of the FA-bovine serum albumin solution was quickly adjusted to 7.4, filter sterilized with a 0.45 μM filter, and stored in small aliquots under an atmosphere of N_2 at −20°C until needed. Thawed aliquots of the stock FA-bovine serum albumin solution were added directly to the culture medium to achieve the desired FA concentration.

Cells were seeded at approximately 25% of confluency in 75-cm^2 tissue culture flasks (for PL analysis) or 35-mm dishes (for measurement of cytotoxicity and GJIC) containing D medium for 24 hr. After 24 hr, cells were rinsed with phosphate-buffered saline and fresh D medium added, supplemented with various FA at a concentration of 50 μM. The cells were allowed to proliferate to confluency for an additional 48 hr period in the FA-supplemented medium. An equivalent quantity of bovine serum albumin (12.5 μM) was added to control cell cultures.

Lipid extraction and phospholipid analysis. Following the appropriate treatment, confluent WB-F344 monolayers were washed several times with phosphate-buffered saline, harvested by trypsinization, and pelleted by low-speed centrifugation (1000 × *g* for 10 min). The cell pellet was rinsed with phosphate-buffered saline and stored under N_2 in a glass centrifuge tube at −20°C prior to further analyses. Frozen cell pellets were resuspended in 3 mL of phosphate-buffered saline and total lipid was

[1] Present address: Molecular Mechanisms of Tumor Promotion Section, Laboratory of Cellular Carcinogenesis and Tumor Promotion, National Cancer Institute, Bethesda, MD 20892.

*To whom correspondence should be addressed.

Abbreviations: ACAS, anchored cell analysis and sorting; 20:4n-6, arachidonate, AA; 18:3n-3, α-linolenate, ALN; 22:6n-3 docosahexaenoate, DHA; 20:5n-3, eicosapentaenoate, EPA; FA, fatty acid(s); FRAP, fluorescence redistribution after photobleaching; GJIC, gap junctional intercellular communication; 18:3n-6, γ-linolenate, GLN; LDH, lactate dehydrogenase; 18:4n-3, octadecatetraenoate, ODA; PL, phospholipid(s); SEM, standard error of the mean; TPA, 12-*O*-tetradecanoyl-phorbol 13-acetate.

extracted with 12 mL of a 2:1 mixture (v/v) of chloroform and methanol (6). PL were separated from total lipids by solid phase extraction with a silica Sep-Pak cartridge (Waters Associates, Milford, MA). Neutral lipids and free FA were eluted with 20 mL chloroform followed by 20 mL chloroform/methanol (20:1, v/v). PL were eluted with 10 mL chloroform/methanol (1:1, v/v). The purity of the PL fraction was checked through the use of radiolabeled FA and thin-layer chromatography. Contamination of the PL fraction with free FA and triglycerides was negligible. The FA of total cell PL were methylated with boron trifluoride (7). FA methyl esters were analyzed by gas-liquid chromatography utilizing a Varian 3700 gas-liquid chromatograph (Sunnyvale, CA) equipped with a flame ionization detector and a Supelcowax 10 capillary column (Bellefonte, PA) 30 meters in length with an inside diameter of 0.53 mm. The oven temperature was increased from 140 to 195°C at 3° per min and then held at 195°C until the last FA methyl ester was eluted. The amount of each FA present was calculated as a percentage of total FA in the sample using a Varian CDS 111 integrator.

Assessment of gap junctional intercellular communication. GJIC was assessed with the ACAS (anchored cell analysis and sorting) 570 interactive laser cytometer (Meridian Instruments Inc., Okemos, MI) (8) *via* FRAP (fluorescence redistribution after photobleaching) analysis as previously described (9). Following the 48-hr treatment period, FA-supplemented medium was removed, and the cell monolayers were rinsed several times with phosphate-buffered saline containing calcium (0.9 mM) and magnesium (0.5 mM) (Ca/Mg phosphate-buffered saline). The cells were incubated for 10 min with the fluorescent dye 5,6-carboxyfluorescein diacetate (7 μg/mL in Ca/Mg phosphate-buffered saline) (Molecular Probes, Inc., Eugene, OR). Cells were washed several times with Ca/Mg phosphate-buffered saline following the labeling period and subjected to FRAP analysis. Five separate confluent cell areas per 35-mm plate were selected and FRAP analysis was performed on 6 labeled cells within each area. Analyses were repeated in duplicate on 3 separate occasions. The tumor-promoting phorbol ester, 12-*O*-tetradecanoyl phorbol 13-acetate (TPA), a potent inhibiter of GJIC *in vitro* (10,11), was utilized as a positive control.

Determination of cytotoxicity. Lactate dehydrogenase leakage into the culture medium was assessed as an indicator of cytotoxicity with an optimized EC 1.1.1.27 UV-test kit (Sigma Chemical Co., St. Louis, MO). Aliquots of the culture medium were analyzed for lactate dehydrogenase activity following FA treatment. Identically treated cultures were exposed briefly (10 min) to a solution of 20% Triton X-100 to solubilize cell contents and an aliquot of the culture medium removed for measurement of total lactate dehydrogenase activity. Lactate dehydrogenase activity was expressed as the amount released from treated cells as a percentage of total cellular lactate dehydrogenase.

In addition to the determination of lactate dehydrogenase release, changes in cell morphology and apparent growth rate due to FA treatment were assessed with the aid of a phase-contrast microscope on a daily basis. Analyses were not conducted on cells which had not achieved complete confluency after 48 hr of treatment.

Statistical analyses. Since dye recovery values from FRAP analysis were obtained as percentages, they were normalized using an arcsin square root transformation procedure (12). Data from all experiments are presented as means ± SEM. Data from all figures were analyzed using a one-way analysis of variance. The F-max test was utilized to assess homogeneity of variance. Statistically significant differences between individual treatment means were determined with the least significant difference test. Criterion for significance was set at $p < 0.05$ for all comparisons.

RESULTS

Effect of α- versus γ-linolenate on GJIC. In a previous investigation (1), we found that α-linolenate (9,12,15 18:3n-3) was a potent inhibitor of GJIC when incorporated into cellular PL. It was of interest therefore, to examine the effect of its positional isomer, γ-linolenate (6,9,12 18:3n-6), on GJIC. As shown in Figure 1, 18:3n-6 does not inhibit GJIC, as fluorescence recovery in cells subsequent to 18:3n-6 treatment did not differ significantly from that of control cells. However, both 18:3n-3 and TPA significantly inhibited GJIC ($p < 0.05$) in WB-F344 cells. The inhibition of GJIC by 18:3n-3 was dose-dependent (Fig. 2) with concentrations of 30, 40, and 50 μM significantly inhibiting GJIC relative to control cells.

Modulation of gap junctional intercellular communication by n-3 fatty acids. It appears that the effect of triunsaturated 18-carbon FA on GJIC is related to the position of the first double bond from the methyl end of the FA. Thus, we examined the ability of various desaturation/elongation products of 18:3n-3 (18:4n-3, 20:5n-3, 22:6n-3) to inhibit GJIC. As shown in Figure 3, each of these n-3 FA were effective inhibitors of GJIC. Interestingly, there was an inverse relationship between the ability of n-3 FA to inhibit GJIC and the degree of carbon chain unsaturation (*e.g.*, 18:3n-3 > 18:4n-3 > 20:5n-3 > 22:6n-3). This is consistent with the ability of these FA to compete with 18:2n-6 and 20:4n-6 for esterification into PL and subsequent competition with 20:4n-6 for eicosanoid

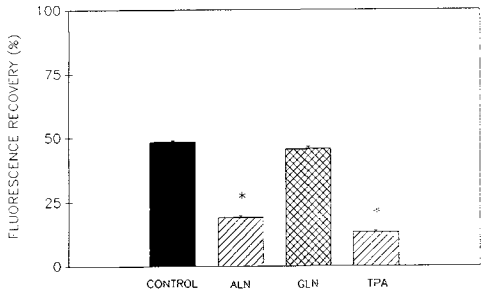


FIG. 1. Modulation of gap junctional intercellular communication (GJIC) in WB-F344 cells by α-linolenate (18:3n-3, ALN), γ-linolenate (18:3n-6, GLN), and 12-*O*-tetradecanoyl phorbol-13-acetate (TPA). Cells were allowed to grow to confluency for 48 hr in medium supplemented with 3% fetal bovine serum plus 50 μM of ALN or GLN. Control cells were treated with 12.5 μM bovine serum albumin. As a positive control, cells were treated with TPA (10 ng/mL) for 1 hr. Percent fluorescence recovery (values normalized using an arcsin transformation procedure) represents GJIC as assessed by fluorescence redistribution after photobleaching (FRAP) analysis following fatty acid or TPA treatment (see Materials and Methods). Values are expressed as means ± SEM. * = significantly less than control, $p < 0.05$, least significant difference test.

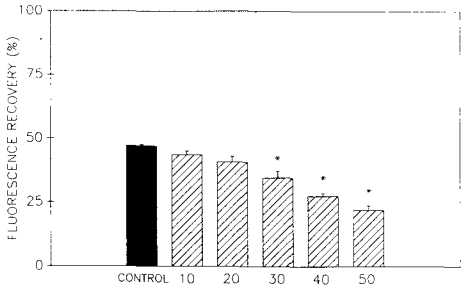


FIG. 2. Dose-dependent inhibition of GJIC in WB-F344 cells incubated with varying concentrations of ALN. Cells were allowed to grow to confluency for 48 hr in medium supplemented with 3% fetal bovine serum plus varying concentrations (10–50 μM) of ALN or 12.5 μM bovine serum albumin (control). FRAP analysis (see Materials and Methods) was performed as an assessment of GJIC 48 hr after ALN treatment. * = significantly less than control, $p < 0.05$, least significant difference test. Definitions are as in legend to Figure 1.

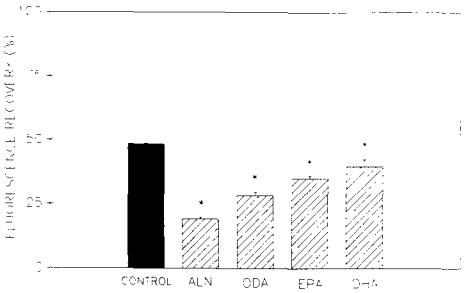


FIG. 3. Effect of n-3 FA on GJIC in WB-F344 cells. Cells were allowed to grow to confluency for 48 hr in medium supplemented with 3% fetal bovine serum plus 50 μM ALN, octadecatetraenoate (18:4n-3, ODA), eicosapentaenoate (20:5n-3, EPA), docosahexaenoate (22:6n-3, DHA), or 12.5 μM bovine serum albumin (control). GJIC was assessed *via* FRAP analysis (see Materials and Methods) at 48 hr following treatment. * = significantly less than control, $p < 0.05$, least significant difference test. Definitions are as in legend to Figure 1.

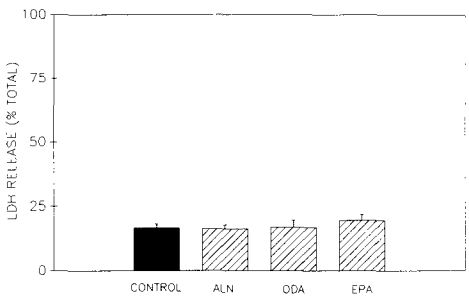


FIG. 4. Percent total lactate dehydrogenase (LDH) release in WB-F344 cells treated for 48 hr with 50 μM ALN or 12.5 μM bovine serum albumin (control). Values represent means ± SEM of triplicate replications obtained on at least two separate occasions. Definitions are as in legend to Figure 1.

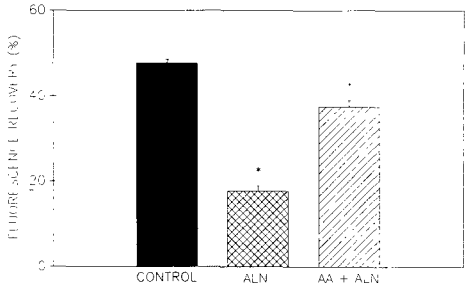


FIG. 5. Attenuating effect of arachidonate (20:4n-6, AA) on ALN-induced inhibition of GJIC in WB-F344 cells. Cells were allowed to grow to confluency in medium supplemented with 3% fetal bovine serum plus 50 μM ALN, 50 μM AA plus 50 μm ALN concurrently, or 12.5 μM bovine serum albumin (control). GJIC was assessed *via* FRAP analysis (see Materials and Methods) at 48 hr following treatment. * = significantly less than control, $p < 0.05$, least significant difference test. Definitions are as in legend to Figure 1.

synthesis (13). The inhibition of GJIC induced by n-3 FA is unlikely to be mediated by cytotoxicity as evidenced by minimal lactate dehydrogenase leakage into the culture medium (Fig. 4). Lactate dehydrogenase release following treatment with 18:3n-3, 18:4n-3, and 20:5n-3 was not significantly different from that of control cells.

Fatty acid modulation of WB-F344 cell phospholipids. Marked changes in the FA composition of total cellular PL were observed after WB-F344 cells were grown for 48 hr in FA-supplemented medium (Table 1). In general, cell PL became enriched in the FA with which they were supplemented, with the extent of enrichment varying with the particular FA employed. For example, the 22:6n-3 content of WB-F344 cell PL following treatment with this FA increased to approximately 23%, *versus* 1.9% in control cells. Conversely, the 18:3n-6 content of cell PL following 18:3n-6 treatment was only elevated to 1.2%, from less than 0.1% in control cells. In addition to the ability of WB-F344 cell PL to incorporate both n-6 and n-3 FA, there was significant elongation and desaturation of these FA. Cells treated with 18:3n-3, 18:4n-3, and 20:5n-3, produced cellular PL highly enriched with 20:5n-3 and 22:5n-3. Treatment with 18:4n-3 elevated the content of 20:5n-3 to the greatest extent (12.7%) compared to controls (0.6%), while incubation with 20:5n-3 increased the 22:5n-3 content of cell PL to 15.6%, *versus* 0.9% in control cells. Similar desaturation/elongation mechanisms were apparent in cells treated with 18:3n-6. In cells supplemented with this FA, the quantity of 20:4n-6 and 22:4n-6 in cell PL was elevated to 20.3% and 12.0%, respectively, compared to 4.6% and 1.1% in control cells. In general, all FA treatments elevated the 16:0 and 18:2n-6 content of cell PL, while reducing 16:1 and 18:1n-9.

Attenuation of α-linolenate-induced inhibition of GJIC by arachidonate. Since 18:3n-3 and 20:4n-6 are from different FA families, and compete for esterification into cell PL, or as substrates for the Δ6 or Δ5 desaturation enzymes, it was of interest to investigate the effect of co-incubating 18:3n-3 and 20:4n-6 on GJIC and the subsequent alterations in cell PL composition. As shown in Figure 5, when cells were grown to confluency in equimolar concentrations of 20:4n-6 and 18:3n-3 for 48 hr, the inhibition of GJIC induced by 18:3n-3 was partially attenuated. This treatment regimen also prevented, to some degree, some of the alterations in the FA composition of cell PL due to 18:3n-3 treatment alone (Table 2). The 20:4n-6 content of control cells, which was reduced to 2.6% following 48 hr of incubation with 50 μM 18:3n-3 (Table 1), was elevated to 18.6% when 20:4n-6 and 18:3n-3 were co-incubated. The direct elongation product of 20:4n-6 (22:4 n-6) was also elevated (11.5%) relative to control cells

TABLE 1

Effect of Various Fatty Acid Supplements on WB-F344 Cell Phospholipid Composition[a]

Phospholipid fatty acids (%)[c]	Fatty acid treatment[b]					
	Control	ALN	GLN	ODA	EPA	DHA
14:0[d]	2.3 ± 0.03	2.0 ± 0.1	3.0 ± 0.5	2.2 ± 0.8	3.3 ± 0.3	3.5 ± 0.4
16:0	18.8 ± 3.3	31.9 ± 2.0	30.3 ± 1.8	26.5 ± 1.5	32.1 ± 2.1	29.7 ± 0.2
16:1	8.2 ± 0.4	2.9 ± 0.3	2.6 ± 0.2	3.0 ± 0.2	4.4 ± 0.3	3.1 ± 0.03
18:0	10.7 ± 0.7	20.5 ± 2.2	12.4 ± 0.6	16.8 ± 1.5	12.2 ± 0.5	18.0 ± 0.7
18:1n-9 + n-7	32.4 ± 0.9	10.9 ± 1.2	8.9 ± 0.7	9.0 ± 0.2	16.8 ± 0.9	11.4 ± 0.0
18:2n-6	0.5 ± 0.2	1.5 ± 0.1	0.7 ± 0.1	1.5 ± 0.2	1.2 ± 0.3	1.7 ± 0.0
18:3n-6	*	0.9 ± 0.5	1.2 ± 0.2	*	*	0.2 ± 0.1
18:3n-3	*	6.8 ± 0.3	*	*	*	0.2 ± 0.1
18:4n-3	*	0.3 ± 0.2	*	0.7 ± 0.04	*	*
20:1n-9 + n-7	3.2 ± 0.1	*	0.1 ± 0.1	0.5 ± 0.4	0.3 ± 0.2	0.2 ± 0.1
20:2n-6	1.5 ± 0.3	*	0.1 ± 0.1	*	0.3 ± 0.2	*
20:3n-9	6.6 ± 1.3	0.5 ± 0.03	0.4 ± 0.1	0.5 ± 0.0	0.3 ± 0.2	0.5 ± 0.04
20:3n-6	0.9 ± 0.3	0.6 ± 0.02	4.5 ± 0.3	0.6 ± 0.1	0.5 ± 0.1	0.5 ± 0.0
20:4n-6	4.6 ± 0.3	2.6 ± 0.6	20.3 ± 0.7	4.5 ± 0.3	2.8 ± 0.2	4.0 ± 0.4
20:3n-3	*	2.4 ± 0.0	*	3.6 ± 0.9	0.2 ± 0.1	*
20:5n-3	0.6 ± 0.1	7.4 ± 3.5	*	12.7 ± 0.9	5.3 ± 0.7	2.7 ± 0.3
22:0[e]	5.5 ± 1.4	0.3 ± 0.1	0.6 ± 0.6	1.5 ± 1.1	0.4 ± 0.3	0.2 ± 0.1
22:4n-6	1.1 ± 0.1	0.3 ± 0.1	12.0 ± 0.4	0.9 ± 0.1	0.6 ± 0.03	0.6 ± 0.04
22:5n-6	0.4 ± 0.1	*	1.0 ± 0.1	0.4 ± 0.3	0.7 ± 0.5	*
22:5n-3	0.9 ± 0.1	9.1 ± 0.8	0.9 ± 0.04	13.7 ± 2.4	15.6 ± 1.9	0.9 ± 0.6
22:6n-3	1.9 ± 0.5	1.1 ± 0.1	0.9 ± 0.04	1.8 ± 0.3	1.1 ± 0.2	23.1 ± 1.8

[a] Abbreviations used: ALN = α-linolenate; GLN = γ-linolenate; ODA = octadecatetraenoate; EPA = eicosapentaenoate; DHA = docosahexaenoate. * = FA present at less than 0.1%.

[b] FA were added directly to culture medium at a concentration of 50 μM for 48 hr (see Materials and Methods).

[c] Values are expressed as weight percent + SEM of two replications, 3 flasks per replication.

[d] Individual fatty acids are abbreviated as number of carbon atoms:number of double bonds. n represents the position of the first double bond from the methyl end of the fatty acid.

[e] 22:0 also includes 22:1 and 22:2.

TABLE 2

Effect of Arachidonate on Alterations in the Fatty Acid Composition of WB-F344 Cell Phospholipids Induced by α-Linolenate[a]

Phospholipid fatty acids (%)[c]	Fatty acid treatment[b]		
	Control	ALN	AA + ALN
14:0[d]	2.3 ± 0.03	2.0 ± 0.1	4.4 ± 0.22
16:0	18.8 ± 3.3	31.9 ± 2.0	33.1 ± 1.3
16:1	8.2 ± 0.4	2.9 ± 0.3	4.2 ± 0.3
18:0	10.7 ± 0.7	20.5 ± 2.2	10.5 ± 0.6
18:1n-9 + n-7	32.4 ± 0.9	10.9 ± 1.2	8.7 ± 1.0
18:2n-6	0.5 ± 0.2	1.5 ± 0.1	0.9 ± 1.0
18:3n-3	*	6.8 ± 0.4	2.0 ± 1.1
20:3n-9	6.6 ± 1.3	0.5 ± 0.03	0.1 ± 0.1
20:3n-6	0.9 ± 0.3	0.6 ± 0.03	0.5 ± 0.04
20:4n-6	4.6 ± 0.3	2.6 ± 0.6	18.6 ± 0.8
20:5n-3	0.6 ± 0.1	7.4 ± 3.5	0.8 ± 0.6
22:4n-6	1.1 ± 0.1	0.3 ± 0.1	11.5 ± 2.5
22:5n-3	0.9 ± 0.1	9.1 ± 0.8	2.2 ± 1.0
22:6n-3	1.9 ± 0.5	1.1 ± 0.1	0.5 ± 0.04

[a] Abbreviations: ALN = α-linolenate; AA = arachidonate. * = FA present at less than 0.1%.

[b] FA were added directly to culture medium at a concentration of 50 μM for 48 hr (see Materials and Methods).

[c] Values are expressed as weight percent ± SEM of two replications, 3 flasks/replication.

[d] Individual fatty acids are abbreviated as number of carbon atoms:number of double bonds. n represents the position of the first double bond from the methyl end of the fatty acid.

(1.1%), and cells treated with 18:3n-3 alone (0.3%). In addition, 20:5n-3 and 22:5n-3, which were markedly increased following 18:3n-3 supplementation, were present at levels similar to controls when 20:4n-6 was co-incubated with 18:3n-3. Furthermore, treatment with 20:4n-6 and 18:3n-3 concurrently, minimized the amount of 18:3n-3 in cell PL (2.0% *vs* 6.8%) compared to cells treated with 18:3n-3 alone.

DISCUSSION

The results of this study demonstrate that growing cells in the presence of the n-3 FA, 18:3n-3, 18:4n-3, 20:5n-3, and 22:6n-3, results in the inhibition of GJIC in WB-F344 cells. The most effective FA in this regard was 18:3n-3, while its positional isomer, 18:3n-6, had no effect. Other investigators have examined the relationship between saturated (14,15), monounsaturated (16), and polyunsaturated FA (17,14,15,18,19) on gap junction function in a variety of cell culture systems with conflicting results. However, the influence of n-3 FA on GJIC has not been reported to date.

The exact mechanism by which n-3 FA inhibit GJIC in WB-F344 cells remains to be elucidated. In a previous report (1) we suggested that the inhibition of GJIC by 18:3n-3 was related to the reduction of 20:4n-6 in cell PL. Similarly, in the present investigation, treatment of cells with 20:5n-3 reduced the amount of 20:4n-6 in cell PL (2.8%), and significantly inhibited GJIC, further

TABLE 3

Influence of Supplemental Fatty Acid Effects on Various Cellular Parameters[a]

Parameter	Supplemental fatty acids[b]					
	Control	GLN	ALN	ODA	EPA	DHA
Fluorescence Recovery (%)	48.4	45.6	19.1	28.3	35.1	39.7
20:4n-6 content of cell PL (%)[c]	4.6	20.3	2.6	4.5	2.8	4.0
Total n-3 FA	3.4	1.8	27.7	32.5	22.2	26.9
Total n-3 FA	9.0	39.5	5.9	7.9	6.1	7.0
n-3/n-6 ratio	0.4	0.04	4.7	4.1	3.6	3.8

[a] Abbreviations: ALN, α-linolenate, 18:3n-3; DHA, docosahexaenoate, 22:6n-3; EPA, eicosapentaenoate, 20:5n-3; FA, fatty acid; GLN, γ-linolenate, 18:3n-6; ODA, octadecatetraenoate, 18:4n-3; PL, phospholipid.

[b] Fatty acids were added directly to culture medium at a concentration of 50 μM for 48 hr (see Materials and Methods).

[c] n represents the position of the first double bond from the methyl end of the fatty acid.

supporting the concept that GJIC was inhibited due to inadequate 20:4n-6 in cell PL. However, GJIC was also significantly reduced following treatment with 18:3n-3 and 22:6n-3, even though the 20:4n-6 content of WB-F344 cell PL was not decreased.

There was a marked elevation in the total n-3 FA content of cell PL subsequent to each FA treatment which resulted in decreased GJIC. A summary of these observations is presented in Table 3. The total n-3 FA in cell PL (ODA > ALN > DHA > EPA) does not correlate well with the inhibitory influence of these FA on GJIC. However, a good relationship exists between the order of the n-3/n-6 FA ratios (ALN > ODA > DHA > EPA ≫ control) and GJIC inhibition.

The elevated n-3/n-6 ratio in cell PL subsequent to n-3 FA treatment could modulate GJIC through an alteration in eicosanoid metabolism. It is well documented that eicosanoids synthesized from n-3 FA are metabolically different than those synthesized from n-6 FA (20). Enrichment of WB-F344 cell PL with n-3 FA might inhibit GJIC by altering the amount and/or type of eicosanoid(s) produced. There is evidence to suggest that eicosanoids do influence gap junction function in a number of different biological systems, although the data are conflicting (19,21-24). Clearly, further research is necessary to determine the role of eicosanoids in gap junction formation and function.

In summary, the results of the present investigation demonstrate that when 18:3n-3, 18:4n-3, 20:5n-3, and 22:6n-3 are incorporated into WB-F344 cell PL, GJIC is inhibited. Although our present findings do not support any simple interpretation, we hypothesize that the inhibitory effect of n-3 FA on GJIC is related to an increase in the total n-3 FA content of cell PL which, in turn, increases the n-3/n-6 FA ratio. The importance of the n-3/n-6 FA ratio and eicosanoid metabolism to GJIC in WB-F344 cells remains to be determined. Future investigations should determine how altering the FA composition of specific PL subclasses in the plasma membranes modulates GJIC. In addition, it should be determined if the inhibition of GJIC by n-3 FA is related to a reduction in the number of gap junction connexins in plasma membrane and the specific connexin(s) affected.

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Rapid Three-Step Purification of a Hepatic Neutral Cholesteryl Ester Hydrolase Which Is Not the Pancreatic Enzyme

Shobha Ghosh and W. McLean Grogan*
Department of Biochemistry and Molecular Biophysics, Medical College of Virginia, Virginia Commonwealth University, Richmond, Virginia 23298-0614

A rat liver cytosolic cholesteryl ester hydrolase (CEH) was purified 12,600-fold by ammonium sulfate precipitation, cation exchange chromatography and gel permeation high-performance liquid chromatography, with an overall yield of 20%. Its properties are compared to those of pancreatic CEH, with which it has sometimes been identified. Liver CEH exhibited a single silver stained band following SDS-polyacrylamide gel electrophoresis (M_r = 66 kDa), was activated by 0.5–10 mM taurocholate but was strongly inhibited by higher levels of taurocholate, which activate pancreatic CEH. Whereas bile salts are known to induce formation of a hexamer of pancreatic CEH, in the current study, 0.5 mM taurocholate dissociated a multimeric form of liver CEH to monomer. Liver CEH did not coelute with pancreatic CEH from cation exchange and chromatofocusing columns, exhibited no immunoreactivity with anti-rat pancreatic CEH IgG in Western blots, was not inhibited by anti-rat pancreatic CEH IgG and had a different amino acid composition from pancreatic CEH. In contrast to liver CEH, which is known to be activated by protein kinases A and C, pancreatic CEH was unaffected by cofactors for protein kinase A and was inhibited by cofactors for protein kinase C.

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Cholesteryl ester hydrolases occur in many tissues and are associated with different functions, depending on location. Whereas the enzyme of pancreas and intestinal mucosa plays an essential role in absorption of dietary cholesterol (1), isozymes in adrenals and gonads provide free cholesterol required for steroid hormone synthesis. Attention recently has been focused on liver cholesteryl ester hydrolase (CEH) and its involvement in cholesterol homeostasis in liver. However, little is known about the structure and function of this enzyme in liver. Although previous reports have demonstrated the presence of more than one CEH with neutral pH optima in both cytosolic and microsomal fractions of the liver (2,3), it is unclear whether a single protein is responsible for these enzyme activities. Camulli *et al.* (4) have recently described affinity purification of cytosolic neutral CEH from rat liver using anti-porcine pancreatic CEH IgG. Inasmuch as liver CEH thus purified had an identical N-terminal amino acid sequence to the rat pancreatic CEH, the authors suggested that CEH in liver and pancreas are very similar and possibly identical proteins. Based on the incomplete neutralization by anti-porcine pancreatic CEH IgG of CEH activity in liver cytosol, the authors suggested the presence of more than one CEH. Other authors report complete inhibition of liver CEH activity by monospecific anti-rat pancreatic CEH IgG and conclude that CEH activity of rat liver may be due to uptake of the enzyme originating in the pancreas (5). In contrast, using the same antibodies, Gallo *et al.* (6) were unable to show any cross-immunoreactivity between solubilized CEH from rat liver, aorta or adrenals.

The present studies describe purification of cytosolic neutral CEH from rat liver using conventional protein purification procedures and comparison of the properties of this enzyme with those of rat pancreatic CEH. The results demonstrate a liver CEH which is very different from rat pancreatic CEH.

MATERIALS AND METHODS

[1-^{14}C]Cholesteryl oleate (56.6 mCi/mmol) was purchased from New England Nuclear (Boston, MA). High-performance liquid chromatography (HPLC) grade solvents were purchased from Fischer Scientific (Columbia, MD). ATP disodium salt, adenosine 3′,5′-cyclic monophosphate (sodium salt), phosphatidylserine, diolein and sodium salts of taurocholic acid, glycocholic acid, cholic acid and deoxycholic acid were obtained from Sigma Chemical Co. (St. Louis, MO). Acrylamide, *bis*-acrylamide, SDS and TEMED were from Bio-Rad (Richmond, CA). All other chemicals used were of analytical grade.

Frozen rat livers from male Sprague-Dawley rats were purchased from Zivic Miller Laboratories, Inc. (Zelienople, PA) and used for all experiments except when effect of protein kinases was studied, in which case fresh livers were used. Tissue was homogenized with a loose teflon pestle in 20 mM Tris HCl buffer, pH 7.5, containing 5 mM 2-mercaptoethanol, 250 mM sucrose and 80 mM KCl (2 mL buffer/g tissue). The 104,000 × *g* supernatant (S104) was obtained as described previously (7). S104 from pancreas (fresh or frozen) was obtained using the same protocol except that the homogenization buffer also contained protease inhibitors, 2 mM 3-methyl propionate, 0.5 mM benzoyl arginine and 5 mM benzamidine.

CEH was assayed as described previously (7) and was purified from S104 as follows: Solid ammonium sulfate was added to S104 to produce 40% saturation and this was allowed to stir on ice for 30 min. The solution was centrifuged at 12,000 × *g* for 20 min. The supernatant was decanted and the pellet stored at −20°C until used. CEH activity was stable under these conditions for several months. The pellet was redissolved in 20 mM Tris HCl buffer, pH 7.5, containing 80 mM KCl, 0.5 mM taurocholate and 5 mM 2-mercaptoethanol (Buffer A) and ammonium sulfate was removed by passing through a Sephadex G-25 column equilibrated with Buffer A. Void volume containing the protein (60 mg) was collected and chromatographed on a Mono S (HR 10/10) cation exchange fast protein liquid chromatography (FPLC) column (Pharmacia, Piscataway, NJ) equilibrated with Buffer A

*To whom correspondence should be addressed.

Abbreviations: cAMP, adenosine 3′,5′-cyclic monophosphate; CEH, cholesteryl ester hydrolase; FPLC, fast protein liquid chromatography; GPHPLC, gel permeation high-performance liquid chromatography; pI, isoelectric point; PKA, cAMP-dependent protein kinase; PKC, protein kinase C; S104, 104,000 × *g* supernatant; SDS-PAGE, sodium dodecylsulfate-polyacrylamide gel electrophoresis.

and eluted with a linear NaCl gradient (0–800 mM). Most of the CEH eluted at 225 mM NaCl. Fractions containing CEH activity were pooled and concentrated using Amicon Centraprep concentrators with a 30,000 molecular weight cut off. Approximately 0.8 mg of the concentrated protein was then chromatographed on gel permeation Protein pak SW 300 HPLC (GPHPLC) column (Waters, Milford, MA) equilibrated and eluted with 200 mM phosphate buffer, pH 7.5, containing 80 mM KCl, 0.5 mM taurocholate and 5 mM 2-mercaptoethanol. CEH activity was eluted in a single (1 mL) fraction corresponding to a molecular weight (M_r) of 60–70 kDa. Purity at all stages was assessed by sodium dodecylsulfate-polyacrylamide gel electrophoresis (SDS-PAGE) using 5–20% gradient gels essentially as described by Laemmli *et al.* (8). After electrophoresis, gels were silver stained using the Bio-Rad silver reagent kit. For Western blots, proteins were blotted on PVDF membranes (Millipore, Bedford, MA) using PolyBlot (American Bionetics Inc., Hayward, CA). Cross-immunoreactivity was assessed using the Immuno-Blot (GAR-HRP) assay kit from Bio-Rad. The protein band at M_r = 66 kDa was cut from the PVDF membrane and used to determine the amino acid composition. CEH inhibition by anti-rat pancreatic IgG was evaluated as described by Harrison *et al.* (5) over the same range of concentrations, with the exception that 0.5 mM taurocholate was used in the final assay buffer. Neutralization of CEH activity in pancreatic cytosol was carried out as described by Gallo *et al.* (6) using 5 mM taurocholate as the bile salt in the assay buffer.

RESULTS

Purification of cytosolic CEH. Rat liver CEH was purified to apparent homogeneity by ammonium sulfate precipitation, cation exchange FPLC and GPHPLC (Table 1). Essentially all activity was recovered from the 40% ammonium sulfate precipitate. From the cation exchange column, most CEH activity eluted with 225 mM NaCl; however, a second peak of activity eluted at 600 mM NaCl (Fig. 1). Activity in this second peak varied from preparation to preparation, but was always less than that associated with the major CEH peak. The apparent yield at this stage was essentially 100% (Table 1). In contrast, pancreatic CEH subjected to the same purification protocol exhibited an entirely different elution profile (Fig. 1).

When concentrated fractions from the cation exchange column were further fractionated by GPHPLC, CEH activity was eluted in a single fraction corresponding to M_r = 60–70 kDa. This purification procedure produced a 12,600-fold increase in specific activity with an overall yield of 20% (Table 1). Purity of the GPHPLC fraction was assessed by SDS-PAGE, which yielded a single silver-stained band, corresponding to M_r = 66 kDa (Fig. 2, lane 1). Purified CEH was unstable in storage at −20°C, however, activity was retained for at least seven days with storage in 250 mM sucrose at 4°C.

Characterization of purified CEH. Activity of fractions from GPHPLC was measured in buffers ranging from pH 4–10. As seen in Figure 3, this enzyme exhibited activity over the range pH 5–9, with optimal activity at pH 7.0–7.5. As seen in Figure 4, enzyme activity rapidly increased between 2.5 to 6.5 μM substrate and gradually increased thereafter, with further increases in substrate up to 300 μM (data not shown). Thus, typical of enzymes with insoluble substrates, this CEH did not saturate with substrate within the range of feasible substrate concentrations.

Effect of bile salts. Among various bile salts tested over the concentration range 0.25–10 mM, taurocholate was found to be most effective in stimulating activity (1.75-fold, at 0.5 mM) of liver CEH in cytosol (data not shown). Glycocholate, cholate and deoxycholate either did not stimulate or only marginally stimulated CEH (data not shown). The purified enzyme was activated by increasing taurocholate concentrations up to 1 mM, remained more or less constant in activity from 1–10 mM taurocholate and declined in activity with further

TABLE 1

Purification of Neutral Cholesteryl Ester Hydrolase from Rat Liver Cytosol[a]

Fraction	Total protein (mg)	Total activity (units/hr)	Specific activity (units/hr/mg)	Yield %	Fold
Cytosol (S104)	125	0.252	0.002	100	—
0–40% Ammonium sulfate precipitate	61.9	0.251	0.004	99.5	2
Cation exchange Mono S FPLC	0.8	0.261	0.327	103.7	164
Gel permeation HPLC SW 300 column	0.002	0.051	25.2	19.8	12,600

[a]One unit is one μmole [1-^{14}C]cholesteryl oleate hydrolyzed per hr at 37°C. Fractions from the Mono S column were assayed in Buffer A. Fractions from the gel permeation HPLC column were assayed in the elution buffer, which gave results equivalent to those of Buffer A.

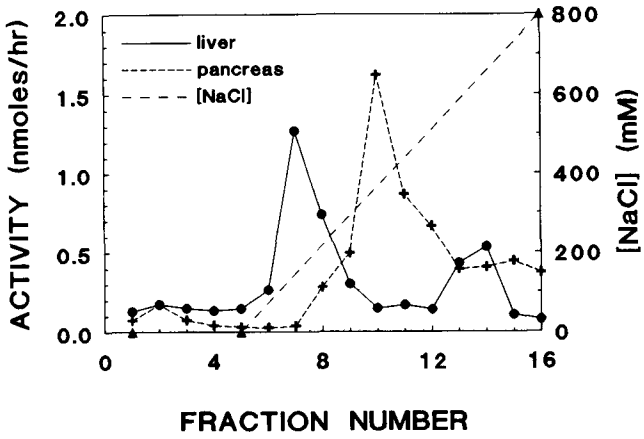


FIG. 1. Elution profile of liver and pancreatic CEH from cation exchange column. 0–40% Ammonium sulfate precipitate was desalted on Sephadex G-25, and 60 mg (or 20–25 mg for pancreas) was loaded on Mono S column. Proteins were eluted using a linear NaCl gradient. Fractions were collected every 2 min and an aliquot was assayed for CEH activity in Buffer A as described in Materials and Methods. Values represent mean of duplicates.

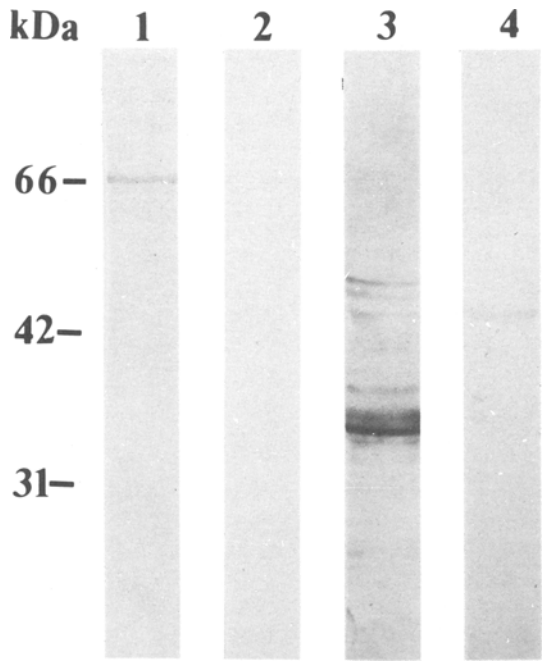


FIG. 2. SDS-polyacrylamide gel electrophoresis of rat liver and pancreatic CEH. Lane 1, silver stained purified liver CEH after GPHPLC; lane 2, immunoblot of lane 1 with anti-rat pancreatic CEH IgG; lane 3, silver-stained rat pancreatic ammonium sulfate precipitate; and lane 4, immunoblot of lane 3 with anti-rat pancreatic CEH IgG.

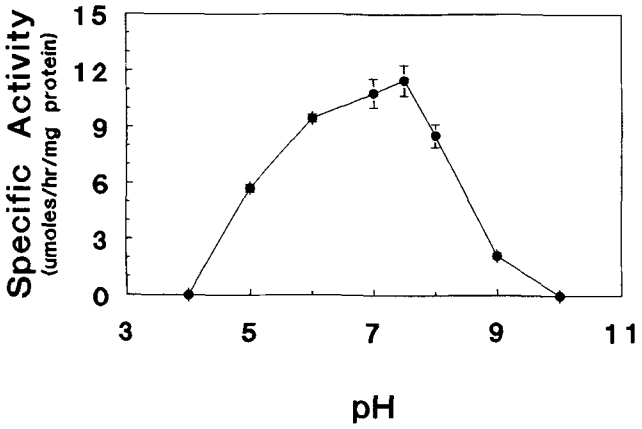


FIG. 3. pH Dependence of purified liver CEH. Fractions from GPHPLC containing purified CEH were assayed in buffers with different pH. Buffers used were: acetate, for pH 4.0, 5.0 and 6.0; phosphate, for pH 7.0, 7.5 and 8.0; and glycine-NaOH, for pH 9.0 and 10.0. All buffers contained 80 mM KCl, 5 mM 2-mercaptoethanol and 5 mM taurocholate. Values represent Mean ± S.E.M. from two experiments, each done in duplicate.

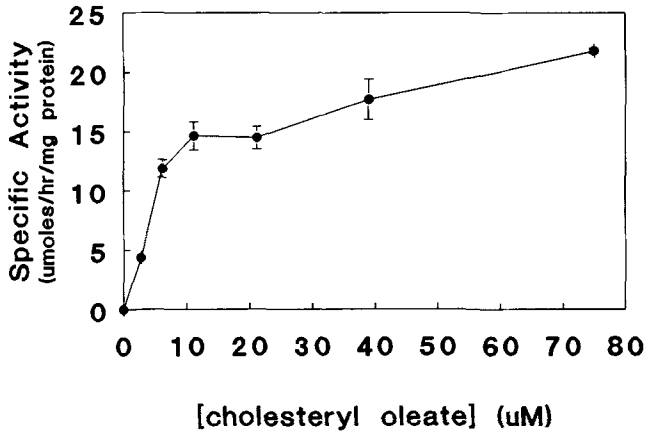


FIG. 4. Effect of cholesteryl ester concentration on catalytic activity of purified CEH. Fractions from GPHPLC were assayed as described in Materials and Methods using indicated concentrations of cholesteryl oleate. Specific radioactivity of the substrate was maintained constant at all concentrations. Values represent Mean ± S.E.M. for duplicate assays. Experiment was repeated with similar results.

increases in concentration (Fig. 5). The liver CEH is strongly inhibited by 20–40 mM taurocholate, in marked contrast to the pancreatic CEH, which is known to be activated by bile salts in this concentration range.

Inasmuch as taurocholate is known to induce formation of an active multimer of pancreatic CEH, purification of CEH was carried out both with and without taurocholate in chromatography eluents. Taurocholate had no apparent effect on elution from the cation exchange column (data not shown). However, an additional peak of activity corresponding to M_r = 400 kDa was eluted from GPHPLC when taurocholate was omitted from the eluent (Fig. 6).

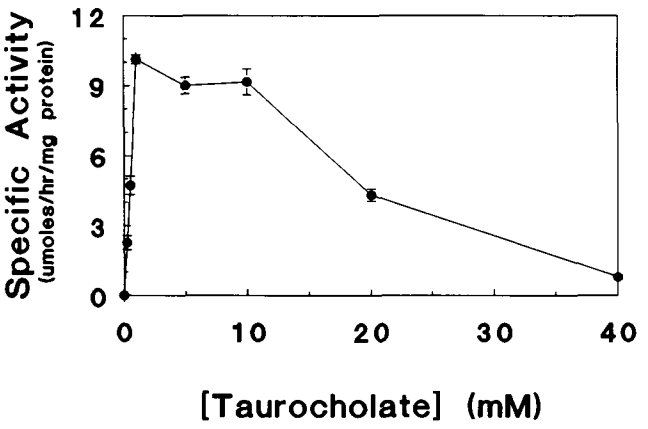


FIG. 5. Effect of varying taurocholate concentration on the activity of purified CEH. Fractions from the GPHPLC purification were assayed in the presence of indicated concentrations of taurocholate. Values represent Mean ± S.E.M. of duplicate assays. Experiment was repeated with similar results.

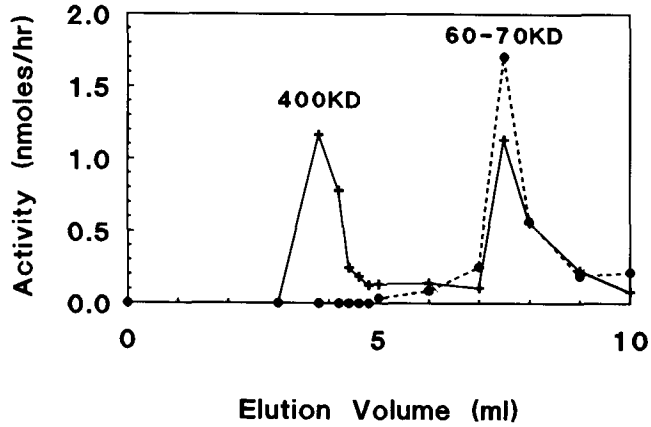


FIG. 6. Effect of taurocholate on apparent molecular weight of rat liver CEH. Pooled and concentrated fractions from cation exchange FPLC were further fractionated by GPHPLC with (—o—) and without (—+—) 0.5 mM taurocholate in the elution buffer. All fractions were equilibrated in elution buffer containing 0.5 mM taurocholate and assayed for CEH as described in Materials and Methods.

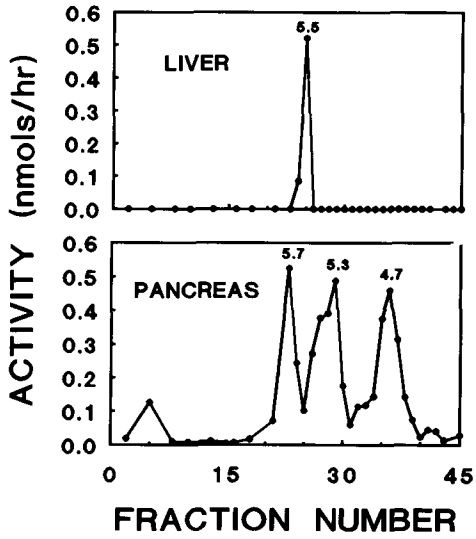


FIG. 7. Elution of liver and pancreatic CEH from Mono P (chromatofocusing) column. Column was equilibrated with 25 mM bis Tris, pH 7.1 containing 80 mM KCl, 0.5 mM taurocholate and 5 mM 2-mercaptoethanol. Proteins were eluted with Polybuffer 74 containing 80 mM KCl, 0.5 mM taurocholate and 5 mM 2-mercaptoethanol. A pH gradient of 7–4 was generated from fractions 5–45. Top panel: fraction containing CEH from GPHPLC was concentrated, brought to 1 mL with equilibration buffer and further fractionated by chromatofocusing. Mono P fractions were neutralized to pH 7.0 with Trizma base and assayed for CEH activity in Buffer A. Bottom panel: 0–40% ammonium sulfate precipitate from pancreatic S104 was desalted and fractionated by cation exchange FPLC. Active fractions were pooled, concentrated and further fractionated by chromatofocusing. CEH was assayed in Buffer A following neutralization. pI Is given for each peak of activity.

These results suggest that liver CEH disaggregates in the presence of bile salts. Whereas both 66 kDa and 400 kDa forms are apparently present in the absence of taurocholate, the activity of the 400 kDa form could not be ascertained.

Determination of isoelectric point (pI). Figure 7 shows the elution of purified rat liver CEH and partially purified rat pancreatic CEH from a FPLC chromatofocusing column. Whereas liver CEH eluted as a single peak corresponding to a pI value of 5.5 (top panel), pancreatic CEH eluted in three distinct peaks corresponding to pI values of 5.7, 5.3 and 4.7 (bottom panel). These results indicate presence of a single species of CEH in the purified fraction from liver and existence of multiple forms in pancreas, none of which are identical to the liver CEH.

N-Terminal analysis and amino acid composition. The purified CEH fraction from HPLC was analyzed by SDS-PAGE, electroblotted on PVDF membrane and proteins stained with Coomassie blue (9). The protein band at 66 kDa was cut out and subjected to protein sequencing in gas phase. Although amino acid analysis showed presence of sufficient protein for sequencing (300 pmoles), no N-terminal residue was detected, suggesting a blocked N-terminus. The amino acid composition, as shown in Table 2, is quite different from the reported compositions for two pancreatic isoenzymes.

Activation by protein kinases. The liver and pancreatic CEH isoenzymes also exhibited differential responses to cofactors for cAMP-dependent protein kinase (PKA) and protein kinase C (PKC). Previously we have demonstrated activation of liver CEH by these cofactors (7). In the current studies, concentrations of cofactors for PKA (cAMP, ATP, Mg^{2+}) which produced optimal activation of liver CEH had no effect on pancreatic CEH (data not shown). Moreover, cofactors for PKC (diacylglycerol, phosphatidylserine, ATP) produced a 33% decrease in pancreatic CEH activity (data not shown).

Immunological properties. Immunoreactivities of purified liver CEH and partially purified pancreatic CEH with anti-rat pancreatic CEH IgG were compared. The liver enzyme showed absolutely no cross-reactivity with antibody to pancreatic enzyme in Western blot analysis

TABLE 2

Amino Acid Composition of Cholesteryl Ester Hydrolases from Rat Liver and Rat Pancreas

Amino acid	Mole (%)		
	Liver	Pancreas[a]	Pancreas[b]
Cys	0.0	18.0	0.007
As(x)	17.8	12.0	11.7
Thr	6.2	6.7	7.4
Ser	5.0	10.5	5.1
Gl(x)	12.9	10.7	8.0
Gly	7.2	11.6	8.3
Ala	7.0	9.0	9.1
Val	6.0	7.0	6.2
Met	Tr[c]	0.3	2.4
Ile	5.2	4.7	5.2
Leu	9.9	7.2	7.3
Tyr	2.6	0.0	3.9
Phe	3.0	4.0	4.6
His	2.1	2.2	2.2
Lys	6.0	5.6	5.9
Arg	4.9	5.2	2.9
Pro	4.0	6.8	7.4
Average molecular weight	66,000	69,000	67,000

[a]Taken from ref. 13.

[b]Taken from ref. 24.

[c]In trace amounts.

(Fig. 2, lane 2). In contrast, the pancreatic cytosol contained a single, strongly immunoreactive band of M_r = 45 kDa (Fig. 2, lane 4). Antibodies to pancreatic CEH had no inhibitory effect on liver CEH activity (90% activity remaining). In our hands, under similar conditions, these antibodies inhibited 88% of CEH activity in pancreatic S104, relative to normal IgG.

DISCUSSION

We have described a three-step, rapid purification of liver CEH yielding a protein associated with a 12,600-fold increase in specific activity and a single band corresponding to 66 kDa on SDS-PAGE. Relatively high yield (20%), which increases confidence that this enzyme makes a major contribution to liver CEH *in vivo*, is attributable to the simplicity and speed of this procedure, which minimizes loss of activity by this relatively unstable enzyme. Based on inhibition of liver CEH activity by antibodies to pancreatic CEH (5) and purification of rat liver CEH with antibodies to porcine pancreatic CEH (4), others have proposed that liver CEH and pancreatic CEH are either very similar or identical enzymes. The present studies provide strong evidence that a major fraction of cytosolic liver CEH is associated with the enzyme we have purified, which has very different properties from pancreatic CEH. We earlier demonstrated that this liver CEH could be distinguished from triglyceride lipase and retinyl ester hydrolase activities by differential purification and regulatory responses (10).

We have shown that this enzyme aggregates to form a multimer of M_r = 400 kDa in the absence of bile salts (Fig. 6). Tuhackova *et al.* (11) also observed aggregation of liver CEH in absence of bile salts. In contrast, Hyun *et al.* (12) and Calame *et al.* (13) have shown that at physiological concentrations of cholic acid or its conjugates, an inactive monomer of pancreatic CEH undergoes aggregation to form an active hexamer. Pancreatic and liver CEH catalytic activities are also affected differentially by bile salts. Like the pancreatic CEH, the enzyme which we have isolated is stimulated by bile salt at 0.25–10 mM. However, unlike pancreatic CEH, liver CEH is strongly inhibited by concentrations greater than 10 mM, used in several of the comparative studies (4,5). Activation by the lower concentrations (<5 mM) of taurocholate was much more pronounced with the purified liver CEH than in cytosol, suggesting that the activation may be a detergent effect rather than a specific effect on the enzyme. In contrast, as noted earlier, activation of the pancreatic CEH by bile salts involves formation of a multimeric form.

Several other properties of liver and pancreatic CEH indicate that the two enzymes are different. Unlike liver enzyme, pancreatic enzyme is apparently not regulated by reversible phosphorylation, a mode of short-term regulation of CEH in liver (7), adrenal cortex (14), testis (15), macrophages (16) and brain (17). Differential elution of the two enzymes from cation exchange columns (Fig. 1) is indicative of different surface charge distributions on the two proteins. This conclusion is further supported by different pI values for the two enzymes (Fig. 7). The amino acid compositions of the liver CEH is unlike those reported for pancreatic isoenzymes (Table 2).

Purified liver CEH did not cross-react with antibody to rat pancreatic CEH in Western blot analysis (Fig. 2, lane 2). These results are consistent with earlier observations of Gallo *et al.* (6), who showed immunological cross-reactivity between pancreatic and intestinal enzyme but no reaction with the solubilized fractions from liver, aorta or adrenals using antibodies to the rat pancreatic CEH. The anti-pancreatic CEH IgG did react with a protein in pancreatic cytosol with M_r = 45 kDa (lane 4). Although this is not one of the molecular weights previously reported for rat pancreatic CEH, this enzyme is reported to exhibit anomalous apparent molecular weights on SDS-PAGE, perhaps due to carbohydrate content (18). Moreover, the porcine pancreatic CEH has been reported to exist in several different molecular weight forms, including one of M_r = 45 kDa (19–21). It is also possible that the antibodies did not recognize higher molecular weight forms of the enzyme in their denatured state as presented in SDS-PAGE. However, that the antibodies did indeed cross-react with active pancreatic CEH was confirmed by neutralization of 88% of pancreatic enzyme activity.

In contrast to complete inhibition of liver cytosolic CEH reported by Harrison (5), antibodies to pancreatic CEH from the same source did not inhibit the liver CEH purified by us. However, it should be emphasized that 20–75 mM bile salts used by these investigators strongly inhibits the liver CEH which we have purified, as well as the isoenzymes from testis and brain (22,23).

Camulli *et al.* (4) obtained the N-terminal sequence for a liver CEH purified by immunoaffinity chromatography, which was identical to the N-terminus of rat pancreatic CEH. Since these authors used anti-porcine pancreatic CEH IgG for purification, it appears that they isolated a component of total liver CEH which is similar to the pancreatic enzyme. Using a different protocol, we have purified a liver CEH which is apparently an entirely

different protein, accounting for at least 20% of the liver cytosolic CEH. Its specific activity is two orders of magnitude higher, it does not cross-react with antibody to the pancreatic CEH and it is blocked in the N-terminus. Together, these results indicate polymorphisms for liver CEH which need to be investigated further.

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Effect of Chylomicron Remnants on Cholesterol Metabolism in Cultured Rabbit Hepatocytes: Very Low Density Lipoprotein and Bile Acid Production

Vladimir A. Kosykh*, Dmitri K. Novikov, Iliya N. Trakht, Eugeni A. Podrez, Alexander V. Victorov, Vadim S. Repin and **Vladimir N. Smirnov**
USSR Cardiology Research Center, Academy of Medical Sciences, Moscow 121552, USSR

The interrelationship between very low density lipoprotein (VLDL) secretion and bile acid production was studied in primary culture of rabbit hepatocytes. Chylomicron remnants (CR) were added to the cultures to study their effect on VLDL secretion and bile acid production. After 24 hr preincubation of cells with CR (10–50 μg protein/mL), intercellular neutral lipid content was increased 1.5–4-fold in a dose-dependent manner. Neutral lipid accumulation was accompanied by a 70–90% reduction of [^{14}C]acetate incorporation into cholesterol, while no stimulation of [^{14}C]oleate incorporation into cholesteryl esters was observed. Incubation of cells with CR increased secretion of free cholesterol, triacylglycerol and apoproteins B and E in VLDL. Stimulation of VLDL cholesterol secretion was accompanied by a reduction of taurocholic acid synthesis. These data demonstrate the existence of an inverse relationship between secretion of VLDL cholesterol and bile acid production under conditions of effective uptake of triacylglycerol-rich CR by hepatocytes.
Lipids 26, 799–805 (1991).

Dietary cholesterol and other lipids are transported to the liver in the form of chylomicron remnants (CR) which contain apoproteins B and E (1). These particles are bound and internalized by way of B and E receptors located on the cell surface (2–4). After hydrolysis, the components of CR are available to the cell for further use. Free fatty acids may become esterified and secreted as triacylglycerol, mainly in the form of very low density lipoproteins (VLDL). Free cholesterol can be utilized *via* a number of metabolic pathways, including the assembly and secretion of lipoproteins, the synthesis and secretion of bile acids, the secretion of biliary cholesterol, and esterification; the latter is dependent upon the activity of acyl-CoA:cholesterol acyltransferase (ACAT) (E.C. 2.3.1.26). On the other hand, plasma cholesterol levels are dependent on the flux of free cholesterol into various pathways of hepatocyte metabolism (1). Recent studies have demonstrated that the direction of cholesterol flux into and out of these pathways may depend on the lipid composition of the lipoprotein particles taken up by liver cells (5,6). Van Zuiden *et al.* (5) showed that the *in vivo* administration of triglyceride-rich chylomicrons or their remnants to perfused rat liver reduces ACAT activity and biliary cholesterol content, while secretion of VLDL cholesterol and triacylglycerol by hepatocytes increases. Other studies have demonstrated that administration of apo E- and cholesterol-rich lipoprotein fraction (d 1.02–1.06 g/mL) from hypercholesterolemic rats in isolated perfused liver produced a significant increase in ACAT activity and in hepatic VLDL secretion without significantly affecting biliary cholesterol output (6). These studies suggest that ACAT activity may regulate preferential channeling of CR cholesterol either towards VLDL cholesterol or biliary cholesterol secretion. Although the effect of CR on hepatic cholesterol metabolism has been studied in several animal models as well as in cultured cells (1,5,6), various aspects still need to be investigated further.

In the present study, we have examined the interrelationship between VLDL secretion and bile acid production in the presence of an excess of CR lipids that were delivered to hepatocytes. The effect of CR on cholesterol synthesis and cholesterol esterification was also determined. Since apo B-48 is a marker of intestinally synthesized lipoproteins in both humans and rabbits, only particles containing apo B-48 and apo E as CR were used.

MATERIALS AND METHODS

Materials. All chemicals used were reagent grade. Minimal essential medium (MEM) with Earle's salts, fetal bovine serum (FBS), MEM nonessential amino acids, kanamycin, L-glutamine, Vitrogen 100™ (purified collagen) and 35- to 100-mm culture dishes were purchased from Flow Laboratories, Inc. (Zwanenburg, The Netherlands). Collagenase 1200 units/mg and trypsin inhibitor were products of Sigma Chemical Co. (St. Louis, MO). L-[4,5-^{3}H]Leucine (spec. act. 120–190 Ci/mmol), L-[^{35}S]-Methionine (spec. act. > 1000 Ci/mmol), sodium [1-^{14}C]acetate (spec. act. 59 mCi/mmol), and sodium [1-^{14}C]oleate (spec. act. 50–60 mCi/mmol) were purchased from Amersham International (Amersham, Buckinghamshire, U.K.).

Male Chinchilla rabbits, weighing 2.5–3 kg, were used throughout and maintained on standard laboratory rabbit chow. Animals were killed between 10 and 11 a.m. for preparation of hepatocytes.

Rabbit hepatocyte preparation and culture. Rabbit liver cells were isolated by perfusion with 0.05% collagenase and 0.005% trypsin inhibitor as described in detail elsewhere (7). Cells were plated in 35-mm or 100-mm dishes coated with purified collagen at a density of 2×10^5 cells/cm^2 and were cultured in MEM supplemented with 10% FBS, 2 mM L-glutamine, kanamycin (100 μg/mL), and nonessential amino acids (1%) at 38°C in a 95% air/5% CO_2 atmosphere as described elsewhere (8). Cells were

*To whom correspondence should be addressed at Institute of Experimental Cardiology, Cardiology Research Center of the USSR, 3rd Cherepkovskaya 15 A, 121552 Moscow, USSR.

Abbreviations: ACAT, acyl-CoA:cholesterol acyltransferase; apo, apoprotein; CR, chylomicron remnants; EDTA, ethylenediaminetetraacetic acid; FBS, fetal bovine serum; HDL, high density lipoprotein(s); HPTLC, high-performance thin-layer chromatography; LPDS, lipoprotein deficient serum; MEM, minimal essential medium; SDS, sodium dodecylsulfate; VLDL, very low density lipoprotein(s).

used for experiments after 24-hr preincubation in MEM supplemented with 10% FBS. To examine the influence of CR on various pathways of cellular lipid metabolism, the experiments were carried out as follows. After 24 hr in culture, cells were transferred in MEM supplemented with 10% lipoprotein deficient serum (LPDS) with or without CR at various concentrations; the cells were incubated at 37°C for 24 hr. After preincubation with or without CR, cells were incubated in serum-free medium, and incorporation of [^{14}C]acetate into cholesterol (at 3 hr), incorporation of [^{14}C]oleate into triacylglycerol and cholesteryl esters (at 6 hr), and secretion rates of VLDL and bile acid (at 18 hr) were measured.

Isolation of lipoproteins and LPDS. Human plasma samples were collected from normal subjects 4–5 hr after ingestion of a fatty meal (2 eggs, 200 g of cream) and were kept at 4°C overnight. The creamy layer, which contained triacylglycerol-rich lipoproteins including chylomicrons, was removed and suspended in 0.15M NaCl containing 0.01% ethylenediamine tetraacetic acid (EDTA) (pH 7). Postheparin plasma (lipoprotein lipase-rich plasma) was used to hydrolyze the triacylglycerol-rich fraction and to obtain CR. Plasma was obtained from a normal human subject 2 hr after injection of heparin (100 units/kg of body weight). The total lipoprotein fraction was incubated with postheparin plasma as described by Mahley *et al.* (9). At the end of the incubation, the solution was adjusted to a density of 1.019 with KBr, and overlayered with an equal volume of saline/EDTA. Lipoproteins were refloated overnight at 130,000 × *g* using an SW 28 rotor. After centrifugation, the top 1-mL containing CR and VLDL remnants was collected. Apo B-48 containing CR particles was isolated by immunoaffinity chromatography according to Milne *et al.* (10). Immunosorbent was prepared by covalent coupling of the anti-apo B-100 monoclonal antibodies 2G8 to Sepharose 4B (11). Preparation of monoclonal antibodies against human apo B-100 has been reported (12). These antibodies did not react with apo B-48. High density lipoproteins (HDL) (1.063<d<1.21 g/mL) and LPDS (d>1.21 g/mL) were isolated from normal plasma by preparative ultracentrifugation at 4°C after a density adjustment with solid KBr (13). HDL_2 was isolated as described previously (14). Protein concentration in the lipoprotein fraction was determined by the method of Lowry *et al.* (15). Lipoprotein total cholesterol was assayed enzymatically using the Total Cholesterol Kit (Medix; Finland). Ten μL of lipoprotein or standard solution were combined with 1 mL of cholesterol enzymatic reagent and incubated at 37°C for 20 min. The absorbance at 500 nm was measured for each sample against a reagent blank.

Analytical methods. Cells were incubated for 18 hr with leucine-free MEM supplemented with [^{14}C]leucine (10 μCi/mL) to study the secretion rates of *de novo* synthesized VLDL apoproteins. The medium was removed after incubation for analysis. VLDL fractions secreted by cells were isolated by preparative ultracentrifugation of the medium with an LP-42 Ti rotor (Beckman Instruments, Palo Alto, CA) at 130,000 × *g* for 2.5 hr at 18°C. VLDL was collected from the centrifugation tubes at d < 1.006 g/mL (50 μL from the top), and then recentrifuged. Aliquots of VLDL fractions were prepared as described by Melin *et al.* (16) and subjected to (SDS)-polyacrylamide gel electrophoresis; the gels contained 0.1% Coomassie blue. The relative quantity of VLDL apoproteins secreted by CR-treated hepatocytes as compared with control cells was estimated by scanning using an LKB 2202 Ultrascan laser densitometer. The isotope incorporation into the apoprotein band was estimated by counting the gel slices. The gels were cut into 2–3 mm slices, placed into separate vials containing 30% H_2O_2, and incubated overnight at 70°C. Radioactivity was determined in a scintillation counter.

Cells were incubated for 3 hr with MEM containing [^{14}C]acetate (5 μCi/mL, 0.1 mM) or for 6 hr with [^{14}C]oleate/albumin complex (final concentration 0.4 mM [^{14}C]oleate, approximately 10,000 cpm/nmol in MEM). Neutral lipids were extracted according to Goldstein *et al.* (17), and individual species were separated by high-performance thin-layer chromatography (HPTLC) (silica gel plates; Merck, Darmstadt, Germany) using heptane/diethyl ether/acetic acid (90:30:1, v/v/v) as developing system. Fractions corresponding to cholesterol and cholesteryl esters were scraped into counting vials, and radioactivity was assayed. Lipoprotein phospholipids were extracted according to Folch *et al.* (18) and separated on a thin layer of silica with methyl acetate/1-propanol/chloroform/methanol/0.25% KCl (25:25:25:10:9, by vol) (19). Neutral lipids and phospholipids were quantitatively determined by densitometry of HPTLC plates (20,21) with an automated Camag HPTLC/thin-layer chromatography (TLC) scanner (Muttens, Switzerland) connected to a recording integrator SP 4100 (Spectra Physics, Darmstadt, Germany). Each determination was done in parallel with a known standard mixture containing cholesterol, triolein, cholesteryl oleate, phosphatidylcholine, phosphatidylethanolamine, phosphatidylinositol, phosphatidylserine and sphingomyelin.

After isolation of the total lipid fraction (22), bile acids were extracted according to Kempen *et al.* (23) using a reverse-phase cartridge (Sepak C-18, Waters Associates, Milford, MA). Bile acids were separated by HPTLC with the developing system chloroform/methanol/glacial acetic acid/water (15:5:1:1, by vol) using unlabeled markers. Bile acids were quantitatively determined by densitometry (564 nm) of HPTLC plates pretreated with 10% phosphomolybdenic acid. A mixture of taurocholate, taurodeoxycholate and glycocholate was used as standard.

Cells were incubated for 18 hr with [^{35}S]methionine (250 μCi/100-mm culture dish) for analysis of re-uptake of secreted lipoproteins. The medium containing labeled lipoproteins was dialyzed for 24 hr against unlabeled medium, and administered to control and CR-treated hepatocytes or to 35-mm culture dishes without cells. After 18 hr of incubation, the media were ultracentrifuged to isolate lipoproteins (d<1.21 g/mL). Following separation of apoproteins on SDS-polyacrylamide gel, the amount of ^{35}S in apo B and apo E was quantified by scintillation counting.

Results were analyzed with Student's *t*-test and expressed as mean ± SD. Values of $P < 0.05$ were considered significant.

RESULTS

As shown in Figure 1a, two major species of human apo B (apo B-48 and apo B-100) were identified in a plasma triacylglycerol-rich lipoprotein fraction after a fatty meal.

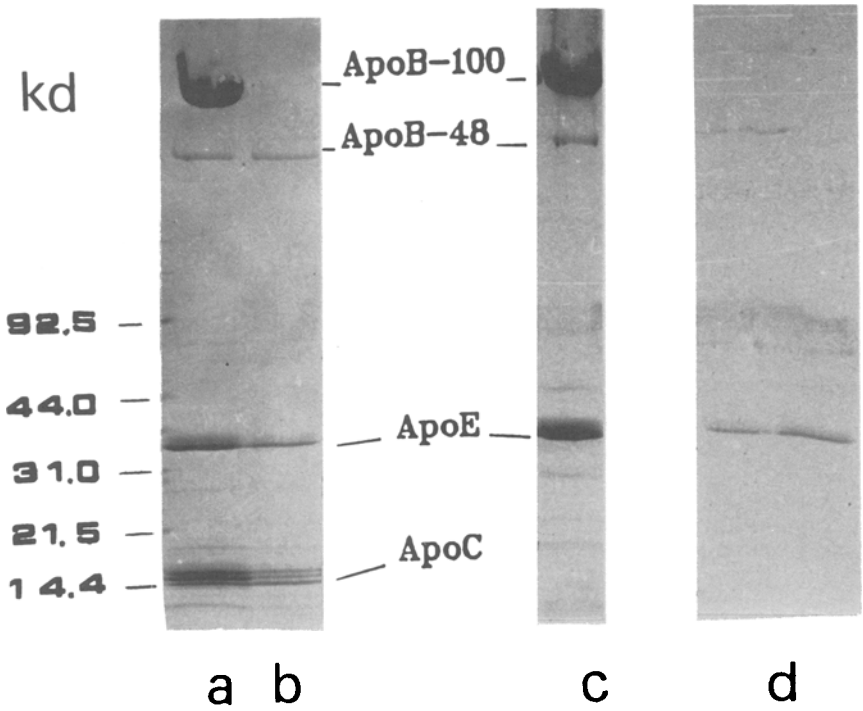


FIG. 1. Polyacrylamide gel electrophoresis of total lipoprotein fraction (a), chylomicrons (b), apo C-deficient triacylglycerol-rich lipoproteins (c), and CR (d). Delipidated apoproteins were applied to 3–20% gradient gel run in the presence of sodium dodecyl sulfate and stained by Coomassie Blue. The vertical axis indicates the molecular weights of the marker proteins.

TABLE 1

Chemical Composition (by the Weight) of Chylomicron Remnants[a]

Preparation	Protein	Triacylglycerol	Cholesterol	Cholesteryl esters	Phospholipids
1	4.1	68.2	2.6	16.3	9.4
2	8.2	61.3	2.2	20.0	8.3
3	6.5	67.7	2.8	16.1	6.9

[a]Various lipid classes were separated by HPTLC and identified by comparison to standards. Lipid concentrations were measured using densitometry. Protein was determined by the method of Lowry *et al.* (15) using bovine serum albumin as standard.

Purified apo B-48 containing chylomicron and CR were isolated by immunoaffinity chromatography (Fig. 1b and d). Lipoprotein particles containing a small quantity of apo C were produced by incubating the total lipoprotein fraction with postheparin plasma (Fig. 1c). Purified CR (Fig. 1d) were used in experiments with cells. Table 1 shows the chemical composition of CR preparations obtained from three donors. Although the protein content varied (4.1 to 8.2 weight%), the lipid composition of three CR fractions was similar.

Figure 2 shows that preincubation of cells with CR up to concentrations of 50 μg protein/mL caused a large rise in cellular neutral lipid content. Sterol synthesis at the high CR dose was decreased by approximately 70–90% (Fig. 3). In addition, incubation of cells with increasing amounts of CR increased, in a concentration-dependent manner, the amount of [^{14}C]oleate incorporated into triacylglycerol, but did not affect [^{14}C]oleate incorporation into cholesteryl esters (Fig. 4).

In these experiments, mass measurements of different classes of VLDL neutral lipids secreted by rabbit hepatocytes also were performed (Table 2). Preincubation with CR increased triacylglycerol and free cholesterol secretion in VLDL, but did not affect the release of VLDL cholesteryl esters.

To examine the effect of CR on the secretion of VLDL apoprotein by hepatocytes after preincubation of cells with CR, the media were changed and hepatocytes were further incubated with L-[4,5-^{3}H]leucine for an additional 18 hr. The VLDL fractions secreted by control and CR-treated cells were analyzed by SDS-polyacrylamide gel electrophoresis; simultaneously [4,5-^{3}H]leucine incorpora-

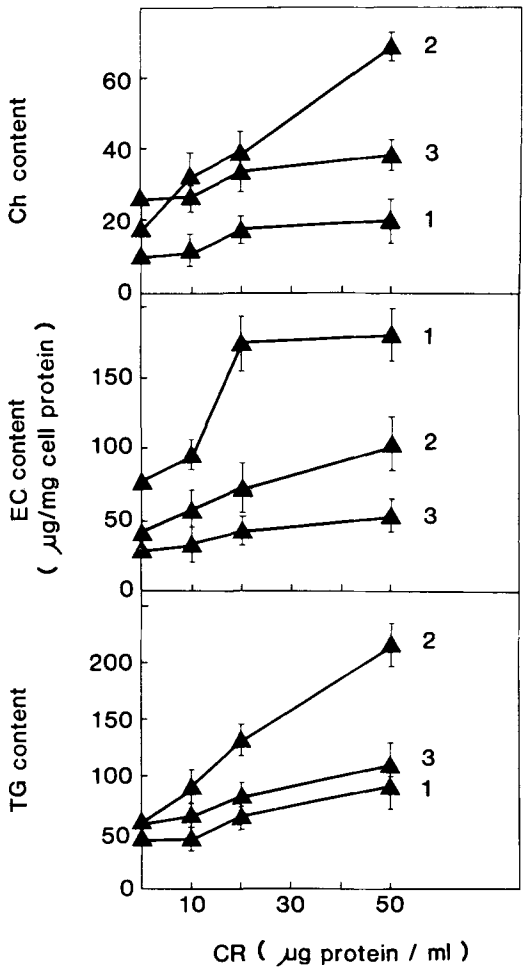


FIG. 2. Hepatocyte neutral lipid accumulation following preincubation with various concentrations of CR. Exp. 1-3 are independent experiments with cells from different rabbit livers. Ch, cholesterol; EC, cholesteryl ester; and TG, triacylglycerol. Values are means ± SD of three replicates.

tion into apo B and apo E was quantified. The results of these analyses are given in Table 3. Preincubation of the cells with CR stimulated secretion of newly synthesized apo B and apo E in the VLDL fraction approximately 1.5-2.0-fold. Analysis of the polyacrylamide electrophoretic gel by densitometry also showed increases (1.5-2.0-fold, two experiments) in VLDL apo B and apo E secretion by the cells preincubated with CR.

To examine the possibility that the results obtained may reflect alterations in the re-uptake of secreted VLDL, control and CR-treated cells were incubated with [^{35}S]methionine-labeled secreted lipoproteins. After 18-hr cultivation, the amount of [^{35}S]methionine-labeled apoprotein remaining in the medium was determined. The results shown in Table 4 demonstrate that cultured control and CR-treated hepatocytes do not actively (at least not more than 10% of total apoproteins added) take up and degrade newly secreted apo B and apo E in the culture medium. Thus, the data show that hepatocytes preincubated with CR secrete more VLDL apo B and apo E than control cells.

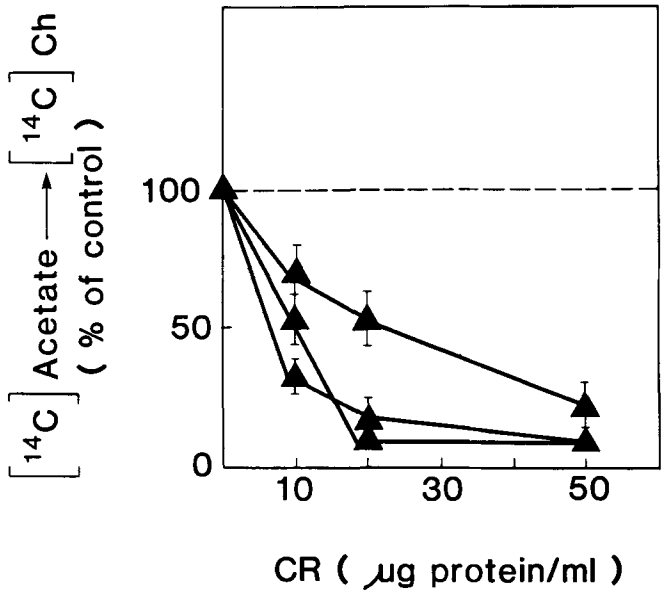


FIG. 3. Effect of CR on [^{14}C]acetate incorporation into cellular cholesterol. Each curve gives the results of one independent experiment. The results shown are based on the radioactivity ranging from 1.4×10^5 to 2.4×10^5 cpm/mg cell protein. Values are means ± SD of three replicates.

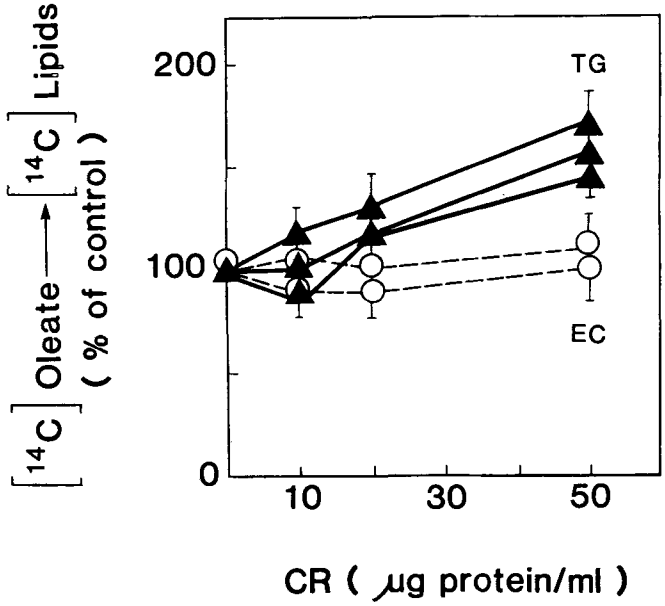


FIG. 4. Effect of CR on [^{14}C]oleate incorporation into cellular lipids. Each curve gives the results of one independent experiment. The results shown are based on the radioactivity ranging: from 5×10^3 to 5.6×10^3 cpm/mg cell protein for esterified cholesterol; and from 2.5×10^5 to 2.8×10^5 cpm/mg cell protein for triacylglycerol. Values are means ± SD of three replicates.

In the following experiments, bile acid formation was measured quantitatively by TLC. After preincubation with or without CR, hepatocytes were incubated in the serum-free medium for 18 hr. TLC analyses of the bile acids accumulated in the medium and in the cells showed

TABLE 2

Effect of Preincubation with Chylomicron Remnants on Lipid Secretion into VLDL[a]

	Exp. 1.		Exp. 2.	
	Control	CR[b]	Control	CR[b]
Triacylglycerol[c]	27.5 ± 2.1	46.7 ± 2.4[d]	15.3 ± 1.1	31.5 ± 2.3[d]
Cholesterol[c]	1.2 ± 0.2	1.8 ± 0.1[d]	2.1 ± 0.2	3.9 ± 0.3[d]
Cholesteryl Esters[c]	0.8 ± 0.2	0.8 ± 0.1	0.8 ± 0.2	0.8 ± 0.2

[a]Hepatocytes were incubated for 24 hr in MEM supplemented with 10% LPDS with or without CR. The cells were washed, and fresh, serum-free MEM was added. Cells were then incubated for 18 hr, and the VLDL fraction (d<1.006 g/mL) was isolated by preparative ultracentrifugation. Values are means ± SD of three replicates.

[b](d<1.006) 40 μg Protein/mL.

[c]μg Lipids/mg cell protein/18 hr; various VLDL lipid classes were separated by HPTLC and identified by comparison with standards. Lipid concentrations were measured by densitometry.

[d]P<0.05, *vs.* control.

TABLE 3

VLDL Apolipoprotein Secretion by Cultured Hepatocytes Following Preincubation with Chylomicron Remnants[a]

	Apo B[b]	Apo E[b]
Exp. 1		
Control	31.2 ± 2.6	16.1 ± 3.1
CR[c]	46.6 ± 4.1[d]	33.2 ± 2.8[d]
Exp. 2.		
Control	52.1 ± 6.2	29.1 ± 4.3
CR[c]	104.2 ± 5.1[d]	55.2 ± 2.6[d]
Exp. 3.		
Control	58.6 ± 4.1	19.6 ± 2.1
CR[c]	138.5 ± 10.4[d]	44.6 ± 3.1[d]
Exp. 4.		
Control	67.2 ± 5.1	32.2 ± 5.1
CR[c]	111.4 ± 8.2[d]	58.1 ± 3.2[d]

[a]Values are means ± SD of three replicates.

[b]10^{-2} × cpm/mg cell protein.

[c]40 μg protein/mL.

[d]P<0.05, *vs.* control.

TABLE 4

Re-uptake of Secreted Radiolabeled Apo B and Apo E by Cultured Hepatocytes[a]

	Apo B[b]	Apo E[b]
Without cells	24219 ± 2584	14325 ± 1240
Control cells	20856 ± 2621	12308 ± 1639
CR-treated cells	22156 ± 1630	11828 ± 2024

[a]Values are means ± SD of three replicates.

[b]cpm/mL Culture medium.

the presence of glycine and taurine conjugates of cholic acid which were identified by comparison with standards. As can be seen from Table 5, the ratio of taurocholic to glycocholic acid was about 3.5:1. During the 18-hr experiment, cultured hepatocytes produced taurocholic and glycocholic acids at a constant rate (data not shown). Addition of CR into the incubation medium suppressed taurocholic acid formation (Table 5), but did not alter the concentration of glycocholic acid. By contrast, addition of HDL_2 markedly stimulated the production of both conjugates of cholic acid in a dose-dependent manner.

DISCUSSION

During the past years, primary monolayer cultures of rat hepatocytes and the Hep G2 cell line have been used to study bile acid metabolism. However, these cells usually exhibit a low rate of bile production in culture (24–27). Recently it was reported that cultured rabbit hepatocytes, unlike rat hepatocytes, maintain a rate of bile acid synthesis similar to that found *in vivo* (28). Therefore, a primary culture of rabbit hepatocytes was used in the present study.

After incubation with CR, cell triacylglycerol and free and esterified cell cholesterol levels were increased. Such CR-induced lipid accumulation may lead to decreased synthesis of cholesterol. At the same time, free cholesterol derived from CR is not esterified, but is secreted in free form with newly synthesized VLDL. Under these conditions, the suppression of cholesterol synthesis is not sufficient to maintain cholesterol homeostasis. Therefore, the processes of apo B and apo E secretion in VLDL are also stimulated. Since each VLDL particle contains only one molecule of apo B (29), the number of VLDL particles secreted by rabbit hepatocytes increases. Our results are consistent with those of others (5,6) who have demonstrated that when exogenous cholesterol and triacylglycerol are available to hepatocytes from lipoproteins, the secretion of VLDL apoproteins and neutral lipids is enhanced. Recently Craig and Cooper (30) have reported that CR also stimulate VLDL apoprotein secretion by Hep G2 cells.

The second part of the study was devoted to the effect of CR on the production of bile acids. Previous studies with cultured rat and rabbit hepatocytes demonstrated that cholesterol availability is an important factor in stimulating the rate of bile acid production (24,31). Newly synthesized cholesterol and cholesterol of different lipoprotein fractions are used for bile acid production by hepatocytes (32,33). Our data confirm the results of Whiting *et al.* (28) that cultured rabbit hepatocytes obtained from normal liver synthesize and secrete mainly

TABLE 5

Effect of Chylomicron Remnants and HDL_2 on Production of Conjugated Bile Acid[a]

	Taurocholic acid[b]	Glycocholic acid[b]
Exp. 1		
Control	3.38 ± 0.17	0.81 ± 0.09
+ CR[c]		
15 μg ch[d]/mL (10 μg protein/mL)	2.88 ± 0.46	0.66 ± 0.06
75 μg ch/mL (50 μg protein/mL)	2.20 ± 0.18[e]	0.85 ± 0.04
+ HDL_2[c]		
90 μg ch/mL (250 μg protein/mL)	4.18 ± 0.35[e]	1.28 ± 0.11[e]
Exp. 2		
Control	2.96 ± 0.14	0.98 ± 0.13
+ CR[c]		
25 μg ch/mL (10 μg protein/mL)	2.66 ± 0.41	0.71 ± 0.12
125 μg ch/mL (50 μg protein/mL)	1.82 ± 0.12[e]	1.01 ± 0.17
+ HDL_2[c]		
100 μg ch/mL (250 μg protein/mL)	3.67 ± 0.07[e]	1.34 ± 0.15[e]
200 μg ch/mL (500 μg protein/mL)	4.39 ± 0.18[e]	2.09 ± 0.23[e]

[a]Values are means ± SD of three replicates.

[b]μg/mg Cell protein.

[c]μg of Lipoprotein total cholesterol added per mL of medium; μg lipoprotein protein/mL are given in parenthesis. Total lipoprotein cholesterol was assayed enzymatically.

[d]ch, Cholesterol.

[e]$P<0.05$, *vs.* control.

cholic acid. However, in our study the accumulation of CR neutral lipids in hepatocytes was not accompanied by the expected stimulation of bile acid production; instead production decreased (Table 5). Two independent mechanisms may be responsible for this effect. The first is suppression of cholesterol synthesis and subsequent decrease in intracellular content of newly-synthesized cholesterol which is mainly used by rabbit hepatocytes for breakdown to bile acid (34). Our data suggest that CR cholesterol is predominantly used for VLDL production but not as precursor for bile acid synthesis. The second mechanism may be related to the direct effect of CR components on the process of bile acid synthesis. For instance, oleic acid in isolated perfused rat liver can suppress the secretion of bile acids through the inhibition of oxidation of the side chain of cholesterol leading to bile acids (35).

Our data, in corroboration with previous reports (25,26), support the idea that HDL_2 is the main source of cholesterol for the synthesis of bile acids and is capable of greatly enhancing bile acid synthesis. Recent reports (24,34) have also indicated that LDL, and cholesterol-rich VLDL added to the primary culture of hepatocytes, can stimulate the synthesis of bile acids. In contrast, triacylglycerol-rich VLDL has no effect on bile acid synthesis.

Our results demonstrate that due to the effective uptake of triacylglycerol-rich CR by hepatocytes, an inverse relationship between secretion of VLDL cholesterol and bile acid production is established. Previous studies have shown that the administration of triacylglycerol-rich CR to perfused rat liver increases VLDL cholesterol and triacylglycerol secretion, but decreases biliary cholesterol content (5). Triacylglycerol-rich CR, which are formed in the bloodstream after feeding, are unable to stimulate hepatic bile acids synthesis and secretion and biliary cholesterol output to compensate for the elevated CR cholesterol content in normal hepatocytes. Evidently, most of the CR cholesterol taken up by hepatocytes is used for VLDL assembly and secretion and, as a result, the plasma cholesterol level is increased. In contrast to this process, hepatocyte HDL_2 cholesterol uptake and subsequent stimulation of bile acids synthesis eliminate excess plasma cholesterol and maintain normal cholesterol level in the bloodstream.

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Similarities in Surface Lipids of Chylomicrons from Glyceryl and Alkyl Ester Feeding: Major Components[1]

Lu-Ying Yang, Arnis Kuksis* and **John J. Myher**
Department of Biochemistry and Banting and Best Department of Medical Research, University of Toronto, Toronto, Canada M5G 1L6

This study tests the hypothesis that the rat chylomicrons are assembled and released into lymph similarly regardless of the site (rough or smooth endoplasmic reticulum) or pathway (phosphatidic acid or monoacylglycerol) of triacylglycerol biosynthesis. For this purpose we determined the lipid class, fatty acid and molecular species composition of the choline, ethanolamine, inositol and serine phospholipids of lymph chylomicrons during absorption of menhaden, mustardseed and corn oil (monoacylglycerol pathway) or the corresponding fatty acid methyl or ethyl esters (phosphatidic acid pathway). The dietary fatty acids were found to be incorporated to various extents into different phospholipid classes, the proportions of which were not affected by the nature of the dietary fat. The chylomicron phospholipids contained 80–82% choline, 8% ethanolamine and 2.5% inositol glycerophospholipids, and much smaller amounts of serine and other minor phospholipids. Administration of a meal of each dietary fat resulted in a retention of approximately 50% endogenous fatty acids in the major glycerophospholipids of the chylomicrons. A minimum of 50% of the molecular species of the choline and ethanolamine glycerophospholipids contained at least one exogenous fatty acid. No significant discrepancies were found in the fatty acid and molecular species composition of the glycerophospholipids between chylomicrons from the oil and corresponding ester feeding. It is concluded that the chylomicrons arising from the monoacylglycerol (oil feeding) and the phosphatidic acid (ester feeding) pathways of triacylglycerol biosynthesis become enveloped in surfactant monolayers containing qualitatively and quantitatively identical classes and molecular species of phospholipids.
***Lipids 26*, 806–818 (1991).**

The digestion products of glyceryl triesters and short-chain alkyl esters of fatty acids are believed to be resynthesized into triacylglycerols by the intestinal villus cells *via* two different pathways (1). The glyceryl esters have been shown to be reassembled largely *via* the monoacylglycerol (MG) pathway (2), while the alkyl esters or free fatty acids are believed to be incorporated into triacylglycerols exclusively *via* the phosphatidic acid (PA) pathway (2,3). The two pathways are known to occur at different microsomal sites and involve acyltransferases of different fatty acid and positional specificity (4,5). Furthermore, there is evidence that the triacylglycerols formed *via* the phosphatidic acid pathway in liver (6) and possibly in intestine (7) are stored and must be hydrolyzed and resynthesized before secretion. It was therefore possible that differences would be found in the chylomicron phospholipids reflecting the different membrane contacts characteristic of the two pathways of triacylglycerol formation and secretion. In previous studies (8), we have demonstrated great qualitative and quantitative similarity in the surface apoproteins of the chylomicrons. This suggested that the two pathways of triacylglycerol formation converge at some stage prior to the final assembly and secretion of the chylomicrons. We have now examined this possibility further in regards to the major surface lipid components of chylomicrons. The present study shows close similarity in the phospholipid classes and near identity in the molecular species of the choline (GPC) and ethanolamine (GPE) glycerophospholipids between chylomicrons from the oil and ester feeding. These results provide a clear indication for a common route of packaging and externalization as chylomicrons of the triacylglycerol products of both the MG and PA pathways. These findings are consistent with the results of stereospecific analyses of the triacylglycerol structure of lymph chylomicrons (9).

MATERIALS AND METHODS

Animals, surgical procedures and diets. The lymph was collected from mature rats over a period of 2–24 hr as previously described (8,10). The experimental meals were administered by stomach tube and consisted of 1–3 mL of menhaden oil and corresponding fatty acid ethyl esters as described (10). Comparable quantities of mustardseed oil and its fatty acid methyl esters (11) were used as oligounsaturated long-chain triacylglycerol controls. Corn oil or its free fatty acids were administered in 1-mL amounts.

Isolation of chylomicrons. Lymph chylomicrons were isolated by ultracentrifugation (Beckman Model L2-65B, rotor 40.3, Palo Alto, CA) at 10°C and characterized as described (8).

Isolation and quantitation of phospholipid classes. Total lipid extracts of chylomicrons were obtained with chloroform/methanol (2:1, v/v) and were resolved into neutral and polar lipids by thin-layer chromatography (TLC) as previously described (11). The phospholipids were recovered from the origin of the TLC plate and were resolved into individual phosholipid classes by TLC using acidic and basic solvent systems (12,13). The phospholipid classes were identified by reference to standards and were quantitated on the basis of the fatty acid content using 17:0 acid as an internal standard. Gel scrapings containing individual phospholipid classes were subjected to acidic methanolysis using 6% H_2SO_4 in methanol at

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*To whom correspondence should be addressed at BBDMR, University of Toronto, 112 College Street, Toronto, Canada M5G 1L6.

Abbreviations: GLC, gas-liquid chromatography; GPC, choline glycerophospholipid; GPE, ethanolamine glycerophospholipid; HDL, high density lipoprotein; HPLC, high-performance liquid chromatography; LPC, lysophosphatidylcholine; LPE, lysophosphatidylethanolamine; MG, monoacylglycerol; PA, phosphatidic acid; PC, phosphatidylcholine; PE, phosphatidylethanolamine; PI, phosphatidylinositol; PS, phosphatidylserine; SPH, sphingomyelin, TMS, trimethylsilyl; TLC, thin-layer chromatography; VLDL, very low density lipoprotein.

80°C for 2 hrs. After addition of the internal standard, the fatty acid methyl esters were analyzed by gas-liquid chromatography (GLC) (10).

Positional analysis of fatty acids. The positional distribution of fatty acids in the glycerophospholipids was determined by digestion with phospholipase A_2 (*Crotalus adamanteus*; Sigma Chemical Co., St. Louis, MO) (14). The released fatty acids and lysophospholipids were resolved by TLC and the fatty acids determined by GLC (10,14).

Preparation of diradylglycerols and derivatization. Diradylglycerol moieties from individual phospholipid classes were obtained by hydrolysis with phospholipase C (*Bacillus cereus*) as previously described (15). The trimethylsilyl (TMS) ethers were prepared by reacting the diradylglycerols with a premixed solution of pyridine/hexamethyldisilazane/trimethylchlorosilane (12:5:2, v/v/v) for 30 min at room temperature (15). The TMS ethers of the diradylglycerol moieties of GPE were resolved into alkenylacyl, alkylacyl and diacylglycerol subfractions by normal phase high-performance liquid chromatography (HPLC) with 0.3% isopropanol in hexane (15).

Gas-liquid chromatography. Diradylglycerol TMS ethers were resolved on the basis of carbon number by means of a fused silica capillary column (8 m × 0.32 mm ID) coated with a permanently bonded SE-54 liquid phase as previously described (15). The analysis of the molecular species of the diacylglycerol moieties of GPC and GPE of chylomicrons from mustardseed oil feeding was performed isothermally at 250°C on a 10-m open tubular glass capillary column wall-coated with SP-2330 liquid phase as previously described (16). The molecular species analyses of the diradylglycerol moieties of GPC and GPE of chylomicrons from menhaden oil feeding were performed isothermally at 260°C on a fused silica capillary column (15 m × 0.32 mm ID) coated with cross-bonded RTx2330 liquid phase as described (17).

Identification and quantitation of GLC peaks. The identity of the GLC peaks of fatty acids, dimethylacetals and diradylglycerols was established relative to standards and by comparison of observed and calculated relative retention times (17). The quantitative composition of the fatty acids, dimethylacetals and molecular species of diradylglycerols was determined from the GLC peak areas obtained by flame ionization. Discrimination against higher molecular weight species resulting from the use of a split injector was corrected by means of analyses made on a nonpolar capillary column using on-column injection. Areas obtained on the polar capillary column were grouped according to carbon number (total number of acyl carbons) and renormalized to match the values obtained on the nonpolar column (17).

RESULTS

Phospholipid class composition. The relative composition of lipid classes of the chylomicrons from menhaden oil feeding averaged 8–10% phospholipids, 87–90% triacylglycerols, 1% cholesteryl esters, 0.5% free cholesterol and 0.7% free fatty acids. The smaller particles resulting from the ester feeding contained correspondingly less triacylglycerols and more phospholipids. Table 1 shows the relative proportions of phospholipid classes in the chylomicrons isolated at peak absorption of menhaden oil or the corresponding fatty acid ethyl esters. There are no significant differences in phospholipid composition between the chylomicrons from the oil and ester feeding. In both instances the major components are GPC (80–82%) and GPE (8%), with smaller amounts of other common phospholipids. In addition, the chylomicron phospholipid fraction contained several minor, previously unidentified components of lower polarity, which accounted for about 5% of total in both instances (18). This composition of the phospholipid classes differs from that of the microsomes of the intestinal villus cells by a higher proportion of GPC relative to other phospholipid classes (19,20).

TABLE 1

Relative Proportions of Phospholipid Classes in Chylomicrons from Rats Receiving Menhaden Oil or Corresponding Fatty Acid Ethyl Esters

Phospholipid classes	Chylomicrons[a]	
	Oil-fed	Ester-fed
	Mole %	
GPC	80.6 ± 1.2	82.4 ± 1.0
GPE	8.3 ± 1.7	7.8 ± 0.3
PS	0.1 ± 0.0	0.1 ± 0.0
PI	2.7 ± 0.5	2.6 ± 0.4
PA	1.1 ± 0.3	0.9 ± 0.2
SPH	0.5 ± 0.2	0.5 ± 0.1
LPC	1.6 ± 0.1	1.4 ± 0.2
Other	5.2 ± 0.5	4.2 ± 0.4

[a]Mean ± S.D. from six determinations.

Fatty acid composition of phospholipids. Figure 1 compares the fatty acid composition of GPC and GPE of chylomicrons isolated during the peak absorption of menhaden oil or its fatty acid ethyl esters. The chylomicron GPC from the oil-fed rats contains 26% 16:0, 14% 18:0, 17% 18:2, 10% 20:4 and 11% 20:5 as major components. This composition is similar to that obtained for the chylomicron GPC of the ester-fed animals. The chylomicron PE from the oil-fed animals contains 11% 16:0, 29% 18:0, 11% 18:2, 13% 20:4, 8% 20:5 and 12% 22:6 as major components, which agrees well with the fatty acid composition of the chylomicron GPE from the ester fed animals. There are marked differences, however, in the fatty acid composition between the GPC and GPE. Specifically, the GPC readily took up the 20:5, but not the 22:6 acid, while the GPE readily took up both 20:5 and 22:6 acids. The chylomicron GPC and GPE retained high proportions of 18:0, 18:2 and 20:4n-6 acids, which were largely absent from the fish oil (17). Furthermore, certain fatty acids (14:0, 16:3, 16:4, 18:4 and 20:4n-3), which occur in moderate concentrations in the fish oil, did not appear at all in the chylomicron phospholipids.

Other analyses showed that the relative proportions of the endogenous fatty acids decreased and those of exogenous (dietary) fatty acids increased with the progress of fat absorption. Thus the 18:2 content of the chylomicron GPC in the oil fed animals decreased to 18% from an initial value of 37%, while the content of 20:4 decreased to 8% from a fasting value of 11% over a period of 0–9 hr. By comparison, the contents of 20:5 and 22:6 increased from 0.7 to 13.5% and 0.6 to 2.2%, respectively, in the same period of time, with other acids showing lesser

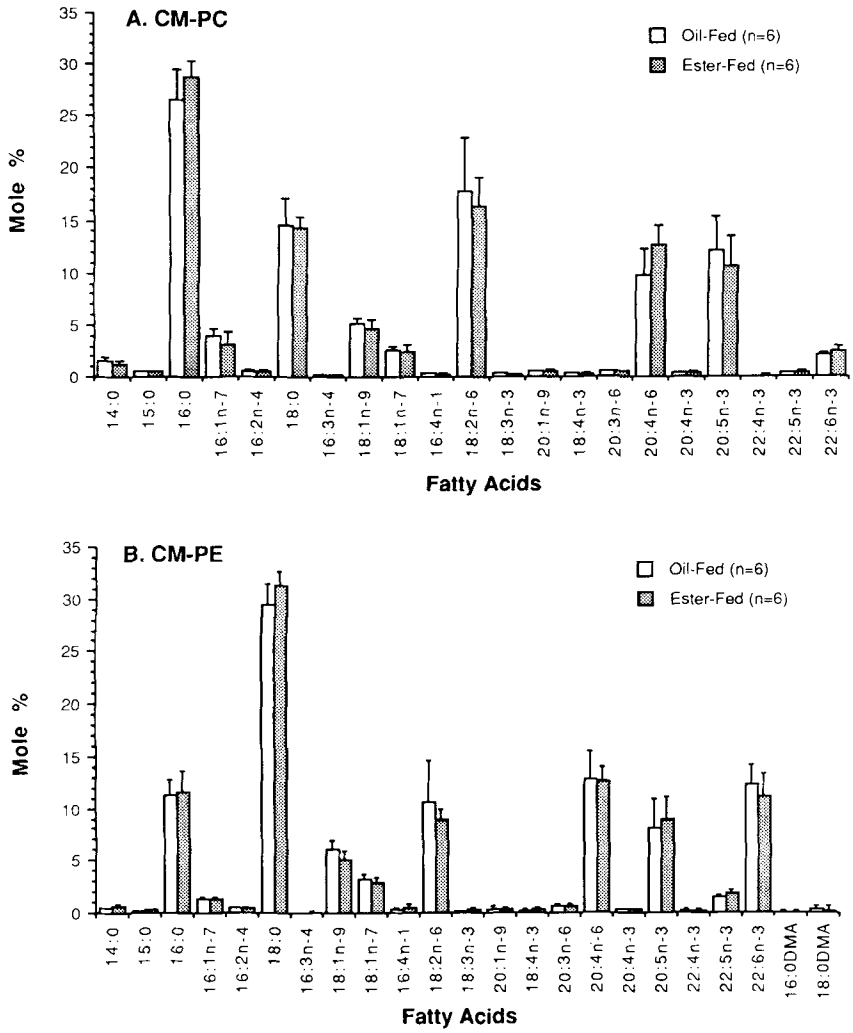


FIG. 1. Fatty acid composition of chylomicron GPC and GPE from rats receiving menhaden oil (open bars) or its fatty acid ethyl esters (hatched bars). The values are means from six determinations for each type of feeding.

differences. Similar trends took place in the composition of the fatty acids of chylomicron GPE from the oil fed animals, and in the GPC and GPE fatty acids of chylomicrons of the ester fed animals (results not shown).

Figure 2 compares the positional distribution of the fatty acids in the chylomicron GPC from the oil and ester fed animals. It confirms the usual location of saturated acids in the *sn*-1 position, a nearly equal distribution of n-9 monoenoic acids between the *sn*-1 and *sn*-2 positions, and the nearly exclusive location of the polyunsaturated fatty acids in the *sn*-2 position. There are a few exceptions among the minor acids. Thus, 14:0 was distributed about equally between the *sn*-1 and *sn*-2 positions. Also, small amounts of long-chain polyunsaturated acids were found in the *sn*-1 position. A similar pattern was seen for the chylomicron GPC from the ester fed animals. On the basis of the association of acids in the molecular species (see below) a similar positional distribution of fatty acids appeared to be present in GPE.

Figure 3 compares the fatty acid composition of PI and PS in the chylomicrons from the two types of feeding. The fatty acids of PI are characterized by a high proportion of 18:0 and 20:4n-6 acids. The fatty acids of PS are somewhat closer in composition to the fatty acids of PE than to any other phospholipid class. The chylomicrons from the oil-fed animals contain a significantly higher proportion of 18:2 in the PI and PS than the ester-fed animals which, in this case, can be attributed to incomplete clearance of the corn oil used in prefeeding. Neither of these phospholipids contains any alkenyl species, as indicated by the absence of dimethylacetals.

Figure 4 gives the fatty acid composition of chylomicron GPC and GPE from rats receiving mustardseed oil or its fatty acid methyl esters. Again, a very close similarity is

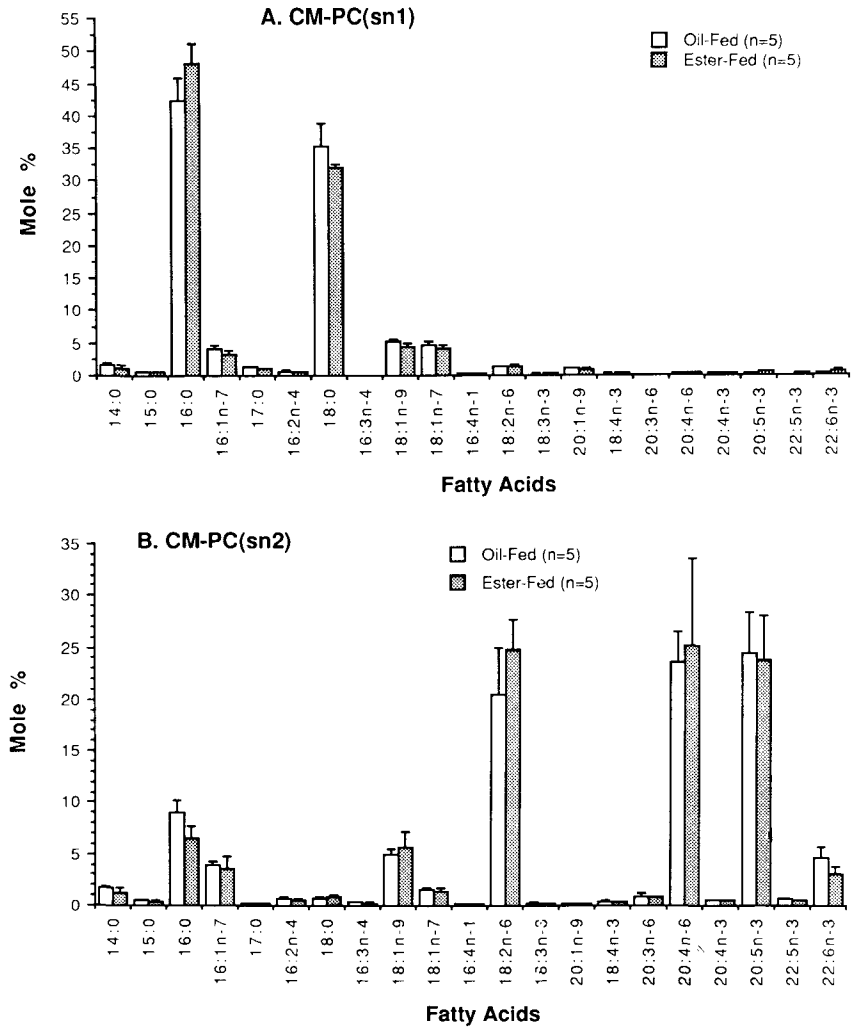


FIG. 2. Positional distribution of fatty acids in chylomicron GPC from rats receiving menhaden oil (open bars) or its fatty acid ethyl esters (hatched bars). The values are means of five determinations from each type of feeding.

found between the GPC samples isolated from the chylomicrons of the oil and the ester fed animals. Likewise, the fatty acids of the GPE are similar for the oil and ester fed animals. In contrast, the fatty acid compositions of the GPC and GPE are markedly different during the oil or ester feeding. Thus, while the GPC contained 25% 16:0, 14% 18:0, 7% 18:1, 32% 18:2 and 9% 20:4, the GPE contained 9% 16:0, 27% 18:0, 13% 18:1, 17% 18:2 and 14% 20:4. Erucic acid, 22:1n-9 (27% of total mustardseed oil), was only sparingly incorporated (less than 2%) into either GPC or GPE. In general, the chylomicron GPC and GPE from the mustardseed oil-fed animals contained the same major fatty acids as the chylomicron GPC and GPE from the fish oil-fed animals, except that those of the mustard seed oil-fed animals contained less 20:5 and 22:6.

Likewise, the fatty acid composition of chylomicron GPC from rats receiving corn oil or its free fatty acids showed only minor differences (Table 2). There was, however, a significant increase in the dietary 18:2 content of both GPC and GPE, when compared to fish oil feeding. On corn oil, the GPC contained about 40% 18:2 and GPE 28% 18:2, with the other acids occurring in their characteristic proportions. The distinct differences between the GPC and GPE were retained again. The GPE samples from corn oil feeding were investigated in detail for differences in the alkylacyl- and alkenylacyl-glycerol moieties following oil feed (see below).

Molecular species of chylomicron GPC and GPE. Figure 5A shows the polar capillary GLC profile of the diacylglycerol moieties of chylomicron GPC from fish oil feeding. A similar GLC profile was obtained for the GPC from ester feeding. The corresponding quantitative values are given in Table 3. The major molecular species of GPC are 16:0-18:2 (11-15%), 18:0-18:2 (11%), 16:0-20:4 (11-13%), 16:0-20:5 (13-15%), 18:0-20:4 (8-11%) and 18:0-20:5 (7-9%). The long-chain polyunsaturated fatty acids have been paired mainly, but not exclusively, with the 16:0 and 18:0 acids. Figure 5B shows the polar

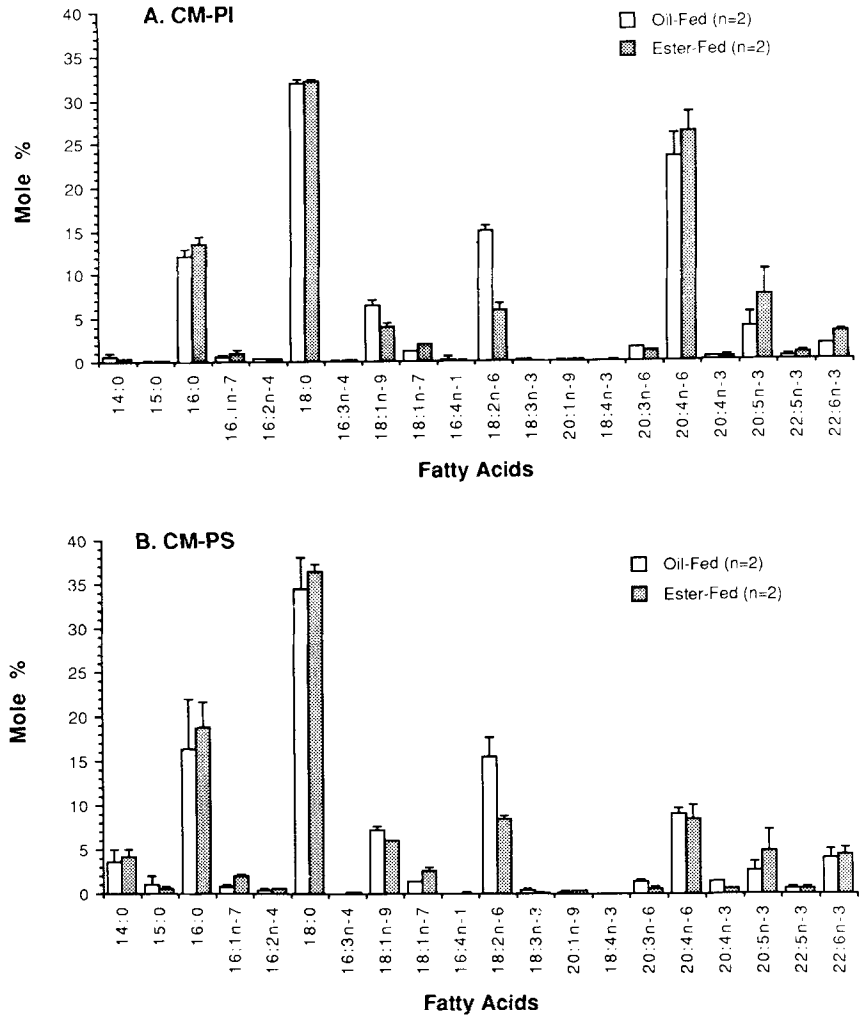


FIG. 3. Fatty acid composition of chylomicron PI and PS from rats receiving menhaden oil (open bars) or its fatty acid ethyl esters (hatched bars). The values are means of two determinations for each type of feeding.

capillary GLC profile of the diacylglycerol moieties of chylomicron GPE from fish oil feeding. Again, a similar profile was obtained for the GPE from ester feeding. The quantitative values are given in Table 3. The major species of GPE are 18:0-18:2 (9-13%), 18:0-20:4 (23-24%), 18:0-20:5 (13-16%) and 18:0-22:6 (14%), with smaller amounts of the 16:0-20:4 (4.4-5.5%), 18:0-18:1 (3-4%), 16:0-20:5 (4%) and 18:1-22:6 (1-4%). A high proportion of GPE, just like GPC, contains molecular species made up exclusively of endogenous fatty acids (18:2 and 20:4 combined with 16:0 and 18:0). There are no significant differences in the molecular species of GPC and GPE between the chylomicrons from the oil- and ester-fed animals. However, in all instances there are large differences between the GPC and GPE species.

Figure 6 depicts the polar capillary GLC profiles of the diacylglycerol moieties of chylomicron PC and PE from mustardseed oil administration. The quantitative values are given in Table 4 along with the corresponding values for the chylomicron phospholipids from the ester feeding. While the oil and ester feeding yielded similar molecular species for the corresponding GPC and GPE, the GPC and GPE differed markedly in their composition. Thus, while the GPC contained about 30% 16:0-18:2, 18% 18:0-18:2 and 6% 18:0-20:4 as major species, the GPE contained 4% 16:0-18:2, 20% 18:0-18:2 and 21% 18:0-20:4 as major species. Other differences were seen in the proportions of the minor species. Both GPC and GPE of the chylomicrons differed greatly from GPC and GPE of the microsomes of intestinal villus cells (19).

A detailed study of the ether lipid content of chylomicron GPE was undertaken only for corn oil feeding, for which microsomal GPE data are already available (19). Corn oil and free acid feeding led to closely similar fatty acid composition of chylomicron GPC and GPE (Table 2). Figure 7 shows the carbon number distribution of the alkenylacyl, alkylacyl and diacylglycerol moieties of chylomicron GPE from the

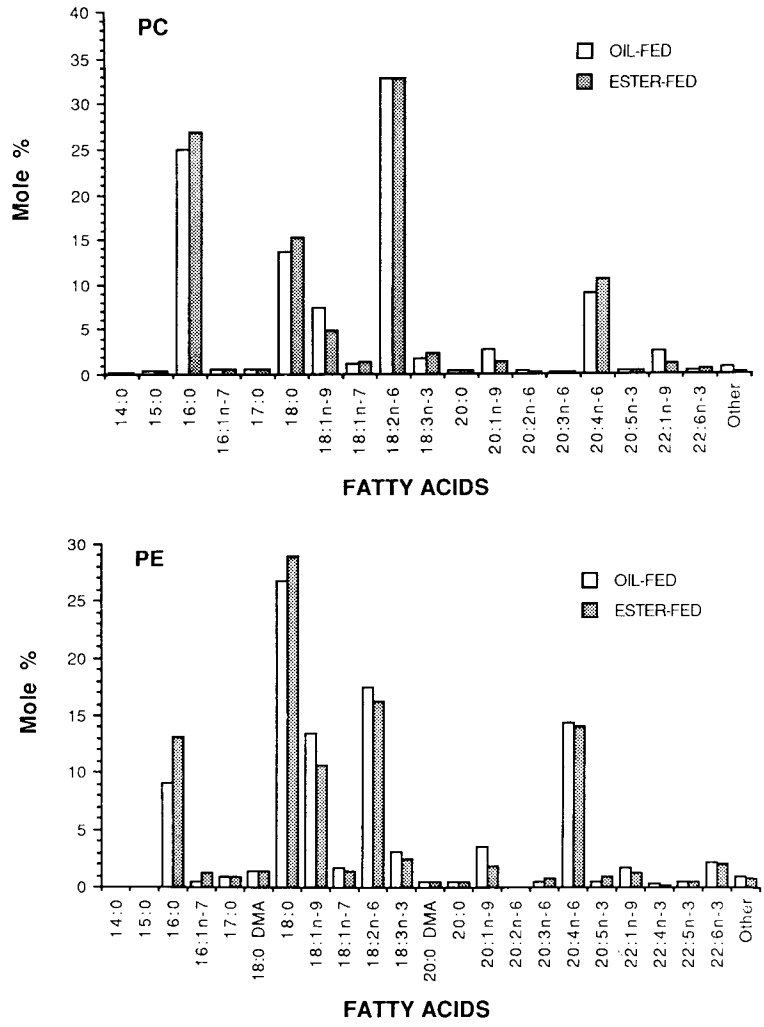


FIG. 4. Fatty acid composition of chylomicron GPC and GPE from rats receiving mustard-seed oil (open bars) or its fatty acid methyl esters (hatched bars). The values are single determinations from each type of feeding.

TABLE 2

Fatty Acid Composition of Lymph Chylomicron GPC and GPE from Rats Fed Corn Oil or the Corresponding Free Fatty Acids[a]

Fatty acids	Oil-fed		FFA-fed	
	GPC	GPE	GPC	GPE[b]
		Mole %		
16:0	26.2 ± 2.0	8.4 ± 0.3	26.9 ± 0.9	12.0
16:1n-7	0.3 ± 0.1	0.1 ± 0.0	0.7 ± 0.1	0.4
18:0	14.2 ± 0.8	28.6 ± 0.5	16.8 ± 1.9	35.4
18:1n-9	8.6 ± 2.2	12.9 ± 0.6	6.2 ± 1.6	10.1
18:2n-6	40.2 ± 3.2	32.7 ± 0.5	34.7 ± 5.6	27.9
18:3n-3	0.3 ± 0.0	0.2 ± 0.0	0.4 ± 0.2	—
20:4n-6	9.5 ± 2.5	10.8 ± 0.4	12.6 ± 4.9	11.5
22:6n-3	0.7 ± 0.1	5.2 ± 0.1	1.6 ± 0.1	2.7

[a]Mean ± S.D. from three determinations.

[b]One determination.

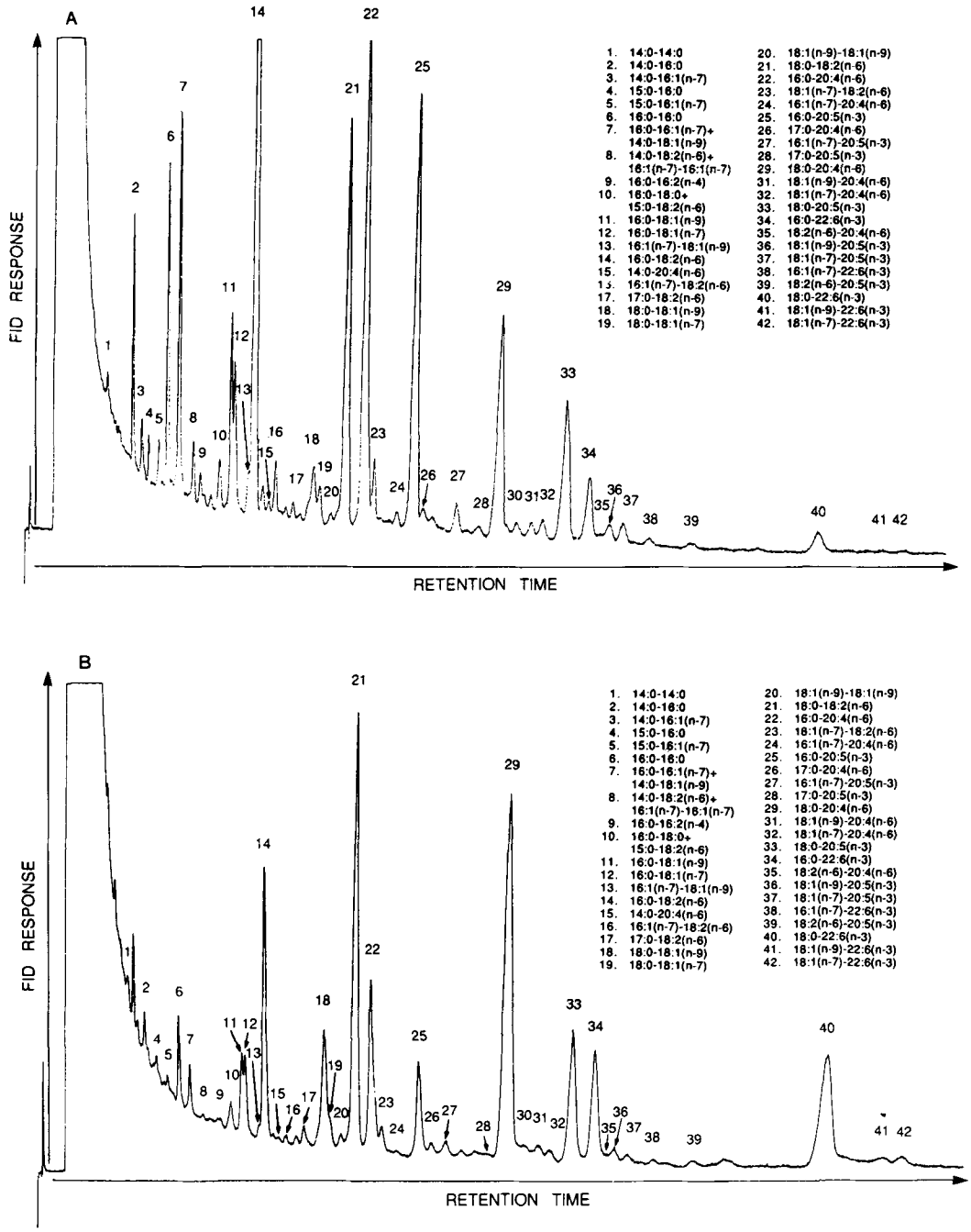


FIG. 5. Polar capillary GLC profiles of the diacylglycerol moieties of chylomicron GPC (A) and GPE (B) from rats receiving menhaden oil. Peak identification as given in Figure. GLC conditions: Column, 15 m × 0.32 mm ID fused silica capillary coated with cross-bonded RTx2330; carrier gas, H_2, 2 psi head pressure; injector split ratio, 7:1; temperature, 260°C, isothermal. Instrument and other GLC conditions are as given in text. Sample: 1 μL of 0.1% diacylglycerol TMS ethers in hexane.

corn oil fed rats as obtained by nonpolar capillary GLC after a prior resolution of the subclasses by normal phase HPLC. The GPE preparation contained 88% diacyl, 6.3% alkylacyl and 5.4% alkenylacyl subclasses. In comparison to the microsomal GPE (19), the chylomicron GPE contains less of the alkylacyl and alkenylacyl species. There are major differences in the carbon number distribution between the diacyl and alkylacyl or alkenylacyl subclasses of GPE. While the diacyl species are made up of 50% C_{36}, 21% C_{38} and 10% C_{34}, the alkenylacyl and alkylacyl species also contain large proportions of the C_{40} (25–34%), C_{42} (14%) and some C_{44} (1%) species. These dif-

TABLE 3

Molecular Species of GPC and GPE of Lymph Chylomicrons from Rats Fed Menhaden Oil or Its Fatty Acid Ethyl Esters (Mole%)[a]

Peak no.	Species	GPC		GPE	
		Oil-fed	Ester-fed	Oil-fed	Ester-fed
1	14:0-14:0	0.1 ± 0.1	0.1 ± 0.0		
2	14:0-16:0	2.6 ± 0.2	1.8 ± 0.3	0.3 ± 0.2	0.2 ± 0.1
3	14:0-16:1n-7	0.4 ± 0.0	0.2 ± 0.1		0.1 ± 0.0
4	15:0-16:0	0.2 ± 0.0	0.2 ± 0.0		0.0 ± 0.0
5	15:0-16:1n-7	0.2 ± 0.1	0.1 ± 0.0	0.2 ± 0.1	0.1 ± 0.0
6	16:0-16:0	2.0 ± 0.1	2.0 ± 0.3	0.3 ± 0.1	0.4 ± 0.1
7	16:0-16:1n-7+ 14:0-18:1n-9	3.8 ± 0.2	3.2 ± 0.3	0.4 ± 0.2	0.5 ± 0.0
8	14:0-18:2n-6+ 16:1-16:1n-7	0.7 ± 0.0	0.6 ± 0.1	0.1 ± 0.1	0.1 ± 0.0
9	16:0-16:2n-4	0.4 ± 0.0	0.2 ± 0.0	0.1 ± 0.0	0.2
	Unknown	0.2 ± 0.0	0.2 ± 0.0		
10	16:0-18:0+ 15:0-18:2n-6	0.8 ± 0.1	0.4 ± 0.1	0.2 ± 0.0	0.2 ± 0.1
11+12	16:0-18:1n-9+ 16:0-18:1n-7	5.4 ± 0.7	4.4 ± 0.2	1.8 ± 0.2	2.3 ± 0.2
13+14	16:1-18:1n-9+ 16:0-18:2n-6	11.0 ± 0.3	15.6 ± 1.1	2.1 ± 0.3	2.8 ± 0.1
	Unknown	0.6 ± 0.2	0.5 ± 0.1		
15	14:0-20:4n-6	0.2 ± 0.1	0.1	0.1 ± 0.1	0.1 ± 0.0
16	16:1-18:2n-6	1.1 ± 0.0	1.0 ± 0.0	0.5 ± 0.1	0.8 ± 0.1
17	17:0-18:2n-6	0.5 ± 0.0	0.6 ± 0.0	0.8 ± 0.1	0.4 ± 0.3
	Unknown	0.5 ± 0.1	0.2 ± 0.1		
18+19	18:0-18:1n-9+ 18:0-18:1n-7	1.9 ± 0.0	1.7 ± 0.4	3.3 ± 0.1	3.8 ± 0.0
20+21	18:1-18:1n-9+ 18:0-18:2n-6	10.8 ± 2.51	11.5 ± 0.0	9.5 ± 2.4	12.3 ± 0.1
22	16:0-20:4n-6+ 18:1n-9-18:2	10.8 ± 1.3	13.3 ± 0.1	5.5 ± 0.4	4.4 ± 0.2
23	18:1n-7-18:2	1.3 ± 0.2	1.3 ± 0.1	0.4 ± 0.0	0.6 ± 0.1
24	16:1-20:4n-6	0.4 ± 0.0	0.3 ± 0.0	0.2 ± 0.1	0.4 ± 0.2
25	16:0-20:5n-3	12.6 ± 3.5	14.8 ± 0.3	4.6 ± 1.4	4.1 ± 0.3
27	16:1-20:5n-3	0.7 ± 0.2	0.5 ± 0.0	0.3 ± 0.2	0.6 ± 0.0
26	17:0-20:4n-6	0.7 ± 0.0	0.6 ± 0.0	0.5 ± 0.0	0.6 ± 0.2
28	17:0-20:5n-3	0.3 ± 0.0	0.2 ± 0.0	0.6 ± 0.0	0.5 ± 0.2
29	18:0-20:4n-6	10.5 ± 3.0	8.2 ± 0.6	24.2 ± 5.9	23.3 ± 2.1
30	Unknown	0.3 ± 0.1	0.6 ± 0.0	0.2 ± 0.0	0.3
31	18:1-20:4n-6	1.2 ± 0.2	1.0 ± 0.1	1.5 ± 0.2	0.6 ± 0.3
33	18:0-20:5n-3	9.4 ± 1.4	7.4 ± 0.4	13.2 ± 2.9	15.9 ± 0.2
34	16:0-22:6n-3	1.8 ± 0.2	1.6 ± 0.1	5.2 ± 0.8	4.4 ± 0.1
36	18:1-20:5n-3	2.0 ± 0.5	1.5 ± 0.0	1.5 ± 0.1	1.5 ± 0.3
38	16:1-22:6n-3	0.3 ± 0.0	0.1 ± 0.0	0.7 ± 0.2	0.5 ± 0.1
39	18:2-20:5n-3	0.4 ± 0.1	0.4 ± 0.1	1.1 ± 0.4	1.0 ± 0.0
	18:1-22:5n-3	0.8 ± 0.2	0.5 ± 0.1	1.2 ± 0.3	0.7 ± 0.2
40	18:0-22:6n-3	2.4 ± 0.2	2.1 ± 0.4	13.6 ± 0.7	13.8 ± 1.6
42	18:1-22:6n-3	0.3 ± 0.0	0.5 ± 0.1	4.2 ± 1.0	1.0 ± 0.1
	C_{42}	0.7 ± 0.2	0.5 ± 0.1	1.5 ± 0.3	1.8 ± 1.1
	C_{44}	0.1 ± 0.0	0.1 ± 0.0	0.1 ± 0.1	0.2 ± 0.0

[a]Average ± range/2.

ferences are best illustrated by the polar capillary GLC profiles shown in Figure 8. The corresponding quantitative values are given in Table 5. While the composition of the molecular species of the diacylglycerol GPE (Fig. 8A) is characterized by the presence of the common 16:0-18:2 (8%), 18:0-18:2 (30%) and 18:0-20:4 (19%) as major components, the molecular species of the alkylacyl GPE (Fig. 8B) are unusual in the high content of the long-chain polyunsaturated acids. Thus, alkylacyl GPE contains 20:0-18:1 (8%), 20:0-20:4 (13%), 18:0-22:4 (7%), 20:0-22:4 (5%) and 20:0-22:5 (22:6) (total of 7%), along with smaller proportions of the other long-chain polyunsaturated species. Alkenylacyl GPE (Fig. 8C) contains high proportions of the long-chain polyunsaturated species such as 16:0-22:4 (4%), 20:0-20:4 (9%), 18:0-22:4 (8%), 18:0-22:6 (7%), 18:0-22:5 (6%), 20:0-22:4 (4%), 20:0-22:6 (4%), and 20:0-22:5 (3%).

DISCUSSION

The phospholipid class composition of chylomicrons found in this study is comparable to that observed earlier

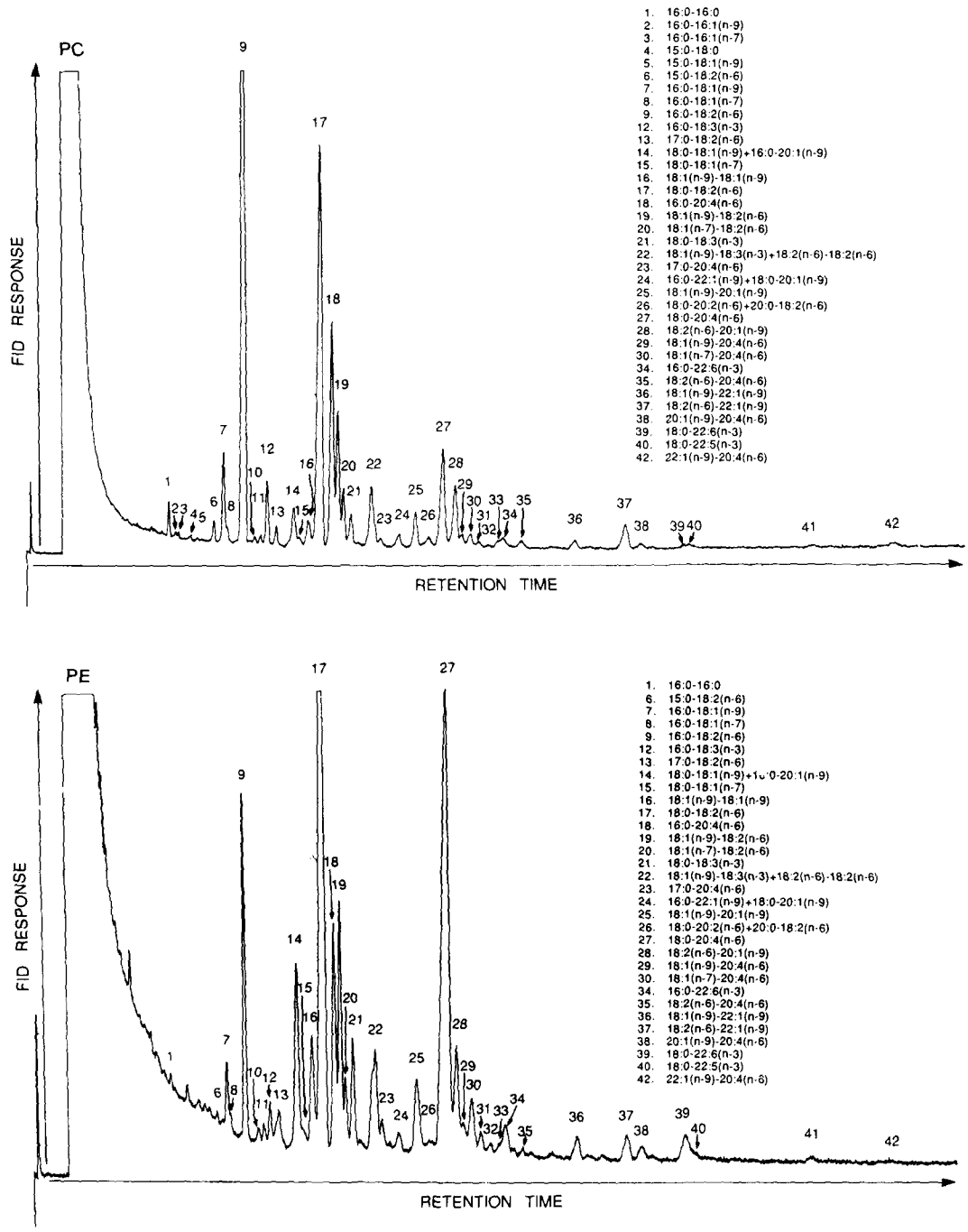


FIG. 6. Polar capillary GLC profiles of the diacylglycerol moieties of chylomicron GPC or GPE from rats receiving mustardseed oil. Peak identification as given in Figure. GLC conditions: Column, 10 m × 0.32 mm ID open tubular glass capillary wall-coated with SP-2330 liquid phase; carrier gas, H_2, 5 psi head pressure; injector split ratio, 7:1; temperature, 260°C, isothermal. Instrument and other GLC conditions are as given in text. Sample: 1 μL of 0.1% diacylglycerol TMS ethers in hexane.

during mustardseed oil feeding (11). The values for the major components are similar to those obtained by Redgrave (21), who, however, reported 6% PA. The PA levels estimated in the present study never exceeded 1%, although other minor polar lipids were isolated from all chylomicron preparations (18). The phospholipid class composition of the chylomicrons differs from that of the villus cells (20), which contain 50% GPC, 27% GPE, 6% PI, 2.7% PS and 2% SPH, and their microsomes (21), which are made up of 45% GPC, 20% GPE, 16% LPC, 8% SPH and 8% PI. Redgrave (21) also reported prechylomicrons to contain 50% GPC, 12% GPE, 11% LPC, 6% SPH, 10% PA and 7% PI. On the basis of these studies, the GPC/GPE ratio changes from 2 to 4 to 10

TABLE 4

Molecular Species of GPC and GPE of Chylomicrons from Rats Receiving Mustardseed Oil or Corresponding Fatty Acid Methyl Esters[a]

Molecular species	GPC		GPE	
	Oil-fed	Ester-fed	Oil-fed	Ester-fed
	Mole %			
16:0-16:0	0.4	0.6		
16:0-16:1n-7	0.1	0.1		
14:0-18:2n-6	0.1	0.1		
16:0-18:0+14:0-20:4n-6	0.2	0.4	0.5	1.5
16:0-18:1n-9	2.6	2.2	0.8	0.8
16:0-18:1n-7	0.4	0.4	0.2	0.3
16:0-18:2n-6	31.8	29.3	4.0	5.0
16:0-18:3n-3	1.1	2.1	0.9	1.3
17:0-18:2n-6	0.6	0.7	0.3	0.4
18:0-18:0	0.5	0.4		
18:0-18:1n-9+				
16:0-20:1n-9	2.4	1.4	4.8	6.0
18:1n-9-18:1n-9	1.7	0.8	3.3	2.7
18:0-18:2n-6	17.8	19.7	19.1	18.5
16:0-20:4n-6	6.9	8.8	5.9	5.9
18:1n-9-18:2n-6	4.5	3.4	6.7	4.2
18:1n-7-18:2n-6	1.7	1.8		1.2
18:0-18:3n-3	1.1	1.1	1.8	1.5
18:2n-6-18:2n-6	1.9	1.9	2.9	2.9
16:0-22:1+18:0-20:1	1.6	1.3	1.8	1.7
18:1-20:1n-9	1.0	0.4	1.9	1.0
18:0-20:4n-6	4.9	7.6	21.1	22.6
18:2-20:1n-9	3.0	2.0	3.4	2.9
18:1-20:4n-6	1.0	1.0	2.8	2.2
16:0-22:6n-3	0.9	0.7	1.4	1.4
18:1n9-22:1n-9	1.0	1.8	1.9	1.7
18:2n6-22:1n-9	4.2	3.6	2.8	0.6
18:0-22:6n-3			1.5	1.7
18:0-22:5n-3			0.7	0.9
Other	4.5	4.5	5	6.8

[a]GPC and GPE from the same preparation of chylomicrons.

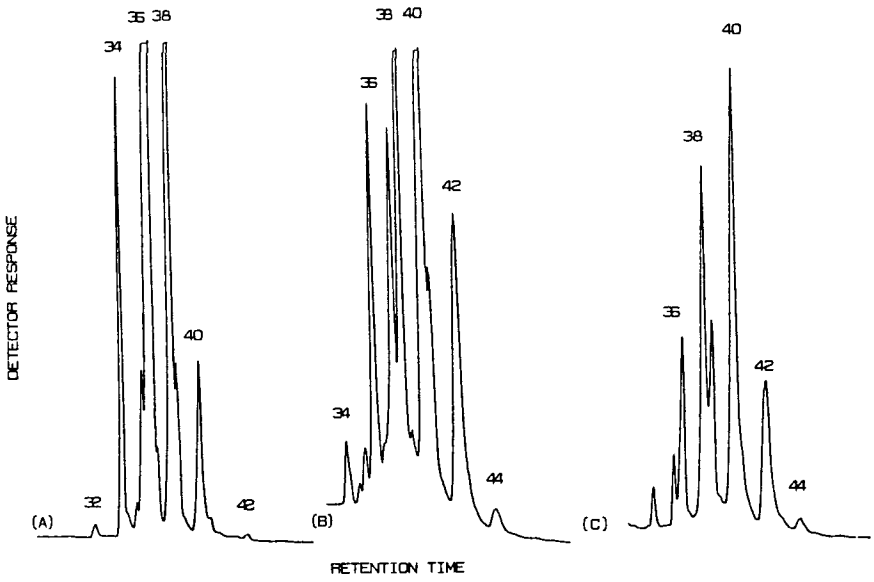


FIG. 7. Nonpolar capillary GLC profiles of diacyl (A), alkylacyl (B) and alkenylacyl (C) glycerol moieties of chylomicron GPE from rats receiving corn oil. Peaks are identified by the total number of carbons in the fatty chains. GLC conditions: Column, 8 m × 0.31 mm ID containing permanently bonded SE-54 liquid phase; carrier gas, H_2, at 6 psi head pressure. Temperature program: 40–150°C at 30°C/min, 150–230°C at 20°C/min, 230–280°C at 10°C/min, then 280–340°C at 5°C/min. Sample, diradylglycerol TMS ethers.

TABLE 5

Molecular Species of GPE of Chylomicrons from Rats Receiving Corn Oil[a]

Molecular species	GPE^0	GPE′	GPE″
		Mole %	
16:0-16:0	0.2		
16:0-16:1	0.3		
15:0-18:1	0.1		
16:0-18:0	0.2	0.7	0.4
16:0-18:1	2.0	1.0	0.9
16:0-18:2	8.0	0.6	0.8
17:0-18:1	0.3		
17:0-18:2	0.6		
18:0-18:0			0.6
18:0-18:1	5.2	4.9	3.1
18:1-18:1	1.6		0.5
18:0-18:2	30.4		5.0
16:0-20:4	3.1	6.6	2.4
18:1-18:2	8.1	0.9	1.0
18:1-18:2	1.3	0.2	0.4
16:0-20:5	0.6		
18:2-18:2	3.8		
17:0-20:4	0.5	0.2	0.2
20:0-18:1	0.6	7.9	2.4
18:0-20:4	18.7	19.9	20.5
16:0-22:4	1.2	1.7	4.1
18:1-20:4	1.9	1.9	0.0
18:1-20:4	0.8		
18:0-20:5	0.9	1.6	1.1
16:0-22:6	1.6	0.9	2.5
16:0-22:5	0.3	1.1	1.6
18:2-20:4	0.5		
22:0-18:1	0.4		
20:0-20:4		13.6	9.6
18:0-22:4		6.7	8.1
18:0-22:6	3.0	3.3	7.4
18:0-22:5	1.5	3.8	5.7
18:1-22:6			1.2
18:1-22:5		0.4	0.8
22:0-20:4		1.5	1.2
20:0-22:4		4.9	4.0
20:0-22:6		2.8	3.7
20:0-22:5		3.9	2.7
20:1-22:6			1.3
C_{44}		1.0	1.4
Other	1.7	4.9	2.9
Total (% of GPE)	88.3	6.3	5.4

[a]GPE^0; 1,2-diacyl-*sn*-GPE; GPE′; 1-alkyl,2-acyl-*sn*-GPE; GPE″; 1-alkenyl, 2-acyl-*sn*GPE. GPE subclasses from the same chylomicron preparation.

when proceeding from the microsomes to prechylomicrons and chylomicrons, respectively. The microsomal GPE also contained 11–16% alkylacyl and 4–8% alkenylacyl subclasses (19), which occurred in the chylomicrons to a lesser extent (5–6% each). The phospholipids of chylomicrons are also different from those of rat plasma, which contains less GPC (63.9%) and GPE (1.3%), but much more LPC (22.7%), SPH (7.7%) and PI (4.3%) (22). These results indicate that lymph chylomicron phospholipids did not originate from microsomal phospholipids or from exchange with the plasma phospholipids that may have been filtered into the lymph.

The pathway of triacylglycerol synthesis may influence the phospholipid composition of the chylomicrons at the originial site of synthesis, or the final stage of assembly, or both. Since the synthesis takes place in the smooth (MG pathway) or rough (PA pathway) endoplasmic reticulum, depending on the nature of dietary fat, the phospholipid composition of the resulting chylomicrons would vary, assuming that the two microsomal membranes differed in their lipid composition (23). Differences in the surface phospholipids could also result if the triacylglycerols from the PA pathway were stored prior to secretion (6). Finally, the two microsomal membranes would be expected to exhibit differences in the utilization of dietary fatty acids due to differences in the enzyme specificity. The present study demonstrates great similarity in the phospholipid class and molecular species composition between the chylomicrons resulting from the oil and ester feeding. Hence these phospholipids must have been derived from the same pool of precursors during the formation and secretion of chylomicrons regardless of the metabolic origin of the triacylglycerols. This study confirms the previously demonstrated (24,25) strong dietary influence on the fatty acid and molecular species composition of GPC of lymph chylomicrons. The dietary influence is also seen in GPE, but not in PS and PI.

Previous work has shown (26–28) that chylomicron GPC originates largely from dietary and bile GPC, which are hydrolyzed to LPC in the intestinal lumen. The absorbed LPC is reacylated to GPC in the microsomes of the villus cells and incorporated into chylomicrons at one or more stages of the assembly. Scow *et al.* (26) have demonstrated that during oil feeding some 40% of the GPC of lymph chylomicrons retain the radio-label present in the lumenal (dietary plus biliary) GPC. Likewise, Parthasarathy *et al.* (27) observed that 52% of dietary GPC could be recovered from intestinal mucosa. Mansbach (28) has suggested that up to 100% of the chylomicron GPC may come from exogenous sources, depending on the dietary condition. Since the bile GPC is converted into LPC during intestinal absorption, a direct comparison is not appropriate between the molecular species of bile and chylomicron GPC. However, a comparison may be made between the ratios of 16:0 and 18:0, which are retained in the *sn*-1 position of the two types of GPC. Patton *et al.* (25) reported that based on the newly synthesized species, at least 20% of GPC is synthesized *de novo* under the condition of pure triacylglycerol feeding, which is similar to the results of the present experiment. We calculated a 16:0/18:0 ratio of 70:30 from rat bile GPC. This compares to 55:45 and 57:43 for the GPC of chylomicrons upon oil and ester feeding, respectively, which suggests that at least 15% of GPC came from *de novo* synthesis in this case as well. Under comparable conditions, the 16:0/18:0 ratio of the microsomal GPC (19) was 60:40. This indicates that neither the chylomicrons nor the microsomes correspond directly to the bile in the 16:0/18:0 ratio of the corresponding GPC. The biosynthesis and utilization of the selected molecular species of GPC for intestinal lipoprotein formation in the rat has been investigated by Arvidson and Nilsson (24) and Patton *et al.* (25). Patton *et al.* (25) have concluded that GPC incorporated into lymph HDL, VLDL and chylomicrons originate from different metabolic pools and/or different mechanisms, including *de novo* synthesis. The selectivity for chylomicron GPC species was the same in both pathways of triacylglycerol synthesis in the present study.

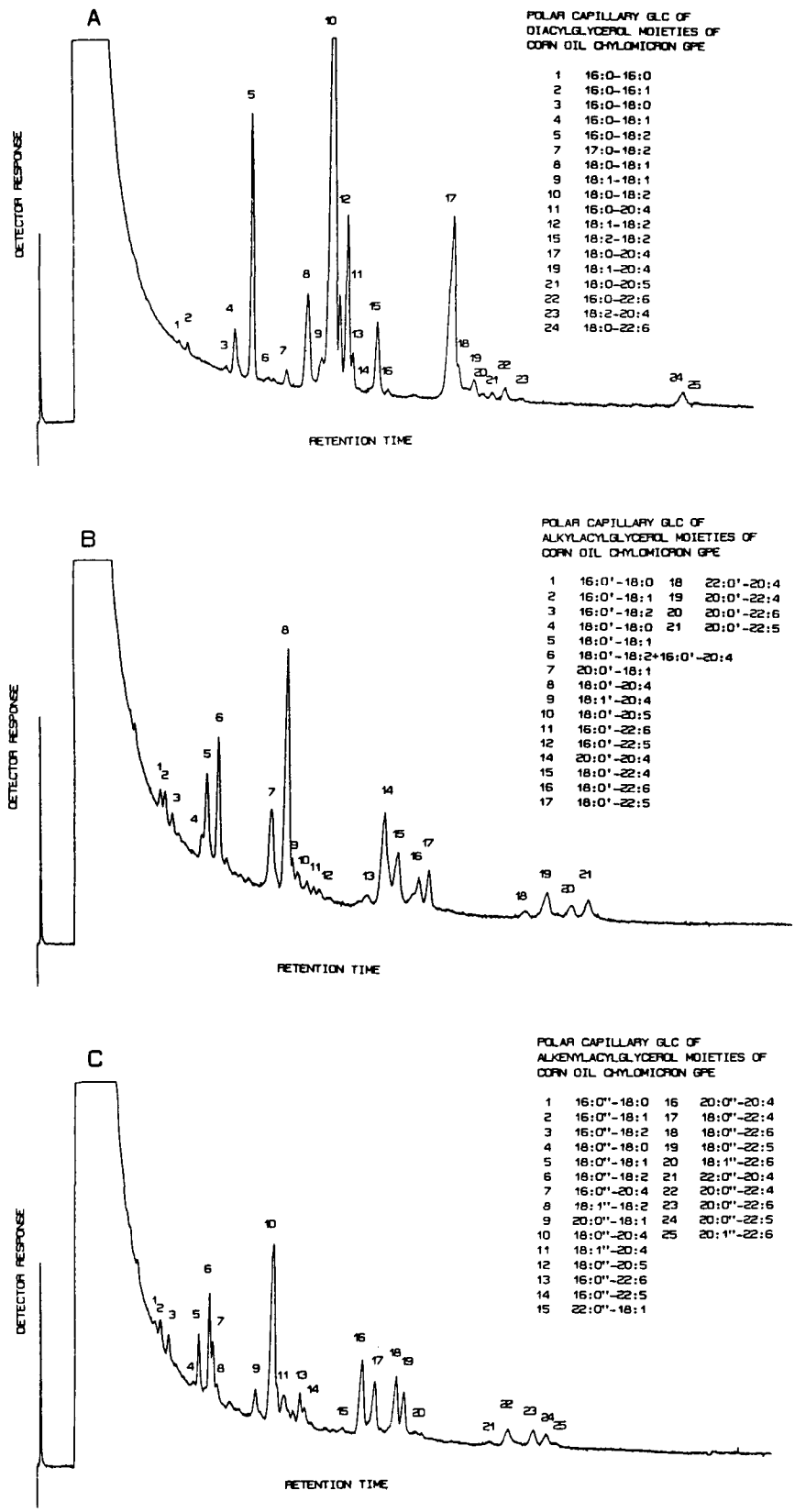


FIG. 8. Polar capillary GLC profiles of the diacyl (A), alkylacyl (B) and alkenylacyl (C) glycerol moieties of chylomicron GPC from rats receiving corn oil. Peak identification as given in Figure. GLC conditions as given in Figure 5.

Patton *et al.* (29) have demonstrated that GPC exchange can occur between chylomicrons and other lymph lipoproteins. Although the individual molecular species of GPC were noted to exchange at different rates, nearly complete equilibration of both radiolabel and mass was found after 24 hr. According to Higgins and Fieldsend (30), only a small amount of phospholipid may become associated with the triacylglycerol particles during transfer to the Golgi. The bulk of GPC would then be added just prior to the secretion of the mature chylomicrons in the Golgi, as suggested for VLDL secretion by rat liver (30-32).

The origin of chylomicron GPE has received much less experimental attention and has remained largely uncertain. The GPE values obtained for chylomicrons by Redgrave (21) and in the present studies are similar to those reported for rat bile by Kawamoto *et al.* (33), who has also detected small amounts of PS, PI and SPH in rat bile. Landin and Nilsson (34) have observed a rapid clearance of radiolabeled GPE from chylomicrons after intravenous infusion, which apparently accounts for the low level of GPE in plasma chylomicrons. Some or all of the chylomicron GPE could have come from absorption and reacylation of LPE or from *de novo* synthesis. Vance and Vance (35) have demonstrated that in hepatocytes, GPE derived from PS is preferentially used as a source of surfactant for lipoproteins, while GPE derived from ethanolamine is rejected. Also, GPE may have served as a precursor of chylomicron GPC, as the methylation pathway has been shown (36) to contribute to lipoprotein phospholipid secretion in hepatocyte cultures deficient in choline. The present work demonstrates that the mass composition of chylomicron GPE is more closely related to that of chylomicron PS than GPC in an apparent agreement with the conclusion of Vance and Vance (35), based on a study on hepatocyte VLDL. The present work also shows that chylomicron GPE contains lesser amounts of the alkylacyl and alkenylacyl species than the microsomal GPE (19).

In conclusion, this study demonstrates that the chylomicron triacylglycerols synthesized during absorption of mixed fatty acids as the glyceryl or ethyl esters possess identical surface phospholipids. This coincides with the earlier demonstrated identity in the apoprotein composition (8) and the recently shown (9) similarity in the fatty acid composition of the *sn*-1 and *sn*-3 positions of the chylomicron triacylglycerols. This work supports the idea that only a single type of chylomicrons is generated by the intestinal villus cells regardless of the nature of the dietary fat.

ACKNOWLEDGMENTS

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Compositional Changes and Apoprotein A-I Metabolism of Plasma High Density Lipoprotein in Estrogenized Chicks

B.H. Simon Cho* and **Jeong R. Park**

Department of Food Science and Division of Nutritional Science, University of Illinois, Urbana, Illinois 61801, and Harlan E. Moore Heart Research Foundation, Champaign, Illinois 61820

The effect of estrogen on compositional changes, apolipoprotein (apo) A-I metabolism and the morphology of plasma high density lipoprotein (HDL) were investigated in chicks. The administration of 17β-estradiol (25 mg/kg body weight) to growing male chicks (8-week-old) markedly reduced the concentrations of plasma HDL components, except for triglyceride (TG). At the same time, levels of TG, total cholesterol (TC) and phospholipid (PL) in plasma were greatly elevated. The respective values for TG, TC, PL and protein in HDL were 13.9, 89.3, 154.1 and 231.7 (mg/dL) in the control, and 39.0, 35.1, 113.8 and 160.0 (mg/dL) in chicks upon estrogen treatment for one day. *In vivo* kinetic studies showed that the fractional catabolic rate of HDL apo A-I was significantly higher ($p<0.05$) in estrogen-treated chicks than in control birds, indicating an increased efficiency of HDL removal in the former. The production rate of HDL apo A-I also was significantly lower ($p<0.05$) in estrogen-treated chicks. Sodium dodecyl sulfate-acrylamide gel electrophoresis followed by laser scanning densitometry of HDL apolipoproteins in estrogen-treated chicks revealed a reduction of apo A-I and the occurrence of new apolipoproteins which had been absent in HDL of untreated birds. The HDL particles showed that the mean particle size of HDL became larger upon estrogen treatment. Particles with diameters between 70 and 123 Å were predominant in HDL of control chicks, while particles with diameters between 97 and 143 Å were most abundant in HDL of estrogen-treated chicks.

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High density lipoprotein (HDL) levels in plasma have received considerable attention because they have been shown to correlate inversely with the risk of coronary heart disease (1,2). The anti-atherogenic effect of HDL has been associated to its ability to remove cholesterol from peripheral tissues, a process generally referred to as reverse cholesterol transport (3,4). The concentration of plasma HDL is related to age and sex of the individual (5), to sex steroids (6,7), hypertriglyceridemia (8), obesity (9), cigarette smoking (10), dietary composition (11), alcohol intake (12), exercise (13), and hypolipidemic drug therapy (14). A significant rise in HDL is also noted when estrogen is given to humans (6,7). The elevation of plasma HDL by estrogen therapy occurs mainly in the HDL_2 subclass (15). In contrast to humans, the administration of estrogen to chickens has been shown to decrease the level of plasma HDL (16,17). Estrogen administration to chickens also causes massive hypertriglyceridemia along with a significant rise in plasma very low density lipoprotein (VLDL) levels, as has also been shown in humans (18).

Since in avian species estrogen administration causes both qualitative and quantitative changes in plasma lipoproteins, the chick is a good model for the study of estrogen effects on lipoprotein metabolism. In the present study, we have examined the turnover of HDL apolipoprotein (apo) A-I in chicks upon estrogen treatment. Plasma HDL was isolated from untreated chicks and then labeled *in vitro* with radioactive iodine. The ^{125}I-labeled HDL was reinjected into control and estrogen-treated chicks, and HDL apo A-I kinetics were studied *in vivo*. In addition, the concentrations of lipids and protein in the HDL fractions were monitored, and the size of HDL particles was determined by electron microscopy.

MATERIALS AND METHODS

Isolation of plasma high density lipoprotein (HDL). Blood samples from male crossbred 8-week-old (New Hampshire male × Colombian female) chickens were drawn *via* wing vein into glass tubes containing ethylenediaminetetraacetic acid tetra sodium salt (EDTA) (1 mg/mL blood). Plasma was separated from the blood by low speed centrifugation at 1,000 × *g* for 30 min at 4°C. Plasma was adjusted to d = 1.063 g/mL by adding NaCl/NaBr solution, and was centrifuged at 115,000 × *g* for 20 hr at 10°C in a Beckman model L5-50 Preparative Ultracentrifuge (Palo Alto, CA). After centrifugation, the top layer containing lipoproteins (d<1.063 g/mL) was removed and the infranatant fraction was recentrifuged to remove the contaminating lipoproteins of d<1.063. The infranatant fraction was then adjusted to d = 1.21 g/mL with a NaCl/NaBr solution and centrifuged for 24 hr. The top layer containing HDL was harvested and washed once by recentrifugation. The HDL isolated was dialyzed against 0.15 M NaCl containing 0.01% EDTA (pH 7.0), using 3–4 changes. The purity of the HDL preparation was examined by agarose gel electrophoresis (19). The protein content of the HDL was determined by the method of Lowry *et al.* (20) as modified later (21).

Radioiodination of HDL. The HDL preparation was labeled with ^{125}I (carrier-free from New England Nuclear, Boston, MA) by the iodine monochloride method of McFarlane (22) as modified by Fidge and Poulis (23). HDL was brought to pH 10 by dialysis against 0.4 M glycine/NaOH buffer (pH 10), and then 2 mL (10 mg protein) was added to a mixture of 50 μL of 60 mM iodine monochloride in 2 M NaCl and 50 μL of ^{125}I in dilute NaOH (100 μCi) at 4°C. The solution was mixed immediately by vortexing. Unlabeled iodine was removed by dialysis against 100 vol of 0.15 M NaCl and 0.01% EDTA solution (pH 7.4) for more than 5 hr, with batches being changed every 20–30 min. After dialysis, small aliquots of labeled HDL were removed to determine radioactivity and its distribution, and the remainder was sterilized

*To whom correspondence should be addressed at Harlan E. Moore Heart Research Foundation, 503 S. Sixth Street, Champaign, IL 61820.

Abbreviations: apo, apolipoprotein; EDTA, ethylenediaminetetraacetic acid; FCR, fractional catabolic rate; HDL, high density lipoprotein; LDL, low density lipoprotein; PL, phospholipid; SDS, sodium dodecyl sulfate; TC, total cholesterol; TG, triglyceride; VLDL, very low density lipoprotein.

by passing through a 0.45 μm Millipore filter (Millipore Corp., Bedford, MA). The efficiency of iodination was 29.4%. Characterization of ^{125}I-labeled HDL showed that 97.4% of radioactivity was precipitated by 10% trichloroacetic acid, and 1.1% was extracted with chloroform/methanol (2:1, v/v). The ^{125}I-labeled HDL had the same electrophoretic mobility on agarose gel electrophoresis as unlabeled HDL.

Kinetic study. One day before the study, 8-week-old chicks were treated subcutaneously with 17β-estradiol (25 mg/kg body weight) in propylene glycol (carrier alone for control chicks). After overnight fasting, blood samples were drawn from control and estrogen-treated chicks and were used for lipoprotein isolation and chemical analysis. Labeled HDL (1-2 μCi, 1-2 mL vol) was then injected into the birds *via* a wing vein. Subsequently, 2-mL blood samples were collected in glass tubes containing EDTA (1 mg/mL blood) at 5, 15, 30, 45, 60, 90, 120, 180 and 210 min. Plasma was separated at 4°C by low speed centrifugation. Small aliquots of plasma were counted for radioactivity in a gamma-ray spectrometer. Plasma was adjusted to d = 1.063 g/mL with a NaCl/NaBr solution and centrifuged for 20 hr at 115,000 × *g* at 10°C. Lipoproteins of d<1.063 g/mL were removed by aspiration of the top 2-mL layer. The infranatant fraction was then adjusted to d = 1.21 g/mL with a NaCl/NaBr solution and centrifuged for 24 hr. The top layer containing HDL was harvested and the HDL isolated was dialyzed against a solution containing 0.15 M NaCl and 0.01% EDTA. HDL apo A-I was then separated by sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis as described below. Apo A-I bands from the gels were sliced and the radioactivity was determined. The radioactivity decay pattern of each sample was plotted on semilogarithmic paper, and the fractional turnover rate (FCR, K) was determined from two time points on the resulting straight line as described by Shipley and Clark (24). The turnover rate of HDL apo A-I was calculated by multiplying K by the total pool of HDL apo A-I per kilogram body weight (K × HDL apo A-I concentration × plasma volume/body weight). The plasma volume was estimated by extrapolating the plasma radioactivity decay curve back to zero time, assuming that the intercept represented total mixing of the injected ^{125}I-labeled HDL.

Sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis. The HDL fraction was delipidized with ice-cold diethyl ether and ethanol. The protein precipitate was washed twice with diethyl ether/ethanol (3:1, v/v), dried under a stream of N_2, and dissolved in 0.2 M phosphate buffer containing 2% SDS and 2-mercaptoethanol. HDL apo A-I was then separated by SDS-polyacrylamide gel electrophoresis using a 10% gel containing 0.1% SDS. Gels were prepared in glass tubes (0.5 cm i.d., 9 cm length) according to Laemmli (25). After electrophoresis, the gel was stained in a solution containing 0.125% Coomassie brilliant blue R-250, 25% methanol and 10% acetic acid. Gels were destained in a solution containing acetic acid (7.5%) and methanol until the background was completely clear. Molecular weights of apoproteins were estimated by plotting log molecular weight *versus* distance of migration of the reference proteins. A typical HDL apoprotein separation is shown in Figure 1. The stained apoproteins were scanned using a laser densitometer (Ultrascan 2022, LKB Instruments, Bromma, Sweden). The percent distribution of apo A-I was calculated as the integrated peak area of the apoprotein divided by the sum of the total peak areas of HDL apoproteins. The apo A-I concentration was calculated by multiplying the total HDL protein concentration by the area percent of apo A-I.

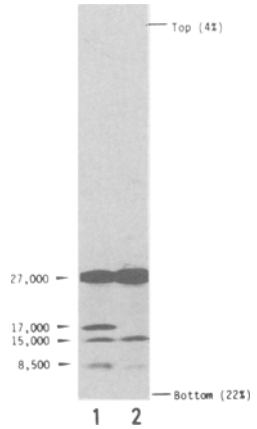


FIG. 1. SDS-polyacrylamide gel electrophoresis of HDL apoproteins of estrogen-treated (lane 1) chicks, and controls (lane 2).

Electron microscopy of HDL. Aliquots of the fresh HDL preparations from control and estrogen-treated chicks were negatively stained with 2% phosphotungstic acid (pH 6.5). The morphology and homogeneity of HDL particles were examined with a Hitachi HU-12 electron microscope (Tokyo, Japan) on carbon-coated Formvar grids.

Lipid analysis. The total and free cholesterol contents of plasma and HDL preparations from control and estrogen-treated chicks were determined using enzymatic cholesterol reagents (Wako Chemical USA, Dallas, TX). Triglyceride values were measured by the method of Foster and Dunn (26). Total phospholipid content was determined by the method of Eng and Noble (27) after digestion of lipid extracts with perchloric acid.

Statistical analysis. Data were analyzed by the difference between means, and statistical significance was based on Student t-test using the SAS program (SAS Institute Inc., Cary, NC).

RESULTS

In the present study we observed that the administration of 17β-estradiol (25 mg/kg body weight) to growing male chicks markedly reduced the plasma HDL concentration (Table 1). At the same time, levels of triglycerides (TG), total cholesterol (TC) and phospholipids (PL) in plasma were greatly elevated. The respective values for TG, TC, PL and protein in HDL were 13.9, 89.3, 154.1 and 237.1 (mg/dL) in the controls, and 39.0, 35.1, 113.8 and 160.0 (mg/dL) in chicks upon estrogen treatment for one day. The ratio of the nonpolar core mass (triglyceride and cholesteryl ester) to the polar surface components of HDL (protein, phospholipid and free cholesterol) was significantly higher ($p<0.05$) in estrogen-treated chicks than in controls (0.25 ± 0.001 *vs* 0.22 ± 0.002), suggesting that the HDL particles in the former may be larger than those

TABLE 1

Effect of Estrogen on Levels of Plasma Lipids and High Density Lipoprotein Components in Chicks[a]

	Triglyceride	Cholesterol			Phospholipid	Protein
		Total	Free	Ester[b]		
Plasma (mg/dL)						
Control	113.3 ± 15.7	123.4 ± 3.0	44.1 ± 2.8	79.3 ± 2.2	94.4 ± 12.7	—
Estrogen	1170.7 ± 73.0[c]	205.1 ± 8.3[c]	77.4 ± 5.9[c]	129.6 ± 7.8[c]	129.6 ± 24.1[c]	—
High density lipoprotein (mg/dL)						
Control	13.9 ± 4.0	89.3 ± 12.4	14.6 ± 2.2	74.7 ± 10.2	154.1 ± 27.7	231.7 ± 30.4
Estrogen	39.0 ± 3.3[c]	35.1 ± 1.5[c]	3.5 ± 0.2[c]	31.6 ± 1.6[c]	113.8 ± 3.0[c]	160.0 ± 8.8[c]

[a]Values are mean ± SD (n=4). The value in the same column bearing a superscript letter is significantly different from the control ($p<0.05$).
[b]Determined as the difference between total and free cholesterol.

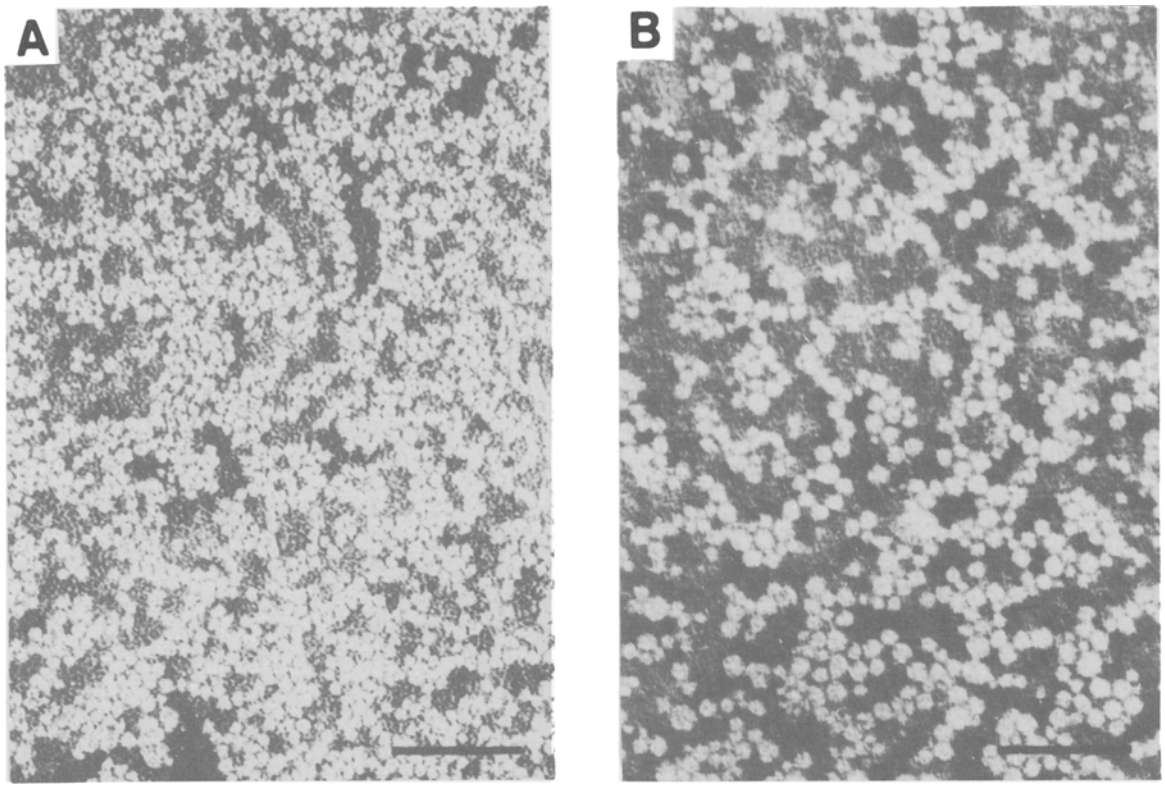


FIG. 2. Electron micrographs of plasma high density lipoproteins from control and estrogen-treated chicks. Panel A, control HDL; Panel B, estrogen-treated HDL. Final magnification is × 150,000.

in the latter. The electron microscopic appearance of HDL particles, as shown in Figure 2, is consistent with the chemical data. The mean particle size of HDL was larger in estrogen-treated chicks than in untreated birds. In control birds, HDL particles with diameters between 70 and 123 Å were predominant, whereas HDL particles with diameters between 97 and 143 Å were abundant in HDL of estrogen-treated chicks.

The separation of HDL apolipoproteins by SDS-acrylamide gel electrophoresis is shown in Figure 1. The HDL apoproteins of untreated birds were resolved into three bands and consisted mainly of apo A-I (molecular weight of 27000), accounting for 81.1% of the total HDL protein as determined by densitometric scanning. In the estrogen-treated chicks, the proportions of HDL apoproteins were significantly altered, with a marked reduction of apo A-I (58.0% of the total HDL protein). New apoproteins, presumably apo VLDL-11, with molecular weights of 8500 and 17000 were also present in HDL of estrogen-treated birds. Consequently, the amount of HDL apo A-I was significantly lower ($p<0.05$) in estrogen-treated chicks than that in controls (83.1 *vs* 174.1 mg/dL). The disappearance of radioactivity from apo A-I of plasma HDL in control and estrogen-treated chicks is shown in Figure 3. The HDL apo A-I metabolism was faster in estrogen-treated chicks than in control birds, with the fractional catabolic rate being significantly higher (Table 2). The production rate of HDL apo A-I was significantly lower

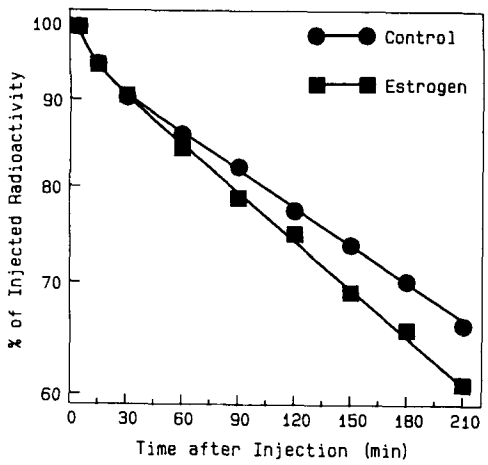


FIG. 3. Radioactivity curves of apoprotein A-I of plasma high density lipoproteins in control and estrogen-treated chicks. Points indicate the average values from 4 birds; control, ●; estrogen-treated, ■.

($p<0.05$) in estrogen-treated chicks than in controls (7.34 *vs* 12.81 mg/hr/kg body weight).

DISCUSSION

Middle-aged women have higher levels of plasma HDL (5) and a lower incidence of coronary heart disease (28) than their male counterparts. Estrogen, a female steroid hormone, has been observed to increase the plasma concentration of HDL, and especially of the HDL_2 subfraction. Estrogen has been shown to suppress hepatic lipase (15), an enzyme thought to be responsible for the catabolism of HDL_2 phospholipid (29).

However, the effect of estrogen on HDL in avian species appears to be opposite to that in humans. Estrogen-induced hyperlipidemia in chicks is characterized by the marked elevation in levels of VLDL and, to a lesser extent, low density lipoprotein (LDL), with a concomitant decrease in HDL (17). The reduction of HDL by estrogen is quite dramatic since HDL comprises almost 90% of the total lipoprotein-associated protein in untreated birds compared to the HDL level of 1–14% in estrogen-treated birds (17,30). The factors causing the decrease in HDL in birds under estrogen treatment are not clear. The decrease in HDL may be a consequence of reduced synthesis of apolipoproteins. Apolipoprotein A-I is a major apolipoprotein in HDL, and the present finding is in agreement with those of others indicating that about 90% of the protein content of HDL is composed of apo A-I in untreated birds (31). Upon estrogen administration, the apolipoprotein composition of HDL undergoes a marked reduction in apo A-I concentration in chick and turkey (30,32). This effect of estrogen in avian species is believed to be the result of decreased apo A-I synthesis causing a reduction in the levels of plasma HDL apo A-I. Since the synthesis of vitallogenin and VLDL are greatly stimulated under estrogenic influence, the synthesis of other plasma proteins, such as apo A-I, may be reduced due to competition at the level of mRNA translation (32). In a mutant strain of chicken which develops a spontaneous HDL deficiency syndrome, the principal metabolic defect has also been attributed to a major reduction in apo A-I production (33). This finding correlates well with a decrease in the concentration of apo A-I in estrogenized chickens (30). However, the reduced apo A-I production rate in the present study does not seem to agree with a previous report by others in which estrogen treatment of chickens did not alter the messenger RNA activity for apo A-I in hepatic polysomes (34). Since HDL apo A-I also originates from intestinal sources, either as nascent HDL or as the surface coat of triglyceride-richy lipoproteins of intestinal origin (35), the mRNA activity for apo A-I in the intestine may also be affected by estrogen.

In addition to reduced synthesis, a reduction in HDL apo A-I could also be due to an increase in apo A-I catabolism. The present finding clearly shows that estrogen-treated birds have a significantly increased fractional catabolic rate for apo A-I in comparison to untreated birds, indicating enhanced removal of apo A-I in estrogenized birds. The enhanced catabolism of apo A-I in estrogenized birds in conjunction with the reduced synthesis of apo A-I may then accentuate the effect of estrogen on the reduction of HDL concentration. This finding of hypercatabolism of HDL is similar to that seen in a mutant strain of chickens which have a deficiency in HDL and apo A-I (33). An increased fractional catabolic rate for triglyceride enriched HDL has also been reported in hypertriglyceridemic patients (36), and the enrichment of HDL with triglyceride may contribute to the increased HDL catabolism. Since estrogen treatment of chickens greatly elevates VLDL triglyceride, the movement of triglyceride from VLDL to HDL may be facilitated by lipid transfer protein (37) resulting in triglyceride enrichment of HDL. The present finding that the TG content of HDL in estrogen-treated chickens was nearly three times higher than that of control birds confirms the enrichment of HDL with triglyceride.

The present study indicates that the overall effects of

TABLE 2

Effect of Estrogen on Plasma Levels, and Fractional Catabolic and Turnover Rates of High Density Lipoprotein Apo A-I in Chicks[a]

Group	Body weight (g)	Plasma volume (mL)	HDL Apo A-I Content (mg/dL)	HDL Apo A-I FCR	HDL Apo A-I Turnover rate (mg/kg/hr)
Control	1423 ± 213	97.6 ± 20.3	174.12 ± 16.22	0.1086 ± 0.0097	12.81 ± 1.39
Estrogen	1570 ± 59	103.2 ± 3.1	83.10 ± 9.47[b]	0.1348 ± 0.0089[b]	7.34 ± 0.72[b]

[a]Values are mean ± SD (n=4).

[b]Significantly different from the control group ($p<0.01$).

estrogen on HDL metabolism are complex, and the reduction of HDL apo A-I concentration in estrogenized male chickens seems to result from decreased production and increased catabolism of this major HDL apolipoprotein.

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In Vivo Determination of Triglyceride Secretion Using Radioactive Glycerol in Rats Fed Different Dietary Saturated Fats[1]

Hui-Chuan Lai[a], John B. Lasekan[a,2], Huan Yang[a], Murray K. Clayton[b] and Denise M. Ney[a,*]
[a]Department of Nutritional Sciences, and [b]Departments of Statistics and Plant Pathology, University of Wisconsin-Madison, Madison, WI 53706

Our objective was to determine the relative rates of *in vivo* triglyceride (TG) secretion and the composition of very low density lipoproteins (VLDL) in rats fed different dietary saturated fats. Male Sprague-Dawley rats (150–200 g) were fed diets containing 16% corn oil, or 14% butterfat, 14% beef tallow, 14% olive oil, or 14% coconut oil plus 2% corn oil for 5 wk. Changes in plasma TG specific radioactivity were determined in individual, unanesthetized fasted rats after injection of 100 μCi [2-^{3}H]glycerol. Nonlinear regression analysis using a 2-compartment model was used to determine the fractional rate constant for TG turnover in plasma. The plasma TG pool was 33–40% larger with beef tallow than with corn, olive or coconut oil feeding ($p<0.05$), and 20% larger with beef tallow than with butterfat feeding. The rate of TG secretion into plasma (mg/min/100 g body weight) was 60% higher in animals fed beef tallow than corn or coconut oil ($p<0.05$) and 26–33% higher in animals fed beef tallow than olive oil or butterfat. Differences in VLDL composition (% wt) were also noted. Our data suggest that greater TG secretion is the primary factor contributing to the larger TG pool with ingestion of beef tallow relative to butterfat, corn or coconut oil. These results suggest that different dietary saturated fats have unique effects on TG metabolism in rats.
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The influence of diet on plasma triglyceride (TG) levels has received recent attention, in part, because of a better appreciation of how TG-rich lipoproteins may promote atherogenesis (2). Ingestion of saturated or monounsaturated fats has been associated with higher plasma TG concentrations compared to ingestion of polyunsaturated fats in humans (3,4) and in rats (5–9). Possible mechanisms contributing to higher plasma TG levels with ingestion of saturated fat relative to unsaturated fat include an increase in very low density lipoprotein (VLDL) secretion (4,10–12) and hepatic lipogenesis (13,14) and a decrease in the rate of clearance of VLDL or chylomicrons from plasma (7,8). An increase in hepatic TG secretion has been demonstrated to be the primary factor associated with the hypertriglyceridemic effect of saturated fats in humans (4,10).

The mechanism(s) underlying high plasma TG levels with ingestion of saturated fats in the rat remain controversial. *In vitro* studies with liver perfusion demonstrated an increase (11,12) or no difference (6) in TG secretion when long-chain saturated fatty acids replaced unsaturated fatty acids in the perfusate. However, *in vivo* studies using Triton WR 1339 demonstrated similar rates of TG secretion in rats fed palm oil relative to sunflower seed oil (8). Other work suggests that saturated TG in chylomicrons (7) and VLDL (8) are cleared more slowly from plasma than unsaturated TG.

The relative effects of different types of dietary saturated fats on *in vivo* rates of TG secretion and the composition of TG-rich VLDL have not been compared concurrently in one study. In addition, previous methods used to determine TG secretion such as liver perfusion and the Triton technique have limitations which may not reflect the *in vivo* response. Moreover, these methods often require the use of anesthesia or restraint which has been shown to significantly decrease TG production (15,16).

Therefore, our objectives were to determine the relative rates of *in vivo* TG secretion and plasma VLDL composition in rats fed corn oil, butterfat, beef tallow, olive oil or coconut oil. Rates of TG secretion were determined using 2-[^{3}H]glycerol and a two-compartment model (17) with sequential blood sampling of individual, unanesthetized animals using minimal restraint.

MATERIALS AND METHODS

Animals and diets. Male, Sprague-Dawley rats (Harlan Sprague-Dawley, Madison, WI) weighing 138–176 g were housed in individual stainless steel cages and maintained at 25°C with a 12-hr (7 a.m. to 7 p.m.) light-dark cycle. Rats were assigned to dietary groups ($n = 15$) by controlled randomization 5 days after delivery so that initial body weights were not different among groups.

Rats were fed *ad libitum* one of five AIN 76A diets containing 16% fat and equal amounts of sucrose and cornstarch (9). The five diets contained 16% corn oil or 14% butterfat, 14% beef tallow, 14% olive oil or 14% coconut oil plus 2% corn oil to prevent essential fatty acid deficiency (Table 1). Cholesterol was added to provide a similar amount of dietary cholesterol based on the cholesterol content of anhydrous butterfat, 0.25 g cholesterol/100 g butterfat (9). The fatty acid composition of the diet was determined (9) using a Varian 3400 gas chromatograph (Varian, Sugarland, TX) equipped with an on-column temperature programmable injector (Table 2). Diets were stored at 4°C and fresh diets were presented to animals biweekly. Food intakes were calculated weekly based on a 4-day period, and body weights were measured biweekly.

After feeding the diets for 5 wk, 7 rats from each group were randomly selected and killed for analysis of plasma, VLDL and liver lipid concentrations. Rats were fasted

[1]Presented in part at the 1990 meeting of the Federation of American Societies for Experimental Biology in Washington, D.C. (see ref. 1).
[2]Current address: Kraft General Foods, Technical Research Center, T22-1, 250 North Street, White Plains, NY 10625.
*To whom correspondence should be addressed at Department of Nutritional Sciences, 1415 Linden Drive, Madison, WI 53706.
Abbreviations: ANOVA, analysis of variance; apo, apolipoprotein; PL, phospholipid; TG, triglyceride; VLDL, very low density lipoprotein.

TABLE 1

Diet Composition

Ingredient[a]	Amount (%)
Casein	20.0
DL-Methionine	0.3
Sucrose	27.0
Cornstarch	27.0
Cellulose	5.0
Fat source[b]	14.0
Corn oil	2.0
Cholesterol[c]	0.035
Mineral mix — AIN 76	3.5
Vitamin mix — AIN 76A	1.0
Choline bitartarate	0.2
Butylated hydroxytoluene (BHT)[d]	0.02

[a]All ingredients were obtained from TEKLAD, Harland Sprague Dawley Inc. (Madison, WI) except for cholesterol and BHT, which were obtained from Sigma (St. Louis, MO).
[b]Anhydrous butterfat, beef tallow, corn oil, coconut oil or olive oil.
[c]Butterfat, 0.035% naturally occurring cholesterol; corn oil, olive oil, and coconut oil, 0.035% added cholesterol; beef tallow, 0.0196% naturally occurring cholesterol + 0.0154% added cholesterol. Cornstarch was adjusted to offset the added cholesterol and BHT.
[d]BHT was not added to the beef tallow diet as it already contained an antioxidant.

TABLE 2

Fatty Acid Composition of the Diets

Fatty acid	Corn oil[a]	Butter-fat[b]	Beef tallow[a]	Olive oil[a]	Coconut oil[b]
			(%)		
4:0		3.1			0.2
6:0		1.8			1.4
8:0		1.2			6.0
10:0		2.7			5.3
12:0		3.1			40.0
14:0	0.1	9.3	2.5		15.1
14:1		0.7			
16:0	12.4	23.9	24.1	9.9	9.5
16:1		1.0	3.6	0.5	0.1
18:0	1.8	17.6	15.8		2.7
18:1	25.9	20.7	38.9	76.0	9.4
18:2	56.5	10.1	12.0	11.8	8.7
18:3	1.0	0.5	0.1	0.6	0.2
20:0		0.7		0.4	0.1
20:4	0.2		0.2	0.3	

[a]Values are % distribution of fatty acid methyl esters.
[b]Values are % distribution of fatty acid butyl esters.

overnight, anesthetized with CO_2, and blood (8–10 mL) was collected by cardiac puncture into syringes containing 1 mg EDTA and 0.1 mg gentamicin sulfate per mL blood. Livers were excised, blotted dry, weighed, and kept at −20°C for analysis. The remaining animals were used for determination of TG secretion.

Determination of in vivo triglyceride secretion. In vivo TG secretion was determined in individual, unanesthetized fasted rats by multicompartment analysis of the changes in plasma TG specific radioactivity (dpm/mg TG) after injection of 100 μCi [2-^{3}H]glycerol (5–10 Ci/mmol; New England Nuclear, Boston, MA). Animals underwent surgical placement of cannula in the left carotid artery (18) after ingesting the diets for 5 wk. After being anesthetized by intramuscular injection of a mixture providing 50 mg ketamine plus 10 mg xylazine per kg body weight, a polyethylene catheter (15 cm, PE-50, ID 0.58 mm, OD 0.97 mm, Dow Corning Corp., Midland, MI) was inserted 2.5 cm into the left carotid artery and sutured securely to the artery (18). The free end of the catheter was tunneled under the skin and secured in the midscapular area with two surgical staples. The catheters were pretreated with 2% TDMAC-Heparin solution (1000 usp units/mL; Polysciences Inc., Warrington, PA) and sterilized. Animals were allowed 1 wk to recover from surgery and to regain their presurgical weights. All experiments were conducted at 8 a.m. on unanesthetized rats that were fasted for 10 hr. Minimal restraint was used during injection of isotope and repeated blood sampling.

Prior to the injection of isotope, a baseline blood sample (300 μL) was collected into a 1-mL syringe containing 0.3 mg EDTA and 0.03 mg gentamicin sulfate. Each animal then received 100 μCi [2-^{3}H]glycerol diluted in 300 μL saline followed by flushing the cannula 3 times with 200 μL of saline to displace any remaining isotope. A preliminary *in vitro* experiment demonstrated little residual isotope after this flushing procedure.

Blood samples (300 μL) were obtained 10, 15, 20, 25, 30, 40, 60, and 80 min after injection of isotope as described for baseline blood samples. These time points were chosen (17) to sample 3–4 times during both the ascending and descending parts of the TG specific activity curve. After each sample was obtained, the cannula was flushed with 300 μL saline which was then removed and discarded prior to obtaining the next sample. After the experiment, cannulae were dissected from selected animals and counted for ^{3}H; results demonstrated minimal residual contamination with isotope. The repeated blood sampling resulted in a 14–18% decrease in blood hematocrit with no significant differences among groups.

Determination of plasma triglyceride specific activity. Plasma was separated by centrifugation at 1200 × *g* for 20 min. Lipids were extracted twice from plasma samples (120 μL) with 2.5 mL chloroform/methanol (2:1, v/v) (19) and triglycerides were separated and identified by thin-layer chromatography on Whatman Silica Gel H (American Scientific Products, Mc Gaw Park, IL) (20). TG concentrations were determined in TG extracts from chromatography plates based on saponification and measurement of free glycerol (21).

Radioactivity was counted with an accuracy of ± 2% in a Packard 2000 CA Tri-carb Liquid Scintillation Analyzer, with Opti-fluor (Packard Instrument Inc., Downers Grove, IL) as scintillation fluid. The TG specific activity was calculated for each sample and expressed as dpm/mg TG. A known amount of tritiated tripalmitin was added to selected plasma samples before lipid extraction; recovery of radioactivity ranged from 85–90%.

Kinetic analysis. The kinetic model used to evaluate changes in plasma TG specific activity was based on a two-compartment system with irreversible flow, time delay between the compartments and equal fractional rate constants (17). The equation describing the model is:

$$SA_{(2)} = A(t-t_{lag})e^{-k(t-t_{lag})} \quad [1]$$

where $SA_{(2)}$ = specific activity of TG in compartment 2;

A = a constant; k = the fractional rate constant; t = time of sampling; t_{lag} = lag time (delay in appearance of labeled TG in plasma).

For each animal, the coefficients A and k were determined by nonlinear regression analysis using the SAS NLIN procedure (22) on a Hewlett-Packard 9000/825 computer. Plots of residual *versus* predicted values were used to assess fit of the model (23). Plasma TG pool size was calculated as baseline plasma TG concentration multiplied by plasma volume, where plasma volume = (body weight × 0.041) − 1.59 (17). The half life ($t_{1/2}$) for plasma TG turnover was calculated from the equation: $t_{1/2} = \ln 2/k$. The rate of TG secretion into plasma was calculated by multiplying k by the plasma TG pool size.

Analyses of liver and plasma lipids. Plasma was obtained by centrifugation at 4°C for 20 min at 1200 × *g*. VLDL were fractionated from 3 mL plasma by sequential ultracentrifugation (9) with the following modifications to improve recovery of TG. The VLDL fraction was initially isolated by spinning at d = 1.006 g/mL for 18 hr. A tube slicer was then used to help remove the top two mL containing VLDL followed by repeated rinsing of the inside wall of the tube with d = 1.006 g/mL solution to recover lipid clinging to the tubes. The VLDL fraction was then ultracentrifuged for another 18 hr to minimize albumin contamination and the top one mL containing VLDL was carefully removed and saved for analysis.

Concentrations of cholesterol and TG (Sigma TG Kit, No. 336, Sigma Chemical Company, St. Louis, MO) were determined in plasma and VLDL (5). Concentrations of VLDL protein, total and free cholesterol and phospholipid were determined (5). The concentration of esterified cholesterol was multiplied by 1.68 when VLDL composition was expressed as % by weight to account for the fatty acid moiety. Lipids were extracted from 1 g hepatic samples with chloroform/methanol (2:1, v/v) (19) and assayed for cholesterol (5) and TG (21) contents.

Concentrations of apolipoprotein B and E in plasma VLDL were determined by the Laurell electroimmunoassay technique (24) and expressed as mg apolipoprotein/dL plasma. Standard curves were calculated using standardized rat plasma of known apolipoprotein concentration.

Statistical analysis. Differences among dietary treatment groups were assessed by one-way analysis of variance (ANOVA) using the SAS general linear models program (22). Group means were considered to be significantly different at $p \leq 0.05$ as determined by the least significant difference technique (25). Values are expressed as mean ± SEM.

RESULTS

Final body weight, liver weight and lipid contents of plasma and liver are summarized in Table 3 for nonsurgical rats fed for 5 wk. Food intake (14.6–15.3 g/d) and final body weight were not significantly different among dietary groups. However, liver weight was significantly higher with ingestion of olive or coconut oil than with ingestion of corn oil, butterfat or beef tallow.

In vivo triglyceride secretion. Figure 1 illustrates the changes in plasma TG specific activity after administration of 100 μCi [2-^{3}H]glycerol to rats fed different dietary fats. Illustrated data were taken from representative animals. Examination of residual plots indicated that our model gave an adequate fit to the data. Steady state plasma TG levels were maintained throughout the experiment satisfying an assumption of the kinetic model.

The magnitude of plasma [2-^{3}H]TG specific activity differed from animal to animal. For example, plasma TG specific activity ranged from 1555 to 39682 dpm/mg TG in one animal fed corn oil and from 833 to 66227 dpm/mg TG in another animal fed beef tallow. Differences within a dietary treatment were also present indicating a large intra animal variation with respect to the extent of tracer incorporation. This suggests the importance of doing compartmental kinetic analysis on sequential samples from individual animals rather than killing multiple animals at specific time points.

The effects of dietary saturated fats on plasma TG pool size, the rate of TG secretion, the fractional rate constant (k), lag time (t_{lag}) and half life ($t_{1/2}$) are shown in Table 4. The plasma TG pool was 33–40% larger with beef tallow than with corn, olive or coconut oil feeding ($p<0.05$), and 20% larger with beef tallow than with butterfat feeding. The rate of TG secretion into plasma (mg/min/100 g body weight) was 60% higher in animals fed beef tallow than corn or coconut oil ($p<0.05$) and 26–33% higher in animals fed beef tallow than olive oil or butterfat. Values for fractional rate constants (0.048–0.060 min^{-1}), t_{lag} (10.9–11.9 min) or $t_{1/2}$ (10.8–15.0 min) were not significantly different among the dietary groups.

TABLE 3

Body Weight, Liver Weight and Lipid Contents of Plasma and Liver[a]

	Corn oil	Butterfat	Beef tallow	Olive oil	Coconut oil
Final body weight (g)	313 ± 7^b	305 ± 9^b	322 ± 5^b	310 ± 6^b	315 ± 5^b
Liver weight (g/100 g body weight)	3.2 ± 0.1^b	3.0 ± 0.1^b	3.2 ± 0.1^b	3.4 ± 0.1^c	3.5 ± 0.1^c
Plasma (mg/dL)					
Total cholesterol	83 ± 3^b	87 ± 2^b	87 ± 3^b	83 ± 1^b	82 ± 3^b
Triglyceride	52 ± 5^b	62 ± 5^{cb}	80 ± 7^c	77 ± 9^c	68 ± 7^c
Liver (mg/g wet weight)					
Total cholesterol	3.5 ± 0.3^{cb}	2.6 ± 0.3^d	2.8 ± 0.1^{bd}	4.0 ± 0.4^c	2.8 ± 0.1^{bd}
Triglyceride	11.2 ± 2.0^c	7.2 ± 1.2^b	7.7 ± 1.8^b	11.1 ± 1.1^c	8.8 ± 1.0^b

[a]Values are means ± SEM, n=7. Means with different superscripts in the same row are significantly different, $p<0.05$.

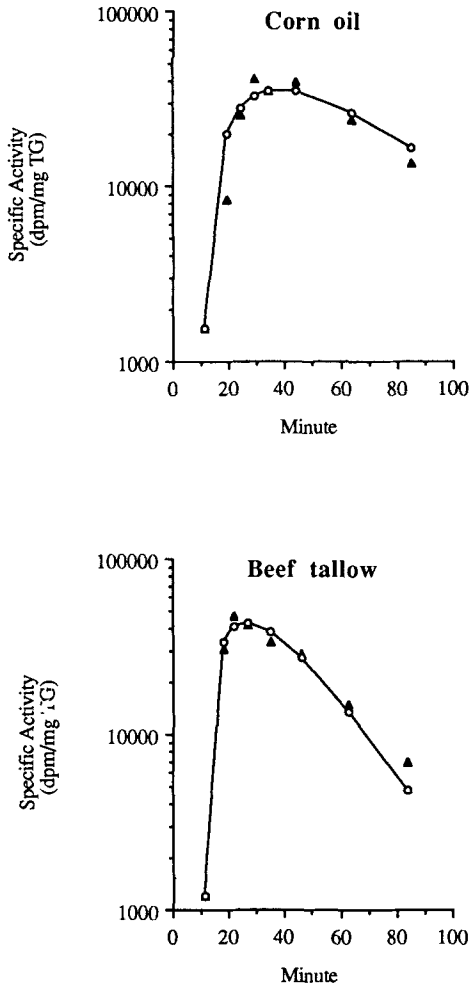


FIG. 1. Plasma triglyceride specific radioactivity after injection of 100 μCi [2-^{3}H]glycerol (5–10 Ci/mmol) into rats fed different dietary saturated fats. Data were taken from representative animals from the corn oil and beef tallow groups. ▲, observed triglyceride specific radioactivity; O, predicted triglyceride specific radioactivity based on the model: $SA_{(2)} = A(t-t_{lag})e^{-k(t-t_{lag})}$.

Analysis of plasma VLDL. VLDL concentration (mg/dL plasma) and composition (% by weight) are shown in Table 5. VLDL total mass was significantly higher with ingestion of beef tallow or olive oil than corn oil, as indicated by significantly higher individual concentrations of VLDL-TG, protein, phospholipid (PL) and cholesterol. VLDL-TG, protein and cholesterol concentrations were not significantly different among corn oil, butterfat or coconut groups. Ingestion of butterfat, olive or coconut oil resulted in VLDL with less % by weight TG than beef tallow.

VLDL from animals fed beef tallow demonstrated a significantly higher ratio of core-to-surface components (TG + esterified cholesterol/free cholesterol + PL + protein) than all fats except corn oil. This suggests the presence of larger TG-enriched VLDL with ingestion of beef tallow than with ingestion of butterfat, olive or coconut oil. Ingestion of butterfat was associated with relatively smaller VLDL particles than beef tallow or corn oil based on a significantly lower ratio of VLDL core-to-surface components.

Concentrations of apolipoprotein (apo) B and E in plasma VLDL are shown in Table 6. Apo B was 3–4 fold higher ($p<0.0001$), and apo E was 1.8–2 fold higher ($p<0.01$) with ingestion of beef tallow or olive oil than corn oil. However, no significant differences in apo B and E levels were noted in rats fed butterfat, coconut or corn oil.

Overall, the data suggest distinct effects on VLDL concentration, composition and relative size with ingestion of different dietary fats. Beef tallow and olive oil feeding appeared to be associated with a greater number of VLDL particles than corn oil because of a significantly higher VLDL mass without a significant difference in VLDL size based on the relative ratios of VLDL core-to-surface components.

Plasma and hepatic cholesterol and triglyceride contents. Fasting total plasma cholesterol levels were not significantly different among the groups after ingestion of the diets for 5 wk. Fasting plasma TG levels were 30–54% higher with ingestion of beef tallow, olive or coconut oil than corn oil ($p<0.05$). Rats fed corn oil or butterfat demonstrated similar plasma TG levels. Consistently lower concentrations of hepatic total cholesterol (25–35% lower) and TG (30–50% lower) were noted with ingestion of butterfat, beef tallow or coconut oil relative to corn oil. Rats fed olive oil demonstrated similar levels of hepatic cholesterol and TG when compared with corn oil feeding but significantly higher levels of hepatic cholesterol and TG when compared with feeding saturated fats.

DISCUSSION

The present study utilized two unique features to determine if a greater rate of hepatic TG secretion contributes to higher plasma TG levels with ingestion of dietary saturated fats rather than unsaturated fats in fasted rats. First, three saturated fats, butterfat, beef tallow and coconut oil, the monounsaturated olive oil and polyunsaturated corn oil were compared concurrently. Second, *in vivo* TG secretion was determined in individual, unanesthetized unrestrained rats using [2-^{3}H]glycerol and a two-compartment model (17). The most pronounced response was noted in the beef tallow group which demonstrated a 60% higher rate of TG secretion than the corn or coconut oil groups ($p<0.05$).

Measurement of plasma TG kinetics by tracer techniques assumes a steady state condition, *i.e.* balanced secretion and clearance of the plasma TG pool (26), as we have confirmed. A greater relative rate of TG secretion due to ingestion of specific dietary fats would result in an expanded TG pool without differences in the fractional rate constant (26). Therefore, our observation of a larger TG pool with no significant difference in fractional rate constants (Table 4) suggests that greater TG secretion is the primary factor contributing to the larger TG pool associated with ingestion of beef tallow relative to butterfat, corn or coconut oil. This conclusion is supported by lower hepatic TG content (Table 3) and higher VLDL concentrations of TG, protein, apo B and apo E (Table 5,6) with ingestion of beef tallow relative to corn or coconut oil.

Further examination of the magnitude of increases in the rate of TG secretion and TG pool size provides indirect

TABLE 4

Effects of Dietary Saturated Fats on Triglyceride Secretion into Plasma[a]

	Final body weight (g)	Plasma TG pool size (mg/100 g body wt)	TG secretion (mg/min/100 g body wt)	k (min^{-1})	t_{lag} (min)	$t_{1/2}$ (min)
Corn oil n=6	310 ± 14^{bcd}	3.10 ± 0.06^{c}	0.15 ± 0.01^{c}	0.048 ± 0.004^{b}	11.0 ± 0.5^{b}	15.0 ± 1.3^{b}
Butterfat n=7	302 ± 15^{cd}	3.41 ± 0.33^{bc}	0.19 ± 0.02^{bc}	0.056 ± 0.004^{b}	11.8 ± 0.7^{b}	10.8 ± 0.6^{b}
Beef tallow n=7	344 ± 9^{b}	4.11 ± 0.70^{b}	0.24 ± 0.03^{b}	0.058 ± 0.004^{b}	10.9 ± 0.2^{b}	12.4 ± 0.8^{b}
Olive oil n=4	289 ± 8^{d}	2.94 ± 0.32^{c}	0.18 ± 0.02^{bc}	0.060 ± 0.003^{b}	10.9 ± 0.5^{b}	11.7 ± 0.5^{b}
Coconut oil n=4	335 ± 11^{bc}	3.03 ± 0.35^{c}	0.15 ± 0.02^{c}	0.050 ± 0.003^{b}	11.9 ± 0.8^{b}	13.9 ± 0.9^{b}

[a]Values are means ± SEM. Means with different superscripts in the same column are significantly different, $p<0.05$. TG, triglyceride; $t_{1/2}$ = ln2/k. Plasma TG pool size was calculated as baseline plasma TG concentration multiplied by plasma volume, where plasma volume equals (body weight × 0.041)−1.59. Rate of TG secretion was calculated from the formula: rate = plasma TG pool size × k.

TABLE 5

Plasma VLDL Concentration and Composition[a]

	Corn oil	Butterfat	Beef tallow	Olive oil	Coconut oil
			(mg/dL plasma)		
Triglyceride	24.3 ± 5.0^{d}	31.7 ± 6.6^{bd}	63.2 ± 8.8^{c}	45.7 ± 7.6^{bc}	42.1 ± 5.3^{bd}
Phospholipid	11.7 ± 1.8^{b}	23.7 ± 6.4^{c}	20.5 ± 2.2^{c}	26.1 ± 1.2^{c}	26.9 ± 0.4^{c}
Protein	10.3 ± 1.8^{d}	13.1 ± 2.0^{bd}	22.4 ± 2.6^{c}	18.0 ± 2.8^{cb}	13.8 ± 2.2^{bd}
Cholesterol					
free	1.7 ± 0.2^{b}	2.2 ± 0.2^{b}	3.0 ± 0.3^{b}	2.9 ± 0.3^{b}	2.1 ± 0.4^{b}
esterified	0.9 ± 0.2^{b}	1.3 ± 0.2^{b}	1.3 ± 0.3^{b}	2.7 ± 0.5^{c}	0.8 ± 0.2^{b}
			(% by weight)		
Triglyceride	50.8 ± 1.6^{cb}	43.3 ± 3.2^{d}	56.3 ± 1.8^{c}	45.4 ± 2.4^{bd}	48.2 ± 1.9^{bd}
Phospholipid	23.1 ± 1.6^{bd}	31.5 ± 4.4^{c}	18.9 ± 1.7^{d}	29.0 ± 3.4^{cb}	32.3 ± 2.4^{c}
Protein	19.5 ± 0.4^{c}	18.7 ± 1.6^{c}	20.2 ± 0.4^{c}	18.0 ± 0.8^{cb}	15.5 ± 1.4^{b}
Cholesterol					
free	3.7 ± 0.3^{c}	3.2 ± 0.3^{cb}	2.7 ± 0.1^{bd}	3.1 ± 0.2^{bc}	2.4 ± 0.3^{d}
esterified	3.0 ± 0.3^{bd}	3.3 ± 0.6^{cb}	1.9 ± 0.3^{de}	4.5 ± 0.6^{c}	1.6 ± 0.3^{e}
			(ratio)		
E/F cholesterol	0.5 ± 0.1^{b}	0.6 ± 0.1^{cb}	0.4 ± 0.1^{b}	0.9 ± 0.2^{c}	0.4 ± 0.1^{b}
(TG+ECH)/(FCH+Pro+PL)	1.15 ± 0.07^{bc}	0.87 ± 0.09^{d}	1.39 ± 0.09^{c}	0.99 ± 0.10^{bd}	0.99 ± 0.07^{bd}

[a]Values are means ± SEM, n=7. Means with different superscripts in the same row are significantly different, $p<0.05$. E/F cholesterol, esterified/free cholesterol; TG, triglyceride; ECH, esterified cholesterol; FCH, free cholesterol; Pro, protein; PL, phospholipid.

evidence of different rates of TG clearance from plasma with ingestion of beef tallow relative to corn or coconut oil. For example, a 60% higher rate of TG secretion but only a 33% larger TG pool size were observed with ingestion of beef tallow relative to corn or coconut oil. This suggests that the rate of TG clearance was also higher in association with the expanded TG pool with ingestion of beef tallow relative to corn or coconut oil.

The TG response to olive oil feeding differed from effects associated with feeding saturated fats or corn oil. The rate of TG secretion into plasma was 20% higher than the rate observed with corn or coconut oil with no difference in plasma TG pool size. This could be related to a higher rate of TG clearance as indicated by the fractional rate constant which was 20% higher with ingestion of olive oil compared to corn or coconut oil. Similar to previous reports (5,27), higher hepatic cholesterol and TG contents were noted with ingestion of olive oil compared to all of the saturated fats.

Proposed mechanism(s) underlying high plasma TG levels with ingestion of saturated fats in the rat provide conflicting evidence which is due, in part, to different experimental conditions (6–8,11–14). Liver perfusion studies by Nestel and Steinberg (11) and by Kohout *et al.* (12) suggested that an increase in hepatic VLDL productions was the primary factor contributing to the high plasma TG levels associated with saturated fatty acids. In their studies (11,12), the secretion of TG into the media and the accumulation of TG by liver were higher when palmitate rather than linoleate was perfused. Higher activities of

TABLE 6

Plasma VLDL Apolipoprotein B and E Concentration[a]

	Apo B	Apo E
	(mg apolipoprotein/dL plasma)	
Corn oil	1.37 ± 0.14^b	0.78 ± 0.09^b
Butterfat	1.73 ± 0.12^b	0.95 ± 0.07^{cb}
Beef tallow	4.44 ± 0.52^c	1.45 ± 0.23^{dc}
Olive oil	5.39 ± 0.53^d	1.55 ± 0.21^d
Coconut oil	1.79 ± 0.20^b	0.72 ± 0.13^b

[a]Values are means ± SEM, n = 4-7. Means with different superscripts in the same column are significantly different, $p<0.05$. Apo, apolipoprotein.

hepatic lipogenic enzymes have also been noted with ingestion of saturated rather than unsaturated fats (13,14). In contrast, Groot *et al.* (8) suggested that less efficient VLDL catabolism is responsible for the higher plasma TG levels with ingestion of palm oil relative to sunflower seed oil. Their conclusion (8) reflected lower lipoprotein lipase activity and slower chylomicron catabolism with no differences in hepatic VLDL production using the Triton technique. Interestingly, Green *et al.* (7) also noted slower chylomicron catabolism with ingestion of palm relative to corn oil using multicompartmental analysis. They concluded (7) that slowly catabolized chylomicrons enriched in saturated fatty acids would increase hepatic uptake of TG, stimulate hepatic production of VLDL-TG and thus, contribute to higher plasma TG levels.

As demonstrated in our study, different types of dietary saturated fats do not increase the TG pool or rate of TG secretion and alter VLDL composition to the same extent when compared to monounsaturated or polyunsaturated fats. For example, ingestion of saturated beef tallow or monounsaturated olive oil resulted in 33-60% higher rates of TG secretion and significant increases of VLDL-mass with little change in VLDL composition (mg %) or relative particle size compared to ingestion of corn oil. On the other hand, ingestion of butterfat resulted in only a 20% higher rate of TG secretion with little change in VLDL mass but relatively smaller VLDL particles than ingestion of corn oil. Based on our observations comparing 5 different dietary fats, it is not surprising that previous reports comparing only 2 or 3 dietary fats provide conflicting results regarding the relative contribution of secretion and clearance to elevated plasma TG levels with ingestion of saturated fats. Moreover, different VLDL compositions as a result of ingesting different dietary saturated fats are likely to affect VLDL-TG clearance and hepatic uptake of VLDL remnants, which may impact on hepatic VLDL secretion (7).

The kinetic model used for our data is a variation of that used by Bird *et al.* (17). Written in a form slightly different from theirs, it is $SA_{(2)} = C[e^{-\alpha(t-t_{lag})} - e^{-\beta(t-t_{lag})}]/(\alpha-\beta)$. Using calculus it is possible to derive our model as a special case of theirs by taking the limit $\alpha \rightarrow \beta$. Preliminary data analyses confirmed that this modification was more appropriate for fitting the data of our experiment. Several differences in our experimental conditions may explain the need for this modified model. Baker (28) has commented more broadly on the effects of experimental conditions on modeling of TG secretion.

First, anesthesia was not given during repeated blood sampling, and minimal animal restraint was used. The effects of anesthesia, restraint and surgery have been reported to lower TG production in anesthetized animals when compared to unanesthetized, unrestrained animals (15,16). Second, the [2-^{3}H]glycerol was injected *via* the left carotid artery into the aorta, which provided more direct and rapid delivery of the tracer to the liver than does injection *via* the jugular vein (17). Given these two differences in experimental approach, it seems likely that the value of β in Bird *et al.* (17), which represents the fractional rate constant of hepatic TG secretion, might very well be increased because of more rapid delivery to, and turnover of, the label in hepatic pools. Our observation of a shorter lag time for appearance of isotope, 10.9-11.9 min *versus* 14-17 min reported by others (16,17,29), supports the idea of less hepatic delay in TG secretion.

Our observation of a greater rate of TG secretion in association with higher plasma TG levels with ingestion of saturated *versus* polyunsaturated fat is consistent with some human studies (4,10). However, consumption of a diet rich in saturated fat rather than polyunsaturated fat does not consistently result in higher plasma TG levels in humans (2,30). In addition, the saturated diets used in human studies often consist of only one type of saturated fat, or a mixture of saturated fats whose fatty acid profile is not always defined (4). We have demonstrated in the rat that ingestion of different types of dietary saturated fats results in unique effects on VLDL composition, TG pool size and TG secretion. These observations challenge generalizations about the hypertriglyceridemic effects of saturated fats in human diets. Further research is needed utilizing controlled diets containing a variety of naturally-occurring fats of defined fatty acid composition in order to better understand how TG-rich lipoproteins may promote atherogenesis.

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Geotrichum candidum NRRL Y-553 Lipase: Purification, Characterization and Fatty Acid Specificity

Mary Welch Baillargeon* and **Stephen G. McCarthy**
U.S. Department of Agriculture, Agricultural Research Service, Eastern Regional Research Center, Philadelphia, Pennsylvania 19118

Lipases from *Geotrichum candidum* NRRL Y-553 are of interest because of their unique specificity for *cis*-9-unsaturated fatty acids relative to both stearic and palmitic acids. The lipases were partially purified by chromatography on Octyl Sepharose, AG MP-1 macroporous anion exchanger, and chromatofocusing resin. The preparation was found to contain multiple, glycosylated lipases varying slightly in pI (pI 4.88, 4.78, 4.65, 4.57 and 4.52) as judged by both activity and silver staining. The molecular mass determined by sodium dodecyl sulfate polyacrylamide gel electrophoresis was 64 kilodaltons for the main species, with minor species of 60 and 57 kilodaltons present as well. The specificity of the crude lipases for hydrolysis of 4-methylumbelliferyl esters of oleic *vs.* palmitic acid was 20-to-1. The specificity of the purified, partially separated lipases was similar to that of the crude preparation. Thus the lipases could be used even in crude form for the hydrolysis and restructuring of triacylglycerols on a large scale.
***Lipids 26*, 831-836 (1991).**

Lipases (EC 3.1.1.3) from *Geotrichum candidum* are known to show preference for unsaturated fatty acids with a *cis*-double bond at the 9-position (oleic, linoleic, linolenic, palmitoleic acids) (1). The extent of specificity for *cis*-9-unsaturated fatty acids in comparison to saturated fatty acids varies between strains. Among the saturated fatty acids, palmitic acid (16:0) is hydrolyzed more readily by some strains than others. Since palmitic acid and stearic acid are among the most plentiful saturated fatty acids of common fats and oils, a lipase which would preferentially react with unsaturated fatty acids in the presence of these saturated fatty acids could be of commercial use for the splitting and restructuring of natural triacylglycerols. The utility of such a process would ultimately depend on the selectivity of the enzyme employed.

We have previously reported the relative specificity constants for the competitive hydrolysis of oleic and palmitic methyl or butyl esters for several strains of *G. candidum* (2). The relative specificity constants ranged from 1.0 (no specificity shown) to 50 (highly specific). Two preparations showed high specificity: strain NRRL Y-553 and lipase 26557 RP, a proprietary enzyme preparation from Rhône-Poulenc. The original strain of *G. candidum* (3), characterized by Jensen and co-workers (4), but no longer available, released 23 ± 14 times as much oleic acid (18:1) as palmitic acid (average of four triacylglycerols containing only these two fatty acids). Of the strains available (5-8), *G. candidum* NRRL Y-553 produces one of the lipases that is most specific for oleic acid relative to palmitic acid.

Continuing our efforts to obtain *G. candidum* lipases that are highly selective against both stearic and palmitic acids, we report here the purification and further characterization of *G. candidum* NRRL Y-553 lipases. We have modified our previous purification scheme for separating *G. candidum* lipases (9) to accomplish the separation of a relatively low concentration of lipases from a complex medium.

MATERIALS AND METHODS

Lipase production. G. candidum NRRL Y-553 was obtained from the Agricultural Research Service Culture Collection (Peoria, IL). The culture was stored on silica gel according to Perkins (10) and plated onto acidified potato dextrose agar (Difco Laboratories, Detroit, MI) as needed. Lipase was produced under the conditions previously optimized (2). A liquid medium was used consisting of 5% peptone (Difco), 0.1% $NaNO_3$, and 0.1% $MgSO_4 \cdot 7H_2O$, adjusted to pH 7.0 before sterilization. Sterile soybean oil (0.75% v/v) was added as a carbon source. A single colony was transferred to 65 mL of medium in a 250-mL Erlenmeyer flask and incubated about 24 hr at 30°C (300 rpm). An aliquot (0.5-1.0 mL) was used to inoculate similar flasks for lipase production. Lipase activity peaked at about 40 hr after inoculation under these conditions.

The crude lipase broth was tested for protease activity using an agar diffusion method with casein as substrate (kit from Bio-Rad Laboratories, Richmond, CA); no protease activity was found even after extended incubation times.

Materials. Endoglycosidase F/N-glycosidase (Endo F), N-glycosidase F and the glycan detection kit were obtained from Boehringer Mannheim (Indianapolis, IN). Purified olive oil, 4-methylumbelliferyl (4-MUMB) esters and concanavalin A (Con A) labeled with peroxidase were obtained from Sigma Chemical Company (St. Louis, MO). Chromatography media were obtained from Pharmacia LKB Biotechnology Inc. (Piscataway, NJ) or Bio-Rad. All other materials were reagent grade or better.

Assays. Lipase activity was quantitated titrimetrically by an initial rate assay at pH 8.2 using an olive oil-gum arabic emulsion containing 11 mM $CaCl_2$, as described previously (11). A unit of lipase activity (U) was defined as the release of one micromole of free fatty acid (FFA)/min at room temperature. Lipase activity of column fractions and native electrophoretic gels were qualitatively measured on Rhodamine-olive oil-agar plates (12), as previously described (9).

Protein was assayed by the bicinchoninic acid (BCA) method (13) (Pierce, Rockford, IL) when detergent was present, or by the Bradford method (14) (Bio-Rad), with bovine serum albumin (BSA) (Sigma) as standard.

The specificity of lipase for oleate *vs.* palmitate was

*To whom correspondence should be addressed at: USDA, ARS, ERRC, 600 East Mermaid Lane, Philadelphia, PA 19118.

Abbreviations: BCA, bicinchoninic acid; BSA, bovine serum albumin; Con A, concanavalin A; Endo F, endoglycosidase F/N-glycosidase; FFA, free fatty acids; IEF, isoelectric focusing; 4-MUMB, 4-methylumbelliferyl; SDS PAGE, sodium dodecyl sulfate polyacrylamide gel electrophoresis; U, unit of lipase activity.

calculated from the initial rate of non-competitive hydrolysis of the respective esters of 4-methylumbelliferone (15), as previously described (9). To avoid loss of enzyme activity, enzyme samples were diluted as needed with cold buffer [0.1 M tris(hydroxymethyl)aminomethane hydrochloride (Tris), 5 mM $CaCl_2$, pH 8.0] containing 0.1 mg/mL BSA.

The specificity for one substrate (A) *vs.* another substrate (B) at one minute reaction time was calculated using Eq. [1] (16):

$$\text{Specificity A/B} = \frac{V_A\,[B]}{V_B\,[A]} \qquad [1]$$

where the rate, V, was multiplied by the substrate concentration at one min. Low values of specificity (around 1) can be measured much more accurately than large values (around 100). This method produces the same values of specificity 18:1/16:0 as obtained by non-competitive hydrolysis of emulsified methyl esters of oleic and palmitic acids (measured by initial rate titration at 35°C), but is much more rapid and sensitive.

Electrophoresis. Polyacrylamide gel electrophoresis was performed using the PhastSystem (Pharmacia) and the gels were silver stained (17) or activity stained as previously described (9,11). Standards for pI measurements were obtained from Sigma (pI 3.6–6.6) and molecular weight standards were from Diversified Biotech (Newton Centre, MA). Molecular weight measurements have a standard deviation of 2 kilodaltons (kDa).

Carbohydrate analysis. Carbohydrate was removed by treatment of native lipase with Endo F (pH 6.0) (18) or of denatured lipase with N-glycosidase F (pH 7.5) according to the instructions of the manufacturer. Two to three units of glycosidase activity were added to the lipase sample in aliquots over an extended incubation period. After IEF or sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS PAGE), samples were transferred to Immobilon transfer membranes (Millipore Corp., Bedford, MA) by diffusion blotting, and glycosylation was detected with the glycan detection kit according to the manufacturer's instructions, or by peroxidase-labeled Con A (18).

Purification. The procedure previously used for purifying commercial *G. candidum* lipases (9) was modified to accommodate dilute lipases in a broth of high peptone concentration. All steps were performed at room temperature (21–25°C). When lipase activity was maximal (about 14 U/mL), the broth was centrifuged for 200,000 $g \times$ min, decanted, and filtered under a slight vacuum through Whatman No. 2 filter paper. The supernatant (approximately 1 L) was incubated with Octyl Sepharose in an affinity rather than hydrophobic interaction mode (80 mL preequilibrated in 0.01 M imidazole buffer, pH 6.8) for 30 min with gentle agitation. The resin was then placed in a Buchner funnel with a fritted disc, and washed with the imidazole buffer to remove the colored peptone; negligible lipase activity was washed from the column by this procedure. A limited amount of water (250 mL) was used to wash the resin, which was then placed into a 1.5 × 50 cm column, and briefly packed with water (flow rate of 25 mL/hr). Lipase activity was eluted by a linear gradient of 250 mL each of water and 0.5% polyoxyethylene 10-tridecyl ether (Emulphogene BC-720, Sigma) in water.

The fractions containing the main lipase activity were pooled and applied directly to an AG MP-1 column (1.5 × 12 cm) equilibrated in 0.05 M histidine buffer (pH 6.0). After washing with the histidine buffer (flow rate of 50 mL/hr), the lipase activity was eluted by a linear gradient of 50 mL each of histidine buffer and histidine buffer with 0.4 or 0.5 M NaCl.

Chromatofocusing was used for separation of the molecular forms of lipases with varying charge as previously described (9), with a pH gradient from 5.4 to 3.8. The pH gradient was later narrowed (pH 5.2 to pH 4.1), resulting in increased resolution of the multiple lipase species.

Attempts to desalt the chromatofocusing buffer from column fractions by gel filtration on Bio-Gel P-30 were abandoned because lipase activity was retained on the column and trailed severely. Activity was also lost during dialysis; this was attributed to binding to the dialysis membrane.

RESULTS

Isoelectric focusing. Analytical IEF gels of *G. candidum* NRRL Y-553 lipases are shown in Figure 1. The lipases consisted of three major forms of pI 4.78, 4.65 and 4.57, named b, c, d; and of two minor forms of pI 4.88 and 4.52, named a and e, respectively. Additional minor species are also identifiable in more concentrated samples. All forms were identified as lipases by lipase activity stain, and were also stained with silver stain and esterase stain. Analysis by two-dimensional electrophoresis (not shown) showed charge heterogeneity among lipases of the same or very similar molecular mass. Glycosylation of the lipases was confirmed with the glycan detection kit and Con A-peroxidase.

Purification. The high concentration of peptone in the growth medium interfered with the purification procedure used previously (9). The peptone bound to the Octyl Sepharose at salt levels that promoted hydrophobic interactions (1 M ammonium sulfate), reducing the capacity of the column. The peptone also bound to the AG MP-1 anion exchanger. We therefore developed a rapid and relatively simple procedure for purification of lipases in peptone broth using Octyl Sepharose, AG MP-1, and chromatofocusing. Affinity chromatography on Octyl Sepharose using low ionic strength buffer allowed lipase to bind while the peptone was washed off. This was followed by anion exchange on AG MP-1, accomplishing a rapid concentration and at least partial removal of the detergent. Chromatofocusing was then used to separate the multiple lipase forms.

Elution of lipases from Octyl Sepharose by a detergent gradient is shown in Figure 2. A contaminant and a small amount of lipase activity are eluted at low detergent concentrations. The main lipase activity is eluted in a broad peak from about 0.25 to 0.50% detergent. Fractions 63 to 121 were pooled and applied directly to the AG MP-1 anion exchanger.

Chromatography on AG MP-1 is shown in Figure 3. The lipase activity eluted as a sharp peak (fractions 40–48), with some trailing which included contaminating proteins. Fractions 40 to 48 were pooled, dialyzed against 0.025 M piperazine (pH 5.4), applied to the chromatofocusing

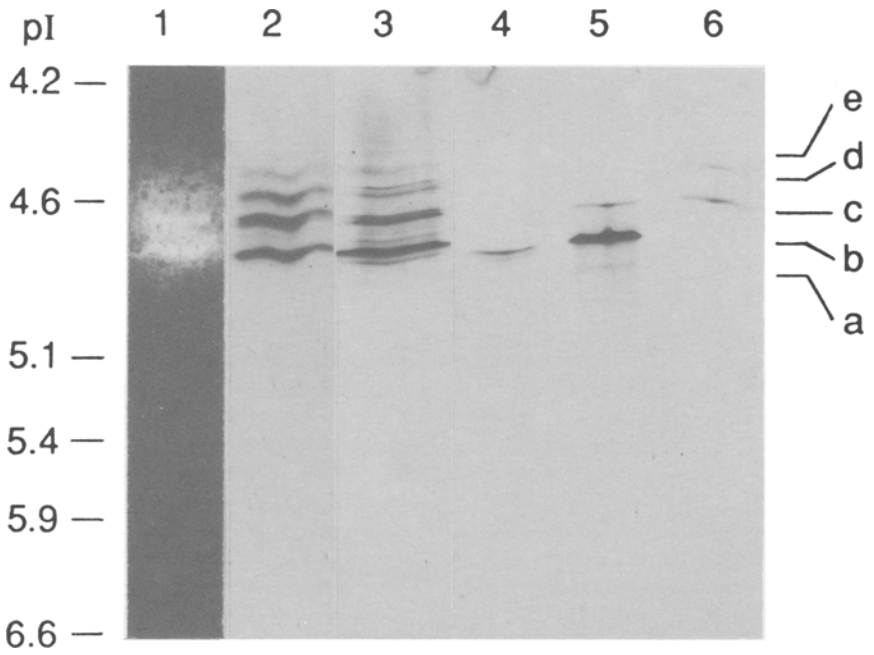


FIG. 1. Isoelectric focusing of *G. candidum* NRRL Y-553 lipases at 15°C. Lipases a through e are identified. Lane 1, AG MP-1 fraction 49 with lipase activity stain photographed under ultraviolet light; the scale is slightly reduced compared to lanes 2–6. Lipases b and c are of much stronger intensity. Lane 2, same sample with esterase stain; lane 3, same sample with silver stain; lane 4, chromatofocusing, fraction 44; lane 5, chromatofocusing, fraction 48; lane 6, chromatofocusing, fraction 57. Lanes 4 through 6 are silver stained.

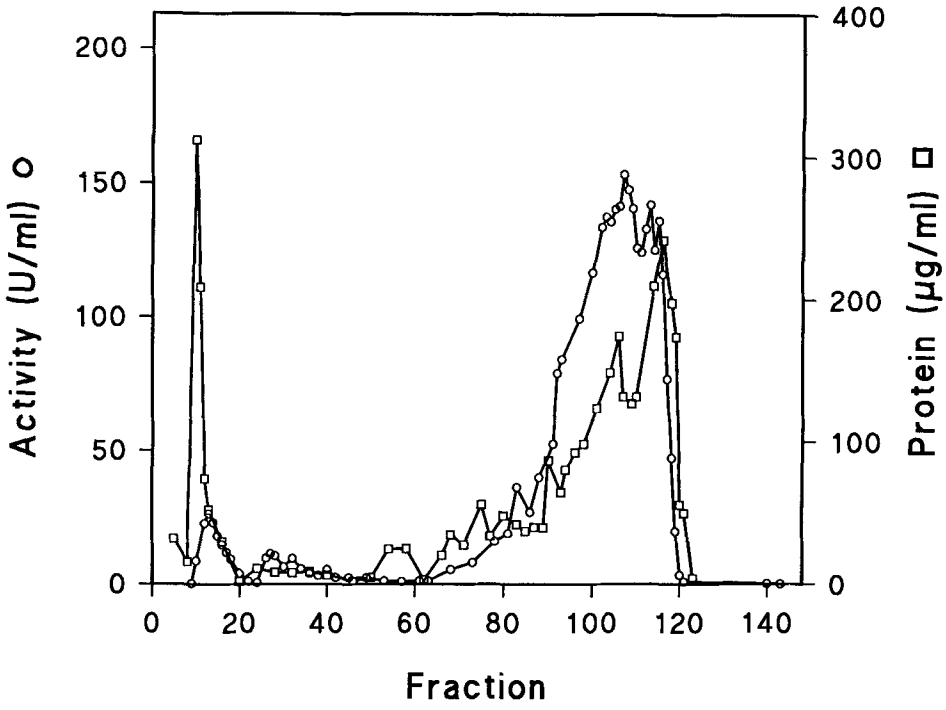


FIG. 2. Chromatography of *G. candidum* NRRL Y-553 lipases on Octyl Sepharose. Lipases were eluted with a detergent gradient from 0 to 0.5% which was started at fraction 1. See text for details.

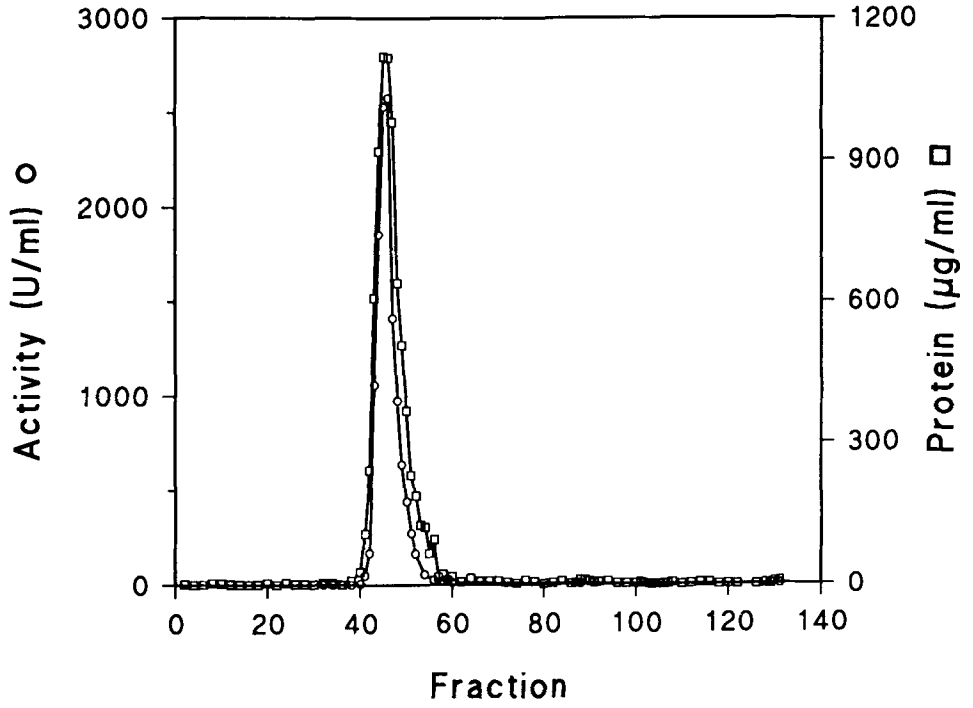


FIG. 3. Anion exchange chromatography of *G. candidum* NRRL Y-553 lipases on AG MP-1. Lipases were eluted with a salt gradient from 0 to 0.5 M NaCl starting at fraction 1.

column, and eluted with Polybuffer (pH 3.8) (Fig. 4). Lipases a and b eluted first (fractions 41–45), followed by lipases b and c at the main peak (fractions 46–52). The small second peak (fractions 53–62) contained lipases c, d, e, and a minor amount of lipase b. The lipases trail together such that no single form is completely isolated from others close in pI. Elution using a narrower pH gradient increases the resolution; however, the lipases are so similar that it is not possible to isolate each one.

The purification achieved is shown in Table 1. A 14-fold purification with 96% recovery of activity was obtained with the Octyl Sepharose column. The anion exchange and chromatofocusing steps resulted in a 23-fold purification with a 46% total recovery of activity.

Figure 5 shows SDS PAGE of the lipases at each purification step. The purified lipases contain a main component of 64 kDa, and minor components of 60 and 57 kDa; glycosylation of all three components was confirmed using both the glycan detection kit and Con A-peroxidase.

Carbohydrate analysis. The various species of *G. candidum* NRRL Y-553 lipase separated by either SDS PAGE or IEF were all found to be glycosylated. In order to study the contribution of glycosylation to lipase heterogeneity, lipases purified by Octyl Sepharose and anion exchange was subjected to enzymatic deglycosylation (data not shown). Endo F was used to deglycosylate the lipases under native conditions. The susceptibility of proteins to deglycosylation by endo F varies; indeed, even after extended incubation, only partial deglycosylation of NRRL Y-553 lipases could be achieved, as measured with Con A-peroxidase detection. The partially deglycosylated lipases contained lipases b and c, with a small amount of lipase d, as detected by IEF gels using lipase and esterase activity stain, and silver stain; however, only lipases b and c were detected with Con A-peroxidase. Two-dimensional electrophoresis of the partially deglycosylated native lipases confirmed the presence of lipases b and c, respectively, at two slightly different molecular masses at constant pI, indicating partial removal of uncharged carbohydrate. The concentration of lipase d was too low to be detected on the 2D gel.

N-Glycosidase was used to deglycosylate denatured *G. candidum* NRRL Y-553 lipases; more complete deglycosylation would be expected under these conditions. Approximately 90% of the lipases were converted to species of lower molecular mass which were no longer detected by Con A-peroxidase or the glycan detection kit, indicating removal of the carbohydrate. A decrease of molecular mass of 2.5 kDa, representing 4% by weight of the main lipase component on SDS PAGE, was obtained. It appeared that the reaction was incomplete, with about 10% of the lipase remaining with unchanged molecular mass and carbohydrate intact.

Specificity. The specificity of *G. candidum* NRRL Y-553 lipases at various stages of purification is summarized in Table 2. The specificity of lipases partially separated by chromatofocusing is given and graphed in Figure 4. The crude lipases have a specificity of 20 ± 4. Fractions eluting from the chromatofocusing column at pH 4.4 (the beginning of lipase elution) and composed of lipases a and b consistently have a slightly higher specificity 18:1/16:0. However, the specificity values are the same within two standard deviations for all stages of purification.

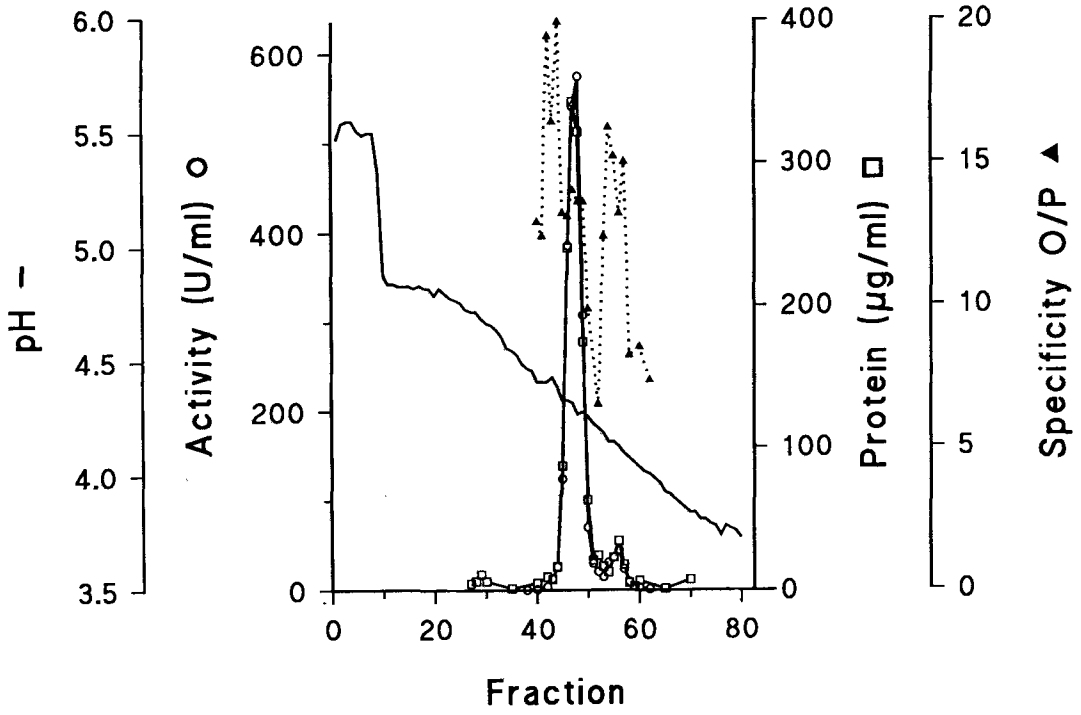


FIG. 4. Chromatofocusing of *G. candidum* NRRL Y-553 lipases. Elution conditions are given in Materials and Methods. The lipase activity closely coincides with the protein concentration.

TABLE 1

Purification of *G. candidum* NRRL Y-553 Lipase

Purification step	Protein (mg)	Activity (U)	Specific activity (U/mg)	Recovered activity (%)	Purification factor[a]
Crude broth	194[b]	13,400	69	100	1
Octyl Sepharose	13.1[c]	12,834	980	96	14
AG MP-1	5.2[b]	6,852	1318	51	19
Chromatofocusing[d]	3.9[b]	6,133	1572	46	23

[a]Based on Bradford assay results (ref. 14).
[b]Determined by Bradford assay (ref. 14).
[c]Determined by BCA assay (ref. 13).
[d]Sum of all lipase peaks.

DISCUSSION

The lipases produced by the fungus *G. candidum* are a complex mixture varying in molecular mass, pI and specificity. The *G. candidum* NRRL Y-553 lipases partially isolated here are quite similar in molecular weight and pI to those recently reported for three other strains—ATCC 34614 (5,19), commercial GC-20 lipases from Amano (9) and an isolate *G. candidum* Link ex Pers. emend. Carmichael from waste water (8,20). However, the specificity varies among the strains—lipases from ATCC 34614 show very little specificity 18:1/16:0 (2,5); while lipase from *G. candidum* Link ex Pers. emend. Carmichael shows intermediate specificity (8). We previously found that although crude GC-20 contains little specificity 18:1/16:0, it contains a low level of an isozyme which is specific (specificity 18:1/16:0 is 29 ± 8) (9). *G. candidum* NRRL Y-553 produces a group of specific lipases (specificity 18:1/16:0 is 20 ± 4) whose specificity approaches that of the original strain of *G. candidum* (specificity 18:1/16:0 is 23 ± 14) (4).

G. candidum NRRL Y-553 lipases consist of five glycosylated species of molecular mass 64 kDa (major component), and 60 and 57 kDa (minor components). Heterogeneity in size due to non-specific proteolytic degradation seems unlikely, since no proteases were detected in the crude lipase broth. Differential glycosylation contributes to the heterogeneity, since partial deglycosylation reduces the molecular mass of the main lipase component to about that of the smaller species, and reduces the number of species from five to three. Additional heterogeneity could be due to actual differences in amino acid sequence; the presence of two genes coding for two closely-related lipases, each containing two potential

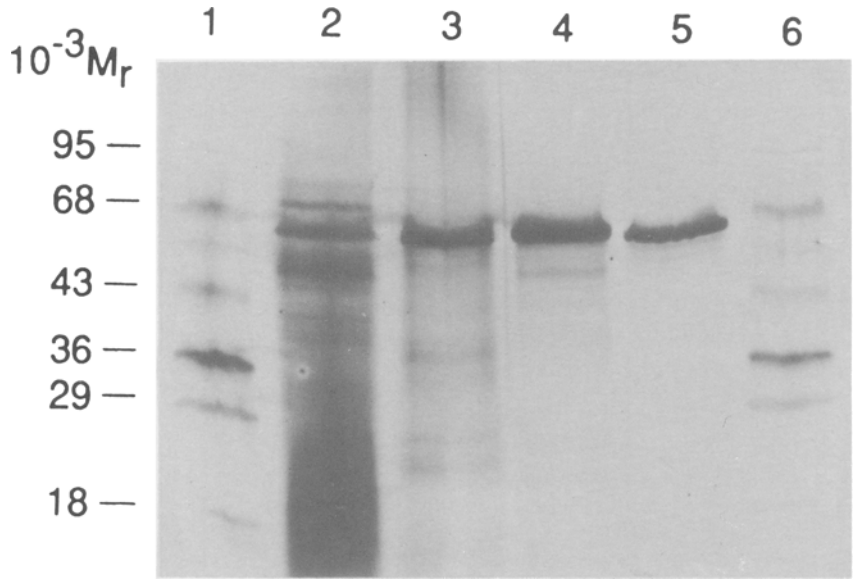


FIG. 5. SDS PAGE of *G. candidum* NRRL Y-553 lipases at various stages of purification with silver staining. Lanes 1 and 6, molecular weight standards; lane 2, crude lipase broth; lane 3, fraction 68 of Octyl Sepharose column (114 ng); lane 4, fraction 46 of AG MP-1 column (140 ng); lane 5, fraction 48 of chromatofocusing column (160 ng).

TABLE 2

Specificity of *G. candidum* NRRL Y-553 Lipases at Various Stages of Purification

Sample	Specificity 18:1/16:0
Crude lipase	20.0 ± 4.4
Lipases (a), b[a]	18.6 ± 1.9
Lipases (a), b, c	13.5 ± 0.4
Lipases b, c	8.2 ± 2.4
Lipases (b), c, d	14.4 ± 1.6

[a]Lipases in parentheses are minor components. Purified lipases are from the chromatofocusing column.

sites for *N*-glycosylation, has recently been reported for *G. candidum* ATTC 34614 (5,21).

G. candidum NRRL Y-553 lipases could be useful for large-scale splitting and restructuring of unsaturated fatty acids from triacylglycerols, and the lipases could be used in crude form without prior purification. We are currently testing the lipases for such practical applications.

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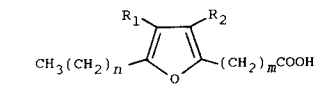
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A Novel Method for the Introduction of a Methyl Group into the Furan Ring of a 2,5-Disubstituted C_{18} Furanoid Fatty Ester *via* a Malonic Acid Function

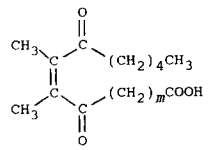
Marcel S.F. Lie Ken Jie* and **Ka-Po Wong**
Department of Chemistry, University of Hong Kong, Hong Kong

Furan ring opening with benzohydroxamic acid of methyl 9,12-epoxy-9,11-octadecadienoate gave a mixture of positional isomers of conjugated methyl 3-phenyl-1,4,2-dioxazolyl C_{18}-enone esters 6a,6b. Michael addition of diethyl malonate anion to the conjugated enone system of 6a,6b furnished the corresponding malonyl intermediates 7a,7b, which upon removal of the dioxazole ring by hydrolysis gave methyl 10- and 11-dicarbethoxymethyl-9,12-dioxooctadecanoate 8a,8b. Cyclization of the latter gave the trisubstituted C_{18} furanoid fatty esters 9a,9b, containing the malonate ester function at the 3-/4-position of the furan ring. Base hydrolysis of compounds 9a,9b gave the corresponding tricarboxylic acid derivatives 10a,10b, which were esterified to the trimethyl esters 11a,11b in BF_3/MeOH. When a mixture of 9a,9b was refluxed with Na_2CO_3/MeOH, hydrolysis of the malonate ester function was followed by decarboxylation to yield a $-CH_2COOH$ substituent at the 3-/4-position of the furan ring (12a,12b). Esterification of the latter with BF_3/MeOH gave the corresponding methyl diester derivatives 13a,13b. When a mixture of tricarboxylic acids 10a,10b was heated at 160–180°C for 6 hr, exhaustive decarboxylation of malonic acid function furnished a methyl group at the 3-/4-position of the furan nucleus. Esterification of the decarboxylated product gave a mixture of trisubstituted furanoid compounds 14a,14b (overall yield 28%). The procedure constitutes a novel method for the introduction of a methyl group *via* a malonic acid group to the 3-/4-position of the furan ring of a 2,5-disubstituted C_{18} furanoid fatty ester.
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Morris *et al.* (1) reported the first isolation of a 2,5-disubstituted C_{18} furanoid fatty acid (1) (Scheme 1) from the seed oil of *Exocarpus cupressiformis*. A trisubstituted C_{18} furanoid acid (2) containing a methyl group at the 3-position of the furan nucleus was identified as the major fatty acid component (95%) in the triglyceride fraction of the latex of the rubber plant (*Hevea brasiliensis*) (2,3). More recently, long-chain tri- and tetrasubstituted furanoid fatty acids have been found in small quantities in a large variety of common plant species (4). Furanoid fatty acids in animals were first recorded by Glass *et al.* (5) to occur in fish, and later were found in soft corals (6), crayfish (7), amphibians and reptiles (8), human blood plasma (9,10) and in bovine liver extracts (11). The role of these natural furanoid fatty acids remains unclear. Biosynthetic experiments by Sand *et al.* (12)



1, R_1 = H, R_2 = H, n = 5, m = 7;
2, R_1 = H, R_2 = CH_3, n = 5, m = 7;
3, R_1 = H, R_2 = COOH, n = 4, m = 2;
4, R_1 = CH_3, R_2 = CH_3, n = 4, m = 6,8,10.



5, m = 6,8,10.

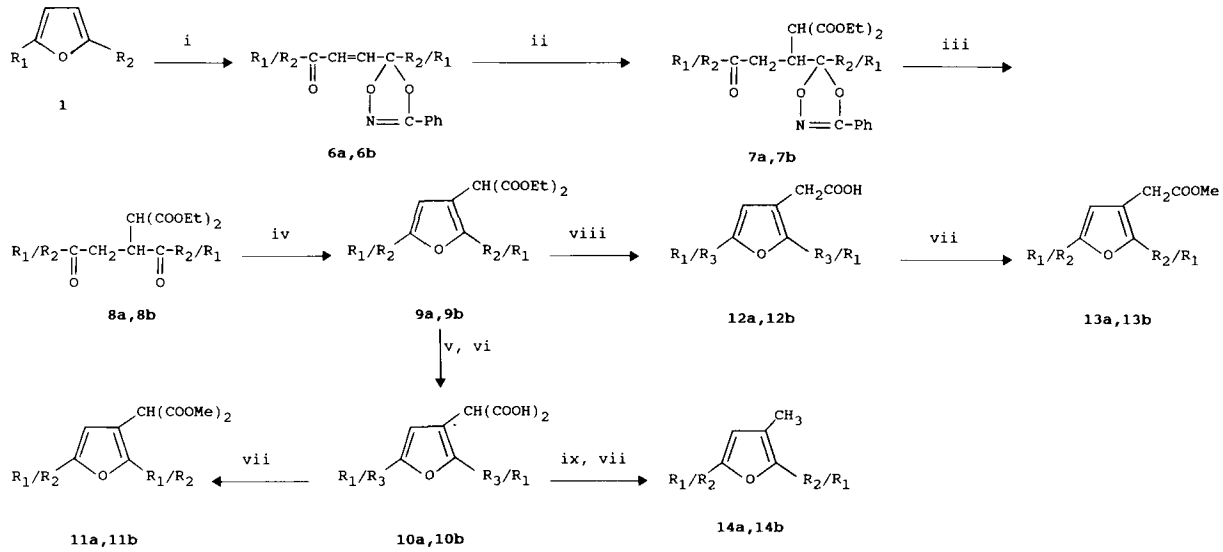
SCHEME 1

failed to show that furanoid fatty acids in fish were produced by acetate *de novo*, while Gorst-Allman *et al.* (13) ruled out 10,13-nonadecadienoic acid as a possible precursor of furanoid fatty acids in animals. Furanoid fatty acids were metabolized to urofuranic acids (3) in human feeding experiments (14). *In vitro* incubation of furanoid fatty acids (4) with lipoxidase gave unstable dioxo ethylenic products (5) (15).

We have synthesized all positional isomers of the 2,5-disubstituted C_{18} furanoid fatty acids (16) and have also produced furanoid fatty acids with one or two methyl groups in the 3- and/or 4-position of the furan ring from ricinoleic acid (17,18). Preliminary screening tests for compound (1) have shown that this compound possesses potent anti-microbial properties against certain microorganisms (Lie Ken Jie, M.S.F., Shortridge, K.F., and Tam, J., unpublished results). In view of the potential therapeutic properties of this class of fatty acids, the aim of this project was to develop chemical means of incorporating different substituents into the 3- or 4-position of the furan ring of the furanoid fatty acid for further antimicrobial investigations. We have selected the malonyl function, as this versatile dicarboxylate system can be readily transformed into an array of other interesting chemical functions. The synthetic route leading to the malonyl substituted C_{18} furanoid fatty ester derivative and the various chemical transformations of this substrate to yield trisubstituted furanoid fatty acid derivatives are presented in Scheme 2 — R_1 = $CH_3(CH_2)_5$; R_2 = $(CH_2)_7COOCH_3$; R_3 = $(CH_2)_7COOH$. Its reagents are: i) benzohydroxamic acid, Pb(IV) oxide, EtOAc; ii) diethyl malonate, NaOMe, Et_2O; iii) H_2O, EtOH; iv) BF_3/etherate, $CHCl_3$; v) KOH, H_2O, EtOH; vi) dilute HCl; vii) BF_3/MeOH; viii) Na_2CO_3, H_2O, MeOH; and ix) 160–180°C, 6 hr.

*To whom correspondence should be addressed at the Chemistry Department, University of Hong Kong, Pokfulam Road, Hong Kong.

Abbreviations: ECL, equivalent chain length; IR, infrared spectroscopy; NMR, nuclear magnetic resonance; R_F, retardation factor; TLC, thin-layer chromatography.



SCHEME 2

MATERIALS AND METHODS

Thin-layer chromatographic (TLC) analyses were carried out on silica gel coated microscope slides (*ca.* 0.1 mm thickness). Column chromatographic purifications were carried out on silica gel and mixtures of light petroleum (b.p. 60–80°C)/diethyl ether were used as eluant. [PE30 denotes a mixture of light petroleum/diethyl ether (70:30, v/v) where the numeral stands for the percentage of diethyl ether in the solvent mixture]. Gas chromatographic analyses were conducted on a Hewlett-Packard 5970 gas chromatograph (Hewlett-Packard, Palo Alto, CA) fitted with a 10-m microbore glass column (0.53 mm diam., 2.65 μm film coating thickness SE-30 stationary phase) and a flame ionization detector. Nitrogen (20 mL/min) was used as the carrier gas and the column temperature was maintained at 190°C. External standards of methyl myristate, palmitate and stearate were used as reference compounds and the equivalent chain length (ECL) value was calculated accordingly for each component (19). Elemental analyses were by Butterworth Laboratories Ltd. (Teddington, Middlesex, England, U.K.); all compounds gave acceptable value determined for C, H, and N (±0.3) percent. Infrared (IR) spectra were obtained with a Perkin-Elmer 577 (Perkin-Elmer, Norwalk, CT) spectrophotometer. Nuclear magnetic resonance (NMR) spectra were recorded with a JEOL FX-90Q (90 MHz) (JEOL Ltd., Tokyo, Japan) Fourier transform spectrometer at 89.56 MHz for protons and at 22.5 MHz for carbon. Alternatively, a JEOL GSX-270 (270 MHz) spectrometer with proton observation at 270.05 MHz and carbon observation at 67.8 MHz was used. Chemical shifts (ppm) are reported relative to Me_4Si as internal standard. Connectivities in the ^1H-^{13}C NMR spectra were established at 270.05 MHz using a combination of spin-spin decoupling, COSY and double quantum filter experiments. Carbon atom types were established in ^{13}C NMR using a combination of broad-band proton decoupling and DEPT experiments. Melting points were measured on a Reichert Thermovar hot-stage microscope (Reichert Co., Vienna, Austria) and are uncorrected. Methyl 9,12-epoxy-9,11-octadecadienoate was prepared from methyl ricinoleate as described elsewhere (20). Benzohydroxamic acid was prepared by the method described by Hauser and Renfrow (21). Diethyl malonate and sodium methoxide were purchased from Aldrich Chemical Co. (Milwaukee, WI).

*Synthesis of methyl 3-phenyl-1,4,2-dioxazolyl C_{18}-enone esters (**6a,6b**).* A mixture of methyl 9,12-epoxy-9,11-octadecadienoate (14 g, 45.5 mmol), benzohydroxamic acid (9 g, 65 mmol), Pb (IV) oxide (60 g, 0.25 mol) and ethyl acetate (150 mL) was stirred at room temperature for 24 hr. The reaction mixture was filtered and the solvent was evaporated under reduced pressure. The residue was chromatographed on a silica gel column (PE30) to give a mixture of compounds **6a,6b** (14.7 g, 73%) as an oil. R_F (PE40) 0.35; IR (cm^{-1}) (film) 1630(m, C=N), 1705(s, C=O), 1740(s, C=O ester). ^1H NMR (δ) 0.9(*t*, 3H, CH_3), 1.2–1.4(*m*, 12H, CH_2), 1.6–1.8(*m*, 6H, CH_2), 2.2–2.5(*m*, 6H, CH_2COOCH_3, $CH_2C{=}O$, $CH_2COOCNPh$), 3.6(*s*, 3H, $COOCH_3$), 6.0(*d*, J = 13Hz, 2H, $CH{=}CH$), 7.3–7.5(*m*, 3H, arom), and 7.6–7.8(*m*, 2H, arom). ^{13}C NMR ($CDCl_3$) 14.0(C-18), 22.5(C-17), 23.4($COCH_2CH_2$), 24.9(C-3), 28.9, 29.0, 29.1, 29.3, 31.7(C-16), 34.0(C-2), 37.8(CH_2 COOCNPh), 43.4($COCH_2$), 51.2($COOCH_3$), 115.4 (*C*OOCNPh), 123.4(arom), 126.8(arom), 128.7(arom), 131.4(arom), 132.4, 134.7(*C*H=*C*H), 158.1(*C*=N), 173.8(C-1) and 203.0 (*C*=O, oxo) ppm.

*Reaction of sodium diethyl malonate anion with compounds **6a,6b**.* A mixture of diethyl malonate (3.2 g, 20 mmol), sodium methoxide (1.2 g, 22 mmol) and diethyl ether (100 mL) was stirred at room temperature for 3 hr. Compounds **6a,6b** (7.3 g, 16.4 mmol) in diethyl ether (20 mL) were added and the reaction mixture stirred for an additional 24 hr. Diluted acetic acid (1M, 10 mL) was added and the reaction mixture was extracted with diethyl ether (3 × 50 mL). The organic extract was washed with water (20 mL), and dried (Na_2SO_4). The solvent was

evaporated; silica gel column chromatography of the residue yielded a mixture of the triesters **7a,7b** (8.5 g, 86%) as an oil. R_F (PE40) 0.25; IR (cm^{-1}) (film) 1626(m, C=N), 1735(s, C=O). ^1H NMR (δ) 0.9(*t*, 3H, CH_3), 1.2-1.4(*m*, 18H, CH_2), 1.4-2.0(*m*, 6H, CH_2), 2.3(*t*, 2H, CH_2COOCH_3), 2.5-2.9(*m*, 8H), 3.6(*s*, 3H, $COOCH_3$), 4.1(*q*, J = 7.2 Hz, 4H, $COOCH_2CH_3$), 7.3-7.5(*m*, 3H, arom) and 7.7-7.8(*m*, 2H, arom). ^{13}C NMR ($CDCl_3$) 14.0(3C, C-18, 2 × $COOCH_2CH_3$), 22.1, 22.5(C-17), 23.8($COCH_2CH_2$), 24.9(C-3), 29.0, 29.2, 29.3, 31.6(C-16), 34.1(C-2), 36.0($CH_2COOCNPh$), 40.6($CHCOOCNPh$), 42.9(CH_2CO), 50.9[$CH(COOEt)_2$], 51.3($COOCH_3$); 61.4, 61.8[2C, 2 × $CH(COOCH_2CH_3)_2$], 119.0($COOCNPh$), 123.1(arom), 126.8(arom), 128.7(arom), 131.5(arom), 158.6(C=N), 168.3(2C, 2 × COOEt), 174.0(C-1) and 207.6(C=O, oxo) ppm.

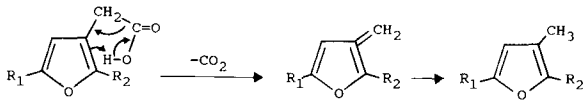
*Hydrolysis of the dioxazole ring in compounds **7a,7b**.* A mixture of compounds **7a,7b** (4.0 g, 6.6 mmol) and aqueous ethanol (50%, 100 mL) was refluxed for 10 hr. Water (100 mL) was added and the reaction mixture was extracted with diethyl ether (3 × 50 mL). The organic extract was washed with water (30 mL) and dried (Na_2SO_4). The solvent was evaporated and the residue chromatographed on a silica gel column (PE30) to give a mixture of methyl 10- and 11-dicarbethoxymethyl-9,12-dioxooctadecanoate (**8a,8b**) (2.6 g, 80%) as an oil. R_F (PE40) 0.4; IR (cm^{-1}) (film) 1368(m), 1736(s, C=O). ^1H NMR (δ) 0.9(*t*, 3H, CH_3), 1.2-1.4(*m*, 18H, CH_2), 1.5-1.8(*m*, 6H, CH_2), 2.3(*t*, 2H, CH_2COOCH_3), 2.5-2.8(*m*, 8H), 3.65(*s*, 3H, $COOCH_3$), 4.16(*q*, J = 7.1 Hz, 4H, $COOCH_2CH_3$). ^{13}C NMR ($CDCl_3$) 14.0(3C, C-18, 2 × $COOCH_2CH_3$), 22.5(C-17), 23.5, 23.8($COCH_2CH_2$), 25.0(C-3), 29.0-29.1(4C), 31.7(C-16), 34.1(C-2), 42.2, 42.9(CH_2CO), 45.2(CHCO), 51.3($COOCH_3$), 53.0[$CH(COOEt)_2$], 61.7 [$CH(COOCH_2CH_3)_2$], 168.1(2C, 2 × COOEt), 174.1(C-1), 207.8(C=O, oxo) and 210.0(C=O, oxo) ppm.

*Synthesis of methyl 9,12-epoxy-10(11)-dicarbethoxymethyl-9-11-octadecadienoate (**9a,9b**).* A mixture of compounds **8a,8b** (6g, 12.4 mmol), boron trifluoride etherate (2.4 mL, 18.6 mmol) and chloroform (50 mL) was stirred at room temperature for 3 hr. Aqueous sodium carbonate (10%, 20 mL) was added and the organic mixture was extracted with diethyl ether (2 × 50 mL). Purification of the organic product on a short silica gel column gave a mixture of compounds **9a,9b** (5.4 g, 94%) as an oil. R_F (PE40) 0.5; IR (cm^{-1}) (film) 1736(s, C=O), 1036(s, furan ring breathing). ^1H NMR (δ) 0.9(*t*, 3H, CH_3), 1.2-1.4(*m*, 18H, CH_2), 1.6-1.8(*m*, 6H, CH_2), 2.30(*t*, 2H, CH_2COOCH_3), 2.54(*t*, J = 7.5Hz, 4H, CH_2-furan), 3.66(*s*, 3H, $COOCH_3$), 4.20(*q*, J = 7Hz, 4H, $COOCH_2CH_3$), 4.41[*s*, 1H, $CH(COOEt)_2$] and 6.06(*s*, 1H, CH furan). ^{13}C NMR ($CDCl_3$) 13.9(C-18), 14.1(2C, $COOCH_2CH_3$), 22.6(C-17), 25.0(C-3), 26.1, 27.9, 28.1, 28.5, 28.9, 29.0, 29.1, 31.6(C-16), 34.1(C-2), 49.4[$CH(COOEt)_2$], 51.3($COOCH_3$), 61.5(2C, 2 × $COOCH_2CH_3$), 106.2(C-4′ of furan, assuming malonate ester substituent at C-3′ of furan ring), 112.0(C-3′ of furan), 151.7, 151.9(C-5′ of furan), 154.2, 154.4(C-2′ of furan), 168.2(2C, 2 × COOEt) and 174.1(C-1) ppm.

*Hydrolysis of compounds **9a,9b** to the tricarboxylic acids **10,10b**.* A mixture of compounds **9a,9b** (4.0 g, 10.1 mmol), KOH (2 g, 35 mmol) and aqueous ethanol (95%, 50 mL) was refluxed for 1 hr. Concentrated HCl (10 mL) was added and the cooled reaction mixture was extracted with diethyl ether (3 × 50 mL). The organic extract was washed with brine (30 mL) and dried (Na_2SO_4). The solvent was evaporated and the residue crystallized from PE40 to give a mixture of compounds **10a,10b** (3.5 g, 88%) as light yellow amorphous solid. Melting point 102-104°C; IR (cm^{-1}) (nujol) 3400(s, br), 1713(s), 925(m). ^1H NMR (δ) 0.89(*t*, 3H, CH_3), 1.2-1.4(*m*, 12H, CH_2), 1.5-1.8(*m*, 6H, CH_2), 2.27(*t*, 2H, CH_2COOH), 2.56(*t*, J = 7.5Hz, 4H, CH_2-furan), 4.4[*s*, 1H, $CH(COOH)_2$], 6.05(*s*, 1H, CH furan) and 10.4(*s*, 3H, 3 × COOH). ^{13}C NMR (CD_3OD) 14.3(C-18), 23.5(C-17), 25.9(C-3), 26.7, 28.7, 29.0, 29.3, 29.6, 29.7, 29.9, 30.0, 32.6(C-16), 34.9(C-2), 50.1[$CH(COOH)_2$], 107.4(C-4′ of furan), 113.9(C-3′ of furan), 152.8(C-2′ of furan), 155.0(C-5′ of furan), 171.8(2C, 2 × COOH) and 177.5(C-1) ppm. Methylation of compounds **10a,10b** using boron trifluoride methanol complex gave the corresponding methyl triester derivatives **11a,11b** (98%) as an oil. R_F (PE40) 0.5; IR (cm^{-1}) 1741(s), 1021(m). ^1H NMR (δ) 0.87(*t*, 3H, CH_3), 1.2-1.4(*m*, 12H, CH_2), 1.5-1.8(*m*, 6H, CH_2), 2.3(*t*, 2H, CH_2COOCH_3), 2.53(*t*, J = 7.3 Hz, 4H, CH_2-furan), 3.64(*s*, 3H, $COOCH_3$), 3.73(*s*, 6H, 2 × $COOCH_3$ or malonate), 4.46[*s*, 1H, $CH(COOMe)_2$], 6.05(*s*, 1H, CH furan). ^{13}C NMR ($CDCl_3$) 14.0(C-18), 22.6(C-17), 25.0, 26.1, 27.9, 28.1, 28.5, 28.8, 29.0, 29.1, 31.6(C-16), 34.1(C-2), 48.9[$CH(COOMe)_2$], 51.2($COOCH_3$), 52.5[2C, 2 × $CH(COOCH_3)_2$], 106.1(C-4′ of furan), 111.8(C-3′ of furan), 151.8, 151.9(C-2′ of furan), 154.3, 154.5(C-5′ of furan), 168.6(2C, 2 × $COOCH_3$ malonate), and 174.0(C-1) ppm.

*Decarboxylation of compounds **9a,9b**.* A mixture of compounds **9a,9b** (4.0 g, 6.4 mmol), methanol (35 mL), water (15 mL) and Na_2CO_3 (10 g) was refluxed for 12 hr. The reaction mixture was acidified with dilute HCl (2M, 10 mL) and extracted with diethyl ether (2 × 30 mL). The organic extract was washed with water (10 mL) and dried (Na_2SO_4). The solvent was evaporated and the residue crystallized from PE50 to give compounds **12a,12b** (1.8 g, 79%) as light yellow amorphous solid. Melting point 63-65°C; IR (cm^{-1}) (nujol) 3400(v.br), 1700, 1220, 1010. ^1H NMR (δ) 0.88(*t*, 3H, CH_3), 1.2-1.4(*m*, 12H, CH_2), 1.5-1.8(*m*, 6H, CH_2), 2.26(*t*, 2H, CH_2COOH), 2.5(*t*, J = 7Hz, 4H, CH_2-furan), 3.3(*s*, 2H, furan-CH_2-COOH), 5.87(s, 1H, CH furan), and 10.5 (*s*, 2H, COOH). ^{13}C NMR (CD_3OD) 14.3 (C-18), 23.4(C-17), 25.9(C-3), 26.6, 28.7, 29.0, 29.4, 29.5, 29.7, 29.9, 31.6, 32.5(C-16), 34.8(C-2), 108.0(C-4′ of furan) 113.1(C-3′of furan), 151.7, 151.8(C-2′ of furan), 154.7, 154.8(C-5′ of furan), 175.2(COOH) and 177.3(C-1) ppm. Methylation of compounds **12a,12b** using boron trifluoride methanol complex gave the corresponding methyl diester derivatives **13a,13b** as an oil. R_F (PE30) 0.5; IR (cm^{-1}) (film) 1740(s), 1580(m), 1010(s). ^1H NMR (δ) 0.88(*t*, 3H, CH_3), 1.2-1.4(*m*, 12H, CH_2), 1.5-1.8(*m*, 6H, CH_2), 2.3(*t*, 2H, CH_2COOCH_3), 2.5(*t*, J = 7.3 Hz, 4H, CH_2-furan), 3.29(*s*, 2H, furan-CH_2COOCH_3), 3.64(*s*, 3H, $COOCH_3$), 3.66(*s*, 3H, furan-$COOCH_3$) and 5.87(*s*, 1H, CH furan). ^{13}C NMR ($CDCl_3$) 14.0(C-18), 22.6(C-17), 25.0(C-3), 26.0, 28.0, 28.5, 28.6, 28.9, 29.0, 29.1, 31.0, 31.7(C-16), 34.1(C-2), 51.2($COOCH_3$), 51.7(furan-$COOCH_3$), 106.9(C-4′ of furan), 111.9(C-3′ of furan), 150.8, 151.0(C-2′ of furan), 154.0, 154.2(C-5′ of furan), 171.9(furan-$COOCH_3$) and 173.9(C-1) ppm.

*Thermal decarboxylation of compounds **10a,10b**.* A mixture of compounds **10a,10b** (2.0 g, 5.1 mmol) was heated at 160-180°C under nitrogen for 6 hr. The resulting reddish oil was refluxed with boron trifluoride methanol com-



$R_1 = CH_3(CH_2)_5$; $R_2 = (CH_2)_7COOH$

SCHEME 3

plex (15%, w/w, 10 mL) and methanol (30 mL) for 10 min. Water (50 mL) was added and the reaction mixture was extracted with light petroleum (3 × 30 mL). The organic extract was washed with water (20 mL) and dried (Na_2SO_4). The solvent was evaporated under reduced pressure and silica gel column chromatography of the residue yielded a mixture of the trisubstituted furanoid fatty ester derivatives **14a,14b** (1.3 g, 83%) as an oil (17,18). R_F (PE10) 0.9; ECL = 18.7 (SE-30); IR (cm^{-1}) (film) 3010(w), 1740(s), 1010(m, furan ring breathing). ^1H NMR (δ) 0.88(*t*, 3H, CH_3), 1.2-1.4(*m*, 14H, CH_2), 1.5-1.8(*m*, 4H,CH_2), 1.88(*s*, 3H, furan-CH_3), 2.28(*t*, 2H, CH_2COOCH_3), 2.49(*t*, J = 7.3 Hz, 4H, CH_2-furan), 3.63(*s*, 3H, $COOCH_3$) and 5.7(*s*, 1H, *CH* furan). ^{13}C NMR ($CDCl_3$) 9.9(furan-CH_3), 14.0(C-18), 22.7(C-17), 25.1(C-3), 26.0, 28.2, 28.3, 28.7, 28.9, 29.0, 29.1, 31.8(C-16), 34.1(C-2), 51.2($COOCH_3$), 107.9(C-3′ of furan), 113.9(C-4′ of furan), 149.3, 149.5(C-2′ of furan) 153.3, 153.5(C-5′ of furan) and 173.9(C-1) ppm.

When a mixture of compounds **10a,10b** (2.0 g, 5.1 mmol) was heated under nitrogen at 160-180°C for 3 hr, silica gel column chromatographic separation of the methyl ester derivatives of the isolated product gave compounds **13a,13b** (1.2 g, 62%) and compounds **14a,14b** (0.44 g, 27%).

RESULTS AND DISCUSSION

As the objective of this project was to incorporate reactive chemical functions to either the 3- or 4-position of the furan ring, no effort was made to develop synthetic methods to obtain regiospecific positional isomers. Thus, treatment of methyl 9,12-epoxy-9,11-octadecadienoate with benzohydroxamic acid in the presence of Pb(IV) oxide gave a mixture of the half-blocked dioxazole-keto esters **6a,6b** in good yield (73%), as described by Mackay *et al.* (22). This reaction was non-regiospecific with the dioxazole ring attached to either C-9 or C-12 of the C_{18} fatty ester chain and the keto group at C-12 or C-9, respectively.

A subsequent 1,4-Michael addition reaction involving compounds **6a,6b** with diethyl malonate anion furnished the malonate ester derivatives **7a,7b**. Hydrolysis of the dioxazole ring in compounds **7a,7b** gave a mixture of the dioxo-malonate ester intermediates **8a,8b**, which was readily cyclized to the corresponding trisubstituted C_{18} furanoid fatty esters **9a,9b**. Hydrolysis of compounds **9a,9b** gave the tricarboxylic furanoid acids **10a,10b**, which were converted to the methyl esters **11a,11b** using boron trifluoride methanol complex. When a mixture of compounds **9a,9b** was refluxed with aqueous sodium carbonate in methanol, hydrolysis of the ester functions was followed by decarboxylation of one of the two carboxylic acid groups of the malonic acid function to yield compounds **12a,12b** containing a -CH_2COOH substituent at the 3-/4-position of the furan ring. Esterification of the latter gave the corresponding methyl diester derivatives **13a,13b**.

When a mixture of compounds **10a,10b** was heated at 160-180°C for 6 hr, an unexpected *bis*-decarboxylation reaction took place, which transformed the malonic acid group to that of a methyl function. Esterification of the isolated decarboxylated product gave the trisubstituted furanoid fatty esters **14a,14b** containing a methyl group at the 3-/4-position of the furan ring in high yield (83%). However, when the same reaction was repeated at the same temperature for 3 hr instead of 6 hr, a mixture of compounds **13a,13b** (62%) and **14a,14b** (27%) was obtained after esterification. The ratio of the products was verified by gas chromatographic analysis.

It appears from these results that the *bis*-decarboxylation process involving compounds **10a,10b** was made possible by the availability of *pi*-electrons in the furan system. The mechanism of this reaction was considered analogous to the decarboxylation process of β,γ-unsaturated carboxylic acids (23) as outlined in Scheme 3. This reaction constitutes a novel method for the introduction of a methyl group *via* a malonic acid group to the 3-/4-position of the furan ring of a 2,5-disubstituted C_{18} furanoid fatty esters.

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Preparation and Some Spectroscopic Properties of *N*-Heterocyclic Derivatives of a Novel 1-Pyrroline C_{18} Fatty Ester

Marcel S.F. Lie Ken Jie* and **M.S.K. Syed-Rahmatullah**
Department of Chemistry, University of Hong Kong, Hong Kong

A C_{18} 1-pyrroline fatty ester, methyl 8-(5-hexyl-2-pyrrolin-1-yl)octanoate (1), was prepared from methyl *iso*-ricinoleate. The C=N bond of the pyrroline ring was oxidized by *m*-chloroperoxy-benzoic acid to yield a mixture of oxaziridine isomers 2a,2b, which decomposed during gas chromatographic analysis to a 2,5-disubstituted pyrrole derivative, methyl 8-(5-hexyl-1*H*-pyrrole-2-)octanoate (3). Compound 3 was also obtained by reaction of 2a,2b with dilute HCl in methanol. Reaction of compound 1 with iodo-methane formed an *N*-methyl iminium iodide intermediate 4, which on reduction with sodium borohydride furnished a mixture of *cis/trans*-*N*-methyl-2,5-disubstituted pyrrolidine derivatives, methyl 8-(*cis/trans*-5-hexyl-*N*-methyl-pyrrolidine-2-)octanoates 5a,5b. Reduction of compound 1 with $NaBH_4$ gave a mixture of *cis/trans*-isomers of 2,5-disubstituted pyrrolidine derivatives, methyl 8-(5-hexyl-pyrrolidine-2-)octanoates 6a,6b. Acetylation of compounds 6a,6b with acetic anhydride furnished the corresponding *N*-acetyl pyrrolidines 7a,7b. When compound 1 was treated with perchloric acid, the corresponding iminium perchlorate derivative, methyl 8-(5-hexyl-1-pyrrolinium perchlorate-2-)octanoate 8 was obtained. The structures of the various derivatives were characterized by a combination of chromatographic, mass spectral and spectroscopic techniques.
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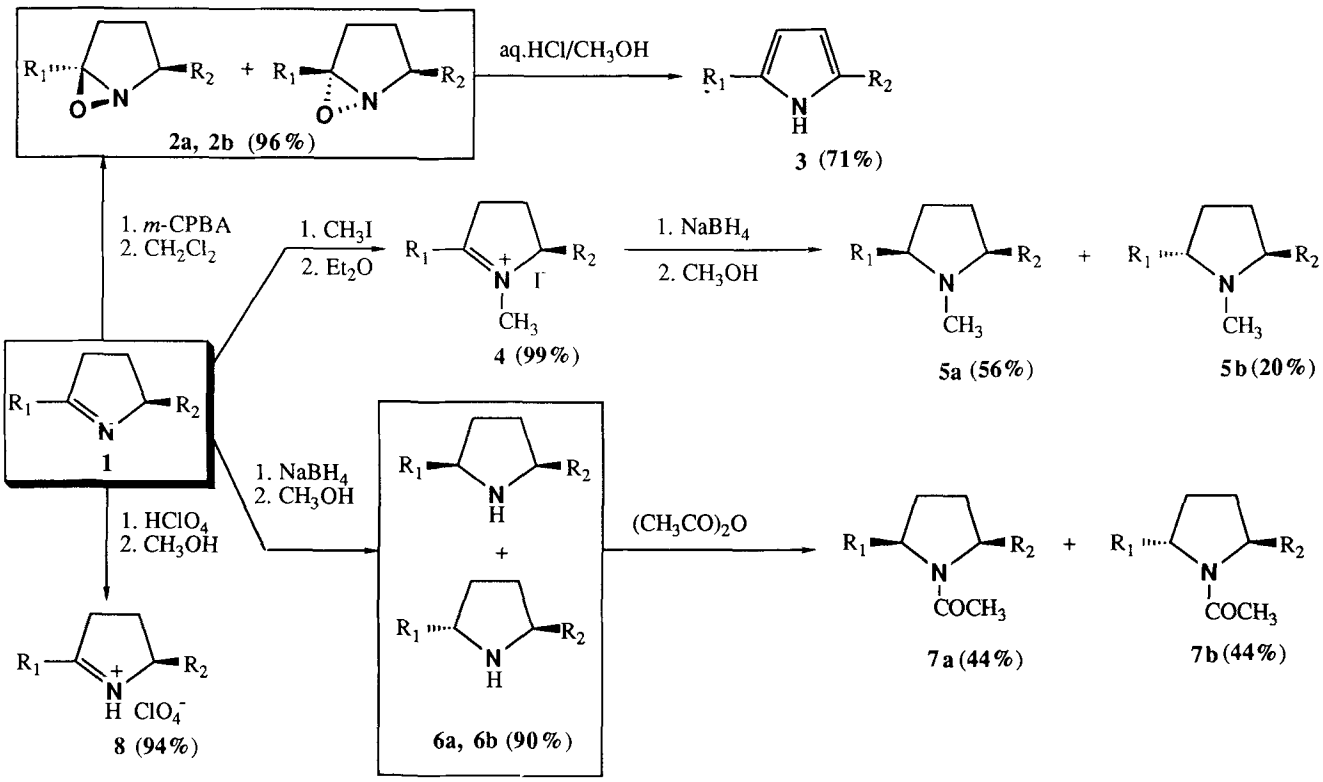
Long-chain *N*-heterocyclic fatty acids have not been found in nature. We have recently reported the synthesis of a novel 1-pyrroline C_{18} fatty ester derivative, methyl 8-(5-hexyl-2-pyrrolin-1-yl)octanoate, from methyl *iso*-ricinoleate (methyl 9-hydroxy-12-*cis*-octadecenoate) (1). The facile method developed for the preparation of this unusual fatty acid derivative provides a unique opportunity to explore the chemical, physical and biological properties of a new class of lipid molecules containing a five-membered *N*-heterocyclic system. 1-Pyrrolines are classified as cyclic imines and possess a chemically reactive C=N bond system. The formation of imine linkages has often been exploited by nature in various biochemical processes, including the condensation of 11-retinal with the amino group of lysine to form a visual pigment (rhodopsin) (2). The chemistry and physical properties of imines have been extensively reviewed (3,4).

In this paper we present some basic reactions involving the C=N (imine) bond of the 1-pyrroline system in the fatty ester derivative to obtain various related *N*-heterocyclic derivatives, in order to study the physical and chemical properties of this new class of five-membered *N*-heterocyclic fatty esters. The reactions carried out include oxidation (*m*-chloroperoxybenzoic acid) and reduction ($NaBH_4$) of the C=N linkage of the 1-pyrroline system, *N*-methylation, *N*-protonation and *N*-acetylation of the derived disubstituted pyrrolidine derivative, and the preparation of a pyrrole fatty ester derivative as outlined in Scheme 1 (derivatives of methyl 8-(5-hexyl-2-pyrrolin-1-yl)-octanoate (1)).

RESULTS AND DISCUSSION

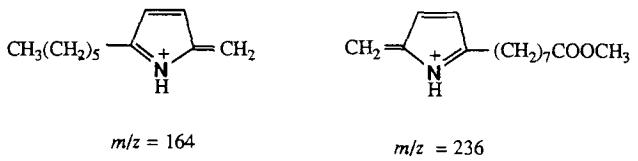
The novel C_{18} 1-pyrroline fatty ester derivative 1 was prepared by reaction of the mesyl derivative of methyl *iso*-ricinoleate (methyl 9-hydroxy-12-*cis*-octadecenoate) with sodium azide, and the structure of compound 1 was unequivocally characterized as reported elsewhere (1). Reaction of compound 1 with *m*-chloroperoxybenzoic acid gave almost quantitative yield of a mixture of the corresponding oxaziridine derivatives **2a,2b.** This oxidation process of the C=N (imine) bond of the 1-pyrroline substrate was non-stereospecific and resulted in the formation of a mixture of *cis/trans*-isomers. The presence of geometric isomers in the product was reflected in the ^1H nuclear magnetic resonance (NMR) spectrum of compounds **2a,2b,** which gave signals at δ3.0(*m*) and 3.3(*m*) for the shift of the proton at the C-9 of the *cis*- and *trans*-isomers, respectively. These values were similar to results reported by Belzecki and Mostowicz (5) for asymmetric oxaziridines. Attempts were not directed to produce optically active oxaziridine derivatives, as such an approach would require optically active (1*S*)-(+)-percamphoric acid (6). The existence of a mixture of stereoisomers was also reflected in the ^{13}C NMR spectrum of compounds **2a,2b,** which showed signals at 67.34 and 65.74 ppm for the chemical shifts of the carbon atom C-9 for the *cis*- and *trans*-isomers respectively. Compounds **2a,2b** were found to be unstable when injected onto a gas chromatographic (GC) column (SE-30, 190°C), forming the pyrrole derivative **3** during the analysis. The latter compound was characterized by gas chromatography/mass spectrometry (GC/MS). Compound 3 was also readily obtained by mild acid-catalyzed dehydration of compounds **2a,2b.** Preparation of the same pyrrole derivative **3** from methyl 9,12-dioxostearate *via* the Paal-Knorr reaction was previously reported by us (7). The pyrrole nucleus in compound **3** was confirmed by ^{13}C NMR analysis, which showed the shift of the hetero-aromatic carbon atoms at 104.6 (C-10, C-11, 2C) and 131.1 (C-9, C-12, 2C) ppm. In the ^1H NMR spectrum of compound 3 the chemical shift of the protons of the hetero-aromatic ring appeared at δ 5.73 (*d*, J = 2.6 Hz). GC/MS confirmed the position of the pyrrole ring between C-9 and C-12 of the alkyl chain from the ion peaks at m/z = 164 (100%, base peak) and m/z = 236 (54%) (Scheme 2). Compound 3 decomposed gradually into a dark red polymeric

*To whom correspondence should be addressed at Department of Chemistry, University of Hong Kong, Pokfulam Road, Hong Kong.
Abbreviations: ^{13}C NMR, ^{13}C nuclear magnetic resonance; GC, gas chromatography; GC/MS, gas chromatography/mass spectrometry; ECL, equivalent chain length; ^1H NMR, ^1H nuclear magnetic resonance; IR, infrared; TLC, thin-layer chromatography; Solvent A, light petroleum (b.p. 40–60°C)/diethyl ether (4:1, v/v); Solvent B, light petroleum (b.p. 40–60°C)/diethyl ether (7:3, v/v); Solvent C, light petroleum (b.p. 40–60°C)/diethyl ether (1:1, v/v); Solvent D, light petroleum (b.p. 40–60°C/diethyl ether (2:3, v/v).



$R_1 = CH_3(CH_2)_5$, $R_2 = (CH_2)_7COOCH_3$

SCHEME 1



SCHEME 2

Substance even at low temperature, but the free acid form could be preserved under nitrogen at 0°C for a considerable period.

Reaction of compound **1** with iodomethane formed an *N*-methyl iminium iodide intermediate **4**, which furnished a mixture of *cis/trans*-*N*-methyl-2,5-disubstituted pyrrolidine derivatives **5a, 5b** when treated with $NaBH_4$. The mixture of compounds **5a** and **5b** was separated by silica gel column chromatography into two fractions. Based on results published on the chromatographic and NMR spectroscopic properties of *cis*- and *trans*-isomers of 2,5-dialkyl and *N*-methyl-2,5-dialkyl pyrrolidines (8,9), the less polar fraction comprised the *cis*-isomer **5a**, while the more polar fraction consisted of the *trans*-isomer **5b**. ^1H and ^{13}C NMR spectral analyses confirmed the structure of these geometric isomers. The chemical shift of the *N*-methyl protons in the *cis*-isomer **5a** appeared at δ2.24(*s*), while that of the *trans*-isomer was found further downfield at δ 2.33(*s*). In the ^{13}C NMR spectrum the shift of the *N*-methyl carbon atom of the *cis*-isomer **5a** appeared at 39.00 ppm, while the same carbon in the *trans*-isomer **5b** was more shielded and appeared at 35.13 ppm instead. The shifts for the chemically equivalent C-9 and C-12 carbon atoms of the *cis*-isomer **5a** appeared at 67.80 ppm, while those of the *trans*-isomer were shifted more upfield to 63.38 ppm. 2,5-Dialkylpyrrolidines were isolated from the venom of ants (10).

Reduction of compound **1** with $NaBH_4$ gave a mixture of *cis*- and *trans*-isomers of 2,5-disubstituted pyrrolidine derivatives **6a,6b**, which could not be separated into individual geometric isomers by silica gel chromatography. However, the presence of the *cis*- and *trans*-isomers in the reaction product was confirmed by ^1H and ^{13}C NMR spectral analyses. By referring to NMR spectroscopic data published (11–13) for the proton chemical shifts associated with the methine groups of the 5-membered *N*-heterocyclic ring of 2,5-dialkylated pyrrolidines, the signals at δ2.93(*m*) and 3.12(*m*) in the spectrum of compounds **6a,6b** were assigned to the protons of the ring methine groups of the *cis*-isomer **6a** and *trans*-isomer **6b** respectively. The ^{13}C NMR spectrum of the mixture of compounds **6a,6b** further confirmed the presence of the *cis*- and *trans*-isomers. The chemical shifts of the ring methine carbon atoms (C-9 and C-12) of the *cis*-isomer **6a** appeared at 59.48, while the

same carbon atoms of the *trans*-isomer **6b** were shifted to 58.21 ppm. Signals at 31.42 and 32.42 ppm corresponded to the shifts of the methylene carbon atoms of the heterocyclic ring (C-10 and C-11) of the *cis*- and *trans*-isomer, respectively.

Acetylation of compounds **6a,6b** was achieved with acetic anhydride to give a mixture of *cis/trans-N*-acetyl pyrrolidine derivatives **7a,7b,** which was readily separated by silica thin-layer chromatography (TLC) into the *cis*-isomer **7a** and *trans*-isomer **7b**. The ^1H NMR analysis gave a characteristic signal at δ 2.05(*s*) for the chemical shift of the protons of the acetyl ($COCH_3$) group, while in the ^{13}C NMR spectrum the shift for the carbon atom of the same group appeared at 23.00 (*q*) ppm. When compound 1 was treated with perchloric acid in methanol, the iminium perchlorate derivative 8 was obtained as a viscous oil. In the ^1H NMR analysis of compound 8, the deshielding effect of the proton on the nitrogen atom of the pyrroline ring caused the chemical shift of the methine proton at C-9 to appear at δ 4.45(*m*). In the ^{13}C NMR spectrum of compound 8, the shifts of the carbon atom at C-12 appeared downfield at 197.67 ppm.

The results of these reactions demonstrate the facile chemical transformations of the 1-pyrroline fatty ester to a range of interesting pyrrolidine and pyrrole fatty ester derivatives.

EXPERIMENTAL PROCEDURES

Iso-ricinoleic acid was isolated from the seed oil of *Wrightia tinctoria* according to the partition method described by Gunstone (14). Methyl *iso*-ricinoleate was prepared by treatment of the acid with methanol in the presence of BF_3/CH_3OH (15). Thin-layer chromatography was performed on microscope glass plates coated with silica gel (*ca.* 0.1 mm thickness) and a mixture of light petroleum (b.p. 40–60 °C)/diethyl ether used as the developing solvent, unless otherwise stated. Column chromatographic purifications were carried out on silica gel using gradient elution of mixtures of light petroleum/diethyl ether as eluant. Gas chromatography (GC) was conducted on a Hewlett-Packard (Hewlett-Packard, Palto Alto, CA) model 5970 gas chromatograph fitted with a 10-m microbore glass column (0.53 mm internal diameter, 2.65 μm film thickness, SE-30 stationary phase) using nitrogen (20 mL/min) as the carrier gas at an isothermal column temperature of 220°C with a flame ionization detector. External standards of methyl myristate, palmitate and stearate were used as reference compounds and equivalent chain length (ECL) values were calculated for each component (16). Elemental analyses were carried out by Butterworth Laboratories Ltd. (Teddington, Middlesex, England, U.K.). Infrared (IR) spectra were obtained on a Perkin-Elmer model 577 (Perkin Elmer, Norwalk, CT) spectrophotometer. NMR spectra were measured on either a Jeol FX-90Z (Jeol Ltd., Tokyo, Japan) Fourier transform spectrometer with proton observation at 90 MHz and carbon observation at 22.5 MHz, or a Jeol GSX-270 instrument with proton observation at 270 MHz and carbon observation at 67.8 MHz. GC/MS analyses were carried out on a Hewlett-Packard model 5970 gas chromatograph fitted with a 12-m capillary glass column (0.2 mm internal diameter, 0.33 μm film thickness, crosslinked methyl silicone gum) using helium (*ca.* 2 mL/min) as the carrier gas under temperature programmed conditions (initial temperature, 140°C; final temperature, 240°C; programmed at a rate of 5°C/min). The outlet of the column was connected to a Hewlett-Packard Mass Selective Detector™.

Methyl 8-(5-hexyl-2-pyrrolin-1-yl)octanoate (1). Methanesulfonyl chloride (1.5 g, 13 mmol) was added to a cooled mixture of methyl *iso*-ricinoleate (2.0 g, 6.4 mmol), triethylamine (2 mL) and CH_2Cl_2 (25 mL) at 0°C. The reaction mixture was stirred at 0°C for an additional 30 min. Water (25 mL) was added and the reaction mixture was extracted with CH_2Cl_2 (2 × 30 mL), washed with dil. HCl (2M, 10 mL), and then with water (2 × 20 mL) and was dried over anhydrous Na_2SO_4. Evaporation of the solvent under reduced pressure furnished methyl 9-mesyloxy-12-*cis*-octadecenoate (2.45 g, 99%) as an oil. The latter compound was dissolved in *N,N*-dimethyl formamide (25 mL) and NaN_3 (0.6 g, 9.2 mmol) was added. The mixture was stirred vigorously at 80°C for 6 hr. Water (40 mL) was added and the reaction mixture was extracted with Et_2O (2 × 50 mL). The ethereal extract was washed with water (2 × 20 mL) and dried over anhydrous Na_2SO_4. Silica gel column chromatography of the extract using Solvent C as eluant yielded compound 1 (1.8 g, 91%) as an oil. Elemental analysis: Found, C, 73.7; H, 11.5; N, 4.8. Calculated for $C_{19}H_{35}NO_2$: C, 73.7; H,11.4, N, 4.5%; R_f 0.5 (Solvent D); ECL 18.4 (SE-30). ^1H NMR (270 MHz, $CDCl_3$) δ 0.89 (*t*, 3H), 1.2–1.5 (*m*, 14H), 1.5–1.8 (*m*, 6H), 2.03 (*m*, 2H, 10-H ring), 2.30(*t*, J = 7.0 Hz, 4H, 2-H, 13-H), 2.45 (*q*, J = 7.0 Hz, 2H, 11-H ring), 3.65 (*s*, 3H, CO_2CH_3) and 3.90 (*m*, 1H, N-CH ring); ^{13}C NMR (67.5 MHz, $CDCl_3$) 14.05(*q*, C-18), 22.60(*t*, C-17), 25.00(*t*, C-3), 26.62(*t*, C-7,C-14 2C), 28.64(*t*, C-10 ring), 29.14(*t*), 29.21(*t*), 29.25(*t*), 29.63(*t*), 31.65(*t*, C-16), 34.07(*t*, C-2 and C-13, 2C), 36.71(*t*, C-8), 36.95(*t*, C-11 ring), 51.34(*q*, CO_2CH_3), 72.50(*d*, C-9 *ring*), 174.13(*s*, C-1) and 177.07(*s*, *C*=N ring) ppm. IR (NaCl) 2925(s), 2860(s), 1740(s,C=O ester), 1640(s, C=N str.), 1215(m) and 1190(m) cm^{-1}; GC/MS *m/z* (relative intensity) 278 (36, M-31), 266 (21), 252 (64), 239 (46), 166 (69), 152 (29), 96 (48), 83 (49), 82 (100).

Reaction of 1 with m-*chloroperoxybenzoic acid. m*-Chloroperoxybenzoic acid (0.6 g, 3.5 mmol) was added to a stirred solution of compound 1 (1.0 g, 3.24 mmol) in CH_2Cl_2 (30 mL), and the reaction mixture was stirred at room temperature for 30 min. Aqueous Na_2SO_3 (5%, 20 mL) was added, and the reaction mixture was extracted with CH_2Cl_2 (30 mL). The organic extract was washed with dil. $NaHCO_3$ (10%, 20 mL), and then with water (3 × 20 mL) and dried over anhydrous Na_2SO_4. The solvent was evaporated and the residue was chromatographed on silica gel using Solvent A as the eluant to give a mixture of methyl 8-[5-hexyl-6-oxa-1-azabicyclo(3.1.0)-hexane-2-]octanoates (**2a,2b,** 1.0 g, 96%). Elemental analysis: Found, C, 70.2; H, 11.2; N, 4.3. Calculated for $C_{19}H_{35}NO_3$: C, 70.1; H, 10.9; N, 4.5%; R_f 0.6 (Solvent A); ECL 20.0 (SE-30); ^1H NMR (90 MHz, $CDCl_3$) δ 0.9 (*t*, J = 7 Hz, 3H), 1.2–1.6 (*m*, 16H), 1.6–1.9 (*m*, 8H), 1.9–2.2 (*m*, 2H), 2.3 (*t*, J = 7 Hz, 2H), 3.0, 3.3 (*m*, broad, 1H, 9-*H*) and 3.6 (*s*, 3H); ^{13}C NMR (22.5 MHz, $CDCl_3$) 14.06(*q*), 22.56(*t*), 24.65(*t*, C-7), 24.96(*t*), 25.65(*t*, C-14), 26.8(*t*), 27.14, 27.55(*t*, C-10), 29.12(*t*), 29.40(*t*), 29.50(*t*), 31.70(*t*), 31.99(*t*), 32.83(*t*, C-11), 32.91(*t*, C-13), 34.07(*t*), 51.38(*t*), 65.74(*d*, C-9 ring, *trans*), 67.34(*d*, C-9 ring, *cis*), 90.00 (*s*, C-12) and 174.17(*s*, C-1) ppm.

*Conversion of compounds **2a,2b** to methyl 8-(5-hexyl-1H-pyrrole-2-)octanoate (**3**).* A mixture of compounds **2a,2b** (0.15 g, 0.46 mmol), dil.HCl (0.1 M, 1 mL) and methanol (15 mL) was refluxed for 20 min. The reaction mixture turned brick red in color. Water (30 mL) was added and the reaction mixture was extracted with Et_2O (2 × 20 mL). The organic extract was washed with water (2 × 20 mL) and dried over anhydrous Na_2SO_4. Silica gel column chromatography using Solvent A as eluant gave compound 3 (0.1 g, 71%) as an oil. R_f 0.6 (Solvent A); ECL 20.0 (SE-30); ^1H NMR (90 MHz, $CDCl_3$)δ 0.9 (*t*, 3H), 1.2–1.8 (*m*, 18H), 2.3 (*t*, J = 7 Hz, 2H), 2.5 (*t*, J = 7.5 Hz, 4H, H-8, H-13), 3.6 (*s*, 3H), 5.73 (*d*, J = 2.6 Hz, 2H, C-*H* pyrrole ring), 7.92 (*s*, br., 1H, N-*H*); ^{13}C NMR (22.5 MHz, $CDCl_3$) 14.08(*q*), 22.64(*t*), 24.95(*t*), 27.85(*t*, C-8), 27.90(*t*, C-13), 29.06(*t*), 29.17(*t*), 29.42(*t*), 29.69(*t*), 29.77(*t*), 31.74(*t*), 34.10(*t*), 51.40(*t*), 104.6(*d*, C-10, C-11, 2C), 131.10(*s*, C-9, C-12, 2C) and 174.25(*s*, C-1) ppm; IR (NaCl) 3400(s, N-H str.), 2925(s), 2860(s), 1740(s), 1580(s), 1420(m), 1380(s), 1200(m), 1180(m) and 770(m) cm^{-1}; GC/MS *m/z*(relative intensity) 276 (9, M-31), 236(54), 178(6), 164(100) and 94(17).

Preparation of N-*methyl 2,5-disubstituted C_{18} pyrrolidine derivatives **5a,5b**.* A mixture of compound 1 (1.0 g, 3.24 mmol), iodomethane (8 mL) and anhydrous Et_2O (20 mL) was refluxed for 1 hr. The solvent and excess iodomethane were removed under reduced pressure to give methyl 8-(5-hexyl-*N*-methyl-pyrrolinium-iodo-2-)octanoate (**4**, 1.44 g, 99%) as a viscous oil. ^1H NMR (90 MHz, $CDCl_3$) δ 0.88 (*t*, 3H), 1.2–1.6 (*m*, 16H), 1.8 (*m*, 2H), 1.95 (*m*, 2H), 2.3 (*t*, J = 7 Hz, 2H), 2.6 (*m*, 2H), 2.9 (*t*, J = 7 Hz, 2H), 3.3 (*m*, 2H), 3.5 (*s*, 3H, NCH_3), 3.6 (*s*, 3H, CO_2CH_3) and 4.5 (*m*, 1H, 9-*H*); IR (NaCl) 3400 (s, br.), 2925(s), 2860(s), 1740(s), 1670(m), 970(m) and 780(m) cm^{-1}.

Compound 4 (0.5 g, 1.1 mmol) was dissolved in MeOH (15 mL) and cooled to 0–5°C. A solution of $NaBH_4$ (0.1 g, 2.6 mmol) in MeOH (5 mL) was added and the reaction mixture stirred at 0–5°C for 10 min. The solvent was evaporated under reduced pressure and water (20 mL) was added to the residue. The aqueous mixture was extracted with Et_2O (2 × 30 mL), and the ethereal extract was washed with water (20 mL) and dried over anhydrous Na_2SO_4. The solvent was evaporated and the residue was chromatographed on silica gel (50 g) using Solvent C (100 mL) followed by a mixture of light petroleum/Et_2O/MeOH (2:2:1, v/v/v) (150 mL) to give methyl 8-(*cis*-5-hexyl-*N*-methyl-pyrrolidine-2-)octanoate (**5a**, 0.2 g, 56%). Elemental analysis: Found, C, 72.6; H, 12.3; N, 4.4; Calculated for $C_{20}H_{39}NO_2$: C, 73.7; H, 12.1; N, 4.3%; R_f 0.5 (solvent D); ECL 19.1 (SE-30); ^1H NMR (90 MHz, $CDCl_3$)δ 0.88(*t*, 3H), 1.2–1.6 (*m*, 22H), 1.7–2.1 (*m*, 6H), 2.24(*s*, 3H, NC_3), 2.3 (*t*, J = 7 Hz, 2H), 3.65 (*s*, 3H); ^{13}C NMR (22.5MHz, $CDCl_3$) 14.08(*q*), 22.64(*t*), 25.00(*t*), 26.63(*t*, C-7), 26.70(*t*, C-14), 29.06(*t*), 29.14(*t*), 29.25(*t*), 29.74(*t*), 29.85(*t*), 31.90(*t*), 34.10(*t*), 34.45(*t*), 39.00(*s*, NCH_3), 51.33 (*s*), 67.80 (*d*, C-9, C-12, 2C) and 174.17(*s*) ppm; IR (NaCl) 2925(s), 2860(s), 2800(m, NCH_3 str), 1740(s), 1430(m), 1230(m) and 1200(m) cm^{-1}; GC/MS *m/z* (relative intensity) 294 (2,M-31), 240 (46, *a*), 168 (100, *b*) (Scheme 3), 96 (12), 55 (16) and 41 (12); and methyl 8-(*trans*-5-hexyl-*N*-methyl-pyrrolidine-2-)octanoate (**5b**, 70 mg, 20%) as an oil. Elemental analysis: Found, C, 73.6; H, 12.6; N, 4.6. Calculated for $C_{20}H_{39}NO_2$: C, 73.7; H, 12.1; N, 4.3%; R_f 0.1 (solvent D); ECL 19.7 (SE-30); ^1H NMR (90 MHz, $CDCl_3$) δ 0.88 (*t*, 3H), 1.2–1.4 (*m*, 16 H), 1.4–1.7 (*m*, 6H), 1.8–2.1 (*m*, 4H), 2.3 (*t*, 2H), 2.33 (*s*, 3H, NCH_3), 2.80 (*m*, 2H, H-9 and H-12, ring) and 3.6 (*s*, 3H); ^{13}C NMR (22.5 MHz, $CDCl_3$) 14.06(*q*), 22.64(*t*), 25.00(*t*), 26.81(*t*, C-7), 26.87(*t*, C-14), 28.77(*t*), 29.12(*t*), 29.25(*t*), 29.69(*t*), 29.80(*t*), 30.58(*t*, C-13), 31.9(*t*), 34.1(*t*), 35.13(*s*, NCH_3), 51.36(*s*), 63.38(*d*, C-9, C-12, 2C), and 174.22(*s*) ppm; IR (NaCl) 2925(s), 2860(s), 2800 (m, NCH_3 str),1740(s), 1439(m), 1230(m) and 1200(m) cm^{-1}; GC/MS *m/z* (relative intensity) 294 (2, M-31), 240(48), 168(100), 96(11), 55(16) and 41(11).



m/z = 168 (*b*) *m/z* = 240 (*a*)

SCHEME 3

*Reduction of compound 1 to methyl 8-(5-hexyl-pyrrolidine-2-)octanoate (**6a,6b**).* A solution of $NaBH_4$ (0.2 g, 5.2 mmol) in MeOH (5 mL) was added to a cooled solution of compound 1 (1.0 g, 3.2 mmol) in MeOH (20 mL) at 0°C. The reaction mixture was stirred for an additional 20 min at 0–5 °C. The solvent was evaporated under reduced pressure, and water (25 mL) was added to the residue. The aqueous mixture was extracted with Et_2O (2 × 30 mL) and the ethereal extract was washed with water (2 × 20 mL) and dried over anhydrous Na_2SO_4. Silica gel column chromatography of the extract gave compounds **6a,6b** (0.9 g, 90%) as an oil. R_f 0.3 (hexane/ Et_2O/MeOH, 2:2:1, v/v/v); ECL 19.4 (SE-30); ^1H NMR (270 MHz, $CDCl_3$)δ 0.9 (*t*, 3H), 1.2–1.6 (*m*, 22H), 1.82 (*m*, 2H, 10-H, 11-H *trans*-isomer), 1.92 (*m*, 2H, 10-H, 10-H, 11-H *cis*-isomer), 2.30 (*t*, 2H), 2.90(*s*, 1H, N-*H*, D_2O exchangeable), 2.93 (*m*, 1H, ring C*H cis*-isomer), 3.12 (*m*, 1H, ring C*H trans*-isomer) and 3.65 (*s*, 3H); ^{13}C NMR (67.8 MHz, $CDCl_3$) 14.0(*q*), 22.64(*t*), 25.03(*t*), 27.25(*t*), 27.41(*t*), 29.20(*t*), 29.25(*t*, 2C), 29.55 (*t*), 29.60 (*t*, 2C), 31.42 (*t*, C-10, C-11, *cis*-isomer, 2C), 31.91(*t*), 32.42(*t*, C-10, C-11 *trans*-isomer, 2C), 34.13(*t*), 36.76(*t*, C-8, C-13, *cis*-isomer, 2C), 36.86(*t*, C-8, C-13 *trans*-isomer, 2C), 51.19(*q*), 58.12 (*d*, C-9, C-12 *trans*-isomer, 2C), 59.48(*d*, C-9 , C-12, *cis*-isomer, 2C) and 173.92 (*s*) ppm; IR (NaCl) 2940(s), 2860(s), 1740(s), 1460(s), and 1165(m) cm^{-1}; GC/MS *m/z* (relative intensity) 180 (M-31, 8), 226(87), 194(17) and 154(100).

*Preparation of the methyl 8-(*cis/trans*-5-hexyl-*N*-acetyl-pyrrolidine-2-)octanoates (**7a,7b**).* A mixture of compounds **6a,6b** (0.18 g, 0.58 mmol) was heated in the presence of acetic anhydride (3 mL) on a steam bath for 20 min. Water (20 mL) was added and the reaction mixture was heated for an additional 5 min to hydrolyze excess acetic anhydride. The reaction mixture was cooled and extracted with Et_2O (2 × 20 mL). The organic extract was washed with water (20 mL) and then with $NaHCO_3$ (20%, 10 mL) and dried over anhydrous Na_2SO_4. The solvent was distilled and the residue separated by preparative silica TLC using solvent B as the developing solvent to yield methyl 8-(*cis*-5-hexyl-*N*-acetylpyrrolidine-2-)octanoate (**7a**, 90 mg, 44%) as an oil. R_f 0.5 (hexane/Et_2O/MeOH, 2:2:1, v/v/v); ECL 22.1 (SE-30); ^1H NMR (90 MHz, $CDCl_3$)δ0.88

(*t*, 3H), 1.2–1.5 (*m*, 16H), 1.5–2.0 (*m*, 10H), 2.05 (*s*, 3H, N-$COCH_3$), 2.3 (*t*, 2H), 3.5–4.1 (*m*, 2H) and 3.6 (*s*, 3H); ^{13}C NMR (22.5 MHz, $CDCl_3$) 14.06(*q*), 22.61(*t*), 23.00(*q*, CH_3, N-$COCH_3$), 24.92(*t*), 26.87(*t*, C-7, C-14, 2C), 28.00(*t*, C-10, C-11, 2C), 29.00(*t*), 29.32(*t*), 29.33(*t*), 31.88(*t*), 32.18(*t*), 34.00(*t*), 35.24(*t*, C-8, C-13, 2C), 51.36(*q*), 57.61, 59.16(*d*, C-9, C-12, 2C), 168.78(*s*, N-$COCH_3$), 174.08(*s*) and 174.19(*s*) ppm; IR (NaCl) 2925(s), 2860(s), 1740(s), 1640(m), 1420(m), 1200(m) and 950(m) cm^{-1}; and methyl 8-(*trans*-5-hexyl-*N*-acetyl-pyrrolidine-2-)octanoate (**7b**, 90 mg, 44%) as an oil. R_f 0.4 (hexane/Et_2O/MeOH, 2:2:1, v/v/v); ECL 22.5 (SE-30). ^1H NMR (90 MHz, $CDCl_3$) δ0.89 (*t*, 3H), 1.2–1.5 (*m*, 16H), 1.5–2.0 (*m*, 10 H), 2.05(*s*, 3H), 2.3 (*t*, 2H), 3.5–4.1 (*m*, 2H) and 3.6 (*s*, 3H); ^{13}C NMR (22.5 MHz, $CDCl_3$) 14.06(*q*), 22.56(*t*), 23.00(*q*, N-$COCH_3$), 24.9(*t*), 26.3, 26.68(*t*, C-7, C-14, 2C), 29.06(*t*), 29.2(*t*), 29.33(*t*), 29.60(*t*), 31.83(*t*), 34.02(*t*), 35.54, 36.10(*t*, C-8, C-13, 2C), 51.33 (*s*), 57.67, 59.54(*d*, C-9, C-12, 2C), 169.02(*s*, N-$COCH_3$) and 174.06(*s*, C-1) ppm; IR (NaCl) 2925(s), 2860(s), 1740(s), 1640(m), 1420(m), 1200(m), 950(s) cm^{-1}.

Preparation of methyl 8-(5-hexyl-1-pyrrolinium perchlorate-2-)octanoate (8). A mixture of compound 1 (3.2 g, 10.3 mmol), perchloric acid (70%, 2 mL) and methanol (50 mL) was refluxed for 1 hr. Water (100 mL) was added and the reaction mixture extracted with dichloromethane (2 × 50 mL). The organic extract was washed with water (3 × 25 mL) and dried over anhydrous Na_2SO_4. The solvent was evaporated under reduced pressure to give compound 8 (4.0 g, 94%) as a viscous oil. ^1H NMR (90 MHz, $CDCl_3$)δ 0.88 (*t*, 3H), 1.2–1.6(*m*, 16H), 1.6–2.1 (*m*, 4H), 2.3 (*t*, 2H), 2.4 (*m*, 10-H, 2H), 2.9(*t*, J = 7.5 Hz, 2H, 13-H), 3.2 (*t*, J = 7.7 Hz, 2H, 11-H), 3.46 (*s*, 1H, N-*H*), 3.6(*s*, 3H) and 4.45(*m*, 1H, C=N-C*H*- ring); ^{13}C NMR (22.5 MHz, $CDCl_3$) 13.73(*q*, C-18), 22.12(*t*, C-17), 24.61(*t*, C-3), 25.00(*t*, C-7), 25.10(*t*, C-14), 25.78(*t*, C-10), 28.65(*t*, 4C), 30.87(*t*, C-16), 32.12(*t*, C-8), 33.36(*t*, C-13), 33.80(*t*, C-2), 36.48(*t*, C-11), 51.27(*s*), 67.28(*d*, C=N-*C*H, C-9), 174.24(*s*) and 197.67(*s*, *C*=N, C-12); IR (NaCl) 3400 (s, br), 2925(s), 2860(s), 1740(s), 1670(C=N-H, str.) and 1200(m) ppm.

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Further Oxidation Products of 2,2,5,7,8-Pentamethyl-6-chromanol

Cacang Suarna, Manuel Baca, Donald C. Craig, Marcia Scudder and **Peter T. Southwell-Keely***
Department of Organic Chemistry, University of New South Wales, Kensington, N.S.W., 2033, Australia

In order to undertake a quantitative study by high-performance liquid chromatography of the rate of oxidation of 2,2,5,7,8-pentamethyl-6-chromanol (1), the model compound of α-tocopherol, a number of potential products were required as standards. Among these compounds were 2,2,7,8-tetramethylchroman-5,6-dione (10) and 2,2,7-trimethyl-6-hydroxychroman-5,8-dione (17), the model compounds of tocored and tocopurple, respectively. Attempts to synthesize 10 and 17 led to the isolation of 8-hydroxymethyl-2,2,7-trimethylchroman-5,6-dione (14) and 1,2-*bis*(2,2,7-trimethylchroman-5,6-dione-8-)ethane (19) a dimer of 10. Purification by thin-layer chromatography of the spirodimer (20) of 1 resulted in an acid-catalyzed decomposition to 1-(2,2,7,8-tetramethyl-6-chromanol-5-)2-[2-(3-methyl-3-hydroxybutyl)-5,6-dimethyl-1,4-benzoquinone-3-]ethane (23), a new chromanol-quinone dimer.
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α-Tocoquinone and tocored were the first oxidation products of α-tocopherol to have been isolated, and they form the bases of the well known Emmerie-Engel and Furter-Meyer colorimetric assays for vitamin E (1,2). According to the literature, the conditions required to produce tocored are more vigorous than those needed for tocoquinone and represent an overoxidation.

Our experience with oxidations of 2,2,5,7,8-pentamethyl-6-chromanol (1), the model compound of α-tocopherol, showed that 2,2,7,8-tetramethylchroman-5,6-dione (10), the model compound of tocored, and 2,2,7-trimethyl-6-hydroxychroman-5,8-dione (17), the model compound of tocopurple, were almost always among the products. Since we desired to make a detailed kinetic study of the rate of formation of various oxidation products of 1 by high-performance liquid chromatography (HPLC), it was necessary to prepare a number of these compounds in a high degree of purity for use as standards. During the preparation of 10 and 17, two new compounds were isolated, one of which was a hydroxylated form of 10, and the second a dimer of 10.

The second part of this work arose from the observation that attempts to purify the spirodimer of 1(20) by repeated chromatography on silica gel thin-layer chromatography (TLC) plates led to its apparent decomposition. It was hypothesized that the decomposition may have been due to acid-catalyzed ring opening of 20 on the TLC plate. This indeed proved to be true and led to the isolation of two new dimers.

*To whom correspondence should be addressed at the Department of Organic Chemistry, University of New South Wales, P.O. Box 1, Kensington, N.S.W., 2033, Australia.

Abbreviations: E.I., electron impact; F_o, observed structure amplitude; HPLC, high-performance liquid chromatography; IR, infrared; I_o, observed intensity; MS, mass spectrum; NMR, nuclear magnetic resonance; σ, standard deviation; θ, diffraction angle; TLC, thin-layer chromatography.

MATERIALS AND METHODS

Infrared (IR) spectra were determined on a Perkin-Elmer (Norwalk, CT) 580B spectrometer, ultraviolet (UV) spectra on a Perkin-Elmer 124 double beam spectrophotometer. ^1H and ^{13}C nuclear magnetic resonance (NMR) spectra were recorded on a Bruker (Karlsruhe, Germany) AM 500 spectrometer. NMR spectra were taken in $CDCl_3$ and are reported in parts per million downfield from tetramethylsilane as internal standard. Electron impact (E.I.) mass spectra were determined at 70 eV on an A.E.I. (Manchester, England) MS 12 spectrometer.

Compound 1 together with its spirodimer (20) and spirotrimer, 5-hydroxymethyl-2,2,7,8-tetramethyl-6-chromanol (5), 5-formyl-2,2,7,8-tetramethyl-6-chromanol (6) and 5-ethoxymethyl-2,2,7,8-tetramethyl-6-chromanol (4) were prepared as reference compounds by known methods (3-8).

Oxidation of 1 by silver nitrate in absolute ethanol. Compound 1 (105 mg, 0.48 mmol) was dissolved in absolute ethanol (5 mL) and heated to 65°C for 5 min. Silver nitrate (358 mg, 2.11 mmol) was then added and the solution kept at 65°C for 2 hr during which time the color changed to blood red, and silver precipitated. The solution was filtered, the silver precipitate washed with chloroform (3 × 10 mL) and the chloroform washings added to the ethanol filtrate. The chloroform caused further precipitation in the combined chloroform/ethanol filtrate which was refiltered, washed with distilled water (4 × 5 mL), dried (Na_2SO_4) and the solvent removed *in vacuo*.

The residue was chromatographed on thin layers of silica gel GF_{254} (solvent, petroleum hydrocarbon (b.p. 60-80°C)/ethyl acetate, 9:1, v/v). The fractions were located under ultraviolet light and eluted with distilled chloroform. The solvent was removed under a stream of nitrogen.

Major bands with the following R_f values were isolated: 0.00 (pink, 1.6 mg), 0.03 (purple, 3.9 mg), 0.13 (red, tocored model compound 10, 39.5 mg), 0.28 (3.3 mg), 0.37 (9.1 mg), 0.44 (1, 10.7 mg), 0.56 (5-ethoxymethyl-2,2,7,8-tetramethyl-6-chromanol, 4, 29.6 mg), 0.62 (5-formyl-2,2,7,8-tetramethyl-6-chromanol, 6, 11.3 mg) and 0.69 (1.0 mg).

The major bands with R_f values of 0.00, 0.03 and 0.13 were combined and rechromatographed on silica gel GF_{254} (solvent, petroleum hydrocarbon (b.p. 60-80°C/ethyl acetate, 1:1 v/v) yielding 5 minor bands with R_f values of 0.02 (pale purple, trace), 0.16 (purple, 3.9 mg), 0.26 (pink, 0.8 mg), 0.40 (pink, 5.4 mg) and 0.72 (red, 36.8 mg).

The minor bands with R_f values of 0.02 and 0.26 were not purified further.

Minor band R_f 0.16 separated as orange crystals (purple on silica gel) from petroleum hydrocarbon and was identified as 2,2,7-trimethyl-6-hydroxychroman-5,8-dione (model compound of tocopurple, 17). Compound 17 had the following spectral data: UV (λ_{max}, methanol) 295 mm; IR (KBr) (cm^{-1}): 3371 (OH), 2976, 2936, 2859 (CH_3), 1732, 1654 (C=O), 1639, 1625, 1612, 1463, 1407, 1376, 1356, 1287, 1267, 1240, 1158, 1120, 1097, 1064 (C—O—C), 983, 943, 883, 837, 764. Mass spectra (MS) (E.I.) (*m/z*) (rel. in-

tensity): 222 (M^+) (24), 207 (75), 167 (13), 139 (26), 123 (15), 109 (23), 95 (32), 81 (33), 69 (44), 55 (64) and 41 (100). ^1H NMR ($CDCl_3$) δ 1.3985 (*s*, 6H, 2 × CH_3), 1.7580 (*t*, 2H, J = 6.6 Hz, =C−CH_2−CH_2−), 1.9100 (*s*, 3H, =C−CH_3), 2.4412 (*t*, 2H, J = 6.6 Hz, =C−CH_2−CH_2−) and 7.23 (*s*, 1H, OH). ^{13}C NMR. ($CDCl_3$) δ 182.205 (2 peaks), 154.988, 151.301, 113.898, 112.133, 79.159, 31.403, 26.495 (2 peaks), 15.540, 7.71.

Minor band R_f 0.40 separated as blood red-purple crystals from absolute ethanol/ethyl acetate (pink on silica gel). The UV spectrum showed that it was contaminated with 2-(3-hydroxy-3-methylbutyl)-3,5,6-trimethyl-1,4-benzoquinone at 268 nm and 258 nm. Purification was achieved by adding n-hexane which dissolved the quinone easily but not the red compound. The compound, which had m.p. 169–171°C, was identified as 1,2-*bis*(2,2,7-trimethyl-chroman-5,6-dione-8-)ethane (**19**) and had UV-Vis (λ_{max}, methanol) 287, 468 nm; IR (KBr) (cm^{-1}) 2926, 2855 (CH_3), 1680, 1644 (C=O), 1581, 1458, 1403, 1375, 1325, 1293, 1264, 1243, 1198, 1172, 1121 (C−O−C), 1078, 938, 882; MS (E.I.) (*m/z*) (rel. intensity): 438 (M^+) (2), 222 (38), 208 (16), 205 (17), 166 (100), 152 (61), 138 (16); ^1H NMR ($CDCl_3$) δ 1.4014 (*s*, 12H, 4 × CH_3), 1.7659 (*t*, 4H, 2 × CH_2, J = 6.7 Hz, =C−CH_2−CH_2−), 2.0741 (*s*, 6H, 2 × CH_3, =C−CH_3), 2.4543 (*t*, 4H, 2 × CH_2, J = 6.7 Hz, =C−CH_2−CH_2−) and 2.6202 (*s*, 4H, 2 × CH_2, =C−CH_2); ^{13}C NMR. ($CDCl_3$) δ 180.929, 177.730, 162.283, 145.355, 134.901, 111.044, 79.758, 31.475, 26.806 (2 peaks), 26.429, 15.809 11.728. As the molecule (**19**) is a symmetrical dimer, each of the peaks actually represents the signals of two carbons.

Crystal data. $C_{26}H_{30}O_6$, M 438.5, monoclinic, space group C2/c, a 26.098 (3), b 6.068 (1), c 18.414 (2)Å, β 129.05 (1)°, V 2264.7 (3)Å^3, D_c 1.29 g cm^{-3}, Z 4, μ_{Cu} 7.00 cm^{-1}. Crystal size 0.04 by 0.48 by 0.46 mm, 2 diffraction angle (θ_{max}) 70°, minimum and maximum transmission factors 0.77 and 0.98. The number of reflexions was 1431 considered observed out of 2156 unique data, with R_{merge} 0.015 for 54 pairs of equivalent hk0 reflexions. Final residuals R, R_w were 0.049, 0.062.

Structure determination. Reflexion data were measured with an Enraf-Nonius (Delft, Holland) CAD-4 diffractometer in $\theta/2\theta$ scan mode using nickel filtered copper radiation (λ 1.5418 Å). Data were corrected for absorption. Reflexions with I>3 standard deviation (σ) (I) were considered observed. The structure was determined by direct phasing and Fourier methods. Hydrogen atoms were located in a difference Fourier, included in idealized positions, and were assigned thermal parameters equal to those of the atom to which they were bonded. Positional and anisotropic thermal parameters for the non-hydrogen atoms and an isotropic extinction parameter were refined using full matrix least squares. Reflexion weights used were $1/\sigma^2$ observed structure amplitude (F_o), with $\sigma(F_o)$ being derived from $\sigma(I_o) = [\sigma^2(I_o) + (0.04I_o)^2]^{1/2}$. The weighted residual is defined as $R_w=(\Sigma w\Delta^2/\Sigma wF_o^2)^{1/2}$. Atomic scattering factors and anomalous dispersion parameters were from *International Tables for X-ray Crystallography* (9). Structure solution was by *MULTAN80* (10). Initial refinement used *BLOCKLS*, a local version of *ORFLS* (11) and was completed using *RAELS88* (12) when disorder was identified. Alternate positions for C(2), and hence for C(10) and C(11), were refined, with slack constraints ensuring that the dimensions of the two components were approximately equal. The proportions of the two components were refined, but their sum was kept at 1.0. As a result of the disorder, ten hydrogen atoms had alternate positions included in the refinement. Thermal parameters of each atom of the minor disorder component were equated to those of the equivalent atom of the major component. *ORTEPII* (13) running on a Macintosh IIcx was used for the structural diagram, and an IBM 3090 computer was used for calculations.

The structure and atom numbering scheme is shown in Figure 1, with the minor disorder component, comprising 16%, omitted. The molecule has a crystallographic center of symmetry in the C(13)—C(13) bond, and only the asymmetric portion is labeled. Material deposited with this journal comprises all atom and thermal parameters, interatomic distances and angles, and observed and calculated structure factors.

Minor band R_f 0.72 separated from petroleum hydrocarbon (b.p. 40–60°C) as blood-red crystals (pink on silica gel) and was identified as 2,2,7,8-tetramethyl-chroman-5,6-dione (**10**). Compound **10** had the following spectral data: UV-Vis (λ_{max}, hexane) 270, 438; (λ_{max}, methanol) 282, 468 nm; IR (KBr) (cm^{-1}); 2975, 2931, 2857 (CH_3), 1676, 1645 (C=O), 1586, 1455, 1401, 1378, 1324, 1288, 1242, 1185, 1121, 1109 (C−O−C) 1047, 923, 897, 880. MS (E.I.) (*m/z*) (rel. intensity): 220 (M^+) (17), 205 (28), 192 (63), 177 (20), 159 (16), 137 (100), 109 (15). ^1H NMR ($CDCl_3$) δ 1.3730 (*s*, 6H, 2 × CH_3), 1.7306 (*t*, 2H, J = 6.7 Hz, =C−CH_2−CH_2−), 1.9431 (*s*, 3H, =C−CH_3), 2.0256 (*s*, 3H, =C−CH_3), 2.424 (*t*, 2H, J = 6.7 Hz, =C−CH_2−CH_2−). ^{13}C NMR ($CDCl_3$) δ 180.825, 177.915, 163.307, 143.724, 134.149, 110.058, 79.088, 31.392, 26.694 (2 peaks), 15.657, 13.721, 11.558. The analysis of the ^1H NMR spectrum was completed by means of a two-dimensional long-range, proton-carbon correlation spectrum (14).

When **10** (36.8 mg) was kept in chloroform solution for a few days, it decomposed into five compounds which were separated on thin layers of silica gel (solvent, petroleum hydrocarbon/ethyl acetate, 1:1, v/v). The compounds had R_f 0.00 (yellow, trace), 0.26 (pink, 1.3 mg), 0.40 (pink, **19**, 3.2 mg), 0.49 (colorless, quinone, trace) and 0.73 (pink-red, **10**, 31.6 mg).

The band with R_f 0.26 (pink) was combined with minor band R_f 0.26 from the silver nitrate reaction and was tentatively identified as 8-hydroxymethyl-2,2,7-trimethyl-chroman-5,6-dione (**14**). It had UV-Vis (λ_{max}, n-hexane): 268, 434; IR (KBr) (cm^{-1}); 3451 (OH), 2958, 2929, 2858 (CH_3), 1734, 1684, 1651 (C=O), 1586, 1458, 1397, 1377, 1326, 1291, 1175, 1117, 1032, 1016, 979, 924, 898, 878; MS (E.I.) (*m/z*) (rel. intensity): 236 (M^+) (13), 220 (55), 207 (23), 205 (100), 191 (13), 180 (24), 177 (33), 165 (32), 149 (16); ^1H NMR ($CDCl_3$) δ 1.4234 (*s*, 6H, 2 × CH_3), 1.7879 (*t*, 2H, J = 6.6 Hz, =C−CH_2−CH_2−), 2.0450 (*s*, 3H, =C−CH_3), 2.4533 (*t*, 2H, J = 6.6 Hz, =C−CH_2−CH_2−) and 4.5175 (*d*, 2H, J = 5.2 Hz, =C−CH_2−OH). On shaking with D_2O the doublet at 4.5175 ppm changed to a singlet peak suggesting that it was bound to an OH.

Hydrolysis of the spirodimer of 1. To the spirodimer of **1** (12 mg) in ethanol/chloroform (3:1, v/v, 2 mL) was added hydrochloric acid in ethanol (150 μL of 1M) and the solution was left at room temperature for 30 min with occasional stirring. The solution was then diluted with

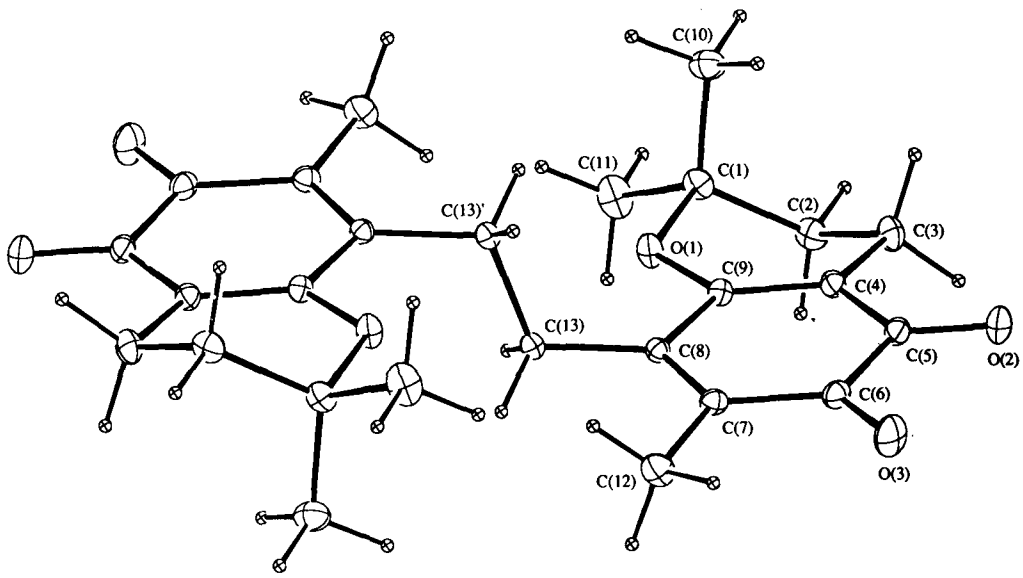


FIG. 1. X-ray crystal structure of 1,2-*bis*(2,2,7-trimethylchroman-5,6-dione-8-)ethane (19).

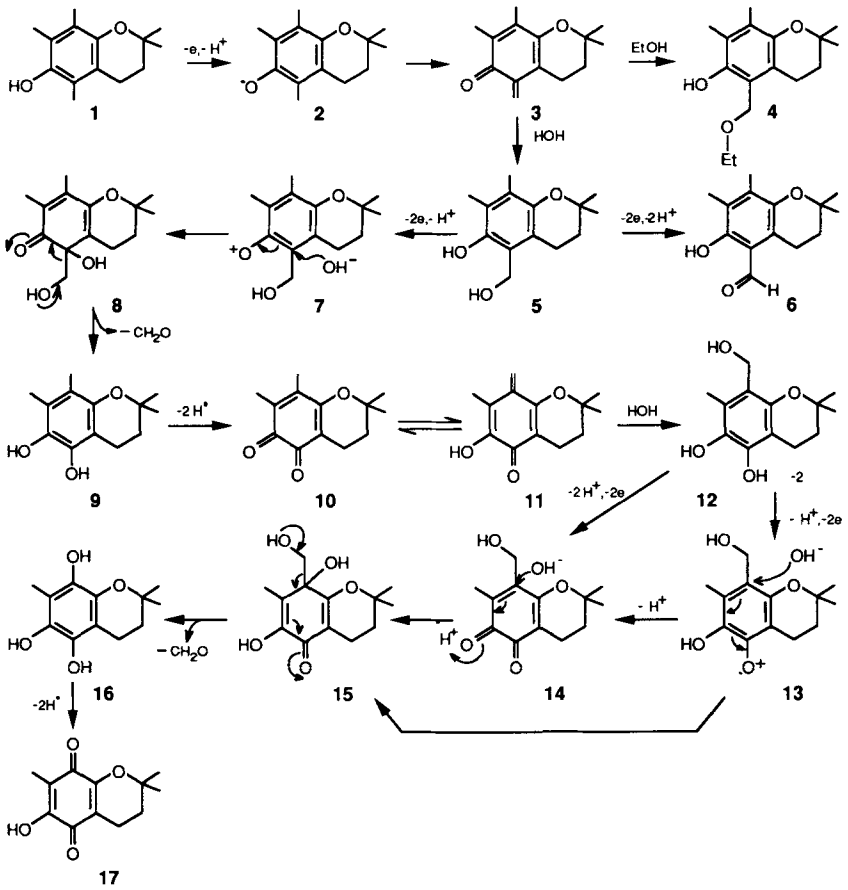
petroleum hydrocarbon (25 mL), washed with water (2 × 10 mL) and the solvent removed *in vacuo*. The residue was chromatographed on thin layers of silica gel GF_{254} (solvent, petroleum hydrocarbon (b.p. 60–80°C)/ethyl acetate 9:1, v/v) yielding bands with R_f values of 0.00 (1.6 mg), 0.05 (6.7 mg) and 0.56 (starting material, 4.0 mg). The compounds with R_f values of 0.00 and 0.05 were combined and rechromatographed on thin layers of silica gel (solvent, chloroform/methanol, 24:1, v/v) to give bands with R_f values of 0.50 and 0.72. Compound R_f 0.72 was identified as 1-(2,2,7,8-tetramethyl-6-chromanol-5-)2-[2-(3-methyl-3-hydroxybutyl)-5,6-dimethyl-1,4-benzoquinone-3-]-ethane (**23**). It had UV λ_{max} (hexane): 263, 270, 294 nm; IR (KBr) (cm^{-1}) 3463 (OH), 2976, 2932 (CH_3), 1726, 1646 (C=C–C=O), 1454, 1384, 1266, 1227, 1170, 1128, 1111, 1091, 1035, 930, 905, 759, 729; MS (E.I.) (*m/z*) (rel. intensity) 454 [M^+] (10), 436 (49), 421 (14), 380 (8), 337, (8), 324 (8), 311 (8), 260 (10), 219 (100); ^1H NMR ($CDCl_3$) δ 1.29 (*s*, 12H, 4 × CH_3), 1.57 (*m*, 2H, =C–CH_2–CH_2–), 1.81 (*t*, 2H, Ar–CH_2–CH_2–, J = 6.8 Hz), 2.04 (*s*, 3H, =C–CH_3), 2.06 (*s*, 3H, =C–CH_3), 2.11 (*s*, 3H, Ar–CH_3), 2.20 (*s*, 3H, Ar–CH_3), 2.65 (*m*, 6H, =C–CH_2–CH_2– & =C–CH_2–CH_2–Ar), 2.78 (*t*, 2H, Ar–CH_2–CH_2–, J = 6.8 Hz), 6.01 (*s*, 1H, OH); ^{13}C NMR ($CDCl_3$) δ 188.53, 187.42, 145.66, 145.23, 144.87, 142.92, 141.17, 140.75, 123.85, 122.86, 122.33, 116.11, 72.58, 71.09, 43.29, 33.12, 29.73, 29.25, 29.25, 26.79, 26.79, 26.17, 21.54, 20.45, 12.45, 12.45, 12.37, 11.95.

Compound R_f 0.5 was identified as 1,2-*bis*[2-(3-methyl-3-hydroxybutyl)-5,6-dimethyl-1,4-benzoquinone-3-]ethane (**24**). Compound **24** had the following spectra data: UV λ_{max} (hexane): 263 nm; IR (KBr) (cm^{-1}) 3396, (OH), 2974, 2930, 2859 (CH_3), 1737, 1648 (C=C–C=O), 1622, 1463, 1448, 1382, 1359, 1294, 1282, 1195, 1147, 1110, 1033, 1007, 941, 888; MS (E.I.) (*m/z*) (rel. intensity) 470 [M^+] (10), 452 (100), 436 (23), 419 (27), 394 (23), 379 (59), 363 (13), 335 (20), 323 (29), 322 (26), 232 (35), 219 (63), 203 (26), 189 (24), 177 (33); ^1H NMR ($CDCl_3$) δ 1.34 (*s*, 12H, 4 × CH_3), 1.67 (*m*, 4H, 2 × =C–CH_2–CH_2–), 2.012 (*s*, 6H, 2 × =C–CH_3), 2.018 (*s*, 6H, 2 × =C–CH_3), 2.56 (*s*, 4H, =C–CH_2–CH_2–C=), 2.73 (*m*, 4H, 2 × =C–CH_2–CH_2–); ^{13}C NMR ($CDCl_3$) δ (each resonance, 2 carbons) 187.86, 187.41, 145.74, 142.57, 141.10, 140.66, 70.71, 43.67, 29.07, 29.07, 26.81, 21.65, 12.43, 12.37.

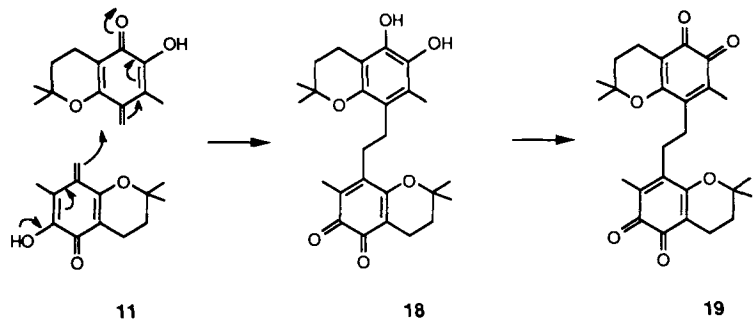
RESULTS AND DISCUSSION

The structure of **14** is tentative since there was insufficient material for a long-range, proton-carbon correlation spectrum which would have fixed the position of the alcohol group. However comparison of the ^1H NMR spectra of **10** and **19** with that of **14** suggests the structure which has been proposed.

Formation of **10** and **17** may occur as outlined in Scheme 1. Oxidation of **1** leads progressively to the chromanoxy radical **2** and the quinone methide **3** (15,16). It seems likely that **3** results from the disproportionation of two molecules of **2** (17). The quinone methide **3** may add ethanol to form **4** (8), the second most abundant product, or water to form **5** (18). The water probably comes from the "absolute ethanol" which contains 0.5% (1.1 mmol). Oxidation of **5** may produce the aldehyde **6**, which is stable and is found as one of the products, or it may form the phenoxylium ion **7**. Addition of OH^- (from water) to **7** could then form **8**. Conversion of **8** to 2,2,7,8-tetramethyl-5,6-dihydroxychroman (**9**) with the loss of formaldehyde is a typical retro-aldol type of reaction where the driving force is the reestablishment of aromaticity in **9**. Compound **9** contains the very labile catechol structure and would be oxidized rapidly to **10**, the major product. This pathway has been proposed previously for the C-demethylation of methyl phenols (19) and is analogous to the C-demethylation of methyl quinones by amines (20). Recent results have shown that compound **4** can also be oxidized



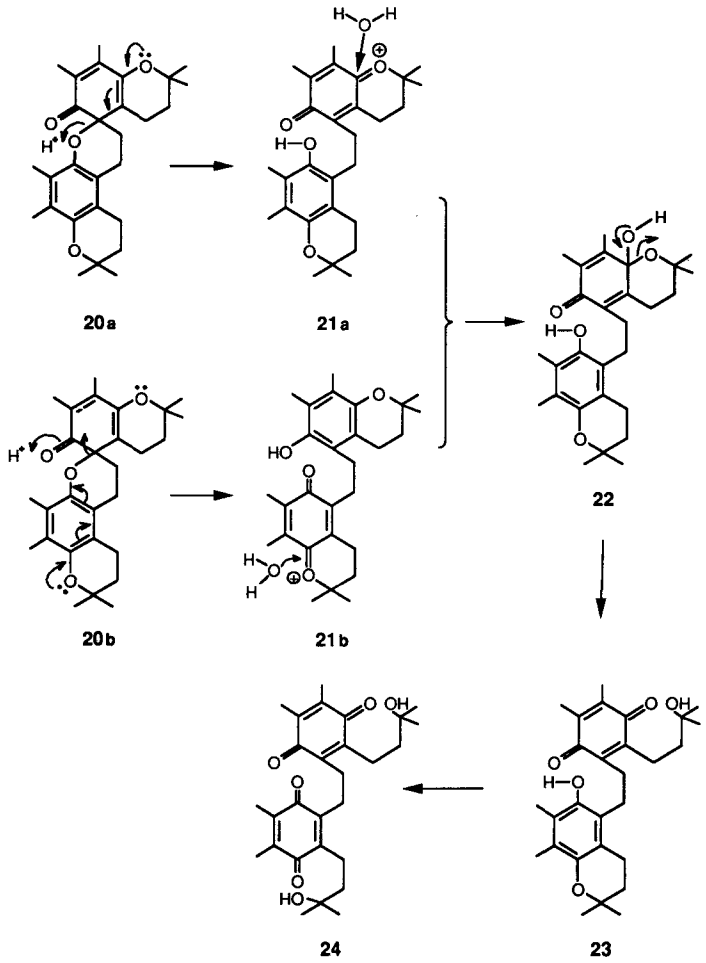
SCHEME 1



SCHEME 2

to **10**, though not as rapidly as can **5** (Kohar, I., and Southwell-Keely, P.T., unpublished results). Thus an alternative, but strictly analogous, pathway (not shown) may be proposed in which **4** substitutes for **5** and either the diethylacetal of formaldehyde or formaldehyde plus ethanol is eliminated instead of formaldehyde itself. In fact it seems likely that both pathways operate simultaneously.

Conversion of **10** into **17** appears to be a repetition of the formation of **10**. Enolization of **10** would produce the *para* quinone methide **11** which could add water as before to form the hydroxymethyl catechol **12**. Oxidation of **12** to 8-hydroxymethyl-2,2,7-trimethylchroman-5,6-dione (**14**) and addition of water would form **15**. Loss of formaldehyde from **15**, as above, would give the trihydroxychroman **16** which could be oxidized readily to **17**. An alternative route from **10** to **17** may involve the production of a second phenoxylium ion **13**. Compound **13** could then either lose



SCHEME 3

a proton to form **14** or add OH^- to form **15**. Compound **15** could then lose formaldehyde as before. Whether **12** goes directly to **14** or first to **13** and then to **14**, or to **13** and then to **15**, remains to be proved. The fact that **14** has been isolated suggests the pathway **12-13-15** may not be a major one.

Formation of **19** (Scheme 2, Fig. 1) may be readily understood as following the enolization of **10** to **11** and its dimerization to **18**, a reaction typical of quinone methides (21). Oxidation of **18** would produce **19**.

Attempted purification of **20** by repeated chromatography on silica gel (solvent, petroleum hydrocarbon/carbon tetrachloride/ethyl acetate, 16:9:1, v/v) failed to produce a single spot and gave, in addition to **20**, an orange band at R_f 0.0. Rechromatography of the R_f 0.0 band (solvent, chloroform/methanol, 24:1, v/v) yielded an orange band at R_f 0.58 whose UV and mass spectra suggested that it was chromanol quinone dimer. It was believed that such a compound could originate by hydrolysis of **20** on the slightly acidic surface of the silica gel. In order to test this hypothesis, **20** was deliberately hydrolyzed in acidic ethanol/chloroform solution. Two compounds were isolated from the hydrolysis, **23**, which was identical to that isolated from the TLC plate, and **24**. The α-tocopherol analogue of **23** has been isolated previously although structural assignment was based on an approximate molecular weight plus UV and IR spectra data (22).

Formation of **23** and **24** may be understood as the direct consequence of an acid catalyzed ring opening of **20** (Scheme 3). Protonation of **20** may occur on the spiropyran oxygen (**20a**) or on the carbonyl oxygen (**20b**) to give the same oxonium ion intermediate (**21a-21b**). Although both sites of protonation are possible, it seems likely that the carbonyl oxygen would be more basic than the spiropyran oxygen particularly because the latter would tend to conjugate with the aromatic ring (23). Addition of water to **21** would form the hemi-ketal **22** which may be thought of as a substituted 8a-hydroxy-α-tocopherone. Compound **22** would be expected to undergo ring opening in the same way as 8a-hydroxy-α-tocopherone and to form **23** (24). Oxidation of **23** would produce **24**.

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METHOD

Lipid Hydroperoxide Measurement by Oxidation of Fe^{2+} in the Presence of Xylenol Orange. Comparison with the TBA Assay and an Iodometric Method

Zhen-Yue Jiang, Alison C.S. Woollard and **Simon P. Wolff***
Toxicology Laboratory, Department of Clinical Pharmacology, University College and Middlesex School of Medicine, London WC1E 6JJ, United Kingdom

Study of the role of hydroperoxides and lipid peroxidation in disease requires simple and sensitive methods for direct hydroperoxide measurement. We report on a technique for measuring hydroperoxide which relies upon the rapid hydroperoxide-mediated oxidation of Fe^{2+} under acidic conditions. Fe^{3+} forms a chromophore with xylenol orange which absorbs strongly at 560 nm, yielding an apparent E_{560} (for H_2O_2, butyl hydroperoxide and cumene hydroperoxide) of 4.3×10^4 M^{-1} cm^{-1}. The assay was validated in a study of liposomal lipid peroxidation and shown to give results comparable with those obtained by an iodometric method or by measuring conjugated dienes. The assay involving thiobarbituric acid, by comparison, underestimates lipid peroxidation and does not measure hydroperoxide *per se*.
***Lipids* 26, 853–856 (1991).**

Lipid peroxidation has been proposed to contribute to various pathophysiological cell and tissue abnormalities. The assessment of oxidative damage in tissue exposed to oxidative stress has been difficult to achieve by direct measurement of oxidative alterations, such as hydroxyl radical damage, or accumulation of lipid peroxidation products (1,2). Methods for the direct determination of hydroperoxides (*e.g.*, based on iodine oxidation, enzymatic or non-enzymatic catalytic oxidation of dyes, or coupled oxidation of NADPH) can often be insensitive and unreliable, particularly if they require strict anoxia (3–5). Methods for measuring hydroperoxide using initiation of the cyclooxygenase reaction (6) or microperoxidase-catalyzed isoluminol luminescence (7) have the potential for excellent sensitivity, but can be subject to various confounding factors, in the former case, and require specialized equipment and materials in the latter. Also, doubts exist about the usefulness and general validity of the widely employed thiobarbituric acid (TBA) assay for lipid peroxidation (8–14).

We have previously modified a method for measuring hydroperoxide initially described by Gupta (see ref. 15) which involves the oxidation of Fe^{2+} by hydroperoxides at low pH in the presence of the Fe^{3+}-complexing dye xylenol orange [*o*-cresolsulfonphthalein-3′,3″-*bis*(methyliminodiacetic acid sodium salt)]. We used this ferrous oxidation/xylenol orange method (which we term FOX) in a study of nanomolar H_2O_2 formation during transition metal-catalyzed glucose oxidation (16). We adapted the method for the study of lipid peroxidation and found that it can be used to quantify lipid hydroperoxides at the nanomolar level. The method is simple and straightforward.

EXPERIMENTAL PROCEDURES

Ammonium ferrous sulfate, hydrogen peroxide, glutathione (GSH), cumene hydroperoxide, butyl hydroperoxide, butylated hydroxytoluene (BHT) and thiobarbituric acid (TBA) were obtained from Sigma (Poole, Dorset, U.K.) or Aldrich (Gillingham, Dorset, U.K.). Phosphatidylcholine (egg yolk, Sigma Type V-E), catalase (Type C-40), glutathione peroxidase (bovine erythrocyte Type G6137) and phospholipase A_2 (porcine pancreas Type P6534) also were obtained from Sigma. Xylenol orange [*o*-cresolsulfonphthalein-3′,3″-bis(methyliminodiacetic acid sodium salt)] was obtained from Aldrich. The fatty acid composition of the phosphatidylcholine used was given by the manufacturer as 16:0 (31.6%), 18:0 (13.4%), 18:1 (29.7%), 18:2 (15.5%), 20:4 (4.3%) and total minor components (5.5%).

Preparation of liposomes and liposome incubations. Two mL of phosphatidylcholine (100 mg/mL) were dried under nitrogen and then hydrated in 10 mL of glucose-free phosphate-buffered saline (10 mM, pH 7.4; PBS) in the dark for 1 hr. The resulting dispersion was ultrasonicated for 3 min while cooling on ice (17). The small liposomes were then incubated at a final concentration of 10 mg/mL in PBS with respective agents in a shaking water bath at 37°C. At defined time intervals, 100 μL of the incubation mixture was removed and 900 μL methanol was added to dissolve the dispersion.

Measurement of stable products of lipid peroxidation by thiobarbituric acid assay and measurement of conjugated dienes. Aldehyde products of lipid peroxidation were measured using TBA. Methanol-solubilized liposomal incubation mixture (200 μL) was incubated with 800 μL of 0.67% TBA (adjusted to pH 4) at 100°C for 5 min. The reaction was complete at this point as judged by comparison with results obtained for malondialdehyde (MDA), prepared by acid hydrolysis of tetramethoxypropane. After cooling, the reaction mixture was centrifuged (12000 × *g*, 10 min) and the absorbance of the supernatant was read at 532 nm. The concentration of MDA was calculated from TBA-reactive material (TBARM) using the extinction coefficient of 1.56×10^5 M^{-1} cm^{-1} given for the MDA-TBA adduct (18). Conjugated diene (CD) concentration was estimated by difference spectroscopy of the methanol extract using an extinction coefficient of 2.8×10^4 M^{-1} cm^{-1} at 233 nm for the methanol extract (19).

*To whom correspondence should be addressed at: Toxicology Laboratory, Dept. of Clinical Pharmacology, University College and Middlesex School of Medicine, 5 University St., London, WC1E 6JJ, U.K.

Abbreviations: BHT, butylated hydroxytoluene; CD, conjugated diene; DTPA, diethylenetriaminepentaacetic acid; FOX, ferrous oxidation/xylenol orange; GSH, reduced gluthathione; MDA, malondialdehyde; PBS, phosphate-buffered saline; TBA, thiobarbituric acid; TBARM, TBA-reactive material; TCA, trichloroacetic acid.

Iodometric determination of hydroperoxide. Hydroperoxide content of liposomes was also determined by an established iodometric method (3). Solution A consisted of a mixture of HPLC-grade methanol (British Drug Houses, Poole, Dorset) and glacial acetic acid (Sigma) (1:1, v/v) containing 1 mg/mL of ethylenediaminetetraacetic acid (disodium salt) degassed with a stream of nitrogen for 20 min. Solution B consisted of HPLC-grade methanol degassed with N_2 for 20 min followed by addition of 20% w/v potassium iodide (Sigma), and degassed for a further 20 min.

One mL of solution A was added to 1.5 mL of solution B in a 3-mL cuvette adapted to permit degassing with nitrogen as well as addition of small volumes of fluid under anoxic conditions. The cuvette contents were treated with N_2 for 5 min and the cuvette was sealed. Absorbance was monitored at 290 nm for 4 min. The solution was discarded if absorbance increased by more than 0.005 AU. A small volume (1–10 μL) of an N_2-treated standard hydroperoxide solution, or methanol-solubilized liposomes, was then injected. The cuvette contents were gently mixed by careful agitation. Absorbance at 290 nm was monitored for 5 min after which time no further absorbance change was observed. A standard curve generated with H_2O_2 gave an extinction coefficient for tri-iodide at 290 nm of 4.7×10^4 M^{-1} cm^{-1}, in good agreement with the value published previously.

Composition of the hydroperoxide-measuring reagent (FOX). The FOX assay described previously (16) requires modification for measurement of lipid hydroperoxides. Sorbitol (which was previously added to stimulate chain-oxidation of Fe^{2+}) causes extensive peroxidation of lipid in the assay itself leading to a false signal (data not shown). This undesirable chain oxidation was prevented by omission of sorbitol and inclusion of the lipid-soluble chain-breaking antioxidant butylated hydroxytoluene (BHT). One hundred μL of methanol-solubilized sample was added to 900 μL of the following reaction mixtures and incubated for 30 min at room temperature prior to measurement at 560 nm: 100 μM xylenol orange, 250 μM Fe^{2+}, 25 mM H_2SO_4, and 4 mM BHT in 90% (v/v) methanol. The reaction is essentially complete after 15 min and the color is stable overnight at room temperature. The apparent extinction coefficients (estimated by curve fitting to a first-degree function) for H_2O_2, cumene hydroperoxide and butyl hydroperoxide at 560 nm were found to be 4.3×10^4 M^{-1} cm^{-1} with little difference in the color yields for the different hydroperoxides. The actual extinction coefficient of the Fe^{3+}-xylenol orange complex at 560 nm (the absorbance maximum) was determined using freshly-made acidic solutions of ferric chloride and determined to be 1.5×10^4 M^{-1} cm^{-1}, indicating that there is some chain oxidation of ferrous by hydroperoxide (approximately 3 mol ferrous ion per mol hydroperoxide) in the assay system.

Unless otherwise indicated, the data are presented as the mean ± standard deviation of duplicate/triplicate determinations and are representative of at least two experiments.

RESULTS AND DISCUSSION

Application of FOX to studies of lipid peroxidation and comparison with TBA and conjugated diene measurements. Figure 1 shows results obtained by application of FOX to the measurement of liposome peroxidation, and compares the results with those obtained using the TBA assay and conjugated diene (CD) measurement. In all instances, peroxidation was catalyzed by Cu^{2+} and inhibited by BHT (Fig. 1). Assuming that lipid hydroperoxides derived from phosphatidylcholine have an apparent extinction coefficient of approximately 4.3×10^4 M^{-1} cm^{-1} (the mean value for those obtained for H_2O_2, butyl hydroperoxide and cumene hydroperoxide), and using an extinction coefficient for the MDA-TBA adduct of 1.56×10^5 M^{-1} cm^{-1}, it is clear that the measurement of TBARM greatly underestimates the extent of lipid peroxidation (Fig. 1). The assumption that the apparent extinction coefficient of the lipid hydroperoxide can be derived directly from that found for the standard hydroperoxides is supported by the close agreement of the zero time CD and lipid hydroperoxide measurements (Fig. 1). After initiation of lipid peroxidation, however, hydroperoxide content increases twice as rapidly as CD content. Hydroperoxide formation in the presence of Cu^{2+} thus appears to occur by a chemical pathway which is antagonistic to CD formation (9).

Confirmation of liposome hydroperoxide content. The material reactive with FOX in the liposomes was established as lipid hydroperoxide by pre-treatment with enzymes known to be involved in hydroperoxide metabolism. To a sample of liposome (pre-incubated with Cu^{2+}) were added catalase alone; catalase; glutathione (GSH) and glutathione peroxidase; catalase, GSH, glutathione peroxidase and phospholipase A_2; or phospholipase A_2 alone (Fig. 2). The yields of FOX-reactive and TBA-reactive material were then compared. Catalase produced an approximately 29% decrease in the yield of color in the FOX assay which indicates the formation of some H_2O_2 during lipid peroxidation. This compares with an 88% decrease when catalase was added together with GSH and glutathione peroxidase. Phospholipase A_2 did not further decrease the color in the presence of these enzymes, but increased it slightly (13%) when present alone, probably due to the presence of contaminating lipoxygenase (Fig. 2). GSH in the absence of glutathione peroxidase caused a slight increase in color yield in the FOX assay at its final concentration (10 μM) as compared to the control (not shown). It is surprising that a combination of glutathione peroxidase and GSH caused a decrease in hydroperoxide signal in the absence of phospholipase because esterified lipid hydroperoxides are generally regarded to be poorly metabolized by glutathione peroxidase in the absence of phospholipase (20). However, esterified hydroperoxides are poor substrates for glutathione peroxidase as opposed to being non-substrates (21). There is also the possibility that the liposomes contained a high proportion of highly peroxidizable, non-esterified polyunsaturated fatty acid.

Hydroperoxides are not precursors of TBARM in the TBA assay. The data show that the FOX assay measures predominantly lipid hydroperoxide. In contrast, the level of malondialdehyde (Fig. 2) was not affected by pretreatment with the enzymes, with the exception of a slight increase produced by phospholipase A_2. Essentially the same results were obtained when the TBA was incubated at 100°C with an acidified methanol extract of the liposomes, or when the liposome incubation mixture was

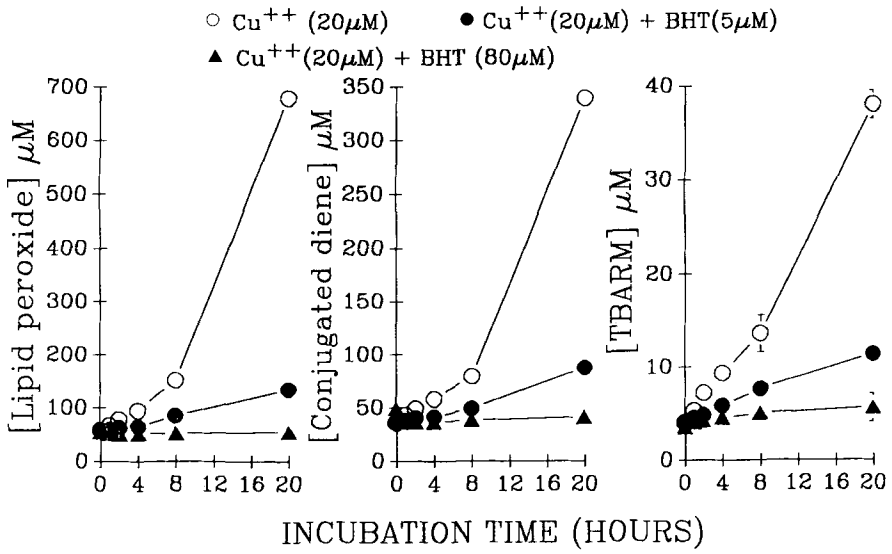


FIG. 1. The accumulation of hydroperoxide and TBA-reactive material in liposomes. Liposomes (10 mg/mL) were incubated with 20 μM Cu^{2+} alone or in the presence of BHT (5 μM or 80 μM) in PBS at 37°C for 20 hr. Samples were taken at zero time (and at various time intervals up to 20 hr) and analyzed for hydroperoxide content. The liposome incubation mixture was diluted 1:4 with methanol and centrifuged at 12,000 × *g* for 2 min at room temperature. TBARM, conjugated dienes and hydroperoxides were measured as described in Materials and Methods.

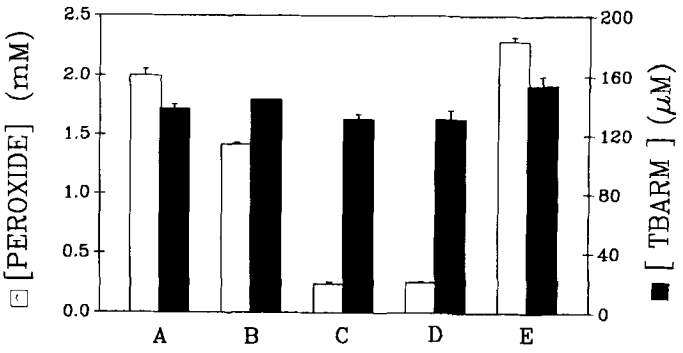


FIG. 2. Enzyme treatment of hydroperoxide and TBA-reactive material in liposomes. Liposomes (preincubated at a concentration of 10 mg/ML for 24 hr with 20 μM potassium phosphate, pH 7.4) were exposed at a concentration of 1 mg/mL in PBS for 20 min at 37°C to (B) catalase (10 units/mL); (C) catalase (10 units/mL, GSH (500 μM) and glutathione peroxidase (5 units/mL); (D) catalase (10 units/mL), glutathione (500 μM), glutathione peroxidase (5 units/mL) and phospholipase A_2 (5 units/mL); or (E) phospholipase A_2 without further additions. (A) represents the control. The phospholipase incubations contained, in addition, 1 mM calcium chloride and 3 mM magnesium chloride. At the end of the incubation period, the samples were diluted 1:4 with methanol and centrifuged at 12,000 × *g* for 2 min. Samples were then analyzed for hydroperoxides and TBARM as described in Materials and Methods.

precipitated with 5% trichloroacetic acid (TCA) prior to boiling of the TCA supernatant with TBA (not shown). Malondialdehyde is thus not formed from hydroperoxide under the conditions of the TBA-assay, as has been postulated (22), but must be present in the sample prior to incubation with TBA.

This suggestion is supported by examination of the rate of decay of hydroperoxide under conditions in which the TBA assay is performed (Fig. 3). When pre-peroxidized liposomes were incubated at 100°C in methanol (adjusted to pH 4) there was a decay in the concentration of material reactive in the FOX assay over a 40-min time period. Consistent with the identity of this material as hydroperoxide, the decay was rapid in the presence of Cu^{2+} but inhibited by BHT and the metal-chelating agent diethylenetriaminepentaacetic acid (DTPA). In contrast, the level of TBARM was unaffected by these treatments (Fig. 3).

Comparison of the FOX assay with an iodometric method. The FOX assay was compared directly with the iodometric method described in Methods and Materials using liposomes incubated in PBS at 37°C for 20 hr in the presence and absence of 10 μM Cu^{2+} (Fig. 4). Assuming that the hydroperoxides present in the incubated liposomes were equally reactive towards iodide and ferrous ions, then the methods are comparable with respect to sensitivity and reproducibility.

We have previously applied this method to the study of lipid peroxidation induced in low density lipoprotein by glucose and have found that measurement of TBARM greatly underestimates hydroperoxide content in this case as well (23). The FOX method also appears suitable for use in more complex biological samples, such as for the

METHOD

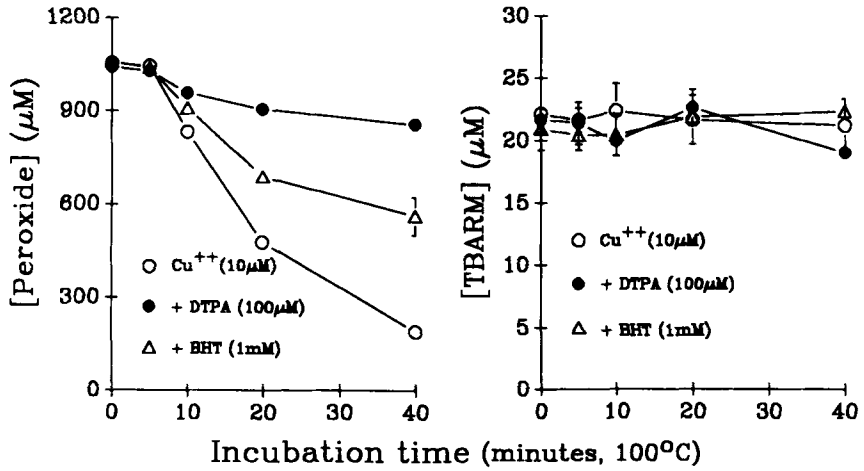


FIG. 3. Hydroperoxides are not precursors of TBARM in the TBA assay. Liposomes (10 mg/mL) were pre-incubated for 22 hr with 10 μM Cu^{2+} in PBS. The liposome incubation mixture was then adjusted to pH 4 and divided into three aliquots. BHT (1 mM) and DTPA (100 μM) were then added separately to two of the aliquots, and the three new liposome incubation mixtures were incubated at 100°C for a total of 40 min. Samples were withdrawn at various time intervals and diluted 1:9 with methanol. Samples were then analyzed for hydroperoxides and TBARM as described in Materials and Methods.

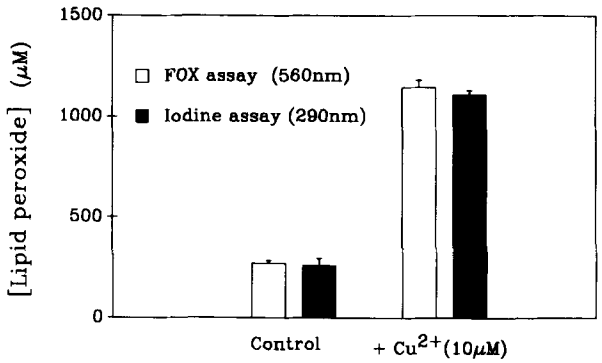


FIG. 4. Comparison of the FOX assay and the iodometric technique. Liposomes (10 mg/mL) were incubated in PBS in the presence or absence of 10 μm Cu^{2+} at 37°C for 20 hr. The samples were then solubilized in methanol and analyzed for material reactive in the FOX assay or the iodometric assay as described in Materials and Methods.

measurement of hydroperoxides in erythrocyte and hepatocyte membranes (Jiang, Z.-Y., Hunt, J.V., Birlouez, I., and Wolff, S.P., unpublished data). However, we find that it is important to perform hydroperoxide recovery experiments in any system of interest and to confirm that the signal is related to hydroperoxide presence using the pre-treatment with enzymes as described above.

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COMMUNICATION

Effects of Perfluorodecanoic Acid on *de novo* Fatty Acid and Cholesterol Synthesis in the Rat

John W. Davis, II[a], John P. Vanden Heuvel[b] and Richard E. Peterson[a,*]
[a]School of Pharmacy and [b]Environmental Toxicology Center, University of Wisconsin, Madison, Wisconsin 53706

Perfluorodecanoic acid (PFDA) is a peroxisome proliferator that causes a dose-dependent (20–80 mg/kg) increase in hepatic triacylglycerol and cholesteryl ester levels in the rat. We hypothesized that PFDA may cause an increase in the *de novo* synthesis of fatty acids and cholesterol in this species, which would explain observed effects. The incorporation of ^3H_2O into tissue lipids was examined 7 days after rats received vehicle or 20 or 80 mg/kg of PFDA. PFDA treatment decreased the rate of synthesis of cholesterol and fatty acids in the liver and in epididymal fat pad. At a PFDA dose (20 mg/kg) that decreased *de novo* synthesis of fatty acids and cholesterol, there was no effect on the concentration of fatty acids and cholesterol in the liver, epididymal fat pads, and plasma. We conclude that PFDA induced fatty liver is due to either a decrease in the oxidation of fatty acids in the liver, or an impairment of triacylglycerol catabolism and/or export from the liver, and is not the result of an increase in *de novo* synthesis of fatty acids and cholesterol.
Lipids 26, 857–859 (1991).

Perfluorodecanoic acid (PFDA) is a straight-chain fatty acid analog representative of the perfluorinated carboxylic acids used industrially as lubricants, wetting agents, plasticizers and corrosion inhibitors (1). We have previously shown that PFDA alters energy utilization (2) and lipid metabolism (3) in the rat. Despite a decrease in caloric intake, PFDA treated rats maintain a relatively high amount of carcass and liver triacylglycerols. There is 17-fold increase in hepatic triacylglycerols in rats treated with 80 mg PFDA/kg body weight when compared to their pair-fed counterparts (3). A similar, but less dramatic increase in cholesteryl esters is observed. This increase in neutral lipids may be due either to decreased catabolism or alternatively to increased synthesis of fatty acids.

This report examines the hypothesis that PFDA causes an increase in the synthesis of fatty acids, which would explain the increase in triacylglycerols and cholesteryl esters observed following treatment with PFDA. The incorporation of ^3H_2O into liver and fat pad lipids was examined 7 days after treating male rats with PFDA. We will demonstrate that PFDA does not increase the synthetic rates of free fatty acids or cholesterol, and that inhibition of fatty acid oxidation is a more likely explanation for the observed alterations in lipid metabolism.

*To whom correspondence should be addressed at School of Pharmacy, University of Wisconsin, 425 N. Charter St., Madison, WI 53706.

Abbreviations: ANOVA, analysis of variation; PFDA, perfluorodecanoic acid; TLC, thin-layer chromatography.

MATERIALS AND METHODS

Chemicals. Perfluorodecanoic acid (PFDA) was purchased from Aldrich Chemical Company (Milwaukee, WI) and was further purified as described previously (4). ^3H_2O (sp. act. 5 mCi/mL), [7(n)-^{3}H]cholesterol (sp. act. 5.5 Ci/mmol), and [9,10(n)-^{3}H]palmitic acid (sp. act. 56 Ci/mmol) were purchased from Amersham (Arlington Heights, IL). All enzymes and cofactors were obtained from Boehringer Corp. (Indianapolis, IN), with the exception of cholesterol oxidase and *o*-dianisidine, which were purchased from Sigma Chemical Co. (St. Louis, MO). Plates for thin-layer chromatography (TLC) and 2′,7′-dichlorofluorescein were purchased from J.T. Baker Inc. (Phillipsburg, NJ). Hionic-Fluor and Soluene-350 were purchased from Packard Instrument Co. (Downers Grove, IL).

Experimental design. Six-week-old male (170–195 g) rats, obtained from Harlan Sprague-Dawley (Indianapolis, IN), were individually housed in suspended stainless-steel cages in a temperature-controlled room with a 12-hr light/dark cycle (lighted 0500–1700 hr). After an acclimation period of one week, rats were administered PFDA (20 or 80 mg/kg) in propylene glycol/water (1/1, v/v; 1 mL/kg, i.p.). All rats were given access to feed (Purina Rat Chow, #5012, Ralston Purina Co., St. Louis, MO) during the meal period (1700–0900 hr). Water was available *ad libitum.*

One week after PFDA treatment, rats were administered ^3H_2O (1 mCi/kg, i.p.). After 60 min, the rats were decapitated and trunk blood was collected in lightly heparinized tubes. The liver and fat pads were removed, weighed, and freeze-clamped. Samples were stored at −70°C until the radioactivity could be quantitated.

Analytical procedures. Total lipids were extracted from the liver, fat pads, and plasma as previously described (5). Simple lipids were separated using a TLC system described by Kelley (6) and visualized under ultraviolet light after plates were sprayed with 2′,7′-dichlorofluorescein (0.1% in methanol). The free fatty acids and cholesterol bands, as determined using authentic standards, were scraped off and counted directly in 10 mL Hionic-Fluor. The synthetic rates of cholesterol and free fatty acids were calculated as described by Andersen and Dietschy (7), and expressed as nmol of ^3H_2O incorporated per g of tissue per hr (nmol/g/hr). The use of ^3H_2O to estimate lipid synthetic rates in the rat has been validated by several investigators (8–10). Incorporation of ^{3}H from ^3H_2O into lipids has been demonstrated to give accurate lipid synthetic rates because the specific activity of body water can be correctly determined as the precursor pool does not change (10). Cholesterol was quantitated in the total lipid extract as described by Salè *et al.* (11). Free fatty acids were measured using a sensitive micro method (12).

Statistical analysis. Differences between treatment groups were analyzed using one-way analysis of variation

(ANOVA) and, if the ANOVA was significant, differences between the means were evaluated by Dunnett's t-test. Significance was set at the $p<0.05$.

RESULTS AND DISCUSSION

The effects of PFDA treatment on body weight change and tissue weights are shown in Table 1. During the 7-day posttreatment period, rats receiving 20 mg/kg of PFDA gained slightly less weight than vehicle control rats. Rats treated with 80 mg/kg of PFDA showed a significant decrease in body weight. Relative liver weight was increased by both doses of PFDA. Although there was a slight increase in absolute liver weight, the weights were not significantly different from controls.

Hepatomegaly and alterations in hepatic lipid metabolism are sensitive indices of PFDA exposure in rats (3,13, 14). PFDA treated rats have increased hepatic triacylglycerol and cholesteryl ester contents, despite a decrease in food intake (3). In addition, PFDA causes alterations in long-chain fatty acid profiles with a relative increase in palmitic and oleic acids (14).

Several explanations for these PFDA induced changes in lipid metabolism are possible, including an increase in the rate of fatty acid synthesis. Increased synthesis of fatty acids has been proposed as the cause of fatty livers in acute ethanol intoxication, phenobarbital treatment, and essential fatty acid deficiency (15). Support for the hypothesis that PFDA treatment increases hepatic fatty acid synthesis is based on the observations that PFDA treatment increases both hepatic triacylglycerol levels (3) and the activity of hepatic malic enzyme [1-malate: $NADP^+$ oxidoreductase (decarboxylating); EC 1.1.1.40] (16). Malic enzyme is a NADPH producing enzyme whose activity has been shown to correlate with lipogenesis (17,18).

Tritium incorporation into free fatty acids and cholesterol of liver and fat pads was much higher than incorporation into triacylglycerol and cholesteryl esters (data not shown). In fact, ^3H incorporation into triacylglycerol and cholesteryl esters within 1 hr was too low to be quantified. Therefore, only the synthetic rates of fatty acids and cholesterol are presented.

The rate of hepatic cholesterol synthesis was not increased by PFDA, as hypothesized, but rather was decreased (Table 2). The rate of hepatic *de novo* synthesis of cholesterol or fatty acids in PFDA treated rats was similarly reduced to about one-third of that of controls. Hepatic cholesterol and fatty acid concentrations were not affected by PFDA treatment (Table 2), except in the high dose PFDA group, where cholesterol was higher.

An increase in fatty acid synthesis in adipocytes and subsequent fatty acid release into the circulation and uptake by the liver may contribute to the hepatomegaly caused by PFDA. For this reason, incorporation of ^3H_2O derived label into fatty acid and cholesterol was examined in epididymal fat pads (Table 3). We found that the rate of cholesterol synthesis in the fat pads was significantly lower in PFDA treated rats, and that the rate of fatty acid synthesis in this tissue followed a similar pattern. However, the levels of cholesterol and free fatty acids in epididymal fat pads were not affected by PFDA treatment.

PFDA had little effect on the concentrations of cholesterol and free fatty acids in plasma (Table 4). Rats receiving 20 mg/kg of PFDA had a lower plasma cholesterol concentration; however, the difference was not significant. The amount of ^3H_2O label incorporated into fatty acids and cholesterol of plasma was not affected by either dose of PFDA (data not shown).

We conclude that the PFDA induced increase in hepatic triacylglycerol and cholesterol concentration in rats treated with PFDA is not caused by an increase in *de novo* synthesis of fatty acids or cholesterol in hepatocytes or adipocytes. Hepatic, epididymal fat pad and plasma concentrations of fatty acids and cholesterol were not altered by 20 mg PFDA/kg, and hepatic cholesterol concentration was increased at 80 mg PFDA/kg. Therefore, *de novo* synthesis of fatty acids and cholesterol is decreased while their tissue concentrations either remain constant or are increased. We conclude that the most likely cause of PFDA induced fatty liver is either a decrease in hepatic fatty acid oxidation and/or a decrease in hepatic VLDL export. PFDA induced fatty liver does not result from increased synthesis of fatty acids, even though the activity of the lipogenic enzyme, malic enzyme, is increased by PFDA (16).

TABLE 1

Effects of PFDA Treatment on Body, Liver and Epididymal Fat Pads Weight[a]

Treatment	Body weight change (g)[b]	Liver weight		Fat pads weight	
		Absolute (g)	Relative[c] (g/kg)	Absolute (g)	Relative[c] (g/kg)
Vehicle	+17 ± 1	8.8 ± 0.5	33.4 ± 1.6	1.8 ± 0.1	7.2 ± 0.2
PFDA (20 mg/kg)	+12 ± 5	10.9 ± 0.2	42.5 ± 1.2[d]	1.8 ± 0.2	6.7 ± 0.8
PFDA (80 mg/kg)	−59 ± 10[d]	10.4 ± 0.8	56.4 ± 2.8[d]	1.5 ± 0.1	8.3 ± 0.3

[a]Rats were sacrificed 7 days after i.p. administration of vehicle or PFDA. Each value is mean ± SE (n = 4).

[b]Change in absolute body weight from time of vehicle or PFDA treatment (day 0) until rat was sacrificed (day 7).

[c]Organ weights expressed relative to whole body weight.

[d]Significantly different from vehicle control ($p<0.05$).

TABLE 2

Effects of PFDA Treatment on the Rate of Synthesis and Concentration of Fatty Acids and Cholesterol in the Liver[a]

Treatment	Hepatic synthetic rate (nmol/g liver/hr)		Hepatic concentration (μmol/g liver)	
	Cholesterol	Fatty acid	Cholesterol	Fatty acid
Vehicle	16.9 ± 2.8	36.9 ± 10.3	3.45 ± 0.17	2.12 ± 0.13
PFDA (20 mg/kg)	5.8 ± 1.1[b]	12.7 ± 3.3[b]	3.08 ± 0.09	1.97 ± 0.21
PFDA (80 mg/kg)	5.2 ± 1.2[b]	10.3 ± 3.9[b]	5.40 ± 0.79[b]	2.29 ± 0.78

[a]Rats were sacrificed 7 days after i.p. administration of vehicle or PFDA. Each value is mean ± SE (n = 4).

[b]Significantly different from vehicle control ($p<0.05$).

TABLE 3

Effects of PFDA Treatment on the Rate of Synthesis and Concentration of Fatty Acids and Cholesterol in Epididymal Fat Pads[a]

Treatment	Fat pad synthetic rate (nmol/g fat pad/hr)		Fat pad concentration (μmol/g fat pad)	
	Cholesterol	Fatty acid	Cholesterol	Fatty acid
Vehicle	29.2 ± 6.2	30.5 ± 3.9	7.13 ± 1.71	2.12 ± 0.47
PFDA (20 mg/kg)	6.6 ± 2.0[b]	5.6 ± 1.1[b]	7.47 ± 1.27	1.49 ± 0.09
PFDA	8.5 ± 2.3[b]	7.0 ± 1.7[b]	9.38 ± 1.74	1.21 ± 0.38

[a]Rats were sacrificed 7 days after i.p. administration of vehicle or PFDA. Each value is mean ± SE (n = 4).

[b]Significantly different from vehicle control ($p<0.05$).

TABLE 4

Effects of PFDA Treatment on Cholesterol and Fatty Acid Concentration in Plasma[a]

Treatment	Plasma concentration (μmol/mL plasma)	
	Cholesterol	Fatty acid
Vehicle	0.46 ± 0.10	0.65 ± 0.12
PFDA (20 mg/kg)	0.23 ± 0.03	0.68 ± 0.11
PFDA (80 mg/kg)	0.48 ± 0.03	0.75 ± 0.21

[a]Rats were sacrificed 7 days after administration of vehicle or PFDA. Each value is mean ± SE (n = 4).

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ARTICLE

Transient Activation of 1-*O*-Alkyl-*sn*-glycero-3-phosphocholine: Acetyl-CoA Acetyltransferase During the Incubation of Macrophages

Takayuki Sugiura*, Teruo Fukuda, Neng-neng Cheng and Keizo Waku
Faculty of Pharmaceutical Sciences, Teikyo University, Sagamiko, Kanagawa 199-01, Japan

The activity of the platelet-activating factor (PAF)-synthesizing enzyme, 1-*O*-alkyl-*sn*-glycero-3-phosphocholine (lysoPAF):acetyl-CoA acetyltransferase (EC 2.3.1.67) in alveolar macrophage lysate was found to be elevated after warming the cells to 37°C. Such an increase in enzyme activity was detectable only when intact cells were warmed. The stimulation was transient, reaching a peak at 2 min, and then gradually decreased to the control level. We could not find increased PAF formation in warmed cells which had increased acetyltransferase activity, even though substantial amounts of lysoPAF were shown to be present within cells. In contrast, considerable amounts of PAF were formed after treatment of the cells with exogenous lysoPAF. These results suggest that the activation of acetyltransferase is not sufficient to induce PAF formation and that the increased availability of substrates, especially lysoPAF, in the cells is indispensable for triggering PAF biosynthesis in this type of cells. *Lipids 26*, 861–865 (1991).

Platelet-activating factor (PAF), originally described as a mediator of acute hypersensitivity (1), is a unique bioactive ether phospholipid whose structure was elucidated in 1979 as 1-*O*-alkyl-2-acetyl-*sn*-glycero-3-phosphocholine (2–4). PAF is known to elicit a variety of biological responses, such as platelet activation, neutrophil and eosinophil activation, and increased vascular permeability and hypotension, and is regarded as an important lipid mediator in various inflammatory and allergic reactions (5,6). Recent evidence specifically suggests that PAF plays a central role in inflammatory or allergic reactions, especially in asthma (7).

Various types of mammalian cells including alveolar macrophages have been reported to produce PAF (8). Several groups have studied the biosynthetic routes for PAF in mammalian tissues (8). Until now, dual synthetic pathways have been proposed, one involving the hydrolysis of 1-*O*-alkyl-2-acyl-*sn*-glycero-3-phosphocholine by phospholipase A_2 to produce lysoPAF followed by the acetylation of this lysophospholipid by acetyltransferase (EC 2.3.1.67), the other involving cholinephosphotransferase (EC 2.7.8.16) using 1-alkyl-2-acetyl-*sn*-glycerol and CDPcholine as substrates. The former pathway is thought to be responsible for the production of PAF in inflammatory cells upon stimulation.

Much attention has been directed to the regulatory mechanism of PAF biosynthesis in white blood cells (9–25). Albert and Snyder (10) suggested that the acetyltransferase activity, which was shown to increase upon stimulation, has a regulatory function in PAF biosynthesis in rat alveolar macrophages. Similar assumptions have been made by various investigators for other cell types (8), although the precise mechanism triggering PAF synthesis is not yet fully understood. Recently, we observed that the addition of lysoPAF (20 μM) *per se* elicits PAF formation in rabbit alveolar macrophages (23) and human polymorphonuclear leukocytes (25) without apparent activation of the acetyltransferase. Thus, the question remains as to the relative importance of the activation of acetyltransferase *versus* increased availability of substrates, such as lysoPAF, in the triggering of PAF biosynthesis.

In the present study we tried to address this question. We found that the enzyme activity of acetyltransferase increased transiently during preincubation of macrophages, although we observed negligible PAF production and negligible increase in lysoPAF levels. The relative importance of substrate availability *versus* activation of acetyltransferase in the induction of PAF synthesis in this type of cells is discussed.

MATERIALS AND METHODS

[1-^{14}C]Acetyl-CoA (50 mCi/mmol) was purchased from ICN Radiochemicals (Irvine, CA). 1-*O*-Hexadecyl-2-[^{3}H]acetyl-*sn*-glycero-3-phosphocholine (GPC) was synthesized from 1-*O*-hexadecyl-GPC and [^{3}H]acetic anhydride (ICN Radiochemicals) in the presence of pyridine. [^{3}H]LysoPAF (1-*O*-[^{3}H]hexadecyl-GPC) was prepared from 1-*O*-[^{3}H]hexadecyl-2-acetyl-GPC (Dupont-New England Nuclear, Boston, MA) by mild alkaline treatment. LysoPAF (1-*O*-hexadecyl-GPC) and acetyl-CoA were from Sigma (St. Louis, MO). PAF (1-*O*-hexadecyl-2-acetyl-GPC) was from Bachem (Bubendorf, Switzerland). A23187 was obtained from Calbiochem (La Jolla, CA) and dissolved in dimethylsulfoxide (DMSO).

Alveolar macrophages were prepared from normal rabbits (Japan white) as described earlier (26). Cells were suspended at 2×10^6 cells/mL in ice-cold 20 mM HEPES-Hanks balanced salt solution (HBSS). In order to examine the effect of warming of cells, the cell suspension (0.5 mL) was warmed for various periods of time in a water bath (37°C) with gentle shaking. The temperature of the cell suspension reached 25°C within 20 sec and 37°C within 45 sec. After warming the cells for the indicated periods (30 sec–5 min), the cells were cooled and sonicated with a Branson Sonifier (setting 3, 20 sec, 50% pulse). In another experiment, the cells were sonicated before warming at 37°C. In order to examine the effect of A23187 on acetyltransferase activity, the cells were first prewarmed to 37°C for 10 min and then challenged with A23187 (final 0.1–2 μM, final DMSO concentration was 0.5%) for 2 min. After the addition of ice-cold HBSS, the

*To whom correspondence should be addressed.

Abbreviations: DMSO, dimethylsulfoxide; GPC, *sn*-glycero-3-phosphocholine; HBSS, Hanks balanced salt solution; lysoPAF, lyso platelet-activating factor, 1-*O*-alkyl-*sn*-glycero-3-phosphocholine; PAF, platelet-activating factor, 1-*O*-alkyl-2-acetyl-*sn*-glycero-3-phosphocholine; PMA, phorbol myristate acetate.

cells were centrifuged at 1200 rpm for 6 min, resuspended at 2 × 10^6 cells/mL in HBSS and sonicated. To compare the effects of A23187 and warming, 2 min-warmed cells (without prewarming and without A23187) were also centrifuged, resuspended and sonicated. Acetyltransferase activity was measured at 37°C, using 20 μM lysoPAF, 100 μM [^{14}C]acetyl-CoA (2 × 10^5 dpm) and 0.5 mL cell lysate as described earlier (23). The incubation was carried out for 5 min and the total lipids were extracted by the method of Bligh and Dyer (27). The radioactivity recovered in the chloroform layer was measured and the enzyme activity was calculated as described in (23,25).

In some experiments, the amount of PAF produced by intact cells was determined. After the extraction of total lipids, the PAF fraction was separated by thin-layer chromatography (TLC) using chloroform/methanol/water (65:35:6, v/v/v) as developing solvent and quantified by bioassay with washed rabbit platelets, using hexadecyl PAF as a standard (28). The lysoPAF content was also measured by bioassay following the chemical acetylation (12) of the 1-radyl-GPC fraction which was treated in advance with 0.5 N NaOH and then with 0.5 N HCl.

RESULTS

Figure 1 shows the effect of warming the cells on the activity of acetyltransferase as a function of time. We found that the enzyme activity of acetyltransferase in cell lysate rapidly increased when the intact cells were warmed to 37°C (Fig. 1 (a)). Similar activation was observed when ^{3}H-labeled lysoPAF (20 μM) and acetyl-CoA (100 μM) were used instead of unlabeled lysoPAF and ^{14}C-labeled acetyl-CoA. This increase in enzyme activity was transient, reaching a peak at 2 min, and then acetyltransferase activity gradually declined to control levels. By contrast, only a gradual decline in enzyme activity was observed when cells were sonicated prior to warming (Fig. 1 (b)).

The effects of warming and A23187-treatment of cells on the activity of acetyltransferase are compared in Figure 2. After warming the cells for 2 min followed by the centrifugation and washing, the acetyltransferase activity in cell lysate was still elevated *ca.* 2-fold over controls. Similar activation was observed for A23187- (0.5 μM and 2μM) treated cells, although the increase was less prominent when a low concentration of ionophore (0.1 μM) was used. In any event, the observation that warming the cells and treatment with A23187 resulted in a similar activation of acetyltransferase prompted us to examine PAF production in these cells; it is assumed that the activation of acetyltransferase is a key event in the regulation of PAF biosynthesis in several types of stimulated cells.

Figure 3 shows the amounts of PAF (cell-associated plus released) in warmed (a) and A23187-treated (b) macrophages. Cells immediately after preparation from lung contain a small amount of PAF (a), which may have been formed during cell preparation or when handling animals. However, we did not find any PAF production following the warming of the cells at 37°C. Only a gradual decrease in the PAF level was observed during the incubation. In contrast, cells produced large amounts of PAF if challenged with A23187 (0.5 μM and 2 μM)(b). The production was detectable within 1 min after stimulation and the PAF level increased almost linearly at least up to 4 min. Thus,

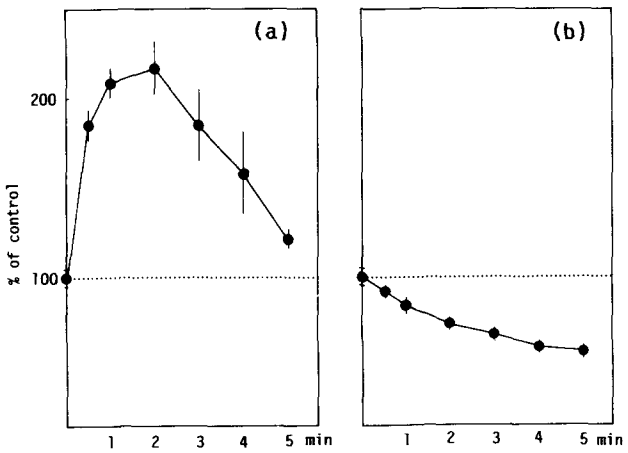


FIG. 1. Effects of warming of intact cells (a) and lysed cells (b) on the activity of acetyltransferase. Cells suspended in ice-cold HBSS were incubated in a water bath (37°C) for the indicated periods of time prior to (a) or after (b) sonication. Cell lysate was then incubated with 100 μM lysoPAF and 20 μM [^{14}C]acetyl-CoA at 37°C for 5 min. Acetyltransferase activity was assessed as described in Materials and Methods. Each value (% of control) is the mean ± SD from three to four determinations. The enzyme activities for controls were (a) 1.13 nmol/5 min/10^6 cell lysate and (b) 1.18 nmol/5 min/10^6 cell lysate.

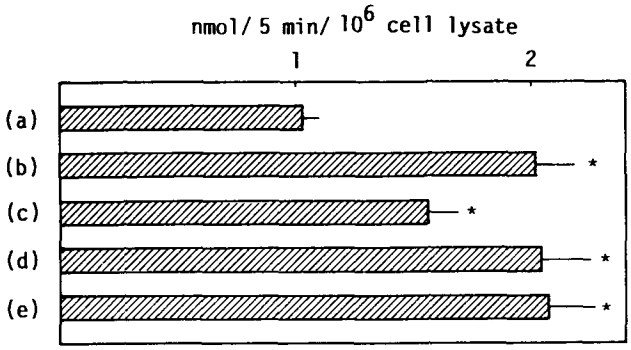


FIG. 2. Comparison of effects of warming and A23187-treatment of cells on cellular acetyltransferase activity. (a) Cells suspended in ice-cold HBSS were washed once with ice-cold HBSS by centrifugation and resuspended in the same solution. (b) Cells suspended in ice-cold HBSS were warmed in a water bath (37°C) for 2 min and then cooled with ice-cold HBSS followed by centrifugation. (c)(d)(e) Cells prewarmed at 37°C for 10 min were further incubated at 37°C in the presence of 0.1 μM (c), 0.5 μM (d) and 2 μM (e) A23187. After 2 min, cells were washed once with ice-cold HBSS. All resuspended cells were sonicated and acetyltransferase activity was measured at 37°C as described in Materials and Methods. Each value is the mean ± SD from five determinations. *P<0.001, compared to (a).

these two cases were quite different in terms of the initiation of PAF synthesis.

We then examined the cellular level of lysoPAF, a substrate for PAF synthesis. As shown in Figure 4, alveolar macrophages contain considerable amounts of lysoPAF. The amounts of lysoPAF did not change markedly during the incubation at 37°C at least for 2 to 10 min. However, the level of lysoPAF increased *ca.* 1.5-fold following stimulation with A23187 for 2 min. The amount of PAF also increased under the same condition as shown in Figure 3. These observations suggest that the increas-

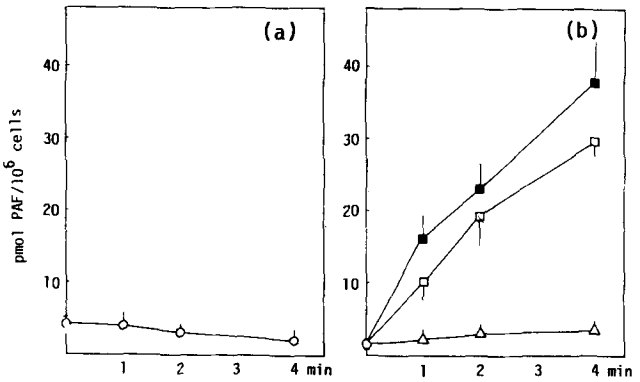


FIG. 3. Amounts of PAF in warmed cells (without preincubation)(a) and in A23187-stimulated cells (b). (a) Cells suspended in ice-cold HBSS were warmed in a water bath (37°C) for the indicated time periods. (b) Cells prewarmed at 37°C for 10 min were further incubated with 0.1 μM (△), 0.5 μM (□) and 2 μM (■) A23187. The incubation was terminated by adding chloroform/methanol (1:2, v/v). PAF levels were determined by bioassay as described in Materials and Methods. Each value is the mean ± SD from four determinations.

TABLE 1

Effects of Acetyl-CoA and LysoPAF on the PAF Level in Alveolar Macrophages[a]

	No addition	+ Acetyl-CoA	+ LysoPAF
	pmol PAF/10^6 cells		
Without preincubation	1.58 ± 0.25 (0)	3.36 ± 0.69[b] (1.78)	41.66 ± 3.97[c] (40.08)
With preincubation	0.51 ± 0.04 (0)	1.44 ± 0.13[c] (0.93)	25.70 ± 3.91[c] (25.19)

[a]Cooled (without preincubation, 4°C) or prewarmed (with incubation, 37°C for 10 min) macrophages (10^6 cells/0.5 mL) were incubated in the presence of acetyl-CoA (100 μM) or lysoPAF (20 μM) at 37°C for 4 min. The incubation was terminated by adding chloroform/methanol (1:2, v/v). The PAF content was estimated as described in Materials and Methods. The values in parentheses represent increments of cellular PAF upon the addition of exogenous substrates. Each value is the mean ± SD from five determinations.

[b]P<0.01, compared to control (no addition).

[c]P<0.001, compared to control (no addition).

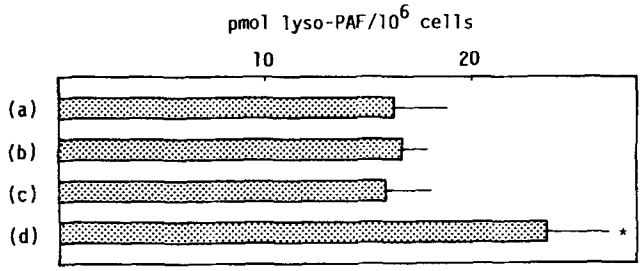


FIG. 4. LysoPAF levels in cells. (a) Cells suspended in ice-cold HBSS. (b) Cells suspended in ice-cold HBSS were warmed in a water bath (37°C) for 2 min. (c)(d) Cells prewarmed at 37°C for 10 min were further incubated in the absence (c) or presence of 2 μM (d) A23187 for 2 min. The incubation was terminated by adding chloroform/methanol (1:2, v/v). The amounts of lysoPAF were estimated by bioassay after conversion of lysoPAF to PAF by chemical acetylation as described in Materials and Methods. *P<0.05, compared to (c). Each value is the mean ± SD from four determinations.

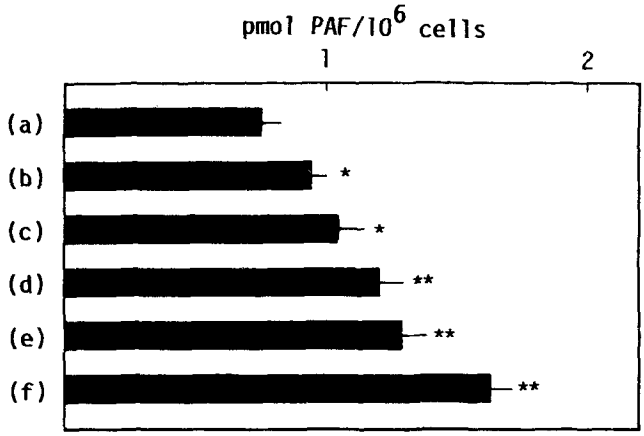


FIG. 5. Formation of PAF in cells treated with various concentrations of lysoPAF. Prewarmed (at 37°C for 10 min) cells were incubated with various concentrations of lysoPAF for 4 min. The incubation was terminated by adding chloroform/methanol (1:2, v/v). The amounts of PAF were estimated by bioassay as described in Materials and Methods. (a) No addition, (b) with 0.2 μM lysoPAF, (c) with 0.5 μM lysoPAF, (d) with 1 μM lysoPAF, (e) with 2 μM lysoPAF and (f) with 5 μM lysoPAF. Each value is the mean ± SD from four to five determinations. *P<0.001.

ed level of lysoPAF within cells is important in the triggering of PAF synthesis.

In order to examine this possibility, we compared the effects of supplementation of substrates on the PAF level in macrophages (Table 1). We found that a small amount of PAF was formed when cells were exposed to a relatively high concentration of acetyl-CoA (100 μM) for 4 min following 10 min preincubation. However, the formation of PAF was more pronounced if cells were incubated with lysoPAF (20 μM) indicating that the increased availability of lysoPAF *per se* is enough to trigger the formation of considerable amounts of PAF. The augmented production of PAF was detectable even after brief treatment of cells with as low as 0.5 μM lysoPAF (Fig. 5). We also found that PAF production induced by the addition of either acetyl-CoA or lysoPAF was remarkably enhanced if the preincubation was omitted (Table 1). Under such a condition the acetyltransferase activity was augmented *ca.* 2-fold as shown in Figure 1, suggesting that the increased acetyltransferase activity is effective in producing larger amounts of PAF.

DISCUSSION

The mechanism of regulation of PAF biosynthesis, including the triggering mechanism, has been studied by many investigators (9–25). It has generally been assumed that acetyltransferase is an important regulatory

enzyme in PAF biosynthesis in various tissues and cells following stimulation (8), although the control mechanism seems to be rather complicated (18) and may somewhat differ from tissue to tissue.

In this study, we found that the activity of acetyltransferase in alveolar macrophages was transiently elevated if the cells were warmed at 37°C. Similar transient activation of acetyltransferase (*ca.* 2-fold) was also observed for human polymorphonuclear leukocytes (Sugiura, T., unpublished data). By contrast, acetylhydrolase activity in cell lysate was not influenced by warming cells (data not shown). The mechanism of the augmentation of acetyltransferase activity after warming is not yet clear. The enzyme activity was increased even when exogenous Ca^{2+} was omitted during the warming of macrophages (data not shown), indicating that the entry of Ca^{2+} into the cell is not the direct cause of the increase of acetyltransferase activity during warming. On the other hand, when cells were sonicated before warming, acetyltransferase was not activated (Fig. 1 (b)). This suggests that the intact organization of cellular components and/or metabolic integrity is required to enhance enzyme activity. Several investigators have already demonstrated that the activation of acetyltransferase in several tissues and cells upon stimulation with appropriate agonists is due to the phosphorylation of enzyme protein (29–32). It would be possible, therefore, that the phosphorylation of the enzyme protein may also account for the transient activation of acetyltransferase in warmed cells. The possible metabolic changes induced by transferring intact cells from 4°C to 37°C may be responsible for the transient modification of enzyme. Further studies are still needed to clarify the exact mechanism of the transient activation of acetyltransferase in warmed cells.

The activation of acetyltransferase is not sufficient to induce PAF formation in this type of cell. We did not find any PAF production in warmed cells in which the activity of acetyltransferase was elevated. Recently, Leyravaud *et al.* (22) also demonstrated that human neutrophils do not produce PAF when challenged with a high concentration of phorbol myristate acetate (PMA), though the activity of acetyltransferase increases under this condition. They suggested that the reduced availability of lysoPAF in a high concentration of PMA-treated cells may be responsible for the diminished production of PAF. Under the condition of the present study, considerable amounts of lysoPAF were present within the cells. A possible explanation for the failure of PAF production in warmed cells shown in this study is that the preexisting lysoPAF is topologically isolated from acetyltransferase or masked with proteins or other macromolecules, preventing acetyltransferase from interacting with lysoPAF. Recently, Burrier and Brecher (33) demonstrated that the fatty acid binding protein obtained from rat liver is also able to bind lysophosphatidylcholine. Furthermore, a protein which binds PAF and catalyzes the transfer of PAF was found in the cytosolic fraction of macrophages (34), although it is not clear whether this protein is capable of binding lysoPAF as well and of interfering with the action of acetyltransferase.

In contrast to warmed cells, the addition of A23187 to cells was shown to increase the cellular level of lysoPAF *ca.* 1.5-fold (Fig. 4). Total amounts of generated lysoPAF may be higher than the actually observed level of lysoPAF, because a portion of generated lysoPAF had rapidly been acetylated to form PAF as shown in Figure 3. Also, large amounts of lysoPAF can be acylated to form long-chain fatty acid-containing choline glycerophospholipids. We have already shown that lysoPAF is rapidly acylated with polyunsaturated fatty acids through a transacylation system in alveolar macrophage microsomes (35). It seems very likely that the increased availability of intracellular lysoPAF, as observed in A23187-stimulated cells but not in warmed cells, is one of the important factors for eliciting PAF synthesis. This was supported by the results of other experiments (Table 1 and Fig. 5) showing that the addition of exogenous lysoPAF to cells induces PAF formation, although the levels of lysoPAF-induced PAF formation in intact cells were considerably lower compared with those observed in A23187-stimulated cells.

From these results we conclude that the activation of acetyltransferase can be favorable for the increase in PAF synthesis in stimulated cells but is not *per se* sufficient to induce PAF formation. Furthermore, it is possible that the increased availability of substrates, especially lysoPAF, is indispensable in the triggering of PAF synthesis, although the involvement of other factors in this process should also be taken into account. More detailed aspects of the regulatory mechanism of PAF biosynthesis including intracellular processing will need to be clarified in the future.

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Effects of an Eicosapentaenoic and Docosahexaenoic Acid Concentrate on a Human Lung Carcinoma Grown in Nude Mice[1]

M.G. de Bravo[2], R.J. de Antueno[2,3,*], J. Toledo, M.E. De Tomás[2], O.F. Mercuri[2] and C. Quintans[4]
Instituto de Investigaciones Bioquimicas de La Plata (INIBIOLP), Facultad de Medicina, UNLP, 60-120 (1900), La Plata, Argentina

The effects of the n-3 fatty acids, eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA), on the growth of a human lung mucoepidermoid carcinoma (HLMC) in athymic mice were studied. The mice were divided into three groups which were given either a control chow diet (C), a chow diet supplemented with EPA/DHA (P) (25 or 50 mg of free n-3 fatty acids/g of pellet/day), or chow diet supplemented with palmitic acid (S) (isocaloric with P). Two independent experimental schedules were followed: i) host mice bearing either tumors that were allowed to reach 4000 mm^3, or only 35 mm^3, were fed C, P or S for 21 or 41 days; ii) animals were fed C, P and S for 9 days before tumor implant and were maintained on these diets throughout tumor growth. Food consumption, mouse weight and liver/body weight ratio showed no significant differences between supplemented diets and chow. Tumor growth was markedly inhibited (45%) in both experiments by the EPA/DHA supplemented diet. In Experiment 2, only 60% of mice fed diet P had tumors. The fatty acid composition of neutral and polar lipids of host liver and tumor reflected the dietary intake of n-3 fatty acids; the content of arachidonic acid was reduced by 50%, and EPA/DHA was increased 3- to 5-fold. Tumor prostaglandin E_2 levels were reduced 7.4-fold in the P group. The reduced PGE_2 content may be a factor in tumor growth inhibition.
Lipids 26, 866-870 (1991).

Fish oil consumption is considered to be a promising nutritional approach in the prevention of cardiovascular diseases (1-4), in the control of inflammation (5), and in the treatment of tumors (6-11). Several studies have indicated that a fish oil supplemented diet significantly decreases the growth rate of rat or mouse mammary (6-8) and colon (9) carcinomas and human melanoma (10), prostatic (11) and mammary carcinomas (12), as well as the size and number of rat pancreatic preneoplastic lesions (13). The reason for these effects may be the high content of n-3 polyunsaturated fatty acids found in fish oil (6,14).

It has recently been proposed that enhanced effects can be observed when eicosapentaenoic (EPA, 20:5n-3) or docosahexaenoic (DHA, 22:6n-3) acids are given as free fatty acids, because polyunsaturated n-3 free fatty acids are absorbed more efficiently than the corresponding triglycerides (15).

EPA and DHA can depress prostaglandin E_2 (PGE_2) production by reducing stored phospholipid arachidonate (20:4n-6). EPA also competes with 20:4n-6 for cyclooxygenase (16). The eicosanoids produced *via* cyclooxygenase from 20:4n-6 may modulate biochemical pathways involved in cancer initiation and promotion, cell proliferation, and differentiation (17,18). In particular, high levels of PGE_2 have been associated with aggressive growth rates of human skin carcinomas (19). PGE_2 has also been shown to suppress the cytotoxic activities of various immune cells, such as natural killer cells (20). Because n-3 fatty acids may affect PGE_2 metabolism and therefore tumor growth, we examined whether a diet supplemented with EPA/DHA concentrate reduces the growth rate of a human lung mucoepidermoid carcinoma (HLMC) grown in athymic nude mice. The tumor content of PGE_2 and the fatty acid composition of liver and tumor were also studied.

MATERIALS AND METHODS

Chemicals. All organic solvents and chemicals were reagent grade and were obtained from Mallinkrodt (St. Louis, MO). Lipid standards and gas-liquid chromatographic supplies were purchased from Nu-Chek-Prep (Elysian, MN) and Supelco (Bellefonte, PA). Palmitic acid was obtained from Riedel de Haen A.G. (Hannover, Germany). Silica gel 60G and 60HR for thin-layer chromatography (TLC) was purchased from Merck (Darmstadt, Germany). Prostaglandin E_2 was measured using a [^{125}I] radioimmunoassay (RIA) Kit obtained from NEN Research Products-DuPont (Wilmington, DE).

EPA/DHA concentrate. A free fatty acid concentrate of EPA and DHA was prepared from South Atlantic hake body oil as described elsewhere (21) employing urea inclusion and further purification by silicic acid chromatography. The free fatty acid concentrate was regularly checked for peroxide content by ultraviolet (UV) absorption spectroscopy and kept under N_2 with 0.02% butylated hydroxytoluene at −20°C until use. The fatty acid composition of the concentrate is shown in Table 1.

Source of tumoral tissue. A human lung mucoepidermoid carcinoma (HLMC) was obtained by surgery from a local hospital. Non-cancerous and obviously necrotic tissues were dissected away before transplantation within 1 to 2 hr after resection. The tumor specimen was finely minced in sterile isotonic NaCl solution and inoculated subcutaneously on the back of the nude mice. Host animals were killed by cervical dislocation 9 mon after the primary implant. Tumor (2 g) was minced through a

[1]Preliminary data were presented at the Annual Meeting of the American Oil Chemists' Society, (1990), Baltimore, MD, (de Antueno, R.J., Bravo, M.G. de, Toledo, J., De Tomas, M.E., Mercuri, O., Quintans, C. (1990) *INFORM 1*, 328 (Abstract HH1).
[2]Members of the Carrera del Investigador Cientifico of the Consejo Nacional de Investigaciones Cientificas y Tecnicas, Argentina.
[3]Member of the Faculty of Cs. Veterinarias, UNLP, La Plata, Argentina.
*To whom correspondence should be addressed at Efamol Research Institute, P.O. Box 818, Kentville, Nova Scotia, B4N 4H8, Canada.
[4]Comision Nacional de Energia Atomica, Secc.Bioterio, Ezeiza, Argentina.

Abbreviations: DHA, docosahexaenoic acid; EPA, eicosapentaenoic acid; PG, prostaglandin; PGE_2, prostaglandin E_2; HLMC, human lung mucoepidermoid carcinoma; RIA, radioimmunoassay; TLC, thin-layer chromatography.

TABLE 1

Fatty Acid Composition of Lipids in Chow Diet and EPA/DHA Concentrate

Fatty acid	Fatty acid [mole %]	
	Chow diet	EPA/DHA concentrate
14:0	0.2	0.9
16:0	11.8	0.6
16:1	0.5	0.8
17:0	—	2.4
18:0	3.3	0.7
18:1	20.5	0.9
18:2n-6	50.8	0.9
18:3n-3	7.3	1.8
18:4n-3	—	7.9
20:4n-6	0.3	3.2
20:4n-3	0.7	2.9
20:5n-3	0.7	23.2
22:4n-3	—	2.5
22:5n-6	—	2.8
22:5n-3	0.7	2.5
22:6n-3	1.1	41.4

stainless steel 1 mm^2 mesh and suspended in 3 mL of isotonic NaCl solution. A volume of 250 μL of this suspension was injected into nude mice. Tumor growth could be recognized between 15–20 days following inoculation. The mass doubling time during the log growth phase was 6–7 days. The tumor was serially transplanted every two months and histopathologically controlled as previously described (22).

The present experiments were performed with tumors weighing between 2.54 g (small tumors) and 5.13 g (large tumors).

Animals and diets. One-month-old athymic Balb-C female mice bearing nu/nu genotype were provided by the Comision Nacional de Energia Atomica, seccion Bioterio (Ezeiza, Argentina). Host animals were maintained *ad libitum* on a gamma-irradiated chow diet until the tumor average size was 35 mm^3 or 4000 mm^3, and then were randomly separated into three dietary groups: C, control chow diet containing 70 mg/g of fat; P, chow diet supplemented with 50 mg/g of EPA/DHA concentrate, and S, chow diet supplemented with 50 mg/g of palmitic acid. The fatty acid composition of the chow diet is shown in Table 1.

At least three animals were used per dietary group in all experiments. Animals with tumors that were allowed to reach 4000 mm^3 or with small tumors of 35 mm^3 were fed for 21 days or 41 days, respectively, with the different diets.

Further experiments were performed with other groups of 5 animals per diet fed either the C diet, the chow diet supplemented with 25 mg/g of EPA/DHA concentrate (P') or the chow diet supplemented with 25 mg of palmitic acid (S') for 9 days before tumor implant and then maintained on the same diets until they were killed by cervical dislocation. Fresh diet was made and fed every two days and leftover feed was discarded.

Testing. When tumors became palpable, they were measured twice weekly with calipers and the volume was calculated for an ellipsoid by the formula $V = a^2 \times b/2$, where a is the width and b is the length, in millimeters. The tumor sizes were expressed as relative tumor volume. This was calculated by the formula Vi/Vo, where Vi is the mean tumor volume at any given time and Vo is the mean initial tumor volume at the start of each experiment (23).

Animals were weighed twice weekly. Relative animal weight was calculated by the formula Wi/Wo, where Wi is the mean animal weight of each group at any given time and Wo is the mean initial animal weight when treatment was begun.

Lipid analysis. Total lipids from 500 mg of liver or tumor were extracted according to Folch-Pi *et al.* (24) and fractionated into phospholipids and neutral lipids by silicic acid column chromatography (25). Further separation into classes was accomplished by TLC on silica gel HR (26) and G (27) for phospholipids and neutral lipids, respectively. Fatty acid composition of individual lipid classes was determined by gas-liquid chromatography of methyl esters as previously described (22).

Prostaglandin (PG) analysis. Tumor prostaglandins (PG) were extracted essentially according to the method of Powell (28) using Sep-Pak C18 cartridges in the presence of indomethacin 10 μg/mL. PG were purified by TLC as previously described (29). The PGE zone was detected by comparing the R value with authentic standard and was eluted from the silica gel with methanol (30).

PGE_2 ^{125}I radioimmunoassay (RIA) was performed on the PGE extract according to the procedure described in the DuPont PGE_2 ^{125}I-RIA Kit.

RESULTS

Animals in all groups consumed similar amounts of food (3.6 g/day on average), and although the mean body weight of the mice fed diet P was lower than of those fed diets C or S, the differences were not significant (Table 2). The ratio of liver to body weight was also not significantly affected by diets P and S (Table 2). EPA and DHA excretion in the feces (data not shown) was not significantly altered.

Tumor growth. The data in Figure 1 for small tumors (35 mm^3) show that feeding 175 mg/day of the EPA/DHA mixture to the host animals significantly depressed the growth rate of the human lung mucoepidermoid carcinoma when compared to C or S groups. After 41 days, the relative volume of the P group tumors was 52% lower than that of the C group, whereas the S group tumors increased in relative volume (147%) when compared to group C. There were no significant differences in animals bearing large tumors (4000 mm^3) during the experiment (data not shown).

TABLE 2

Effect of Different Diets on Food Consumption, Body Weight and Liver Weight[a]

Diet	Food consumption (g/day)	Body weight (g)	Liver weight/body weight × 100
C	3.70 ± 0.23	21.50 ± 0.75	5.60 ± 0.29
P	3.40 ± 0.23	17.51 ± 1.09	7.32 ± 1.27
S	3.60 ± 0.29	20.53 ± 0.69	6.20 ± 0.63

[a]Each value represents the mean ± SE from 3 animals. Differences are not significant. Diets: chow (C), chow + EPA/DHA (50 mg/g diet) (P), chow + palmitic acid (50 mg/g diet) (S).

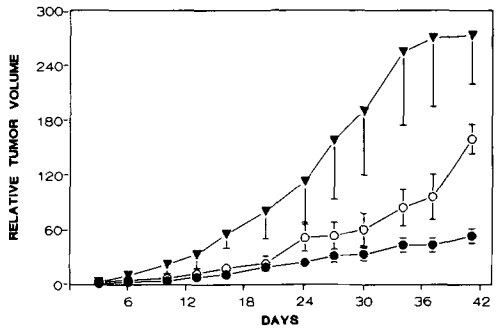


FIG. 1. Growth rate of human lung mucoepidermoid carcinoma xenografts in nude mice. Mice were fed the control and supplemented diets when the tumor size was 35 mm^3. Diets: ○, Control chow (C); ▼, Control chow + palmitic acid (50 mg/g diet) (S); ●, Control chow + EPA/DHA (50 mg/g diet) (P). Values are the mean of 3 animals ± SE. After day 36 and day 16, tumors in P group were significantly smaller than in C and S groups, respectively (P<0.05) (Student t-test).

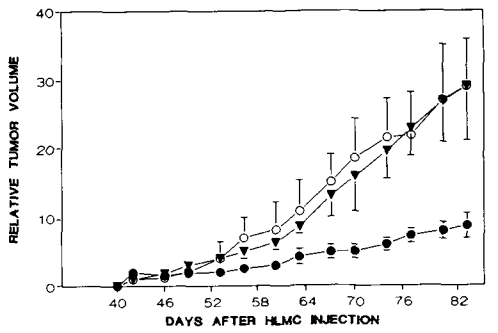


FIG. 2. Growth rate of human lung mucoepidermoid carcinoma xenografts in nude mice previously fed supplemented diets. Mice were fed for 9 days: ○, Control diet (C); ▼, Control chow diet + palmitic acid (25 mg/g diet) (S'); ●, Control chow diet + EPA/DHA (25 mg/g diet) (P') before tumor injection. Tumor size was measured 40 days thereafter. Values are the mean of 5 animals ± SE. After day 46 and day 67, tumors in P group were significantly smaller than in S and C groups, respectively (P<0.05) (Student t-test).

When animals were fed the diets before tumor implantation, the tumor was detected in all mice of groups C and S' but only in 60% of those in the P' group (3 out of 5) receiving 87.5 mg/day of EPA/DHA mixture. As can be seen in Figure 2, the relative volume of tumors in the P' group was 80% lower than in group C or S' after 42 days of tumor development and no differences were seen between groups S' and C.

Liver and tumor fatty acid analysis. Table 3 shows that feeding the EPA/DHA supplemented diet markedly increased the content of both EPA and DHA in host liver lipids. This increase appeared to be at the expense of 20:4n-6 and eicosatrienoic acid (20:3n-6) as well as hexadecenoic acid (16:1) and ocatadecenoic acid (18:1). A similar trend is seen for linoleic acid (18:2n-6) though these data were significant at P<0.10. The content of 20:3n-9 was decreased below detectable levels. Fatty acid composition of total tumor lipids also reflected the dietary intake of EPA/DHA. Significant increases in the content of EPA/DHA were found in phosphatidylcholine, phosphatidylethanolamine and triglycerides (Fig. 3) when compared to controls. Phosphatidylinositol was also analyzed, but showed no significant differences compared to groups C or S.

TABLE 3

Fatty Acid Composition of Total Liver Lipids (μg/g wet tissue)[a]

Fatty acid	Diet		
	C	P	S
16:0	3800.9 ± 76.9	3200.5 ± 435.2	4244.4 ± 516.8
16:1	365.0 ± 20.8	261.7 ± 28.3[b]	320.9 ± 56.3
18:0	2243.8 ± 61.8	2533.9 ± 268.1	2262.0 ± 223.1
18:1	3737.9 ± 205.2	2310.2 ± 408.7[b]	3301.7 ± 592.5
18:2n-6	2355.6 ± 64.4	1762.9 ± 293.1	2263.9 ± 283.2
18:3n-6	145.4 ± 29.4	142.4 ± 16.8	127.1 ± 22.5
20:3n-9	57.0 ± 5.8	TA	69.8 ± 1.6
20:3n-6	265.4 ± 0.6	135.2 ± 16.5[c]	237.1 ± 19.9
20:4n-6	1873.8 ± 29.8	1432.6 ± 156.3[c]	2219.1 ± 176.3
20:5n-3	154.6 ± 9.1	784.6 ± 64.8[c]	147.6 ± 10.7
22:5n-3	105.1 ± 8.4	230.4 ± 26.8[b]	146.2 ± 4.5
22:6n-3	2213.3 ± 42.8	4234.3 ± 559.1[b]	2841.1 ± 155.5

[a]Each value represents the mean ± SE from 3 animals. Comparison was made between P and C diet and between P and S diet (Student t-test). TA, trace amounts.

[b]P<0.05 P *vs* C.

[c]P<0.05 P *vs* C and P *vs* S.

Prostaglandin analysis. Measurements of urinary or plasma levels are likely to have little relevance because PG act locally. As already proposed by Turini *et al.* (31), these measurements are not representative of a specific site of synthesis and thus may explain why frequently no correlations have been observed. Therefore, we have focused our studies on the tumor content of PGE_2. Table 4 illustrates that in tumors of the P group the PGE_2 content was 14% of that measured in groups C or S.

DISCUSSION

In this study we have examined the effects of an EPA/DHA concentrate obtained from hake body oil on the development of a human lung mucoepidermoid carcinoma grown in nude mice. As already reported for mammary, pancreatic and colon tumors (6–13), we have shown that n-3 fatty acids provided as free fatty acids can inhibit the growth of a human lung carcinoma. This was true not only in animals with small tumors fed the EPA/DHA supplemented diet but also, more noticeably, when animals were fed the EPA/DHA diet prior to tumor implantation. High fat diets are rich in calories, and it is interesting to consider whether the effects on tumor development are related to the quality of the fats or to an increased supply of calories (32,33). In the experiments described here, diets P (chow supplemented with 50 mg/g of EPA/DHA) and S (chow supplemented with 50 mg/g of palmitic acid) exhibited opposite effects although they are isocaloric. Our results indicate that fatty acid supplemented diets can depress or enhance the development of this human carcinoma depending on both the dose and type of fatty acid supplied.

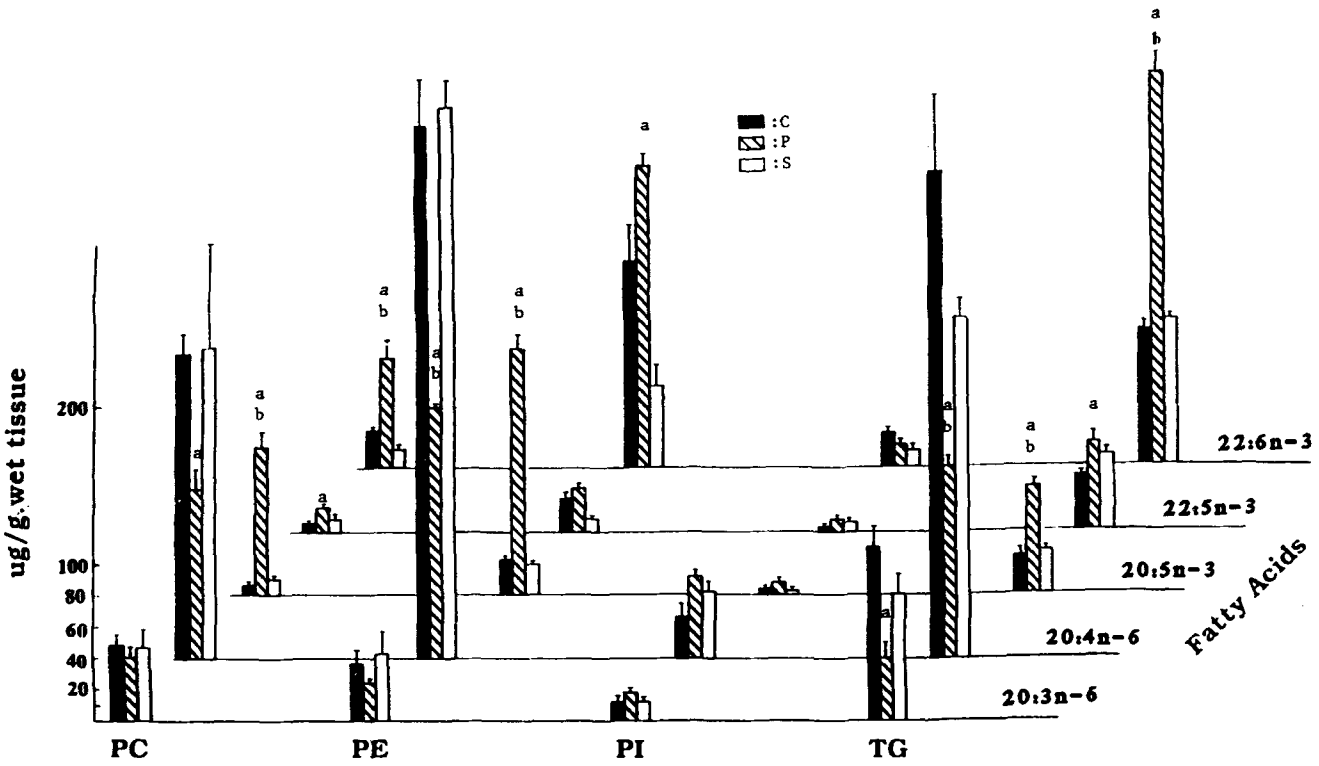


FIG. 3. Polyunsaturated fatty acid content of tumor phospholipids and triglycerides from mice fed control and supplemented diets. Diets: C, Control chow; P, Control chow + EPA/DHA (50 mg/g diet); S, Control chow + palmitic acid (50 mg/g diet). PE, phosphatidylethanolamine; PC, phosphatidylcholine; PI, phosphatidylinositol; TG, triglycerides. Values are the mean of at least 3 animals ± SE. a: P<0.05 P *vs* C; b: P<0.05 P *vs* S.

TABLE 4

Prostaglandin E_2 Tumor Content in Mice on Chow and Supplemented Diets[a]

Diet	PGE_2 ng/g wet tissue
C	30.72 ± 4.73
P	4.20 ± 0.56
S	36.00 ± 9.80

[a]The values are the mean of 3 tumors ± SE. P<0.01 P *vs* C and P *vs* S (Student t-test). Diets: chow (C), chow + EPA/DHA (50 mg/g diet) (P), chow + palmitic acid (50 mg/g diet) (S).

We have also demonstrated that an n-3 fatty acid supplemented diet changes the pattern of 20:4n-6, 20:5n-3 and 22:6n-3 in liver and tumor lipids. The reduced content of 20:4n-6, together with the disappearance of 20:3n-9 may also be caused by the inhibition of Δ6 and/or Δ5 desaturase activity as reported *in vitro* (34) and *in vivo* for rat liver following fish oil feeding (35,36). However, Borgeson *et al.* (12) found an increase in Δ6 and Δ5 desaturase in a human mammary tumor and liver of nude mice fed fish oil. This difference may lie in the genotype of the mice, the tumor origin, as well as the precursors used for the enzyme measurements. The reduced 16:1 and 18:1 content of the liver lipids (shown in Table 3) may be a consequence of reduced Δ9 desaturase activity as previously demonstrated (12,37).

The change in fatty acid profile certainly modifies the substrate for prostaglandin synthesis. The decreased 20:4n-6 content of tumor phosphatidylcholine and phosphatidylethanolamine probably resulted in a decrease of 2-series PG synthesis.

We have previously described the capacity of HLMC homogenates to synthesize PGE_1 and PGE_2 when ^{14}C-labeled fatty acid precursors were added to the incubation medium (21). Now, we find a marked decrease in PGE_2 content of tumors from animals fed an EPA/DHA supplemented diet which may be beneficial to the host. This diminished ability of the tumor to synthesize PG *in vivo* may be the result of the competition between n-3 fatty acids and n-6 fatty acids for cyclooxygenase (17,38). Furthermore, EPA and DHA could probably act synergistically in the inhibition of this enzyme (21). PGE_2 has been shown to elicit unfavorable effects in tumor-bearing animals through the suppression of lymphocyte proliferation and the inhibition of both macrophage and natural killer cell cytotoxicity toward tumor cells (30,39). In the present studies, we have used athymic nude mice which lack T cells but have natural killer (NK) cells (23). The latter cells may be a key factor in the immunosuppressive status of the tumor-bearing host fed n-3 fatty acids. It should be pointed out that Bennett *et al.* (40) have shown that fish oil enriched diets did not modify NK functions in mice even when given carcinogens. However, in our model, the presence of a PG secreting tumor may alter the sensitivity of the host NK

cells. Further data is needed to conclusively state that the slower HLMC development in nude mice fed EPA/DHA results from a decrease in PGE_2 levels. It is also possible that endogenous lipid peroxidation in the presence of increased n-3 polyunsaturated fatty acids could be responsible for the slower tumor growth observed (12).

In summary, the present study demonstrates that supplementing the host diet with EPA/DHA as free fatty acids leads to significant growth inhibition of this human lung carcinoma. The use of free n-3 fatty acids instead of fish oil causes significant effects, suggesting that free fatty acid administration may be helpful in future human experiments by reducing the volume of active components to be ingested.

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Specific Accumulation of Docosahexaenoic Acid (22:6n-3) in Brain Lipids During Development of Juvenile Turbot *Scophthalmus maximus* L.

Gabriel Mourente[1], **Douglas R. Tocher*** and **John R. Sargent**
NERC Unit of Aquatic Biochemistry, Department of Molecular and Biological Sciences, School of Natural Sciences, University of Stirling, Stirling FK9 4LA, Scotland

The changes in the lipid class and the fatty acid compositions of total lipids and individual glycerophospholipids which occur in brain during development of juvenile turbot (*Scophthalmus maximus* L.) were investigated. Fish were sampled during a 10-week period immediately following weaning from a live feed to a pellet diet. During this period, brain dry weight increased over 6-fold. The percentages of protein, cholesterol and galactolipids increased in brain during development, presumably reflecting increased membrane maturation and myelination processes. The percentages of docosahexaenoic acid (DHA; 22:6n-3) were low at the beginning of the study period. However, DHA specifically accumulated in juvenile turbot brain during development. The percentages of DHA increased in total lipid, total diradyl glycerophosphocholine (GPC), total diradyl glycerophosphoethanolamine (GPE), phosphatidylserine (PS) and phosphatidylinositol (PI) reaching 26.1%, 25.8%, 40.8%, 47.1% and 17.9% of the total fatty acids, respectively, by the end of the 10-week period. The percentages of other n-3 polyunsaturated fatty acids (PUFA) and n-6 PUFA generally decreased during this period, as did that of monoenes; the percentages of saturated fatty acids remained relatively constant. Non-linear regression analysis showed that the increase in DHA in total lipid, GPC, GPE and PS fitted 1st order rate kinetics (plus offset) allowing maximum values for the percentages of DHA in each lipid class to be estimated.
Lipids 26, 871–877 (1991).

Recent research has established a critical role for n-3 polyunsaturated fatty acids (PUFA) and docosahexaenoic acid (DHA) in neural and retinal tissue functions in mammals (1,2). During dietary n-3 PUFA deprivation studies, DHA is tenaciously retained by mammalian neural tissues (3) and at least two generations of deprivation are required to significantly reduce DHA levels in brain or retina (4–7). Depletion of neural DHA levels is accompanied by alterations in physiological parameters and functional defects such as reduced visual acuity and impaired learning abilities (4–7). As evidence of the important role of DHA in development of neural tissues grows, so does support for the nutritional requirement for n-3 PUFA in mammals (8,9).

Tissue lipids in most fish generally contain high levels of n-3 PUFA, especially eicosapentaenoic acid (EPA; 20:5n-3) and DHA (10,11). A recent study has suggested that fish accumulate more DHA in neural tissues (12) than in non-neural tissues (13). Although fish neural tissue lipids can have extremely high levels of n-3 PUFA and DHA (12,14,15), the accretion of DHA by fish brain has not been demonstrated. In fish, considerable growth and development of neural tissues occurs after hatching and during independent feeding of the offspring. Therefore, the vast majority of n-3 PUFA and DHA required for normal development of the brain must be obtained in the diet. The individual contributions of dietary 18:3n-3, EPA and DHA to the final DHA content of adult fish brain are not known. Some marine fish, such as the turbot (*Scophthalmus maximus* L.), have incomplete desaturase pathways and therefore require preformed EPA and DHA in their diet for growth and development (16). Therefore, dietary 18:3n-3 cannot contribute to the DHA content in turbot brain. The developmental stage and diet of turbot can be precisely known because turbot are cultured commercially. Thus, the turbot is a particularly useful species in which to study the role of DHA during development.

Our aims were to determine (i) whether DHA was specifically accumulated in the brain during development of fish and, if so, (ii) what, if any, preferences occurred for individual glycerophospholipid classes and (iii) what, if any, fatty acids were discriminated against in the accumulation processes. The lipid class and fatty acid compositions of total lipid and individual glycerophospholipid classes were determined in juvenile turbot over a 10-week period immediately after weaning from a live feed to a dry pellet diet. The results are discussed in relation to current understanding of the role of lipids in neural development obtained from studies in other vertebrates and in relation to existing data on fatty acid metabolism in fish.

MATERIALS AND METHODS

Materials. Turbot (*Scophthalmus maximus* L.) were obtained from Golden Sea Produce Ltd. (Hunterston, Scotland, United Kingdom). Larvae were fed live foods through metamorphosis until they were large enough to accept dry food. The batch of fish used in this study was weaned from enriched *Artemia* to a dry pellet diet on day 37 post-hatch. Enriched *Artemia* had been supplemented with n-3 PUFA, according to a commercial protocol, to increase the n-3 highly unsaturated fatty acid (HUFA, $\geq C_{20}$ and with at least 3 double bonds) content. The lipid contents and fatty acid compositions of the *Artemia* and the pellet diet are given in Table 1. Triplicate samples of weaned fish

[1]Current address: Departamento de Biología, Facultad de Ciencias del Mar, Universidad de Cádiz. Poligono Rio San Pedro, 11510-Puerto Real (Cádiz), Spain.

*To whom correspondence should be addressed.

Abbreviations: BHT, butylated hydroxytoluene; CL, cardiolipin; DHA, docosahexaenoic acid; DMA, dimethyl acetal; EPA, eicosapentaenoic acid; GPC, total diradyl glycerophosphocholine; GPE, total diradyl glycerophosphoethanolamine; HPLC, high-performance liquid chromatography; HPTLC, high-performance thin-layer chromatography; HUFA, highly unsaturated fatty acid ($\geq C_{20}$ and with at least 3 double bonds); PA, phosphatidic acid; PI, (diacyl)phosphatidylinositol; PS, (diacyl)phosphatidylserine; PUFA, polyunsaturated fatty acid; TLC, thin-layer chromatography.

TABLE 1

Total Lipid and Fatty Acid Contents in Diets Used for Rearing Turbot[a]

Diet	*Artemia*	Pellet diet
Dry weight (percentage of fresh wt)	7.6 ± 0.2	93.3 ± 0.3
Total lipid content (percentage of dry wt)	12.9 ± 1.3	12.8 ± 1.0
Fatty acid (μg/mg dry wt of diet)		
14:0	0.9 ± 0.2	4.0 ± 0.3
15:0	0.2 ± 0.0	0.6 ± 0.1
16:0	8.1 ± 0.8	14.9 ± 0.5
16:1n-7	1.9 ± 0.2	5.9 ± 0.2
16:2	0.5 ± 0.3	0.8 ± 0.1
16:3	tr	0.6 ± 0.1
18:0	3.9 ± 0.3	3.7 ± 0.3
18:1n-9	13.7 ± 1.0	17.2 ± 0.4
18:1n-7	6.5 ± 1.1	3.7 ± 0.3
18:2n-6	4.0 ± 0.5	4.0 ± 0.2
18:3n-3	10.4 ± 1.0	2.2 ± 0.1
18:4n-3	1.5 ± 0.2	2.6 ± 0.1
20:0	0.3 ± 0.1	0.8 ± 0.4
20:1n-9	1.0 ± 0.2	5.9 ± 0.0
20:4n-6	0.6 ± 0.1	1.2 ± 0.1
20:3n-3	0.3 ± 0.0	0.2 ± 0.1
20:4n-3	0.3 ± 0.0	0.8 ± 0.0
20:5n-3	4.4 ± 0.3	9.2 ± 0.6
22:1n-11	0.4 ± 0.1	5.2 ± 0.3
22:5n-6	0.4 ± 0.2	0.2 ± 0.0
22:5n-3	0.2 ± 0.0	1.4 ± 0.1
22:6n-3	1.0 ± 0.1	12.8 ± 2.9
Total saturated	14.1 ± 1.6	24.2 ± 1.1
Total monounsaturated	24.1 ± 2.5	40.7 ± 0.8
Total polyunsaturated	23.8 ± 2.3	38.5 ± 3.0
Total n-6 PUFA	5.6 ± 0.7	7.2 ± 0.2
Total n-3 PUFA	18.2 ± 1.6	31.2 ± 2.8
Total n-6 HUFA	1.1 ± 0.1	2.0 ± 0.2
Total n-3 HUFA	6.2 ± 0.4	24.8 ± 2.4

[a]*Artemia* were enriched with n-3 PUFA for 60 hr prior to being fed to fish. Data are means ± SD of three samples. Totals include some minor components (<0.1%) not shown. PUFA, polyunsaturated fatty acids; HUFA, highly unsaturated fatty acids ($\geq C_{20}$ and with at least 3 double bonds); tr, trace.

(varying from 20 fish/sample for 38-day fish to 10 fish/sample for 105-day fish) were collected at 38, 56, 70, 90 and 105 days post-hatch. Fish were killed by decapitation, and the brains were dissected out on ice, frozen in liquid nitrogen and stored at −80°C until analysis. Butylated hydroxytoluene (BHT), fatty acid-free bovine serum albumin (BSA) and nonadecanoic acid (19:0) were purchased from Sigma Chemical Co. (Poole, Dorset, United Kingdom). Thin-layer chromatographic (20 × 20 cm × 0.25 mm) and high-performance thin-layer chromatographic (10 × 10 cm × 0.15 mm) plates precoated with silica gel 60 (without fluorescent indicator) were obtained from Merck (Darmstadt, Germany). Analar-grade glacial acetic acid, sulfuric acid and isopropanol were purchased from BDH Ltd. (Poole, Dorset, United Kingdom). All other solvents of high-performance liquid chromatography (HPLC) grade were from Rathburn Chemicals (Walkerburn, Scotland, United Kingdom).

Dry weight determination. Pre-weighed samples of 5 brains each were heated at 110°C for 24 hr. Dry weight was determined after cooling the sample *in vacuo* for at least 1 hr.

Protein determination. Protein concentration was measured in fresh brain by the Folin-phenol reagent method, according to Lowry *et al.* (17).

Total lipid extraction. Brain samples (50–500 mg wet wt) were homogenized in a Teflon pestle glass homogenizer in 10 mL of chloroform/methanol (2:1, v/v) containing 0.05% BHT. Total lipids were extracted according to the method of Folch *et al.* (18) as detailed previously (12).

Lipid class and fatty acid analysis. Lipid classes were separated by high-performance thin-layer chromatography (HPTLC) using a single-dimension double-development method described previously (12,19) and were quantified by calibrated densitometry after charring using a Shimadzu CS-9000 dual-wavelength flying spot scanner and DR-13 data handling system (Kyoto, Japan) (19). For fatty acid analyses, glycerophospholipid classes were separated according to the method of Vitiello and Zanetta (20). Fatty acid methyl esters were prepared by acid-catalyzed transmethylation overnight at 50°C using nonadecanoic acid (19:0) as internal standard (21). Methyl esters were extracted and purified by thin-layer chromatography (TLC) as described previously (12) and analyzed in a Packard 436 gas chromatograph equipped with a chemically bonded CP Wax 52 CB fused silica capillary column (50 m × 0.34 mm i.d.; Chrompack UK Ltd., Millharbour, London, United Kingdom). Chromatography was performed with on-column injection, hydrogen as carrier gas and a two-stage thermal gradient from 50°C to 235°C. Individual methyl esters were identified as described previously (12) and quantified using a Shimadzu CR-3A recording integrator. The percentage by weight of total unidentified peaks was 7.7 ± 2.9 (n = 30 analyses).

Statistical analysis. All results are expressed as means ± SD (n = 3), and the differences between means were analyzed by a paired t-test (small group of samples), indicating with an asterisk (*) when $P < 0.05$. The correlations between developmental age and the levels of various fatty acids were studied by linear regression analysis. The evolution of the levels of DHA in various classes was analyzed by non-linear regression and fitted 1st order rate equations, plus offset of the type (Equation 1):

$$WP = \text{limit} \times (1-e^{-kt}) + \text{offset} \quad [1]$$

Where WP = weight percentage; limit = the theoretical maximum value for WP calculated from the intercept with the y-axis; k = rate ($days^{-1}$); t = time (days); offset is the value of the ordinate from the y intercept to y = zero. The offset is necessary as the data which start on day 38 do not pass through zero. This equation simplifies to (Equation 2):

$$WP = a - be^{-kt} \quad [2]$$

where; a = limit value + offset value, and represents the actual maximum percentage; b = limit value.

RESULTS

Total lipid contents and fatty acid compositions of enriched *Artemia* and the pellet diet used to wean turbot larvae are shown in Table 1. The dry weight percentage is ~12 times higher in the pellet diet but the total lipid contents, as percentages of the dry weight, were identical in the diets. *Artemia* total lipid contains relatively large proportions of cholesterol and pigments. Therefore, the *Artemia* diet supply only 62.0 μg total fatty acid/mg diet dry weight compared to 103.4 μg total fatty acid/mg diet dry weight for the pellet diet (Table 1). Only 18:3n-3 and 18:1n-7 were more abundant in the *Artemia* diet. The absolute amount of DHA was almost 13-fold greater in the pellet diet.

The dry weights of the turbot brains increased by a factor of 6.4 from day 38 to day 105 (Table 2). Between 38 and 70 days the protein content as a percentage of the dry weight increased with a concomitant decrease in the percentage of total lipids. However, from 70 days the percentages of protein and lipids in the brain remained generally constant. The total polar lipid content of turbot brain was approximately 1.5 times that of total neutral lipids although there was a trend toward decreasing percentages of polar lipids as development proceeded (Table 2). The predominant polar lipid classes were total diradyl (includes diacyl, alkylacyl and alkenylacyl) glycerophosphocholine (GPC) and total diradyl (includes diacyl, alkylacyl and alkenylacyl) glycerophosphoethanolamine (GPE) (each approximately 20% of total lipids), followed by phosphatidylserine (PS) (~7–10%), phosphatidic acid/cardiolipin (PA/CL) (2–3%) and phosphatidylinositol (PI) (~2.5%). The percentages of sulfatides and cerebrosides were more variable. Cholesterol was the predominant neutral lipid and tended to increase and was primarily responsible for the increasing percentage of total neutral lipids (Table 2). The concomitant decrease in the percentage of polar lipids was due to decreases in GPC, PS and PA/CL.

The fatty acid compositions of total lipids and individual glycerophospholipids in brains of turbot on days 38 and 105 post-hatch are shown in Table 3. In total lipids, saturated fatty acids did not show significant differences between day 38 and day 105, whereas total monoenes decreased significantly. Fatty acids such as 18:3n-3, 18:4n-3, 20:4n-6, 20:3n-3 and EPA decreased significantly whereas DHA increased over 3-fold. Total dimethylacetals (DMA) in total lipid showed a slight, but significant increase.

The percentage of total saturated fatty acids in GPC was higher than that in total lipids, but was not significantly different between day 38 and day 105, whereas the percentage of total monoenes decreased (Table 3). GPC had the lowest percentage of total PUFA of all the glycerophospholipids at both days 38 and 105, but the percentage on day 38 was almost doubled by day 105. The percentages of 18:2n-6, 18:3n-3 and 20:3n-3 decreased in GPC, whereas 20:4n-6, 20:4n-3 and 22:5n-3 increased. However, the largest effect observed in GPC was a 10-fold increase in DHA.

In GPE, the percentage of total saturated fatty acids significantly increased and total monoenes significantly decreased (Table 3). Total PUFA accounted for ~50% of total fatty acids, but there was no significant difference between days 38 and 105. However, the percentages of most PUFA significantly decreased, whereas that of DHA increased from day 38 to day 105.

In PS, the percentage of total monoenes decreased and the percentage of total PUFA increased significantly by day 105. The percentages of PUFA in PS significantly

TABLE 2

Gross Composition and Lipid Class Composition of Brains of Turbot *Scophthalmus maximus* L.[a]

Composition	Time post-hatch days				
	38	56	70	90	105
Dry weight per brain (mg)	0.5 ± 0.0	0.9 ± 0.0	1.5 ± 0.1	2.1 ± 0.1	3.2 ± 0.2
Dry weight, percentage of fresh wt	17.2 ± 0.9	18.6 ± 1.2	18.6 ± 0.8	17.7 ± 0.9	18.6 ± 1.2
Protein, percentage of dry wt	42.8 ± 4.3	42.7 ± 6.7	50.9 ± 5.3	49.1 ± 3.6	51.2 ± 6.5
Lipid, percentage of dry wt	40.8 ± 2.3	36.7 ± 1.2	29.5 ± 0.4	27.2 ± 1.2	29.2 ± 0.7
Lipid class, percentage of total lipid					
Total polar	62.6 ± 5.2	66.2 ± 3.2	62.5 ± 1.2	60.7 ± 2.6	57.6 ± 5.7
Diradyl glycerophosphocholine[b]	22.9 ± 2.5	24.3 ± 1.0	23.3 ± 0.7	20.0 ± 2.3	19.1 ± 4.7
Diradyl glycerophosphoethanolamine	20.9 ± 1.7	21.8 ± 0.8	21.2 ± 0.6	21.3 ± 1.3	21.9 ± 1.4
Phosphatidylserine	9.9 ± 0.7	8.9 ± 0.5	7.5 ± 1.4	7.3 ± 0.4	7.4 ± 0.8
Phosphatidylinositol	2.5 ± 0.3	2.8 ± 0.2	2.1 ± 0.4	1.9 ± 0.4	2.3 ± 0.2
Phosphatidic acid/cardiolipin	3.1 ± 0.2	3.0 ± 0.3	2.5 ± 0.5	2.4 ± 0.3	2.0 ± 0.4
Cerebrosides	2.6 ± 0.2	4.0 ± 0.7	4.3 ± 1.0	3.6 ± 0.7	3.8 ± 1.3
Sulfatides	0.6 ± 0.0	1.2 ± 0.3	1.5 ± 0.3	1.3 ± 0.3	0.8 ± 0.5
Total neutral	37.4 ± 5.2	33.9 ± 3.2	37.5 ± 1.2	39.3 ± 2.6	42.4 ± 5.7
Cholesterol	30.1 ± 2.9	29.1 ± 4.0	31.6 ± 1.2	34.3 ± 5.4	38.6 ± 6.7
Free fatty acids	1.2 ± 0.1	0.3 ± 0.3	0.5 ± 0.6	0.2 ± 0.2	0.2 ± 0.2
Triacylglycerol	1.4 ± 0.5	1.2 ± 0.3	1.5 ± 0.7	0.7 ± 0.1	0.7 ± 0.4
Cholesteryl esters	4.7 ± 1.7	3.3 ± 0.3	3.9 ± 0.4	4.1 ± 0.2	3.0 ± 0.5

[a]Data are means ± SD of three samples. [b]Contains sphingomyelin in less than 1%.

TABLE 3

Fatty Acid Compositions of Total Lipids and Individual Glycerophospholipids from Turbot Brain[a]

	Total lipids		GPC		GPE		Phosphatidylserine		Phosphatidylinositol	
Fatty acid	38	105	38	105	38	105	38	105	38	105
14:0	0.7 ± 0.2	0.8 ± 0.1	0.7 ± 0.0	0.6 ± 0.0	0.3 ± 0.0	0.2 ± 0.0	0.6 ± 0.2	0.1 ± 0.1*	0.8 ± 0.1	0.9 ± 0.1
15:0	0.2 ± 0.0	0.2 ± 0.0	0.3 ± 0.0	0.2 ± 0.0	0.1 ± 0.0	0.1 ± 0.0	0.4 ± 0.2	0.1 ± 0.1	0.2 ± 0.2	0.3 ± 0.0
16:0DMA	0.3 ± 0.0	0.5 ± 0.0*	nd	nd	0.8 ± 0.0	1.4 ± 0.1*	nd	nd	nd	nd
16:0	15.5 ± 0.9	17.2 ± 1.3	26.7 ± 1.2	24.7 ± 0.5	6.5 ± 0.2	9.7 ± 0.1*	3.7 ± 0.8	2.6 ± 0.0*	4.0 ± 1.6	9.4 ± 0.2*
16:1n-7	5.4 ± 1.5	3.2 ± 0.2*	4.1 ± 0.3	1.1 ± 0.0*	1.3 ± 0.5	1.7 ± 0.2	1.4 ± 0.9	0.3 ± 0.0*	9.4 ± 2.3	1.8 ± 0.0*
16:2	0.9 ± 0.2	0.3 ± 0.2	0.6 ± 0.3	0.3 ± 0.0	0.6 ± 0.0	0.3 ± 0.0*	0.2 ± 0.2	tr	0.1 ± 0.1	tr
16:3	0.5 ± 0.2	0.3 ± 0.0	1.1 ± 0.0	0.3 ± 0.0*	0.6 ± 0.0	0.3 ± 0.0*	0.4 ± 0.1	0.1 ± 0.1*	0.5 ± 0.2	0.6 ± 0.0
18:0DMA	0.7 ± 0.0	1.4 ± 0.1	nd	nd	2.0 ± 0.2	3.9 ± 0.4*	nd	nd	nd	nd
18:1n-9DMA	0.7 ± 0.1	1.0 ± 0.1	nd	nd	2.1 ± 0.1	2.9 ± 0.2*	nd	nd	nd	nd
18:1n-7DMA	0.5 ± 0.0	0.6 ± 0.0	nd	nd	1.5 ± 0.1	tr	nd	nd	nd	nd
18:0	8.4 ± 0.8	8.7 ± 0.8	5.4 ± 0.3	5.6 ± 0.1	11.3 ± 0.1	13.2 ± 0.2*	21.5 ± 1.1	24.6 ± 0.4*	17.8 ± 2.9	23.2 ± 0.6*
18:1n-9	15.5 ± 1.3	14.3 ± 1.2	24.3 ± 1.1	15.3 ± 0.5*	8.2 ± 0.1	7.5 ± 0.2*	6.9 ± 0.4	6.0 ± 0.1	10.4 ± 1.3	7.8 ± 0.2*
18:1n-7	4.9 ± 0.3	2.6 ± 0.3*	3.4 ± 0.1	1.6 ± 0.0*	5.6 ± 0.0	2.2 ± 0.1*	3.2 ± 0.1	1.2 ± 0.0*	3.3 ± 0.5	3.3 ± 0.3
18:2n-6	1.1 ± 0.0	0.9 ± 0.4	3.6 ± 0.4	0.5 ± 0.0*	2.1 ± 0.0	0.3 ± 0.1*	1.0 ± 0.0	0.2 ± 0.0*	0.9 ± 0.1	0.5 ± 0.2
18:3n-3	3.0 ± 0.3	0.7 ± 0.1*	2.8 ± 0.0	0.1 ± 0.0*	2.1 ± 0.1	0.2 ± 0.0*	1.1 ± 0.0	tr	0.8 ± 0.1	tr
18:4n-3	1.7 ± 0.2	0.6 ± 0.2*	0.4 ± 0.5	0.1 ± 0.0	0.3 ± 0.0	0.3 ± 0.1	0.1 ± 0.0	tr	0.8 ± 0.2	0.6 ± 0.0
20:0	0.5 ± 0.3	0.3 ± 0.0	0.1 ± 0.0	0.2 ± 0.0	0.2 ± 0.0	0.1 ± 0.0	0.3 ± 0.1	0.3 ± 0.1	0.2 ± 0.0	tr
20:1n-9	0.8 ± 0.1	0.8 ± 0.1	0.5 ± 0.2	0.8 ± 0.1	1.0 ± 0.0	1.0 ± 0.1	1.1 ± 0.1	1.2 ± 0.1	0.8 ± 0.2	0.7 ± 0.0
20:1n-7	0.1 ± 0.0	0.1 ± 0.0	0.1 ± 0.0	0.1 ± 0.0	0.2 ± 0.0	0.1 ± 0.0	0.3 ± 0.0	0.1 ± 0.1	0.2 ± 0.1	0.4 ± 0.0
20:2n-6	0.5 ± 0.3	0.1 ± 0.0*	0.3 ± 0.0	0.1 ± 0.0	0.3 ± 0.0	0.0 ± 0.0	0.4 ± 0.0	tr	0.7 ± 0.2	tr
20:3n-6	0.3 ± 0.0	0.1 ± 0.0*	0.1 ± 0.0	0.1 ± 0.0	0.6 ± 0.0	0.3 ± 0.1*	0.5 ± 0.0	tr	0.6 ± 0.0	0.1 ± 0.1*
20:4n-6	1.8 ± 0.2	1.2 ± 0.1*	0.6 ± 0.0	1.0 ± 0.0*	3.3 ± 0.0	1.0 ± 0.0*	0.9 ± 0.0	0.6 ± 0.2	8.4 ± 1.7	10.1 ± 0.4
20:3n-3	1.2 ± 0.1	0.1 ± 0.0*	0.6 ± 0.0	0.1 ± 0.0*	2.4 ± 0.0	0.1 ± 0.0*	1.8 ± 0.1	0.1 ± 0.0*	0.8 ± 0.0	0.1 ± 0.1*
20:4n-3	0.7 ± 0.1	0.4 ± 0.0*	0.4 ± 0.0	0.5 ± 0.0	1.2 ± 0.0	0.5 ± 0.0*	1.3 ± 0.0	0.4 ± 0.0*	0.4 ± 0.0	0.2 ± 0.1
20:5n-3	8.2 ± 1.0	2.9 ± 0.3*	4.2 ± 0.3	4.9 ± 0.1	17.5 ± 0.2	2.5 ± 0.1*	5.8 ± 0.4	2.2 ± 0.4*	13.3 ± 2.6	12.1 ± 0.4
22:1n-9	0.5 ± 0.2	0.4 ± 0.0	0.1 ± 0.0	0.6 ± 0.0*	0.1 ± 0.0	0.1 ± 0.0	0.3 ± 0.0	tr	0.4 ± 0.0	0.5 ± 0.5
22:4n-6	0.1 ± 0.1	0.1 ± 0.0	0.3 ± 0.3	tr	0.1 ± 0.0	0.3 ± 0.1	0.3 ± 0.0	0.1 ± 0.1	tr	tr
22:3n-3	0.2 ± 0.0	tr	0.3 ± 0.2	tr	0.1 ± 0.0	0.1 ± 0.0	0.4 ± 0.0	tr	0.1 ± 0.0	0.1 ± 0.1
22:5n-6	0.1 ± 0.0	0.2 ± 0.0	0.1 ± 0.0	0.1 ± 0.0	0.2 ± 0.0	0.2 ± 0.0	0.6 ± 0.0	0.4 ± 0.0*	0.2 ± 0.1	tr
22:5n-3	2.8 ± 0.3	1.7 ± 0.1*	0.9 ± 0.1	1.9 ± 0.0*	5.1 ± 0.1	2.1 ± 0.1*	8.5 ± 0.7	4.9 ± 0.1*	2.5 ± 0.1	1.4 ± 0.1*
22:6n-3	8.2 ± 1.0	26.1 ± 2.5*	2.2 ± 0.2	25.8 ± 0.9*	15.1 ± 0.2	40.8 ± 0.2*	23.1 ± 2.1	47.1 ± 0.2*	8.4 ± 0.7	17.9 ± 1.5*
24:1n-9	1.3 ± 0.1	2.7 ± 0.3*	1.2 ± 0.1	2.8 ± 0.2*	0.1 ± 0.0	0.2 ± 0.0	0.8 ± 0.1	1.7 ± 0.2*	0.6 ± 0.0	0.3 ± 0.0*
Total SFA	26.0 ± 1.1	27.6 ± 2.1	33.7 ± 1.7	32.5 ± 0.4	18.7 ± 0.3	24.0 ± 0.3*	27.2 ± 0.2	28.2 ± 0.3	23.3 ± 2.1	33.7 ± 0.3*
Total MUFA	30.8 ± 0.4	25.6 ± 0.6*	34.4 ± 1.1	24.3 ± 0.8*	16.6 ± 0.4	13.1 ± 0.1*	14.6 ± 1.0	11.3 ± 0.3*	25.7 ± 0.7	15.7 ± 0.5*
Total PUFA	32.0 ± 3.3	36.0 ± 2.4	18.9 ± 0.9	36.9 ± 1.0*	53.0 ± 0.7	50.4 ± 0.4	48.1 ± 3.5	56.6 ± 0.2*	39.4 ± 4.5	44.1 ± 1.7
Total DMA	2.1 ± 0.2	3.5 ± 0.2*	nd	nd	6.5 ± 0.1	8.2 ± 0.2*	nd	nd	nd	nd
n-6 PUFA	4.9 ± 0.3	2.5 ± 0.5*	5.2 ± 0.6	1.9 ± 0.0*	7.2 ± 0.2	2.6 ± 0.4*	4.1 ± 0.2	1.5 ± 0.3*	11.1 ± 1.9	10.8 ± 0.4
n-3 PUFA	26.5 ± 3.1	32.9 ± 2.8*	12.1 ± 0.5	33.7 ± 1.0*	44.6 ± 0.5	47.2 ± 0.2*	43.4 ± 3.4	55.0 ± 0.3*	27.6 ± 2.6	32.7 ± 1.9

[a]Data are expressed as wt percentage of total fatty acids and represent the mean ± SD (n=3). SD = 0.0 implies an SD of <0.05. Totals include some other minor components not shown. *, significantly different ($P < 0.05$); nd, not detected; tr, trace < 0.1%; GPC, total diradyl glycerophosphocholine; GPE, total diradyl glycerophosphoethanolamine; SFA, saturated fatty acid; MUFA, monounsaturated fatty acid; PUFA, polyunsaturated fatty acid; DMA, dimethyl acetal.

decreased, except that of DHA which more than doubled by day 105.

The percentage of total saturated fatty acids increased significantly in PI, whereas that of total monoenes decreased significantly from day 38 to day 105. The percentage of DHA in PI more than doubled over this period. PI was characterized by the highest percentage of 20:4n-6 of all the glycerophospholipids and also the highest percentage of EPA other than GPE on day 38.

Brain dry weight was not correlated directly with changes in proportions of 20:4n-6 in total lipid during development ($r = -0.50$; $p = 0.08$) but, in contrast, brain dry weights and the percentages of DHA in total lipid presented a significant, direct correlation ($r = 0.79$; $p < 0.01$) during the study period.

The time courses for the most significant fatty acid changes observed during the period from 38 to 105 days post-hatch are shown in Figures 1-3. In total lipid the largest increase in the percentage of DHA occurred between days 38 and 56 (Fig. 1). In contrast, the percentages of EPA and total monoenes decreased between days 38 and 56 (Fig. 1). The 1st order rate equation (Equation 3) that fitted the evolution of DHA in total lipid was:

$$WP = 27.7 - 69.1e^{-0.032\,t} \quad (k = 0.032 \pm 0.001) \quad [3]$$

where the initial rate at day 38 was very high and the net forward rate decreased, approaching zero at the equilibrium position, and 27.7 is the estimated maximum value for the percentage of DHA in total lipid at the end of development.

In GPC, the percentages of DHA and total monoenes were inversely correlated ($r = -1.1$; $p < 0.01$) during the observed period of development (Fig. 2). The 1st order rate equation (Equation 4) fitting the changes in the percentages of DHA in brain GPC was:

$$WP = 24.9 - 131.5e^{-0.046\,t} \quad (k = 0.046 \pm 0.016) \quad [4]$$

showing a very high initial rate and defining a maximum percentage of DHA in GPC of approximately 25%.

The percentages of EPA and DHA in GPE were inversely correlated ($r = -0.73$; $p = 0.017$) with the initial value of EPA higher than that of DHA (Fig. 2). The

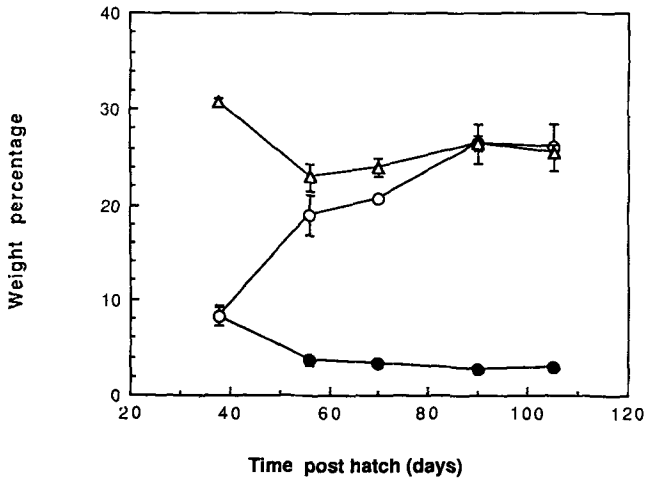


FIG. 1. Variation in the proportions of fatty acids of brain total lipids from turbot during development. (●) 20:5n-3; (○) 22:6n-3; (△) total monounsaturated fatty acids.

proportion of EPA decreased throughout the remaining period. In contrast, the proportion of DHA was increased reaching 40% of GPE fatty acids by day 105. The 1st order rate equation (Equation 5) defining the evolution of DHA in GPE during development was:

$$WP = 40.2 - 193.2e^{-0.054\,t} \quad (k = 0.054 \pm 0.017) \quad [5]$$

showing an estimated maximum percentage of DHA in brain GPE of 40.2% with a higher initial rate than GPC.

PS showed the highest levels of DHA, which increased throughout development, whereas other n-3 PUFA decreased between days 38 and 70 (Fig. 3). The equation (Equation 6) representing the evolution of DHA levels in PS was:

$$WP = 51.0 - 74.4e^{-0.026\,t} \quad (k = 0.026 \pm 0.013) \quad [6]$$

giving an estimated maximum percentage of DHA retained in PS of 51%.

The percentages of 20:4n-6 and EPA in PI remained relatively constant during the study period (Fig. 3). The

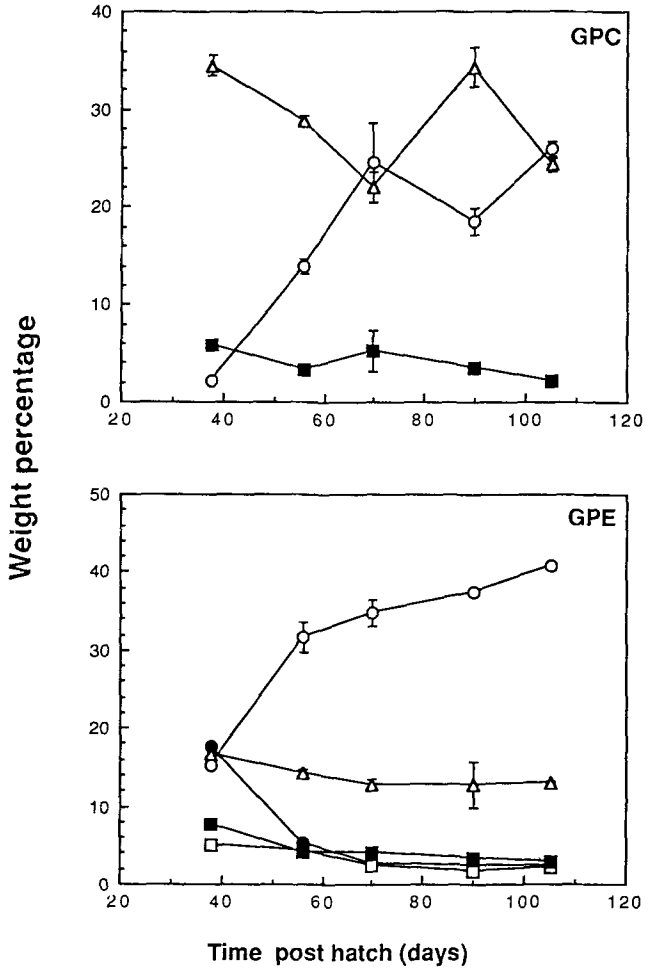


FIG. 2. Variation in the proportions of fatty acids in total diradyl glycerophosphocholine (GPC) and total diradyl glycerophosphoethanolamine (GPE) from turbot brain during development. (●) 20:5n-3; (□) 22:5n-3; (○) 22:6n-3; (△) total monounsaturated fatty acids; (■) total n-6 fatty acids.

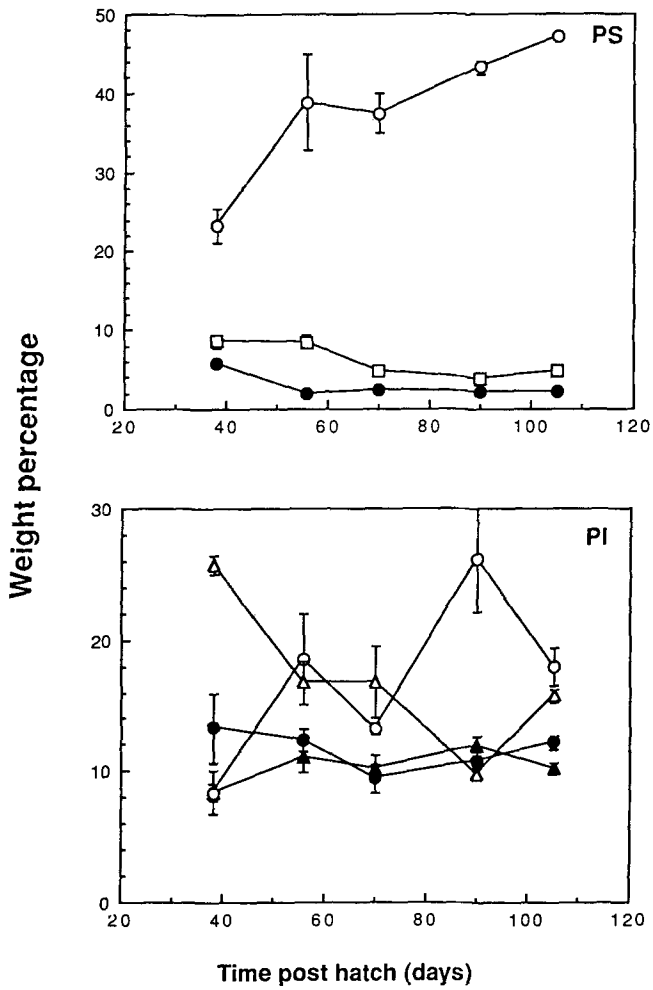


FIG. 3. Variation in the proportions of fatty acids in phosphatidylserine (PS) and phosphatidylinositol (PI) from turbot brain during development. (●) 20:5n-3; (□) 22:5n-3; (○) 22:6n-3; (▲) 20:4n-6; (△) total monounsaturated fatty acids.

percentage of DHA in PI fluctuated, but was inversely correlated to that of total monoenes ($r = -0.7$; $p < 0.01$).

DISCUSSION

Anatomical and biochemical development of brain are sequential processes characterized by specific stages of growth and maturation. The period of brain development under study in the present work may correspond to Period IIa as described by Ansell (22) in mammalian species. This period or "growth spurt" includes the processes of axonal and dendritic growth, glial multiplication, myelination and the development of enzyme systems (22,23). Therefore, the increases in the proportions of total protein, total cholesterol and galactolipids observed in the present study may be due to these processes of growth, restructuring and maturation of brain membranes combined with increasing myelination. Myelination in trout occurs over several months and is accompanied by an increasing ratio of galactolipids to phospholipids, although the ratio of cholesterol to phospholipids in developing trout brain is more constant than the values observed here (24). Trout are similar to turbot in that the proportions of phospholipid classes do not change throughout development (24). Although the data in the present study are consistent with Period IIa of development, it is not clear when Period I, characterized by neuronal multiplication, is complete in fish.

The most significant aspect of lipid metabolism in the developing brain of juvenile turbot is the accumulation of DHA. Accumulation of PUFA during the development of mammalian brain occurs during periods of cell division (25) and, although it is unclear at what stage of development the turbot brains are (see above), both Periods I and IIa are characterized by cell division (22). However, the comparatively low level of DHA in the turbot brain at day 38 and its subsequent enrichment in brain lipids may also reflect factors other than development. The pellet diet contained more DHA, gram for gram, than the *Artemia* diet, the fish may consume more of the pellet diet and there may be differences in the relative ability of turbot to digest the different diets.

The accumulation of DHA was extremely specific, with no other fatty acid increasing in percentage in total lipid other than a small increase in 24:1. In particular, there was no evidence for selective accumulation of arachidonic acid in the fish brain; no correlation was found between this fatty acid and brain dry weight. In comparison, both DHA and arachidonic acid specifically accumulate during development of mammalian brains (25–28).

The accumulation of DHA in turbot brain was rapid and quantitatively very large. From the data for brain dry weights, brain lipid contents, fatty acid content of lipid classes (*e.g.* approximately 35% of the weight of glycerophospholipids is due to the fatty acid components, for triacylglycerols the figure is 90%, etc.) and the percentage of DHA in total lipid, we can calculate the DHA content per brain. On day 38, an individual brain contained <10 μg DHA, whereas on day 105 each brain contained almost 125 μg DHA. This represents an accretion rate of, on average, 1.7 μg DHA per day. The kinetic data show that the rate of accumulation is initially much higher and indicate that the brain DHA content was increasing by >20% every day in the first few days on the pellet diet.

The percentage of DHA had attained its estimated maximal value in total lipid by approximately 7 wk and in GPC and GPE by 10 wk, after placing turbot on the pellet diet. In comparison, the DHA levels in brain cells of rats switched to a soybean oil diet at 60 days of age (after being fed sunflower oil for 4 generations) had not reached control values after 10 wk (29). In n-3 PUFA-deficient rhesus monkeys, brain DHA levels recovered to the levels of control animals after 12 wk on a diet supplemented with fish oil (30). However, the initial levels of brain DHA before changing to n-3 PUFA-containing diets and the dietary compositions (*e.g.* DHA content) in these studies are not directly comparable to those in the present study. Furthermore, no evidence for n-3 PUFA deficiency was found in the turbot at the start of the present study. Nevertheless, the accumulation of DHA in the developing turbot was not dramatically different from the accumulation of DHA in the brains of n-3 PUFA-deficient mammals.

The accumulation of DHA in turbot brain was accompanied by decreases in the percentages of all other fatty acids except saturated fatty acids and, in PI, n-6 PUFA. All the individual monoenes and PUFA generally contributed to the decreases, although to different extents in different glycerophospholipids. In contrast, saturated fatty acids were generally conserved relative to DHA. This is different from the situation in n-3 PUFA-deficient mammalian brains during dietary n-3 PUFA supplementation. There the accumulation of DHA is compensated almost solely by decreasing levels of 22:5n-6 (29,30). Furthermore, other n-3 PUFA also increase, particularly EPA and 22:5n-3, especially when fish oil is fed (29,30). Changes in total saturated fatty acids and monoenes in the mammalian studies are not dramatic and are variable, depending upon the fatty acid compositions of the diets fed.

The data in the present study suggest that juvenile turbot have low Δ5 and limited Δ4 desaturase activities. The *Artemia* diet contained a high level of 18:3n-3 and this was reflected in the levels of 18:3, 18:4, 20:3 and 20:4n-3 observed in day-38 fish, consistent with very low Δ5 desaturase activity. In addition, *Artemia* also contained 4.4 μg EPA/mg diet dry weight which would avoid the Δ5 block. However, the levels of EPA in the brains of day-38 fish were much higher than expected and exceeded the levels of DHA in all glycerophospholipid classes except PS. The levels of EPA in brains were reduced after 10 wk on the pellet diet, although this diet contained higher levels of EPA than the *Artemia* diet. These data suggest that preformed DHA is required by the brain and that liver, as well as brain, in turbot has low Δ4 desaturase activity. Therefore, only DHA present in the diet can contribute to the accumulation of DHA in turbot brain.

The rank order of the glycerophospholipid classes in initial rates of accumulation of DHA was GPE > GPC > PS. However, the decrease in DHA at day 90 in GPC affected the fit of the line and tended to lower the estimated initial rate value. Therefore, there is no evidence in this study that any glycerophospholipid class, either *via de novo* biosynthesis and/or *via* turnover (deacylation/reacylation), is preferentially acylated with dietary DHA. Ether-linked glycerophospholipid may have a role in the initial incorporation of long-chain PUFA, especially DHA, into neural tissues (31,32). However, further analyses of ether-linked ethanolamine and choline glycerophospholipids are required.

By day 105, GPE and PS contained the highest percentages of DHA in the turbot brain. High levels of DHA in GPE and PS are common characteristics of mammalian cerebral cortex (1) and vertebrate retinal photoreceptors (1,2,14,33). The total PUFA contents of GPE and PS in turbot brain, at >50%, suggest the presence of di-PUFA molecular species and possibly of di-docosahexaenoyl GPE and PS. Di-PUFA and di-22:6n-3 species of GPE and PS are found in relatively large amounts in both trout brain (14) and cod brain (34). The properties that di-22:6n-3 phospholipids confer on membrane structure and the precise nature of their role(s) in the specialized functions of these tissues remain to be elucidated (14). However, among subcellular fractions of mammalian neural tissue, the highest levels of DHA and di-22:6n-3 molecular species are found in synaptosomes and synaptic vesicles, as well as in mitochondria and microsomes (1,2). Therefore, DHA may be involved in action potentials or fast signal transmission. However, the signal transduction process is mainly associated with the rapid turnover of inositol phospholipids in which 20:4n-6 is a major component in mammals. PI in fish brain contains the highest level of 20:4n-6 of all the glycerophospholipids and substantial levels of EPA (12). Turbot brain PI also had relatively high levels of both 20:4n-6 and EPA, which remained relatively constant during development.

DHA in neural tissues may have diverse roles related to the biophysical properties of membranes, (1,2,35), as a modulator of lipid-protein interaction and neural enzyme activity (1), and as a precursor of lipoxygenase products (36). The results of the present study have shown that DHA was rapidly accumulated by the developing fish brain during development post-hatch. The accumulation was specific for DHA, and no selective accumulation of 20:4n-6 was apparent. DHA was accumulated at the expense of monoenes and other n-3 and n-6 PUFA, but the percentage of saturated fatty acids was maintained. The accumulation of DHA was maximal 10 wk after transfer to a diet supplying excess DHA. No individual glycerophospholipid class played any specific role in the accumulation process.

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Fatty Acid Composition of Subcellular Particles from Sheep Platelets and Topological Distribution of Phosphatidylethanolamine Fatty Acids in the Plasma Membrane

J. Sánchez-Yagüe, J.A. Cabezas and M. Llanillo*
Department of Biochemistry and Molecular Biology, Faculty of Biology, University of Salamanca, 37008 Salamanca, Spain

The fatty acid composition of individual phospholipids in subcellular fractions of sheep platelets and the asymmetrical distribution of phosphatidylethanolamine (PE) fatty acyl chains across the plasma membrane were examined. The main fatty acids of total lipid extracts were oleic (18:1; 32–41%), linoleic (18:2, 10–17%), stearic (18:0; 13–15%), palmitic (16:0; 11–15%) and arachidonic (20:4; 8–12%) acids, with a saturated/unsaturated ratio of about 0.4. Each phospholipid class had a distinct fatty acid pattern. Sphingomyelin (SM) showed the highest degree of saturation (50%), with large proportions of behenic (22:0), 18:0 and 16:0 acids. The main fatty acid in PE, phosphatidylserine (PS) and phosphatidylcholine (PC) was 18:1n-9. Our findings suggest that fatty acids are asymmetrically distributed between the choline *versus* the non-choline phospholipids, and also between plasma membranes and intracellular membranes. The transbilayer distribution of PE fatty acids in plasma membranes from non-stimulated sheep platelets was investigated using trinitrobenzenesulfonic acid (TNBS). A significant degree of asymmetry was found, which is a new observation in a non-polar cell. The PE molecules from the inner monolayer contained higher amounts of 18:2 and significantly less 18:1 and 20:5 than those found in the outer monolayer, although no major differences were detected in the transbilayer distribution of total unsaturated *versus* saturated PE acyl chains.

Lipids 26, 878–883 (1991).

The platelet plasma membrane plays an important role in hemostatic phenomena (1,2). In human platelets, changes in saturated/unsaturated fatty acid ratios have been shown to be associated with platelet hyperfunction (3) and antithrombotic action (4,5). Interest has increased considerably in recent years in determining the nature of the relationship between fatty acid group composition, membrane physical properties and membrane function. Fatty acid compositions of red blood cell phospholipids in many animal species (6) and also of several purified membrane fractions of human platelets have been reported (7–10). Additionally, the fatty acid distribution between the two halves of the bilayer has been described in plasma membranes from different sources (11–14). The fatty acid asymmetric location found in these studies raises the possibility that membrane properties may be modulated in different ways in the bilayer leaflets.

Prediction of the physical and dynamic properties of a biological membrane requires previous knowledge of the distribution of different lipid classes and fatty acid moieties in the two halves of the bilayer. However, the distribution of the aminophospholipid fatty acids in the platelet plasma membrane, a subcellular fraction where aminophospholipids constitute a very important lipid class involved in the coagulation process (15), has not yet been elucidated. The fatty acid composition of the different subcellular fractions isolated from sheep platelets is also not known, and only limited data are available on the fatty acid composition of individual phospholipids from platelet plasma membranes.

The aim of the present study was to examine the phosphatidylethanolamine (PE) fatty acid distribution in platelet plasma membranes, using trinitrobenzenesulfonic acid as a probe. The fatty acid composition of lipid extracts and of individual phospholipids of intact sheep platelets and their subcellular fractions were also established.

MATERIALS AND METHODS

Materials. Essentially fatty acid- and globulin-free bovine serum albumin (BSA), phenylmethylsulfonyl fluoride (PMSF) and tris(hydroxymethyl)aminomethane were purchased from Sigma (St. Louis, MO). D-Sucrose was obtained from Aldrich-Europe (Beerse, Belgium). Phospray, phospholipid and fatty acid methyl ester (FAME) standards, and methanolic-base were obtained from Supelco (Gland, Switzerland); 2,4,6-trinitrobenzenesulfonate (TNBS) was purchased from Serva Feinbiochemica (Heidelberg, Germany). Thin-layer chromatographic plates (Silica gel G, type 60) were obtained from E. Merck (Darmstadt, Germany). Other products were from Probus or Panreac (Barcelona, Spain). All organic solvents were of high quality and glass-distilled twice.

Preparation of platelets and isolation of subcellular fractions. Platelets were isolated from freshly collected blood of adult sheep (*Ovis aries* L. var. *domestica*) according to the procedure of Barber and Jamieson (16), slightly modified as indicated previously (17). Platelet preparations were sedimented at 3,000 × *g* for 30 min, then gently suspended in solution 1 (5 mM $Na_2PO_4H/NaPO_4H_2$, 0.154 M NaCl pH 7.4 containing 0.2% glucose, 0.05% BSA and 0.05 mM PMSF), and washed once in the same solution. Three main subcellular fractions were isolated from whole platelets using a discontinuous sucrose gradient under conditions similar to those used by Llanillo and Cabezas (18). The characterization of the fractions was described in previous papers (18,19). The upper fraction in the gradient was enriched in plasma membranes. The middle fraction consisted of endoplasmic reticulum, and the lower fraction comprised mitochondria and most of

*To whom correspondence should be addressed at the Department of Biochemistry and Molecular Biology, Faculty of Biology, Pza. de la Merced 1, 37008 Salamanca, Spain.

Abbreviations: ANOVA, analysis of variance; BSA, bovine serum albumin; CGLC, capillary gas-liquid chromatography; FAME, fatty acid methyl esters; PC, phosphatidylcholine; PE, phosphatidylethanolamine; PS, phosphatidylserine; SM, sphingomyelin; TLC, thin-layer chromatography; TNBS, trinitrobenzenesulfonic acid.

the granules. The protein content of the different fractions was determined by the method of Lowry *et al.* (20), using BSA as standard.

Trinitrophenylation of the aminophospholipids. The platelet labeling procedure was carried out under conditions identical to those described by Sánchez-Yagüe and Llanillo (21). TNBS (150 mM solution buffered with 5% $NaHCO_3$) was added to whole platelets (5-6 μmol TNBS/mg protein) to a final TNBS/lipid phosphorus molar ratio of 35. Labeled platelets were washed twice with solution 1 to remove free TNBS and then the plasma membrane fraction was obtained as indicated above. The supernatant from the labeling incubation system was used to measure platelet lysis by assay of lactate dehydrogenase activity (22).

Lipid extraction, fractionation and identification. Total lipid extraction from labeled or unlabeled materials, separation and identification of phospholipid classes, determination of their lipid phosphorus, and calculation of labeled phosphatidylserine and phosphatidylethanolamine were carried out as reported by Sánchez-Yagüe and Llanillo (21). Special care was taken in maintaining a nitrogen atmosphere during all steps of the extraction and fractionation procedures.

Capillary gas-liquid chromatography (CGLC). An aliquot of lipid extracts from each fraction (100 μg lipid phosphorus) was transmethylated using a sodium methoxide/methanol reagent (methanolic base), as reported elsewhere (23). Phosphatidylcholine (PC), phosphatidylserine (PS), phosphatidylethanolamine (PE), and sphingomyelin (SM), separated by thin-layer chromatography (TLC) as indicated above, were localized by staining with "phospray." Wet silica gel areas containing the phosphatides were rapidly scraped into dry tubes and treated with methanolic base. The fatty acid methyl esters (FAME) of unfractionated lipids and individual phospholipids were partitioned in a water/petroleum hydrocarbon system, brought to dryness under nitrogen in small conical tubes, and dissolved in 10-μL aliquots of isooctane for analysis by CGLC. FAME were determined in a Konik (model 3000, Barcelona, Spain) apparatus equipped with a flame ionization detector (FID) and a capillary column of the chemical bond type (BP5, SGE, 50 m × 0.25 mm) programmed as follows: initial 5 min hold at 180°C, 2°C/min 180-230°C, final 20 min hold at 250°C; carrier gas, H_2 (inlet pressure 2 atmospheres); splitless 1:60. Injector port and detector temperatures were 240°C and 280°C, respectively. Appropriate blank areas of silica gel or aliquots of chloroform/methanol (2:1, v/v) without lipids were transmethylated; the products were also gas chromatographed. The areas of the peaks were subtracted from those of the sample under study to account for background contamination. The fatty acid peaks were identified by comparison of their retention times with those of known FAME standards. FAME (23:0 and 24:0; 22:6, 24:0 and 24:1) with similar retention times in CGLC, were resolved into individual esters by argentation TLC (24), extracted with diethyl ether, and determined by CGLC as indicated above. Peak areas were measured with an electronic integrator (Konik model SP 4290).

Statistical analysis. Analysis of variance (ANOVA I) complemented with the Scheffé and Newman-Keuls multiple-range tests were used as statistical methods on an IBM-PS/2 computer.

RESULTS AND DISCUSSION

Fatty acid composition of sheep platelets and their subcellular fractions. Table 1 shows the fatty acyl chain profiles of lipids extracted from sheep platelets and their subcellular fractions. The major fatty acids, representing about 90% of total, were 18:1n-9 (32-41%), 18:2 (10-17%), 16:0 (11-15%), 18:0 (13-15%) and 20:4 (8-12%). Other fatty acids were present at percentages below 5%. These compositions were quite similar in whole platelets, in granule-mitochondria fractions, and in membranes. However, statistically significant differences ($p<0.05$) were found in the percentages of 18:1n-9 and 18:2, which increased progressively in the following order: whole platelets, plasma membranes, endoplasmic reticulum and granule-mitochondria fractions. Other fatty acids (16:1, 18:3, 20:1 and 20:0) occurring in low percentages (<2%) also showed significant differences. Total fatty acids were mainly unsaturated (68-73%), with saturated/unsaturated ratios of about 0.4. Previously published data indicate that the acyl chains of platelet total lipids from different animal species, such as the guinea pig (25), the miniature pig (26), humans (9,10,27) and the rat (28), are composed of the same fatty acids (16:0, 18:0, 18:1, 18:2 and 20:4) as those found in sheep platelets. However, the relative content of individual fatty acid changes somewhat with the species studied. The similarities in the phospholipid composition and in the individual phospholipid fatty acid composition between megakaryocytes and platelets (25) suggest that megakaryocyte are responsible for the overall phospholipid and fatty acid content of the platelets. The greatest difference between these two cell types is the higher concentration of arachidonic acid and a lower concentration of oleic acid in the platelets. This difference is probably due to an accumulation of arachidonic acid by the megakaryocyte in membranes destined to become part of the platelet, *i.e.*, the plasma and certain granule membranes (25).

The fatty acid composition of phospholipid classes from sheep platelets shows more pronounced differences than those of the total lipid extracts; the aminophospholipids show the largest variation (Table 2). The main fatty acid components of PE in all fractions are 18:1n-9 (42.5-47%), 18:0 and 18:2 (12-16%), 20:4 (11-18%) and 16:0 (4-5%). The most significant differences were found in the percentage increase ($p<0.05$) of 18:0 and 20:4 in PE of the plasma membranes compared to the percentage increase of these fatty acids in PE from intracellular membranes. The same five acids accounted for approximately 90% of the total in PS. However, PS had considerably more 18:0, less 20:4, slightly less 18:1n-9, and similar 16:0 and 18:2 levels as compared to PE. Stearic and 18:1n-9 acids increased ($p<0.05$) in PS from plasma membranes and endoplasmic reticulum. PE had the highest unsaturation index compared to the other phospholipids and a saturated/unsaturated ratio of about 0.2, while in PS this ratio was increased to 0.6. PE of subcellular fractions shows a significant increase in the unsaturation index compared to the PE of whole platelets. By contrast, this index decreases in the PS fractions. Table 3 shows the fatty acid composition of choline phospholipids. The major PC fatty acids, representing about 90% of the total, are 16:0 and 18:1n-9 (30-38%), 18:2 (11-13%) and 18:0 (9-11%), with a significant increase ($p<0.05$) in 16:0 in the plasma

TABLE 1

Fatty Acid Composition of Lipid Extracted from Sheep Platelets and Their Subcellular Fractions[a]

Fatty acid	Whole platelets	Subcellular fraction		
		Plasma membranes	Endoplasmic reticulum	Mitochondria + granules
16:0	13.0 ± 1.8	15.0 ± 0.4	14.1 ± 2.2	11.4 ± 1.7
16:1	1.4 ± 0.3[b]	N.D.[b]	1.6 ± 0.8[b]	0.7 ± 0.1[c]
18:0	13.0 ± 2.8	12.7 ± 2.3	15.4 ± 1.6	13.9 ± 1.8
18:1n-7	2.6 ± 0.6	1.7 ± 0.6	2.1 ± 1.2	1.3 ± 0.3
18:1n-9	31.9 ± 4.7[c]	32.3 ± 4.8[c]	37.6 ± 2.8[b]	41.1 ± 5.9[b]
18:2	10.0 ± 2.1[c]	12.5 ± 2.9[c]	15.1 ± 1.2[b]	17.0 ± 3.0[b]
18:3	1.7 ± 0.4[c]	1.4 ± 0.3[b,c]	N.D.[b]	N.D.[b]
20:1	5.0 ± 2.0[b]	4.1 ± 0.8[b]	0.1 ± 0.1[c]	1.1 ± 0.6[c]
20:2	N.D.	N.D.	1.2 ± 0.8	N.D.
20:4	10.4 ± 0.8	12.3 ± 2.3	10.8 ± 1.8	8.0 ± 1.9
20:5	1.9 ± 0.6	2.1 ± 0.2	2.3 ± 1.2	2.9 ± 1.8
22:0	1.9 ± 0.5[b]	0.9 ± 0.2[c]	0.6 ± 0.2[c]	1.7 ± 0.4[b]
22:1	1.2 ± 0.3	0.8 ± 0.2	0.4 ± 0.2	1.1 ± 0.2
22:5	1.6 ± 0.4	N.D.	N.D.	N.D.
Sat.	27.4 ± 1.3	29.0 ± 4.1	28.5 ± 3.1	28.2 ± 1.7
Polyunsat.	26.1 ± 2.1	27.3 ± 3.5	28.4 ± 1.5	28.4 ± 3.0
Monounsat.	41.3 ± 3.8	37.5 ± 2.5	40.4 ± 3.9	44.6 ± 3.2
Total unsat.	68.2 ± 5.3	67.7 ± 7.8	70.3 ± 7.1	73.0 ± 7.7
Sat./unsat.	0.40	0.43	0.40	0.39
U.I.[d]	119.4 ± 20.5	130.4 ± 18.0	121.0 ± 19.5	117.3 ± 30.1

[a]Data shown are given in percentages of total fatty acid content and are means ± S.D. (n = 5, at each point). CGLC determinations were done in triplicate on each sample. By rows, the significance of the results was assessed by ANOVA I of compounded data (superindices without coinciding letters [b,c]: $p<0.05$) as well as by the Scheffé and Newman-Keuls tests. Trace amounts (less than 1%) of 14:0, 18:4, 20:0, 20:3, 23:0, 22:4, 22:6 and 24:0 fatty acids were present. N.D. not detected; Sat., saturated; Unsat., unsaturated.

[d]Unsaturation Index (U.I.) was calculated as the sum of the percentage by weight of each fatty acid times the number of olefinic bonds.

membrane fraction. SM contains a large proportion of long-chain saturated fatty acids (50%) and has the smallest unsaturation index among the different phospholipid classes. SM has less 18:1n-9 (14–18%), 18:2 (8–9%) and 16:0 (8–11%) compared to the PC fractions and a similar 18:0 (11–14%) content; 22:0 (16–19%) and 22:1 increased ($p<0.05$) in the plasma membrane fractions. SM was the only phospholipid class in which 23:0 and 24:0 were identified. This appears to be a general characteristic of SM that has also been observed in other sources (6,9,10,29). The individual fatty acid patterns in sheep platelets are very different from those found in human platelets and erythrocytes (6,7,9,10,27,30). Certain fatty acids of human platelets and erythrocytes are predominant in specific phospholipids: 20:4 in PE, 18:0 in PS, 20:4 and 18:0 in phosphatidylinositol, and 16:0 and 18:1 in PC. The highest abundance of 20:4 in sheep platelets is found in PE (11–18%); however, this percentage is significantly lower than that found in platelets from other sources (7,9,25,26).

The present study demonstrates that fatty acids of sheep platelets are asymmetrically distributed among the phospholipids of the different particulate fractions. The concentration of 20:4, 18:0 and 18:1n-9 was twice as high in aminophospholipids than in choline phospholipids while the concentration of 16:0 was five times smaller. The amounts of linoleic acid were identical in both phospholipid classes. The saturated/unsaturated ratios of the non-choline phospholipids were considerably smaller than those of the choline phospholipids. However, the asymmetry of sheep platelet plasma membrane is not as pronounced as that described by Broekman *et al.* (10) for human platelets. Differences in some fatty acids, such as 20:4 from PE (significantly more enriched in the plasma membrane), exist between plasma membranes and intracellular membranes of sheep platelets. The data are in agreement with those reported for human platelets (7). By contrast, PC from the plasma membrane of human platelets is less enriched in 20:4 than are intracellular membranes (7). However, these latter differences have not been found in sheep platelets.

Studies on the contribution of each phospholipid to the saturation level in whole sheep platelets and their subcellular particles show that aminophospholipids lead to the smallest saturation percentage in each membrane fraction. The data can be calculated by the following expression: % "a" from "b," where "a" represents the saturated fatty acid percentage of a phospholipid class in a specific subcellular fraction (data given in Tables 2 and 3), and "b" is the relative percentage of this phospholipid in the same fraction [values given in Table 2 of our previous paper (21)]. Thus, the phospholipid yielding the highest percentage of saturation is PC

TABLE 2

Fatty Acid Compositions of Aminophospholipids from Sheep Platelets and Their Subcellular Fractions[a]

Fatty acid	Phosphatidylethanolamine				Phosphatidylserine			
	Whole platelets	Plasma membranes	Endoplasmic reticulum	Mitochondria + granules	Whole platelets	Plasma membranes	Endoplasmic reticulum	Mitochondria + granules
14:0	N.D.	0.5 ± 0.2	N.D.	N.D.	1.5 ± 0.6	N.D.	0.1 ± 0.1	N.D.
16:0	5.0 ± 0.6	4.4 ± 1.4	3.7 ± 1.1	4.1 ± 0.5	6.0 ± 1.0	3.9 ± 0.8	4.3 ± 0.9	5.3 ± 1.4
16:1	0.2 ± 0.2	0.2 ± 0.2	N.D.	N.D.	0.1 ± 0.1	0.6 ± 0.2	0.1 ± 0.1	N.D.
18:0	16.2 ± 1.7^b	14.0 ± 2.8^b	11.8 ± 2.0^c	12.0 ± 1.8^c	26.0 ± 2.0^c	33.2 ± 4.6^b	31.9 ± 8.6^b	23.0 ± 4.3^c
18:1n-7	3.9 ± 1.5^b	1.7 ± 1.1^c	2.4 ± 0.5^b	1.0 ± 0.8^c	1.3 ± 0.8	1.8 ± 0.6	1.2 ± 0.6	N.D.
18:1n-9	45.4 ± 3.4	42.5 ± 4.3	45.8 ± 2.7	46.7 ± 2.1	30.2 ± 0.7^c	41.7 ± 3.5^b	41.4 ± 4.6^b	32.5 ± 2.7^c
18:2	12.6 ± 1.7	14.1 ± 3.0	11.6 ± 2.9	12.8 ± 2.3	15.7 ± 2.1	8.4 ± 0.9	9.4 ± 1.0	10.0 ± 2.0
18:3	N.D.^{c}	0.2 ± 0.1^c	0.6 ± 0.1^{b,c}	0.7 ± 0.2^b	1.0 ± 0.2^c	N.D.^{c}	N.D.^{c}	4.5 ± 1.0^b
18:4	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.	1.0 ± 0.3	N.D.
20:1	0.4 ± 0.2^b	0.2 ± 0.2^c	0.3 ± 0.2^b	0.1 ± 0.1^d	0.3 ± 0.2^c	0.7 ± 0.3^c	0.2 ± 0.2^c	3.8 ± 0.9^b
20:2	0.3 ± 0.1^c	0.2 ± 0.2^c	0.3 ± 0.2^c	2.0 ± 1.0^b	1.9 ± 0.9^b	0.1 ± 0.1^c	0.6 ± 0.3^c	N.D.^{c}
20:3	0.3 ± 0.1	0.5 ± 0.5	0.8 ± 0.2	0.5 ± 0.2	0.7 ± 0.2^b	0.3 ± 0.1^c	0.9 ± 0.4^{b,c}	N.D.^{c}
20:4	10.6 ± 1.5^c	18.2 ± 3.4^b	13.9 ± 1.4^d	12.3 ± 1.7^c	5.5 ± 1.0^b	5.3 ± 1.3^b	5.3 ± 1.3^b	N.D.^{c}
20:5	1.2 ± 0.2^c	3.0 ± 1.5^d	4.8 ± 1.5^b	3.1 ± 1.4^d	3.6 ± 0.3	2.4 ± 0.6	1.4 ± 0.8	4.0 ± 1.0
22:0	N.D.	N.D.	N.D.	N.D.	1.6 ± 0.4^c	0.2 ± 0.1^d	1.1 ± 0.4^c	7.8 ± 1.5^b
22:1	N.D.	N.D.	N.D.	N.D.	2.0 ± 0.4^b	0.3 ± 0.1^c	0.6 ± 0.4^b	3.7 ± 0.7^b
22:4	N.D.	0.2 ± 0.2	0.5 ± 0.4	N.D.	1.3 ± 0.2^b	0.2 ± 0.1^c	N.D.^{c}	N.D.^{c}
22:5	N.D.	N.D.	0.5 ± 0.3	N.D.	0.6 ± 0.1	N.D.	N.D.	N.D.
22:6	0.4 ± 0.2	N.D.	N.D.	0.8 ± 0.3	N.D.	N.D.	N.D.	N.D.
Sat.	20.8 ± 1.6	16.1 ± 3.1	16.7 ± 2.1	16.2 ± 4.3	36.0 ± 3.4	38.3 ± 2.7	38.4 ± 3.3	37.4 ± 3.6
Monounsat.	48.6 ± 3.2	45.3 ± 6.4	47.3 ± 5.2	48.5 ± 6.8	32.9 ± 4.7	44.5 ± 6.8	42.8 ± 5.0	40.5 ± 4.8
Polyunsat.	25.9 ± 4.3^b	35.4 ± 3.4^c	32.5 ± 3.6^{b,c}	33.4 ± 4.5^{b,c}	30.8 ± 1.2^b	18.9 ± 3.4^c	17.6 ± 2.1^c	19.0 ± 3.5^c
Total unsat.	75.3 ± 6.2	78.0 ± 8.0	80.1 ± 5.5	81.3 ± 5.7	64.2 ± 6.9	59.6 ± 2.7	60.6 ± 4.3	61.6 ± 6.6
Sat./unsat.	0.28	0.21	0.21	0.20	0.56	0.64	0.63	0.61
U.I.[e]	126.6 ± 10.5^b	160.3 ± 20.8^c	163.4 ± 16.1^c	150.8 ± 15.6^c	120.2 ± 17.3^b	98.4 ± 10.1^c	97.4 ± 8.5^c	92.5 ± 9.5^c

[a]Data (mean ± S.D.; n = 5 at each point) are given in percentages of total fatty acids in each phospholipid. CGLC assays were carried out in triplicate on each sample. By rows, the significance of results was assessed by ANOVA I of compounded data (superindices without coinciding letters [b,c,d]: $p<0.05$) as well as by the Scheffé and Newman-Keuls tests. N.D., not detected; Sat., saturated; Unsat., unsaturated.

[e]Unsaturation Index (U.I.) was calculated as in Table 1.

TABLE 3

Fatty Acid Composition of Choline Phospholipids from Sheep Platelets and Their Subcellular Fractions[a]

Fatty acid	Phosphatidylcholine				Sphingomyelin			
	Whole platelets	Plasma membranes	Endoplasmic reticulum	Mitochondria + granules	Whole platelets	Plasma membranes	Endoplasmic reticulum	Mitochondria + granules
14:0	0.6 ± 0.2	N.D.	0.8 ± 0.3	N.D.	1.7 ± 0.5	1.0 ± 0.4	N.D.	4.7 ± 0.8
16:0	35.0 ± 3.2^b	33.5 ± 4.9^b	30.7 ± 2.7^c	30.0 ± 2.5^c	11.0 ± 3.0	10.5 ± 2.0	10.5 ± 2.3	8.0 ± 0.4
16:1	0.7 ± 0.5	N.D.	N.D.	N.D.	2.0 ± 0.5	N.D.	4.5 ± 0.5	N.D.
18:0	9.2 ± 0.9	11.3 ± 2.9	11.5 ± 1.5	10.0 ± 1.3	14.2 ± 1.9	10.7 ± 1.3	14.2 ± 3.7	14.0 ± 2.1
18:1n-7	3.8 ± 1.4	2.7 ± 1.1	2.6 ± 1.2	2.0 ± 0.7	2.2 ± 0.6^b	1.0 ± 0.8^c	N.D.^{c}	N.D.^{c}
18:1n-9	30.2 ± 2.6^c	36.6 ± 5.2^b	37.1 ± 3.1^b	37.9 ± 4.7^b	17.0 ± 2.5	18.3 ± 2.0	16.5 ± 3.0	14.3 ± 2.5
18:2	13.1 ± 0.9	10.6 ± 3.3	11.3 ± 3.7	13.1 ± 2.0	9.2 ± 1.8	7.7 ± 0.5	9.5 ± 1.5	8.0 ± 1.0
18:3	0.1 ± 0.1	0.1 ± 0.1	N.D.	N.D.	0.1 ± 0.1	N.D.	N.D.	N.D.
18:4	0.1 ± 0.1	0.1 ± 0.1	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.
20:1	1.2 ± 0.5	1.0 ± 0.5	1.5 ± 0.7	1.0 ± 0.3	1.0 ± 0.3	N.D.	N.D.	N.D.
20:2	0.6 ± 0.2^c	0.3 ± 0.3^c	1.0 ± 0.3^{b,c}	1.4 ± 0.5^b	2.0 ± 0.4^b	2.3 ± 0.6^b	5.0 ± 1.0^c	4.5 ± 1.0^c
20:3	0.2 ± 0.1	0.1 ± 0.1	N.D.	0.4 ± 0.2	3.0 ± 0.7^b	1.1 ± 0.5^b	6.0 ± 1.1^c	8.4 ± 1.1^c
20:4	3.1 ± 0.9	2.5 ± 0.9	2.8 ± 0.8	2.2 ± 0.4	N.D.	N.D.	N.D.	N.D.
23:0	N.D.	N.D.	N.D.	N.D.	4.7 ± 0.8^{b,c}	6.0 ± 1.5^b	2.3 ± 0.7^c	3.0 ± 1.0^{b,c}
20:5	0.4 ± 0.1^c	1.1 ± 0.3^b	1.4 ± 0.2^b	N.D.^{c}	1.0 ± 0.2	2.7 ± 0.7	3.2 ± 0.6	1.0 ± 0.5
22:0	N.D.	N.D.	N.D.	N.D.	16.0 ± 1.0	19.5 ± 1.5	16.5 ± 1.5	16.0 ± 2.0
22:1	N.D.	N.D.	N.D.	N.D.	10.0 ± 1.8^b	15.0 ± 2.0^b	6.5 ± 1.0^c	6.7 ± 1.0^c
22:5	N.D.	N.D.	N.D.	N.D.	N.D.	1.8 ± 0.6	N.D.	N.D.
24:0	N.D.	N.D.	N.D.	N.D.	2.8 ± 0.7^b	2.0 ± 0.5^b	5.8 ± 1.0^c	7.8 ± 2.1^c
Sat.	45.9 ± 5.9	43.1 ± 3.1	43.0 ± 3.0	41.1 ± 4.3	48.2 ± 3.5	50.3 ± 5.6	49.9 ± 5.0	52.1 ± 4.3
Monounsat.	34.3 ± 4.1	38.5 ± 5.1	40.8 ± 6.1	39.4 ± 2.5	33.4 ± 4.7	35.1 ± 3.9	30.4 ± 4.8	25.0 ± 5.5
Polyunsat.	16.4 ± 2.0	15.3 ± 1.7	16.3 ± 2.5	16.9 ± 1.8	14.7 ± 1.3	16.0 ± 0.8	21.0 ± 1.9	20.6 ± 1.7
Total unsat.	54.3 ± 5.8	57.6 ± 2.1	57.0 ± 3.0	58.9 ± 4.3	46.5 ± 5.6	40.8 ± 3.7	45.6 ± 5.1	46.9 ± 5.0
Sat./unsat.	0.84	0.75	0.75	0.70	1.04	1.23	1.09	1.11
U.I.[d]	78.3 ± 6.5	79.4 ± 5.2	82.3 ± 9.4	80.9 ± 7.5	66.5 ± 5.3	78.3 ± 8.5	77.8 ± 9.3	78.1 ± 5.3

[a]Data (mean ± S.D.; n = 5 at each point) are given in percentages of total fatty acids in each phospholipid. CGLC assays were carried out in triplicate on each sample. By rows, the significance of results was assessed by ANOVA I of compounded data (superindices without coinciding letters [b,c]: $p<0.05$) as well as by the Scheffé and Newman-Keuls tests. N.D., not detected; Sat., saturated; Unsat., unsaturated.

[d]Unsaturation Index (U.I.) was calculated as in Table 1.

(12-14%), followed by SM (7-8%), PE (5-8%) and PS (5-6%) (data not shown). Both aminophospholipids contribute about 5-6% to the total saturation, even though the percentage of PE is 2.5-3.0 times greater than that of PS in all subcellular fractions (21). The smallest amount of saturated fatty acids from PE with respect to PS is consistent with these values.

Topological distribution of PE fatty acids across the sheep platelet plasma membranes. These studies were carried out using TNBS to separate the PE of the outer monolayer (trinitrophenylated aminophospholipids) from those associated with the inner monolayer (non-reactive with TNBS). The fatty acyl chains of the isolated individual PE were analyzed. In our previous study (21), the topological distribution of aminophospholipids was determined with regard to the polar head groups. Whole sheep platelets were incubated with TNBS under conditions identical to those described in the present paper. This treatment essentially conserves platelet integrity (below 7% of lactate dehydrogenase found in the supernatant), thus preventing TNBS penetration into the platelets, as has been demonstrated previously (21,31). Accordingly, only external aminophospholipids are trinitrophenylated. After labeling, plasma membranes were obtained, and lipids were extracted. The plasma membrane separation procedure that was applied after labeling did not modify the fatty acid pattern of PE located on the external leaflet of the lipid bilayer (data not shown). All PS were located on the internal leaflet (21); therefore, only the distribution of the PE fatty acyl chains across the lipid bilayer were studied by analyzing the fatty acyl chains from trinitrophenylated PE and non-reactive PE. The PE fatty acid composition of the outer leaflet corresponded to the fatty acids found in the labeled PE. The fatty acyl chains from non-reactive PE were located in the inner leaflet of the lipid bilayer. These data are given in Table 4. The results demonstrate, for the first time, that in the plasma membrane of non-stimulated platelets, the two PE pools are different in their fatty acid composition. The PE molecules from the inner monolayer contain a higher amount of 18:2 and significantly ($p<0.05$) less 18:1n-9 and 20:5 than those from the outer monolayer. Nevertheless, the PE unsaturation indices are similar across the plasma membrane, a value not statistically different from that obtained when the total PE from the plasma membrane fraction was analyzed (Table 2).

Perret *et al.* (14) have shown that 20:4 is mostly located on the inside of human platelets in both intracellular membranes and in the inner leaflet of the plasma membranes. Thus the arachidonic acid used as a precursor for prostaglandin and thromboxane synthesis should come from intracellular phospholipids (4). Pelletier *et al.* (13) have found that in the posterior trout intestine, PS and PE fatty acid chains from the brush-border membrane are asymmetrically distributed, the inner phospholipid species having a higher content of 22:6n-3 than the outer ones. By contrast, the PC from the external leaflet is enriched in 20:5n-3 and 22:6n-3 (12). The important fluidizing role played by 22:6n-3 (32) suggests that it may regulate and possibly equalize the fluidity of each of the membrane leaflets. In other systems (11,33,34), an asymmetric distribution in saturated fatty acids between the two leaflets of the plasma membranes has also been reported, although with different results in function. Recently, the molecular species composition of PE on the inner and outer leaflet of the human erythrocyte membrane has been postulated (35), suggesting an asymmetric distribution across the plasma membrane. The PE pool in the outer monolayer displays a higher degree of unsaturation than the inner one, which may be compensated for by the relatively low degree of unsaturation of SM and PC in this bilayer (36,37).

The topological distributions described have numerous repercusions on structure (38) and membrane physical properties (39), and may help membrane enzymes to be modulated in different ways in the two bilayer leaflets. This has been explored in detail for adenylate cyclase (34). Plasma membrane acetylcholinesterase of sheep platelets, an enzyme associated with the external surface, changes its catalytic properties by progressive inclusion of free cholesterol into the plasma membrane (40). This increase in cholesterol causes a gradual shift of aminophospholipids from the inner to the outer side of the lipid bilayer (41), which may also produce changes in the distribution of individual phospholipid fatty acyl chains across the plasma membrane. These modifications in the lipid environment in which the enzyme is embedded may be responsible for the changes observed in acetylcholinesterase, as has been reported for several enzymes (42).

TABLE 4

Fatty Acid Composition of Phosphatidylethanolamines Distributed Between the Two Leaflets of the Lipid Bilayer from Sheep Platelet Plasma Membranes[a]

Fatty acid	External leaflet	Internal leaflet
16:0	5.0 ± 0.5	4.5 ± 1.0
18:0	10.1 ± 1.0	9.7 ± 0.3
18:1n-9	39.3 ± 0.5[b]	37.5 ± 1.2[c]
18:2	16.5 ± 1.1[c]	19.8 ± 1.2[b]
20:4	19.9 ± 1.1	19.9 ± 2.0
20:5	4.8 ± 0.3[b]	4.0 ± 0.3[c]
U.I.[d]	163.0 ± 14.5	170.3 ± 18.5

[a]Results (mean ± S.D.; n = 5 at each point) are given in percentages of total fatty acids in each sample. CGLC determinations were carried out three times on each sample. By rows, the significance of the results was assessed by ANOVA I of compounded data (superindices without coinciding letters [b,c]:$p<0.05$) as well as by the Scheffé and Newman-Keuls tests. 18:1n-7, 20:1, 20:2, 20:3, 22:4, 22:5 and 22:6-fatty acids were also detected at percentages less than 2%. The remaining fatty acids found in trace amounts in the plasma membrane (14:0, 16:1, 18:3, 18:4) were not detected in these assays.

[d]Unsaturation Index (U.I.) was calculated as in Table 1.

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Effect of Ethanol on Low Density Lipoprotein and Platelet Composition

Jerome L. Hojnacki[a,*], Joanne E. Cluette-Brown[a], Randall N. Deschenes[a], John J. Mulligan[a], Thaddeus V. Osmolski[a], Nicholas J. Rencricca[a], Joseph J. Barboriak[b] and Joseph A. Jakubowski[c]

[a]Lipid Research Laboratory, Department of Biological Sciences, University of Lowell, Lowell, Massachusetts 01854, [b]Zablocki Veterans Administration Medical Center, Milwaukee, Wisconsin 53295, and [c]Eli Lilly and Co., Indianapolis, Indiana 46285

The present study was designed to investigate the effect of ethanol (EtOH) dose on low density lipoprotein (LDL) and platelet composition. Male squirrel monkeys were divided into three groups designated Control, Low, and High EtOH, and fed isocaloric liquid diets containing 0%, 12%, and 24% of calories as EtOH, respectively. After four months of treatment, monkeys fed the 12% alcohol dose had LDL and platelet cholesterol concentrations similar to Controls. By contrast, platelet membranes from High EtOH animals contained significantly more cholesterol which was associated with higher levels of plasma LDL cholesterol and apolipoprotein B. Blood platelet count, size, and mass were similar for all groups and circulating platelet aggregates were absent in the two alcohol cohorts. Despite elevations in platelet cholesterol mass and thromboxane A_2 (TXA_2) precursor, phospholipid arachidonate, platelet responsiveness, measured as thromboxane formed in response to a collagen challenge *in vitro*, and the cholesterol/phospholipid molar ratio, were not significantly altered by high dose alcohol. Normal platelet activity in High EtOH monkeys may have resulted from a significant increase in the platelet phospholipid polyunsaturated/saturated fatty acid ratio and a non-significant increase in platelet phospholipid mass, both of which would have a fluidizing effect on platelet membranes. Our data indicate that low EtOH intake has no effect on platelet composition and function while unfavorable platelet cholesterol enrichment following consumption of high dose ethanol may arise from elevations in plasma LDL. Potentially thrombogenic (TXA_2 formation, platelet aggregate formation) consequences of increased platelet cholesterol in squirrel monkeys fed high levels of alcohol may be averted by compensatory physicochemical changes in platelet membrane lipid composition although persistent elevations in circulating LDL may contribute to increased risk of cardiovascular disease. *Lipids 26,* 884-890 (1991).

While moderate ethanol consumption may protect against coronary heart disease (CHD) (1,2), chronic alcohol abuse is associated with hypertension (3), vasospasm (4), stroke (5), and cardiomyopathy (3,6). In an effort to define the underlying mechanisms responsible for these disparate effects of ethanol on the cardiovascular system as related to its lipid modifying properties, we developed a non-human primate model which exhibits dramatically different lipoprotein profiles when these animals are fed low *vs* high dose ethanol (7-10). At low (12% of calories) alcohol levels, atherosclerosis susceptible squirrel monkeys show a coronary protective lipoprotein response in terms of an elevated plasma high density lipoprotein$_2$ (HDL_2/high density lipoprotein$_3$ (HDL_3) ratio and low concentrations of atherogenic low density lipoprotein (LDL) cholesterol and apolipoprotein B (9,10). When this species is given higher alcohol doses (24% calories), they demonstrate a further increment in HDL_2 along with elevations in HDL_3 and significant increases in LDL cholesterol and apolipoprotein B (10). As a point of reference, population surveys have estimated that nonalcoholics may consume 5-18% ethanol calories/day while EtOH intake, as a percentage of total calories, may range from 35-50% in alcoholics (11,12). While these observations may partially explain the differences in coronary risk between moderate drinkers *vs* alcoholics, other studies suggest that ethanol may also exert its cardiovascular effects by modifying the concentration of vasoactive prostanoids and by altering platelet function (13-17). Depending on dose and treatment period, ethanol produces variable alterations in vascular prostacyclin (PGI_2) and platelet thromboxane A_2 (TXA_2) (4,13,16-19). The relative production of these prostanoids may be of clinical importance because of the potent platelet anti-aggregatory, vasodilatory (PGI_2) *vs* pro-aggregatory/vasoconstrictory (TXA_2) properties of these compounds (20,21).

Very recently, several studies have implicated lipoproteins as important modulators of prostanoid production (22-26). Specifically, HDL may provide precursor (arachidonic acid) for PGI_2 synthesis and reduce platelet aggregation (27-31), while LDL may inhibit PGI_2 production (22,32-34) and contribute to platelet enrichment with cholesterol, hyperactivity, and enhanced TXA_2 formation (30,35). While some recent effort has been directed at understanding the relationship between alcohol, HDL, and PGI_2 (31), virtually nothing is known about the effect of ethanol on LDL levels and subsequent changes in platelet composition and function. Consequently, since our non-human primates show divergent responses to low EtOH (low LDL) *vs* high EtOH (high LDL) intake, the present study was designed to employ the squirrel monkey dietary ethanol model to examine the lipoprotein-platelet relationship as a function of alcohol dose.

EXPERIMENTAL PROCEDURES

Adult male squirrel monkeys *(Saimiri sciureus)* were maintained on isocaloric Primate Liquid Diet #19 (Bio-Serv, Inc., Frenchtown, NJ) for 5 mon after which a series of baseline measurements was performed to ensure initial between-group homogeneity. There were no significant differences between animals in platelet count, or plasma lipid parameters (cholesterol, triglyceride, HDL, and LDL

*To whom correspondence should be addressed at Department of Biological Sciences, University of Lowell, Lowell, MA 01854.

Abbreviations: ACD, acid citrate dextrose; CHD, coronary heart disease; C/PL, cholesterol/phospholipid; EDTA, ethylenediamine tetraacetate; EtOH, ethanol; GGT, gamma glutamyltranspeptidase; HDL, high density lipoprotein; LDL, low density lipoprotein; P/S, polyunsaturated/saturated; PGI_2, prostacyclin; PRP, platelet rich plasma; SGOT, serum glutamate oxaloacetate transaminase; TLC, thin-layer chromatography; TXA_2, thromboxane A_2; VLDL, very low density lipoprotein.

cholesterol). Animals were then randomly assigned to three treatment groups consisting of six to eight monkeys per group: (i) Controls fed isocaloric liquid diet; (ii) Low Ethanol monkeys given liquid diet with 100 proof vodka substituted isocalorically for carbohydrate at 12% of calories (2.36 g EtOH/day); and (iii) High Ethanol animals fed diet plus vodka at 24% of calories. Vodka was chosen as the source of ethanol to mimic an alcoholic beverage typically consumed by humans and because of its low concentration of congeners. The control diet had a caloric density of 0.87 kcal/mL, a caloric distribution of 18.4% protein, 52.2% carbohydrate, and 29.4% fat with a polyunsaturated/saturated (P/S) fatty acid ratio of 1.00 and a cholesterol content of 0.14 mg/kcal. Corn oil, coconut oil, chicken fat and soy lecithin were the sources of fat, and casein was the protein component of Liquid Diet #19. Diet consumed was measured after each feeding.

All measurements were performed after four months of treatment because we have previously determined that ethanol-fed squirrel monkeys exhibit stable lipoprotein profiles at this time (10), and because other studies indicate that the short-term (several weeks) platelet response to alcohol may differ appreciably from more long-term (months) exposure suggesting a process of "physiological adaptation" (4,17,18,36,37).

Lipoprotein measurements. After an overnight fast, blood was collected from the femoral vein of unanesthetized monkeys and transferred to tubes containing disodium EDTA. Plasma was isolated and aliquots were removed for triglyceride (Sclavo Diagnostics, Wayne, NJ) and cholesterol analyses (38). Following precipitation of very low density (VLDL) and low density (LDL) lipoproteins with heparin-manganese (39), the supernatant was collected and HDL cholesterol (38) was measured colorimetrically. LDL cholesterol was determined using the formula: (VLDL + LDL cholesterol) − plasma triglyceride/5 which provides values identical to those obtained for LDL separated by ultracentrifugation (40,41). Since VLDL lipid mass represents a minor component of the lower density lipoproteins in squirrel monkeys at these alcohol doses (42), alcohol-related alterations in the VLDL-LDL pellet were taken to represent quantitative changes in LDL. Lipids in the pellet were subsequently extracted (43), phospholipid was separated by thin-layer chromatography (44), and phospholipid fatty acids were transesterified (45) using methanolic HCl (Alltech Associates, Inc., State College, PA). Fatty acid methyl esters were analyzed by gas-liquid chromatography as described earlier (46). Phospholipid mass in another aliquot was determined by the Bartlett procedure (47).

Plasma apolipoprotein B-100 was measured by the Tago radial immunodiffusion method (Tago, Inc., Burlingame, CA) using human anti-apolipoprotein B sera which cross-reacts with squirrel monkey apolipoprotein B antigen. Sample apolipoprotein B was determined from a standard calibration curve prepared by microscopic measurement of ring diameters. The intra- and inter-assay coefficients of variation for this procedure were 6.23% and 6.25%, respectively.

Platelet parameters. Platelet counts and average size were measured in whole blood collected in 15% disodium EDTA using a Coulter S+4 counter (Coulter Electronics, Inc., Hialeah, FL). Platelet mass was calculated by multiplying count by size.

Platelet isolation and extraction. Blood was collected from the femoral vein of unanesthetized monkeys with a 23 gauge butterfly cannula into plastic tubes containing 1/6 volume acid citrate dextrose (ACD). After mixing, samples were centrifuged (150 × *g*) for 15 min and platelet-rich plasma (PRP) was collected, placed in polypropylene conical tubes and centrifuged (1500 × *g*) for 15 min at 22°C to sediment the platelets. The platelet pellet was washed twice and the final pellet resuspended in 0.5 mL platelet washing buffer (pH 6.5) (48). Separate aliquots were then removed to measure platelet counts (Coulter Counter Model ZBI equipped with a 70 μm aperture), to test for the presence of apolipoprotein B using the Tago radial immunodiffusion procedure, and to extract lipids (43). Phospholipid mass in one aliquot of the lipid extract was measured by the Bartlett procedure (47). Lipids in the remainder of the extract were resolved by thin-layer chromatography (44), nonesterified cholesterol was quantitated colorimetrically (38), and phospholipid fatty acid methyl esters were prepared and analyzed as described above for LDL.

TXB_2 formation by collagen-stimulated platelets. Free flowing blood was collected into 1/10 vol 3.8% trisodium citrate *via* a 23 gauge cannula. Aliquots of citrated whole blood were then incubated with 10 μg/mL collagen in a shaking water bath at 37°C for exactly 5 min after which 10 μM indomethacin was added. Following centrifugation (2500 × *g*) at 4°C for 15 min, plasma was aspirated and TXB_2 (the stable hydration product of TXA_2) was quantitated by radioimmunoassay using rabbit anti-TXB_2 antisera (49). Antiserum and tritiated TXB_2 were added to samples and known standards, followed by an overnight incubation (4°C). Unbound [^{3}H]TXB_2 was subsequently adsorbed and sedimented on dextran-coated charcoal, and antibody-bound radioactivity was measured by liquid scintillation spectroscopy. Detection limit of the assay was 500 pg/mL serum. Cross-reactivity with other eicosanoids was 0.1% or less.

Circulating platelet aggregates. Circulating platelet aggregates were measured by the method of Wu and Hoak (50). Briefly, 0.5 mL of blood was collected (as described above for platelet isolation) into two separate tubes, one containing 2 mL of buffered EDTA solution only; the other containing 2 mL of buffered EDTA-formalin solution. Both solutions were isotonic and at pH 7.4. Samples were mixed, maintained at 22°C for 15 min, and then centrifuged (150 × *g*) for 8 min to generate PRP. Platelet counts in the mid-fraction of the PRP were determined using a Coulter Counter Model ZBI. The platelet count ratio was calculated as follows: PRP count in EDTA-formalin/PRP count in EDTA. When platelet aggregates are present, they are fixed with formalin and sediment during centrifugation, thus producing a platelet count ratio of less than 1.0 (50).

Liver function tests. Serum glutamate-oxaloacetate transaminase (SGOT) and gamma-glutamyltranspeptidase (GGT) were measured with Dade test kits (Dade Diagnostics, Inc., Miami, FL).

Statistical analyses. Data were expressed as the mean plus or minus the standard error of the mean. Mean values were analyzed for significant differences ($P<0.05$) by one-way analysis of variance and Duncan's multiple range test. Correlation coefficients and corresponding P values were calculated for the platelet lipid *vs* LDL relationship.

RESULTS

There were no significant differences between the treatment groups for SGOT (Control, 54 ± 3, Low EtOH, 56 ± 5, High EtOH, 58 ± 7 U/mL). However, High EtOH primates showed a significant increase in GTT (27 ± 3 IU/L) compared to Control (14 ± 2), but not compared to Low EtOH monkeys (21 ± 2). All serum liver enzymes were within the normal range for human subjects (51).

As shown in Table 1, platelet parameters (count, size, mass) were similar for the three regimens, and platelet responsiveness (TXB_2 formation) to a collagen challenge was not significantly altered by ethanol dose (Controls 61 ± 15, Low EtOH 66 ± 6, High EtOH 72 ± 5 ng/10^8 platelets). The platelet count ratio for Control primates tended to be the lowest while the ratios for both alcohol groups approximated unity indicating an absence of circulating platelet aggregates (Fig. 1). However, neither measure of platelet function (TXB_2 formation, circulating platelet aggregates) was significantly altered by alcohol treatment. As indicated in Figure 2, platelets from monkeys fed high levels of EtOH had significantly more

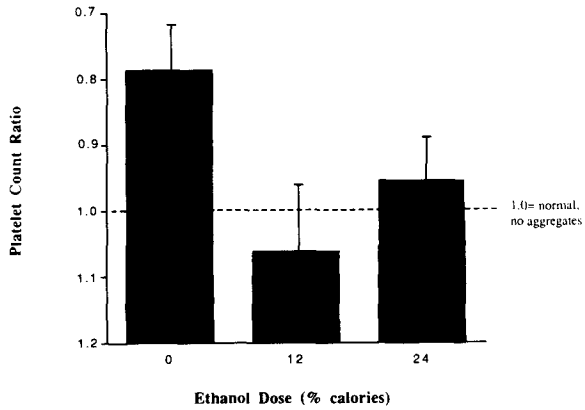


FIG. 1. Effect of ethanol dose on circulating platelet aggregates. Values represent mean ± SEM for 6–8 monkeys/group expressed as platelet-rich plasma count in EDTA-formalin divided by the platelet-rich plasma count in EDTA (50). A platelet count ratio of less than 1.0 indicates the presence of circulating platelet aggregates.

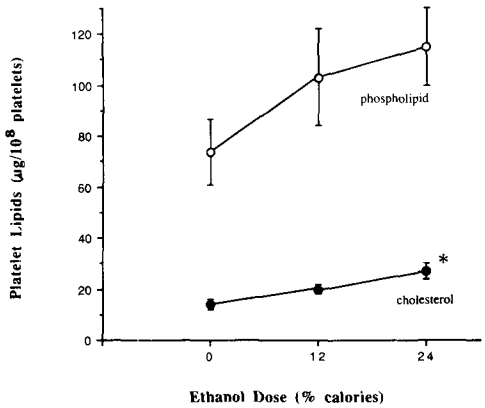


FIG. 2. Effect of ethanol dose on platelet cholesterol and phospholipid concentrations. Values represent mean ± SEM for 6–8 monkeys/group expressed as μg/10^8 platelets. * indicates significant difference (P<0.05) between 24% and 0% EtOH means.

TABLE 1

Effect of Ethanol Dose on Platelet Parameters[a]

Parameter	Treatment groups		
	Control (0%)	Low ethanol (12%)	High ethanol (24%)
Platelet count (10^8/mL)	3.0 ± 0.4	3.4 ± 0.2	3.4 ± 0.2
Size (mean platelet vol, μm^3)	11.7 ± 0.7	12.2 ± 0.6	11.3 ± 0.4
Mass (count × size)	33.8 ± 4.1	41.5 ± 3.1	38.8 ± 3.1

[a]Values represent mean ± SEM for 6–8 monkeys per group.

TABLE 2

Comparison of Platelet and Low Density Lipoprotein Lipid Composition as a Function of Ethanol Dose[a]

	Treatment groups		
	Control	Low ethanol	High ethanol
Platelet component			
Phospholipid 18:2 (μ/10^8 platelets)	4.4 ± 0.6	5.0 ± 0.3	6.3 ± 1.2
Phospholipid 20:4 (μg/10^8 platelets)	7.2 ± 0.6	9.6 ± 0.4	11.3 ± 1.5
Phospholipid 20:4 (% distribution)	17.7 ± 0.3[b,c]	22.0 ± 0.7[c]	25.4 ± 0.4[b]
Phospholipid polyunsaturated/saturated fatty acid ratio	0.623 ± 0.003[b]	0.805 ± 0.005	0.985 ± 0.094[b]
Low density lipoprotein component			
Phospholipid (mg/dL)	52 ± 4[b]	53 ± 2[b]	68 ± 6[b]
Phospholipid 18:2 (mg/dL)	9.0 ± 0.6	8.5 ± 0.5	8.9 ± 0.2
Phospholipid 20:4 (mg/dL)	6.2 ± 0.7[b]	5.6 ± 0.4[b]	8.3 ± 0.7[b]
Phospholipid 20:4 (% distribution)	18.2 ± 1.4[b]	17.6 ± 0.4[b]	21.9 ± 1.6[b]

[a]Values represent mean ± SEM for 6–8 monkeys per group.
[b]High ethanol mean significantly different (P<0.05) from means of other groups with superscript b.
[c]Low ethanol mean significantly different (P<0.05) from means of other groups with superscript c.

cholesterol, a non-significant increase in phospholipid mass, a significant elevation in the percentage of phospholipid arachidonate (Table 2), and the highest polyunsaturated/saturated (P/S) fatty acid ratio (Table 2). Platelet cholesterol/phospholipid molar ratios for Control (0.34 ± 0.08), Low (0.48 ± 0.10), and High (0.49 ± 0.04) EtOH primates were not significantly different. No apolipoprotein B was detected in washed platelet pellets. In addition, there was no evidence of essential fatty acid deficiency as indicated by the similar concentration of arachidonic acid and its precursor, linoleic acid, in all treatment groups for both platelets and LDL phospholipid (Table 2).

Monkeys fed the 24% EtOH dose exhibited a significant increase in LDL cholesterol while consumption of alcohol at 12% of calories had no effect on this parameter (Fig. 3). As noted in Figures 4 and 5, respectively, animals with the highest plasma LDL cholesterol and apolipoprotein B (High EtOH group) also had the greatest amount of platelet cholesterol. HDL cholesterol values for Control, Low, and High EtOH primates were 114 ± 7, 120 ± 10, and 167 ± 21 mg/dL, respectively, with significant differences appearing between the High alcohol group *vs* the other two treatments.

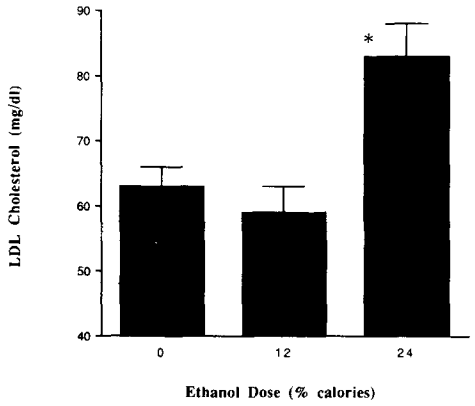


FIG. 3. Effect of ethanol dose on low density lipoprotein cholesterol concentration. Values represent mean ± SEM for 6–8 monkeys/group expressed as mg/dL. * indicates significant difference (P<0.05) between 24% EtOH dose and both 0 and 12% means.

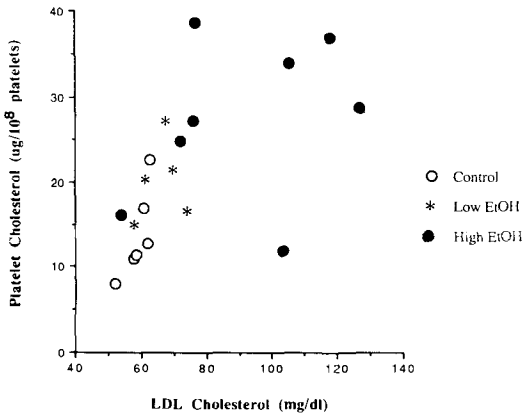


FIG. 4. Relationship between low density lipoprotein cholesterol and platelet cholesterol as a function of ethanol dose. Values for platelet cholesterol ($\mu g/10^8$ platelets) plotted against LDL cholesterol (mg/dL). P = 0.007, r = 0.601.

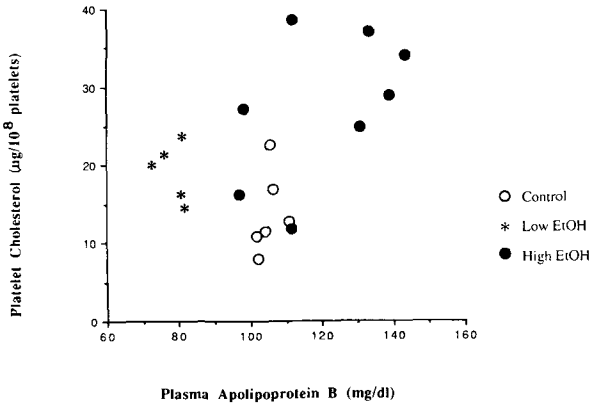


FIG. 5. Relationship between plasma apolipoprotein B and platelet cholesterol as a function of ethanol dose. Values for platelet cholesterol ($\mu g/10^8$ platelets) plotted against plasma apolipoprotein B (mg/dL). P = 0.058, r = 0.441.

DISCUSSION

Several pathophysiologic mechanisms may be responsible for increased or decreased risk of atherosclerosis and cardiovascular disease associated with alcohol consumption. Plasma lipoproteins can either act as a "circulating insult" to the vascular wall and initiate and promote atherogenesis (low density lipoproteins), or participate in the removal of cholesterol from arteries and its transport to the liver for excretion (high density lipoproteins) (52,53). There is a variety of evidence that demonstrates that moderate ethanol consumption raises HDL resulting in (at least in part) a "coronary protective" effect (2, 54–56) while excessive intake may elevate plasma triglyceride and LDL and thus increase coronary risk (57–61). A second potential mechanism involves the hemostatic process and the relationship between the generation of vascular prostacyclin (PGI_2), a potent vasodilatory, platelet antiaggregatory eicosanoid, and platelet thromboxane A_2 (TXA_2), which has proaggregatory/vasoconstrictory properties (20,21). A predominant number of studies in the literature indicate that a moderate alcohol dose favors a shift to an increased PGI_2/TXA_2 ratio (14–18,21,62,63), while long-term, excessive consumption patterns may retard PGI_2 synthesis (64,65) and contribute to hyperactive platelets and enhanced TXA_2 formation (19,66). The latter sequence may thus explain the correlation between vasospasm/stroke and alcohol abuse (3,4,13).

Recently, a number of investigations have shown that lipoproteins may influence eicosanoid production (22–24). Moreover, both LDL and HDL are capable of altering platelet function (26,67–70). However, virtually no studies have examined the relationship between alcohol consumption, alterations in LDL, and changes in platelet activity. Consequently, we hypothesized that since alcohol causes dose-dependent modifications in atherogenic LDL in squirrel monkeys (Fig. 3), these alterations would influence platelet composition/function, which in turn might explain alcohol's observed effects on cardiovascular disease risk. Since platelets do not retain their capacity

for *de novo* sterol synthesis, they may obtain their membrane cholesterol from the surrounding circulatory environment *via* receptor mediated binding and uptake of the LDL ligand, apolipoprotein B-100 (30,71-73). Abnormal platelet cholesterol/phospholipid ratios and concomitant platelet dysfunction have been observed in patients with atherosclerosis and in individuals with familial hypercholesterolemia (69,74-76), both of which may result from excessively high levels of circulating LDL. In addition, the cholesterol/phospholipid (C/PL) ratio in normal platelets is increased following *in vitro* incubation with serum from patients with liver disease which contains a high LDL C/PL ratio (77). Consequently, the elevation in platelet cholesterol in High EtOH primates may be related to a concurrent increase in the circulating pool of LDL (Fig. 3) as suggested by the data presented in Figures 4 and 5. Alternatively, the increased platelet sterol mass in our monkeys could have originated from elevated concentrations of cholesterol in precursor megakaryocytes (78).

The greater amount of platelet cholesterol present in High EtOH primates (Fig. 2) should cause increased membrane rigidity, increased platelet TXA_2 formation and promote aggregation (35,74,76,79-81). However, increased platelet responsiveness was not observed during the collagen stimulated platelet-TXB_2 experiments (Results). Consequently, some type of adaptive response(s) may have taken place to offset the elevated platelet membrane cholesterol. For example, repeated exposure of circulating platelets to plasma ethanol (49-68 mg/dL, Low EtOH primates, 142-159 mg/dL, High EtOH animals) (10,82) would have a fluidizing effect on their membranes over the short-term and could thus influence platelet activity (79,81,83,84). However, chronic exposure of cell membranes to EtOH may, by contrast, increase their rigidity (85). Therefore, of greater importance may be the compensatory physicochemical changes in platelet membrane lipid composition which occurred in our high alcohol primates after four months of treatment. Indeed, with increasing alcohol dose, there was also a commensurate increase in membrane phospholipid (Fig. 2), which although not significantly different from Controls, apparently was elevated enough to modulate the cholesterol/phospholipid molar ratio which did not change appreciably (Results). Therefore, it is possible that the elevated platelet phospholipid in High EtOH monkeys would offset the cholesterol increase, maintain membrane fluidity, and thus contribute to an equilibrium between these lipids in the membrane structure (74,76). And finally, an increase in the platelet phospholipid polyunsaturated/saturated (P/S) fatty acid ratio in the High EtOH treatment group (Table 2) would render the membrane bilayer structure less rigid, increase membrane molecular motion (fluidity) (76,81), and thus counterbalance the cholesterol increase.

Another interesting finding was that High EtOH platelets tended to have elevated absolute and relative concentrations of phospholipid arachidonic acid (20:4), a precursor in TXA_2 synthesis (Table 2). Increased incorporation of 20:4 into phospholipids of cerebral cortical membranes has also recently been reported in mice fed ethanol for chronic periods (86). The source of the platelet 20:4 may be circulating LDL since High EtOH monkeys showed significant elevations in LDL phospholipid arachidonate (Table 2) and because earlier work has documented that platelets may acquire phospholipid components *via* transfer from plasma lipoproteins (87). This observation is significant for two reasons. First, the presence of adequate amounts fo 20:4 and its precursor (linoleic acid, 18:2) (Table 2) in platelets indicates that our animals did not exhibit essential fatty acid deficiency or prostanoid precursor depletion as has been reported in other studies involving long-term alcohol consumption (88-90). Second, although *in vitro* platelet aggregation, and presumably TXA_2 formation, is positively correlated with platelet 20:4 concentration (91), High EtOH platelets showed normal collagen-stimulated TXB_2 formation (Results) which was probably related to the lipid matrix fluidizing effects described above. The observed platelet lipid alterations in High EtOH primates (Table 2) would thus attenuate a potentially adverse increase in TXA_2 formation which has been observed in rats fed alcohol for chronic periods (19), and temper changes in phospholipase A_2 activity which may be stimulated in response to elevated concentrations of low density lipoproteins (Fig. 3) (35).

In conclusion, results from our experiments are consistent with a growing body of evidence that a relationship exists between platelet composition and plasma lipoproteins (22-35,68-73,75,92-105). Our study has advanced present knowledge of this relationship by showing that alterations in the former may be linked to alcohol-mediated changes in the latter. In general, the low alcohol dose (12%) fed to squirrel monkeys had little effect on plasma lipids or platelet parameters. By contrast, although high level alcohol (24%) consumption over a chronic drinking period increased platelet cholesterol and produced an elevation in plasma LDL cholesterol and apolipoprotein B (10), potentially thrombogenic effects (platelet aggregate and TXB_2 formation) may have been averted by adaptive changes in platelet lipid composition. Nevertheless, persistent, high circulating levels of LDL may be particularly detrimental not only because of their well documented role in arterial lipid deposition (52) and their adverse effect on platelet and endothelial cell function (30,35,71,106), but also because this lipoprotein class has recently been shown to be capable of inducing aortic vasoconstriction (107).

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Metabolic Transformation of Intracraneally Injected [1-^{14}C]Linoleic and [1-^{14}C]α-Linolenic Acids in Malnourished Developing Rats

Maria Elena De Tomás*, Osvaldo Mercuri[1] and Cristina Serres
Instituto de Investigaciones Bioquimicas de La Plata (INIBIOLP), Facultad de Ciencias Medicas, (1900) La Plata, Argentina

The effect of a low protein diet during pregnancy and lactation on the fatty acid composition of phosphatidylcholine (PC) and phosphatidylethanolamine (PE) from brains of ten-day-old rats was studied. The results indicated that partial deprivation of protein during early development was associated with an increase in the fatty acids of the n-9 family in PC. The fatty acids of the linoleic acid series decreased in PE but were not modified in PC. These minor changes did not affect the double bond index values either in PC or in PE. The effect of protein depletion on the *in vivo* metabolic transformation of intracraneally injected [1-^{14}C]linoleic and [1-^{14}C]α-linolenic acids was also studied. The percentage distribution of the labeled precursors and their derivatives among PC and PE differed from that of mass distribution. These results indicate that the direct uptake of polyunsaturated fatty acids from the blood and/or the low turnover rate of these acids incorporated into PC and PE might be involved in maintaining the fatty acid pattern of these brain lipids. *Lipids 26,* 891-894 (1991).

Neither linoleic (18:2n-6) nor α-linolenic (18:3n-3) acid can be synthesized by animals; therefore, they are defined as essential fatty acids (EFA). Nevertheless, these acids are precursors of longer chain endogenously synthesized n-6 and n-3 fatty acids which are important constituents of tissue lipids.

Long-chain polyunsaturated fatty acids (PUFA) of both series are synthesized through processes which include chain desaturation and elongation. The desaturation steps are influenced by dietary and hormonal changes described previously (1).

In earlier experiments (2) we demonstrated that liver fatty acid desaturase activities are greatly affected by maternal protein deficiency and suggested that this fact would alter the normal supply of PUFA to the fetus. Furthermore, the adverse influence of protein restriction during pregnancy and lactation upon the activities of the desaturases may contribute to the lack of PUFA observed in the liver of the early developing rat (3,4). Narce *et al.* (5) reported that the effects of protein deprivation in the adult rat on the activities of liver desaturases were not strictly paralleled by changes in the lipid fatty acid composition.

The desaturating enzymes were also studied in brain, because PUFA constitutes the major proportion of the brain phospholipid fatty acids. It was demonstrated that the activity of desaturating enzymes in brain falls very rapidly in the rat after the first week of post-natal life (6-11). Thus, malnutrition during the critical stage of brain growth can cause defects in the metabolism of EFA. Moreover, *in vitro* experiments using brain microsomes (12) clearly demonstrated a deleterious effect of protein deficiency on EFA metabolism when the enzymatic systems were still active.

We undertook this study to determine the effect of a low protein diet during both the prenatal and postnatal periods (until 10 days after birth) on the *in vivo* brain metabolism of intracraneally injected [1-^{14}C]linoleic and [1-^{14}C]α-linolenic acids.

MATERIALS AND METHODS

[1-^{14}C]linoleic acid (specific activity 52 mCi/mmol) and [1-^{14}C]α-linolenic acid (specific activity 41.5 mCi/mmol) were purchased from Radiochemical Centre (Amersham, England). They were 98% radiochemically pure. Gas-liquid chromatographic supplies were purchased from Nu-Chek Prep (Elysian, MN) and Supelco (Bellefonte, PA). All organic solvents were reagent grade and were obtained from Mallinkrodt (St. Louis, MO).

Fatty acid methyl esters used as standards in gas-liquid chromatography were purchased from Sigma Chemical Company (St. Louis, MO).

Animals and diets. Female Wistar rats weighing 150-170 g were divided into two groups after mating. The dams were fed the diets given in Table 1 throughout gestation and lactation. On parturition, five pups of both sexes from each group were allowed to remain with the mother.

In vivo *tracer experiments employing 10-day-old pups.* Five μCi of [1-^{14}C]linoleic acid or 10 μCi of [1-^{14}C]α-linolenic acid as an albumin complex in 5 μL of saline solution was injected intracraneally slightly above and between the eyes by means of a Hamilton microsyringe as described by Dhopeshwarkar and Subramanian (7). The pups were returned to the mother and 8 hr later they were sacrified. The brains were excised, washed in distilled water, and blotted. The brains were then cut through the olfactory lobes and the cerebral peduncles and weighed. Only the cerebrum was used for analyses.

Cerebral lipid analyses. The cerebral lipids were extracted according to Folch-Pi *et al.* (13), and the mixture was stirred overnight at room temperature under a N_2 atmosphere (7). Lipid classes of the extract were fractionated by thin-layer chromatography according to Wagner *et al.* (14). The plates were scanned in a Berthold II Scanner to obtain the distribution of radioactivity in the lipid fractions, and by comparing their R_f values with those of authentic standards.

Phosphatidylcholine (PC) and phosphatidylethanolamine (PE) fractions were isolated and extracted from the silica-gel using the method of Arvidson (15). Plasmalogen-free PE was obtained according to Waku and Lands (16). Fatty acid methyl esters were obtained by transesterifica-

*To whom correspondence should be addressed at Instituto de Investigaciones Bioquímicas de La Plata (INIBIOLP), Facultad de Ciencias Médicas, Calle 60 y 120, (1900) La Plata, Argentina.
[1]Member of the Carrera del Investigador Científico of the Consejo Nacional de Investigaciones Científicas y Técnicas, Argentina.

Abbreviations: EFA, essential fatty acids; GLC, gas-liquid chromatography; PC, phosphatidylcholine; PE, phosphatidylethanolamine; PUFA, polyunsaturated fatty acid(s).

TABLE 1

Diet Composition

Ingredients	Dietary group (%)	
	Control	Low protein
Casein[a]	25	5
Dextrin[b]	65	85
Corn oil	5	5
Salt mixture[c]	5	5
Vitamins[d]		

[a]Molinos Albers, Buenos Aires, Argentina.

[b]Refinerías de Maíz SAICF, Baradero, Argentina.

[c]Percent composition: calcium lactate, 28.0; calcium phosphate monobasic, 16.0; potassium phosphate dibasic, 28.3; sodium phosphate monobasic, 10.3; sodium chloride, 5.2; magnesium sulfate anhydrous, 7.9; ferric citrate, 3.5; and oligo-elements, 0.2.

[d]Each kilogram of diet contained the following vitamins (in milligrams): thiamin, 10; riboflavin, 10; pyridoxine, 10; Ca pantothenate, 50; niacin, 25; inositol, 1000; biotin, 20; choline chloride, 1000; vitamin B-12, 0.1; dl-α-tocopherol, 30; menadione, 50; vitamin A, 3000 IU; and vitamin D, 800 IU.

tion (17) and analyzed (before and after hydrogenation over platinum oxide) in a Panax radio-gas chromatograph with a glass column (1.50 m × 4 mm) packed with 10% SP 2330 and programmed temperature from 140–220°C at 3°C/min.

The fatty acid compositions of PC and PE were determined by gas-liquid chromatography (GLC) using a Shimadzu GC-RIA chromatograph (Kyoto, Japan).

RESULTS

Table 2 shows the fatty acid composition of PC and PE fractions from brains of control and malnourished ten-day-old pups. A striking finding was the similarity in the fatty acid composition of PC from the two dietary groups with the exception of a small but significant increase in the fatty acids from the n-9 family in the malnourished group. The effect of protein deficiency on the n-6 fatty acids was apparently different between PE and PC, because the concentration of fatty acids of the linoleic acid series decreased in PE but was not modified in PC. Minor changes in the n-3 fatty acid concentrations were observed in the brain PC and PE from rats on the low protein diet compared to the control diet. These minor changes in the long-chain unsaturated fatty acid composition promoted by protein depletion did not affect the double bond index values either in PC or in PE.

Table 3 shows the percent distribution of radioactivity of [1-^{14}C]18:2n-6 and its derivatives incorporated into PC and PE. In the PC fraction, the linoleic acid fraction retained the major radioactivity in both groups of rats. Nevertheless, the malnourished group showed a significant increase in the proportion of this radioactive substrate that remained without metabolic transformations. The n-6 acids, other than 18:2n-6, incorporated in PC and PE were the desaturation products of Δ6, Δ5 and Δ4 desaturases and/or of chain elongation (Fig. 1). Gamma-linolenic acid (γ-18:3n-6), the product of Δ6 desaturation of 18:2n-6 and dihomogamma linolenic acid (20:3n-6), the product of elongation of γ-18:3n-6, were decreased in the malnourished group. Arachidonic acid (20:4n-6), the product of Δ5 desaturation of 20:3n-6, was also decreased as a consequence of protein malnutrition. The activity of the Δ4 desaturase was practically negligible because no radioactivity was detected in the 22:5 n-6 incorporated into the PC fraction. Despite the decrease in the radioactivity incorporated into 20:2n-6, ^{14}C in the other fatty acids derived by the elongation pathway, 22:3n-6 and 22:4n-6, was increased in the malnourished group. The ratio ^{14}C in 18:2n-6/Σn-6 − 18:2n-6, increased from 1.47 in the control group to 2.38 in the malnourished group. The [1-^{14}C]18:2n-6 that was not metabolically transformed was drastically increased in the brain PE of malnourished animals. Meanwhile, the desaturation products of 18:2n-6 were significantly diminished with the exception of 22:4n-6 which remained at the same level as in the control group. The ratio ^{14}C in 18:2n-6/Σn-6 −

TABLE 2

Comparison of the Fatty Acid Composition of Phosphatidylcholine and Phosphatidylethanolamine from Brains of Control and Malnourished 10-Day-Old Rats[a]

Fatty acid	Control (5)[b]		Malnourished (10)	
	Phosphatidylcholine	Phosphatidylethanolamine	Phosphatidylcholine	Phosphatidylethanolamine
Σ Saturated	58.3 ± 4.2[c]	36.8 ± 3.3	56.4 ± 1.3	37.1 ± 2.6
Σ Monoethylenic	21.1 ± 0.6	11.7 ± 1.9	22.7 ± 0.8	11.1 ± 1.3
Σ Polyethylenic	19.9 ± 3.4	51.2 ± 4.3	21.2 ± 2.7	51.5 ± 2.7
Σ n-9	23.6 ± 0.9	18.1 ± 2.1	25.5 ± 1.3[d]	18.3 ± 1.4
Σ n-9 − 18:1n-9	7.9 ± 0.9	10.6 ± 1.2	9.1 ± 2.1	11.6 ± 1.6
Σ n-6	13.0 ± 1.5	30.7 ± 3.6	12.2 ± 1.6	26.7 ± 1.9[d]
Σ n-6 − 18:2n-6	12.1 ± 1.5	29.6 ± 3.2	11.2 ± 1.5	26.4 ± 1.9[e]
Σ n-3	4.8 ± 2.1	16.2 ± 1.8	5.5 ± 1.1	16.3 ± 1.7
Σ n-3 − 18:3n-3	4.0 ± 1.9	15.2 ± 2.7	4.8 ± 1.1	15.7 ± 1.7
Double bond index	0.9 ± 0.1	2.0 ± 0.1	0.9 ± 0.1	2.0 ± 0.1

[a]Significant differences between experimental group and control indicated by P values.

[b]Number in parentheses indicates the number of animals in each group.

[c]Values represent the mean ± SD.

[d]$P<0.02$.

[e]$P<0.05$.

TABLE 3

Percent Distribution of Radioactivity in Brain Phosphatidylcholine and Phosphatidylethanolamine from Control and Malnourished Rats After Intracraneal Injection of [1-^{14}C]Linoleic Acid[a]

Fatty acid	Phosphatidylcholine		Phosphatidylethanolamine	
	Control (12)[b]	Malnourished (16)	Control (12)	Malnourished (16)
16:0[c]	12.2 ± 3.7[d]	3.0 ± 2.3[e]	4.2 ± 0.8	4.3 ± 1.2[c]
18:2n-6	52.3 ± 7.8	68.3 ± 2.8[e]	22.3 ± 2.6	55.6 ± 2.8[e]
18:3n-6	3.6 ± 0.2	2.6 ± 1.1[f]	6.4 ± 1.2	4.3 ± 0.7[f]
20:2n-6	6.5 ± 0.9	3.2 ± 1.2[e]	6.8 ± 0.9	4.9 ± 0.5[f]
20:3n-6	5.2 ± 1.5	4.3 ± 0.6[f]	13.1 ± 3.0	2.5 ± 0.3[e]
20:4n-6	4.8 ± 1.8	3.8 ± 1.3[g]	19.2 ± 1.3	7.0 ± 0.8[e]
22:3n-6	4.9 ± 1.2	7.1 ± 0.8[f]	17.2 ± 2.1	11.0 ± 0.7[e]
22:4n-6	4.8 ± 0.7	7.6 ± 2.2[f]	10.8 ± 0.8	10.2 ± 1.1
22:5n-6	5.7 ± 1.3	ND	ND[h]	ND
Ratio $\frac{18:2n-6}{\Sigma n-6 - 18:2n-6}$	1.47	2.38[e]	0.30	1.39[e]

[a]Significant differences between experimental group and control group indicated by P values.
[b]Number in parentheses indicates the number of animals in each group.
[c]Chain length: number of double bonds.
[d]Values represent the mean ± SD.
[e]P<0.001.
[f]P<0.02.
[g]P<0.05.
[h]ND, not detectable.

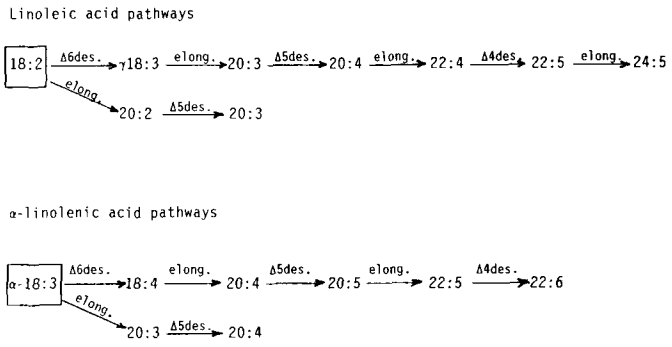


FIG. 1. Steps in the metabolism of linoleic and α-linolenic acids.

18:2n-6 increased in the PE fraction from 0.30 in the control group to 1.39 in the malnourished group.

Table 4 shows the incorporation of the intracraneally injected [1-^{14}C]α-linolenic acid into brain PC and PE fatty acids. Of the total radioactivity incorporated into brain fatty acids in the control group, sixty percent in PC and thirty percent in PE were in the fatty acids synthesized *de novo* from labeled acetate, a product of the β-oxidation of [1-^{14}C]α-18:3n-3. The radioactive tracer that was not metabolically transformed was significantly increased in the PC fraction from the malnourished group. A decrease in the radioactivity incorporated in PE as 22:6n-3 and an increase in the incorporation of labeled 22:4n-3 were observed. Protein depletion promoted an increase in the ratio ^{14}C in α-18:3n-3/Σn-3 − α18:3n-3 in PC and PE from 0.40 to 0.69 and 0.24 to 0.36, respectively.

DISCUSSION

It is clear from the data presented that only minor changes in the fatty acid pattern of brain PC and PE were observed in the malnourished rats. These findings indicate the stability of these structural lipids in this highly differentiated tissue.

A question addressed by our study was the degree to which intracraneally injected [1-^{14}C]18:2n-6 was desaturated and chain elongated. The increase in the ratio 18:2n-6/Σn-6 − 18:2n-6 in the PC and PE fractions promoted by protein depletion indicated that the amount of *in vivo* desaturation/elongation products from 18:2n-6 was significantly diminished in the brain of malnourished animals. Nevertheless the ^{14}C in the fatty acids derived by the elongation pathway, 22:3n-6 and 22:4n-6, was increased in the malnourished group.

The present data and results from other laboratories using *in vivo* techniques (18–20) do not as yet permit conclusion as to the elongation pathways that lead to the formation of PUFA in brain. The major uncertainty in our study is the extent to which malnutrition contributes to the alteration of the normal pattern of fatty acid elongation.

^{14}C-Incorporation of the intracraneally injected [1-^{14}C]α-18:3n-3 into PC and PE fatty acids illustrated the special susceptibility of α-18:3n-3 to oxidation as was previously noted (21,22). The results are consistent with those reported by Anderson and Connor (23) and Crawford and Harbige (24). Only the radioactivity of 18:0 decreased in PC of the malnourished group, while an increase in radioactivity in PE 16:0 was promoted by protein depletion during early development.

The precursor/product ratio of PUFA derived from [1-^{14}C]α-18:3n-3 supports the assumption that the activities of the enzymes involved are diminished in the

TABLE 4

Percent Distribution of Radioactivity in Brain Phosphatidylcholine and Phosphatidylethanolamine from Control and Malnourished Rats After Intracraneal Injection of [1-^{14}C]α-Linolenic Acid[a]

Fatty acid	Phosphatidylcholine		Phosphatidylethanolamine	
	Control (5)[b]	Malnourished (10)	Control (5)	Malnourished (10)
16:0[c]	24.5 ± 3.7[d]	31.4 ± 5.7	7.9 ± 0.8	11.9 ± 1.9[e]
16:1	8.5 ± 1.9	4.8 ± 2.0	4.0 ± 0.9	2.5 ± 0.1[e]
18:0	15.1 ± 2.2	6.9 ± 2.2[f]	11.5 ± 6.0	13.5 ± 2.5
18:1	12.5 ± 1.3	8.2 ± 3.3	7.3 ± 4.2	8.8 ± 2.2
α 18:3n-3	11.4 ± 1.6	20.1 ± 3.0[g]	12.6 ± 2.7	16.9 ± 2.5
20:3n-3	5.4 ± 2.6	5.6 ± 1.0	ND[h]	ND
20:4n-3	4.5 ± 1.3	7.1 ± 2.1	9.9 ± 5.4	5.6 ± 0.6
20:5n-3	4.1 ± 1.2	5.3 ± 0.6	8.8 ± 5.3	7.1 ± 1.5
22:4n-3	7.5 ± 0.4	3.9 ± 2.2[e]	2.1 ± 0.5	9.9 ± 3.1[g]
22:5n-3	6.3 ± 0.9	4.8 ± 1.3	14.3 ± 0.8	18.0 ± 3.9
22:6n-3	0.5 ± 0.1	2.0 ± 1.4	17.2 ± 7.8	6.3 ± 1.6[e]
Ratio $\frac{\alpha 18:3n-3}{\Sigma n-3 - \alpha 18:3n-3}$	0.40	0.69[f]	0.24	0.36[e]

[a]Significant differences between experimental group and control indicated by P values.
[b]Number in parentheses indicates the number of animals in each group.
[c]Chain length: number of double bonds.
[d]Values represent the mean ± SD.
[e]P<0.05.
[f]P<0.001.
[g]P<0.02.
[h]ND, not detectable.

brain of malnourished animals. On the other hand, the decreased amount of radioactivity incorporated in PE as 22:6n-3 was apparently compensated in part by an increase in the incorporation of labeled 22:4n-3, the product of elongation of 20:4n-3.

The present data do not allow conclusion as to the extent to which EFA metabolism would contribute to the maintenance of the fatty acid pattern of brain PC and PE. It is also not clear to what extent the direct uptake of PUFA from the blood (25) and/or a low turnover rate of these acids would be involved.

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Arachidonic Acid, Prostaglandin E_2 and Leukotriene C_4 Levels in Gingiva and Submandibular Salivary Glands of Rats Fed Diets Containing n-3 Fatty Acids[1]

S.Q. Alam*, B.M. Bergens and **B.S. Alam**
Department of Biochemistry and Molecular Biology, LSU Medical Center, New Orleans, Louisiana 70119

The effect of dietary n-3 fatty acids on prostaglandin E_2 (PGE_2) and leukotriene C_4 (LTC_4) levels in rat salivary glands and gingiva was examined in two separate nutritional studies. In the first set of experiments, two groups of male weanling Sprague-Dawley rats were fed semipurified diets containing 10% corn oil (control group) or 10% menhaden oil (experimental group). Rats were killed after 8 wk on the diets; the fatty acid composition of total phospholipids and the concentrations of PGE_2 and its precursor, arachidonic acid, were measured in gingiva and submandibular salivary glands (SMSG). Dietary n-3 fatty acids were incorporated into the tissue phospholipids. Arachidonic acid levels were reduced by 56% in gingiva and SMSG of rats fed menhaden oil compared with the control rats fed the diet containing corn oil. The concentrations of PGE_2 in SMSG and gingiva of rats fed the diet containing menhaden oil were reduced by 74% and 83%, respectively. In a subsequent nutritional study, we tested whether the diet-induced reduction in tissue arachidonic acid levels would also result in a corresponding decrease in LTC_4 production. Three groups of rats were fed diets containing 5% corn oil (group 1), 4% ethyl ester concentrate of n-3 fatty acids plus 1% corn oil (group 2), or 5% ethyl ester concentrate of n-3 fatty acids (group 3). After 6 wk of feeding, gingiva and SMSG were analyzed for arachidonic acid content and *in vitro* production of LTC_4. Arachidonic acid content of total phospholipids was about 60% lower in gingiva and 69% lower in SMSG of rats fed the ethyl ester concentrate of n-3 fatty acids (groups 2 and 3) than those of the control group fed the corn oil diet (group 1). Upon incubation with calcium ionophore, gingiva and SMSG from rats fed the n-3 fatty acids rich diet produced significantly less LTC_4 than those from rats of the control group. Because PGE_2 and LTC_4 are believed to be important biochemical mediators of periodontal disease, one may speculate that a diet-induced reduction in their levels may have a beneficial effect upon the course of the disease. The function of salivary glands may also be altered because of the role of these eicosanoids in salivary secretions.
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Arachidonic acid is released from cell membrane phospholipids mainly *via* the action of a calcium-dependent phospholipase A_2 (1). To a lesser extent, some arachidonic acid is released by diacylglycerol lipase action upon diacylglycerol, which is one of the products of phospholipid degradation by phospholipase C. There are basically two pathways for arachidonic acid metabolism. One is the cyclooxygenase pathway which produces prostaglandins (PG), thromboxanes (TX), and prostacyclins (2). The other pathway is the lipoxygenase pathway which results in the production of a variety of biologically active hydroxyeicosatetraenoic (HETE) acids and the leukotrienes, which are known mediators of the acute vascular changes that accompany the process of inflammation (3,4). Many reports show that cyclooxygenase products of arachidonic acid are present in much higher concentrations in inflamed than in healthy tissue (5,6). The products of arachidonic acid metabolism also contribute to bone resorption (7). A several-fold increase in the content of PGE_2 in gingival fluid and gingival tissue from patients with periodontal disease has been observed compared to the PGE_2 content in normal healthy gingiva (8-10).

An excess of arachidonic acid metabolites can be potentially injurious to gingival tissues. The n-3 fatty acids have been shown to reduce or counteract the effects of arachidonic acid metabolites. A previous study from our laboratory had shown an incorporation of these fatty acid into the submandibular salivary gland lipids (11). The n-3 fatty acids give rise to the series of prostaglandins 3 and thromboxane 3, which inhibit the release of arachidonate from phospholipids and the formation of PGE_2 and thromboxane 2 (12). The eicosanoids derived from n-3 fatty acids exhibit a spectrum of biological activity that differs from that of the arachidonic acid metabolites. Thus, research into the effects of n-3 fatty acids on arachidonic acid and its metabolites in gingiva is important. These fatty acids from the dietary sources could possibly improve gingival health by decreasing inflammation and bone loss in periodontal disease. One of the objectives of the present investigation was to determine if the levels of prostaglandin E_2 (PGE_2), leukotriene C_4 (LTC_4) and their precursor, arachidonic acid, can be altered in gingiva as a result of feeding diets rich in n-3 fatty acids.

Prostaglandins and leukotrienes have also been shown to influence the secretion of saliva. An increase in cAMP content of rat salivary acini *in vitro* (13), and a decrease in salivary flow (14,15) have been reported by PGE_1 and PGE_2. Similarly, it was reported that physiological levels of PGI_2, PGE_2 and $PGF_{2\alpha}$ stimulated the release of rennin from the mouse submandibular salivary gland (SMSG) slices *in vitro* (16). The products of the lipoxygenase pathway of arachidonic acid metabolism, such as LTC_4 and LTD_4, reduced methacholine- and substance P-induced salivary flow in rats (17). It has been reported that among several murine tissues, the SMSG, had the highest ability to synthesize PGE_2 *in vitro* (18). The presence of

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*To whom correspondence should be addressed at the Department of Biochemistry and Molecular Biology, LSU Medical Center, 1100 Florida Avenue, New Orleans, LA 70119.

Abbreviations: EEC, ethyl ester concentrate; FAME, fatty acid methyl esters; GC/MS, gas chromatography/mass spectrometry; HETE, hydroxyeicosatetraenoid(s); L, leukotriene; PG, prostaglandin(s); SMSG, submandibular salivary glands; TX, thromboxane(s).

PGE_2, $PGF_{2\alpha}$, PGI_2, TXB_2 and HETE in rat, rabbit and human saliva has been reported (19–21). In view of the important role of eicosanoids in salivary gland secretion, another objective of our studies was to determine the effects of feeding diets rich in n-3 fatty acids on the levels of arachidonic acid, PGE_2 and LTC_4 in SMSG.

MATERIALS AND METHODS

Experiment 1. Male weanling Sprague-Dawley rats (Holtzman Co., Madison, WI) were fed regular rat ration for 7 days to acclimate them to the laboratory conditions. Rats were weighed and randomly divided into two groups with the same average body weight per group. Rats were housed individually in suspended stainless-steel cages in a temperature and light-controlled room (22–23°C, 12-hr light-dark cycle) and were given diets and tap water *ad libitum*. Rats were weighed weekly and their food intake was also measured. Diets were prepared every week and stored at 4°C. Unused diet before each feeding was discarded to minimize rancidity of the fed oils. The diets were similar to the American Institute of Nutrition (AIN-76A) diet (22,23), with 10% corn oil added to the control group and 10% menhaden fish oil added to the experimental group. Composition of the diets was as follows (in wt %): casein, 20.0; DL-methionine, 0.3; choline, 0.2; mineral mixture, 3.5; cellulose, 5.0; sucrose, 45.0; starch, 15.0; oil, 10.0; vitamin mixture (AIN-76A), 1.0. Menhaden oil, which was obtained under the Fish Oil Test Material Program, National Institutes of Health (NIH), Bethesda, MD, contained supplements of antioxidants to prevent autooxidation. Therefore, in order to equalize the antioxidant content of the two diets, α-tocopherol (0.1%), γ-tocopherol (0.1%) and *t*-butylhydroquinone (0.02%) were added to the diet containing corn oil. The fatty acid composition of the two diets has been previously reported (11). The corn oil diet contained 58% of 18:2n-6 as the main fatty acid and only trace amounts of n-3 fatty acids. The menhaden oil-diet had high levels of n-3 fatty acids (20:5, 15.5%; 22:5, 2.2%, and 22:6, 8.6%; all values are as % of the total fatty acids) and low levels of n-6 fatty acids (1% 18:2).

Rats were killed at the end of 8 wk of feeding the diets. Gingivae and SMSG were quickly dissected while the tissues were immersed in ice-cold physiological saline-indomethacin solution (5 μg/mL) to inhibit prostaglandin synthesis. The tissues were blotted dry, weighed, and immediately frozen in liquid nitrogen.

Extraction and measurement of PGE_2. Gingivae and SMSG from each rat were separately homogenized in glass homogenizer with ethanol (4 × 1 mL). The ethanolic extracts were filtered and the filtrates were dried under nitrogen. Water (1 mL) was added and the pH was adjusted to 3.5 with 2 N HCl. The acidified extracts were passed through a C-18 column (Sep-Pak, J.T. Baker Chemical Co., Phillipsburg, NJ) and PGE_2 was eluted with 0.5 mL of methanol. The solvent was evaporated under nitrogen and the residue was dissolved in an assay buffer. Suitable aliquots were assayed by radioimmunoassay (Prostaglandin E_2 [^{125}I] RIA Kit, NEN, Dupont, Boston, MA). According to the supplier, the stated cross-reactivity of the antiserum was 100% for PGE_2, 3.7% for PGE_1 and <1% for other prostaglandins. The cross-reactivity against PGE_3 was not measured. In separate experiments, the recovery of PGE_2 was also measured in triplicate samples after the addition of [^{3}H]PGE_2 to the tissue homogenates following the above procedure. Methanolic eluates containing [^{3}H]PGE_2 from the C-18 columns were counted for radioactivity using a liquid scintillation counter (Beckman LS 6800, Fullerton, CA). The recovery of [^{3}H]PGE_2 was 38% for gingiva and 43% for SMSG. The recovery of PGE_2 was not influenced by the previous dietary treatment.

Fatty acid composition and arachidonic acid levels (Experiment 1). Lipids were extracted from aliquots of tissue homogenates by homogenizing with chloroform/methanol (2:1, v/v) containing butylated hydroxytoluene (5 mg/100 mL) as an antioxidant. The concentrations of arachidonic acid in the total lipid extracts were measured by gas chromatography, using an internal standard (6,9,12,15-octadecatetraenoic acid, 18:4), which was added prior to lipid extraction. Phospholipids were separated by column chromatography (Bio-Sil A, 100-200 mesh, Bio-Rad Laboratories, Richmond, CA), and fatty acid methyl esters (FAME) were prepared using boron trifluoride/methanol (24). The fatty acid composition was determined by gas chromatography on a 15% Silar 10 C column (3.66 m × 2 mm i.d.) using isothermal conditions as previously described (25).

Experiment 2. The purpose of this study was to determine whether a diet-induced reduction in arachidonic acid concentrations of phospholipids would result in a concomitant reduction in the *in vitro* production of leukotriene C_4 by gingiva and the SMSG. Three groups of male weanling Sprague-Dawley rats were fed semipurified diets (Basal diet was AIN-76A) containing 5% corn oil (group 1), 1% corn oil plus 4% ethyl ester concentrate of n-3 fatty acids (group 2), or 5% ethyl ester concentrate of n-3 fatty acids (group 3). The ethyl ester concentrate (EEC) of n-3 fatty acids, which was obtained from the Fish Oil Test Material Program, NIH, had 78.5% of the total fatty acids as n-3, with 20:5 (43.9%) and 22:6 (24.0%) as the major fatty acids. The EEC also contained 3.4% of the total fatty acids as n-6, with 20:4 constituting 2.3% of the total. Our reason for using the EEC n-3 instead of menhaden oil, which was used in Experiment 1, was that the EEC n-3 contained 3-fold higher levels of n-3 fatty acids than menhaden oil. In this nutritional study we included an additional group (4% EEC n-3 plus 1% corn oil) to exclude the possibility of inadvertently inducing essential fatty acid deficiency. Although EEC of n-3 fatty acids contained only 0.1% 18:2n-6, the addition of 1% corn oil supplied adequate amounts (1.5% of total calories) of 18:2n-6.

Rats were killed after 6 wk of feeding the various diets, SMSG and gingivae were dissected. About one-half of the tissue was frozen immediately in liquid nitrogen, the remaining (0.05–0.1 g for gingiva and 0.2–0.3 g for SMSG) was cut into small pieces with a razor blade and incubated at 37°C for 10 min in 1 mL of Tris buffer, pH 7.4, 0.05 M, in the presence of calcium ionophore, A23187 (10 μM). After incubation, 20 μL of 4.75 M formic acid was added and the contents were frozen at −75°C until analyzed.

Extraction and assay of LTC_4. The contents (tissue plus the incubation medium) were homogenized with 1 × 3 mL of ice-cold absolute ethanol in a ground glass tissue homogenizer. After centrifugation at 12,000 × *g* for 20 min in a refrigerated centrifuge, the supernatant was transferred to a graduated tube. Ethanol was evaporated under N_2 by heating the tube at a temperature less than

40°C. The volume was reduced to 1 mL, and 2.3 mL of H_2O was added to bring the ethanol concentration to about 15% according to the method of Powell (26). The pH of the extract was between 3–3.5.

Partial purification of LTC_4 was obtained by column chromatography over Amprep Ethyl C2 minicolumns (100 mg size, Amersham Corp., Arlington Heights, IL). The columns were washed with 2 mL methanol, followed by 2 mL of H_2O. The tissue extracts containing LTC_4 were applied to the column and then washed sequentially with 1 mL of 15% ethanol, 1.5 mL of H_2O, 2 mL of hexane, and 1.5 mL of 10% ethanol/8% HCl. The LTC_4 fraction was collected by eluting the column with 2 mL of methyl formate. Aliquots of the methyl formate fraction containing LTC_4 were dried under nitrogen, the residue was dissolved in the assay buffer and analyzed by radioimmunoassay. The recovery of a standard of [^{3}H]-LTC_4, when added to duplicate samples, by using the above column chromatographic procedure, was 35% for gingiva and 40% for SMSG. The recovery was not affected by the dietary treatment.

The LTC_4 concentrations in methyl formate fractions eluted from the columns were measured by using the leukotriene C_4 specific [^{3}H]assay system (monoclonal antibody) purchased from Amersham and the radioactivity was measured using a liquid scintillation counter. The stated cross-reactivity of the LTC_4 antibody, as provided by the supplier, was 5.4% against LTD_4, 0.5% against LTE_4 and <0.001% against LTB_4. The cross-reactivity against LTC_5 was not measured.

Fatty acid composition and arachidonic acid concentrations of phospholipids (Experiment 2). Lipids were extracted by the method of Folch *et al.* (27) from the tissues which were frozen in liquid nitrogen immediately after dissection. Total lipid extracts were filtered under nitrogen over a sintered glass funnel, extracts were washed with 0.2 vol of 0.9% KCl, and the volume was adjusted to 6 mL with chloroform. Aliquots (2 mL) of this total lipid extract were subjected to column chromatography on silicic acid columns, and the phospholipid fraction was isolated. The fatty acid composition of total phospholipids was determined after transesterification with 14% BF_3/methanol (24). To quantitate the levels of arachidonic acid in total phospholipids, 10–20 μg of an internal standard (6,9,12,15-octadecatetraenoic acid, 18:4n-3) was added prior to transesterification. This fatty acid could be safely used as an internal standard because it behaves like arachidonic acid in terms of its chemical properties and it was not found in tissue phospholipids or in blood plasma phospholipids of rats fed diets containing corn oil or n-3 fatty acids (Alam, S.Q., unpublished observations).

For gingival phospholipids, FAME were analyzed by gas chromatography on a megabore column (15 m × 0.53 mm FSOT, DB-Wax, 1.0 μm, J & W Scientific, Folsom, CA). Column temperature was programmed from 70°C (initial temperature for 1 min) to 210°C at 8°C/min. The detector temperature was 250°C and the injection port temperature was 225°C. Under these conditions, all FAME could be readily separated within 30 min. The identification was based upon relative retention data using standards.

The fatty acid composition of phospholipids from SMSG was determined by gas chromatography/mass spectrometry. A Hewlett-Packard gas chromatograph, Palo Alto, CA, (Model 5890) with a mass selective detector (Model 5970) was used. FAME were analyzed on a fused silica capillary column (DB-23, 20 m × 0.172 mm, i.d., J & W Scientific). The samples were injected in a split mode. The column temperature was programmed from a 3-min hold at 140°C to 220°C at 5°C/min. The detector and injector temperatures were 280°C and 250°C, respectively. Electron impact spectra were obtained at 70 electron volt. FAME were quantitated by monitoring total ion currents.

Statistical analyses. Data on the fatty acid composition, arachidonic acid level and prostaglandin E_2 levels were analyzed for significance between the corn oil group and the menhaden oil group by using Student's t-test (Experiment 1). Similar comparisons were made for phospholipid fatty acid compositions, arachidonic acid levels and LTC_4 production in SMSG and gingiva between the corn oil group *versus* the two experimental groups fed diets containing EEC of n-3 fatty acids using Student's t-test (Experiment 2).

RESULTS

Two separate sets of animal studies were carried out during the last two years. The purpose of the first study (Experiment 1) was to determine the effects of feeding a diet rich in n-3 fatty acids on arachidonic acid and PGE_2 levels in gingiva and SMSG. The second study (Experiment 2) dealt primarily with the *in vitro* production of LTC_4 in these tissues as modulated by altering the *in vivo* concentration of arachidonic acid.

The fatty acid composition of phospholipids in the SMSG and gingiva of rats fed menhaden oil diet (Experiment 1) is shown in Table 1. Dietary n-3 fatty acids from menhaden oil were incorporated into the SMSG and gingival phospholipids. In phospholipids from tissue of

TABLE 1

Fatty Acid Composition of Phospholipids in Submandibular Salivary Gland and Gingival Homogenates of Rats Fed Diets Containing 10% Corn Oil or Menhaden Oil (Experiment 1)[a]

Fatty acid	SMSG		Gingiva	
	Corn oil	Menhaden oil	Corn oil	Menhaden oil
14:0	2.0 ± 0.1	3.2 ± 0.2[c]	tr	tr
16:0	22.9 ± 0.5	24.3 ± 1.0	14.0 ± 0.6	15.9 ± 0.7
16:1n-7	1.4 ± 0.2	3.4 ± 0.7[b]	tr	tr
18:0	16.7 ± 0.1	17.2 ± 1.1	20.1 ± 0.1	21.0 ± 1.1
18:1n-9[e]	11.5 ± 0.2	15.4 ± 1.4[b]	12.2 ± 0.3	16.5 ± 0.5[d]
18:2n-6	13.6 ± 0.6	3.9 ± 0.2[d]	20.4 ± 2.3	1.6 ± 0.2[d]
18:3n-3	0.6 ± 0.1	0.7	tr	1.8 ± 0.3[d]
20:3n-6	3.8 ± 0.6	1.6 ± 0.3[b]	tr	tr
20:4n-6	26.2 ± 1.2	8.7 ± 1.1[d]	22.9 ± 0.7	12.4 ± 0.3[d]
20:5n-3	tr	16.5 ± 0.1[d]	tr	7.8 ± 0.4[d]
22:4n-6	0.6 ± 0.1	tr	3.5 ± 0.6	tr
22:5n-3	0.3 ± 0.1	1.8 ± 0.3[c]	tr	4.1 ± 0.2[d]
22:6n-3	0.8 ± 0.1	5.0 ± 0.1[d]	tr	19.9 ± 0.9[d]

[a]Values are area percent (mean ± SEM of 3 rats/group for SMSG and 4–5 rats/group for gingiva). Values in the menhaden oil group are significantly different ([b]P<0.05, [c]P<0.01, [d]P<0.001) from the corresponding values in the corn oil group, using Student's t-test. FAME were analyzed by gas chromatography on a packed column. [e]May contain some 18:1n-7 isomer since it was not resolved from 18:1n-9 on this column; tr, indicates trace.

TABLE 2

Concentrations of Arachidonic Acid and PGE_2 in SMSG and Gingiva of Rats Fed Diets Containing 10% Corn Oil or Menhaden Oil (Experiment 1)[a]

Dietary fat	SMSG		Gingiva	
	Arachidonic acid	PGE_2	Arachidonic acid	PGE_2
	(mg/g)	(pg/g)	(mg/g)	(ng/g)
Corn oil	2.78 ± 0.21	220 ± 34	1.42 ± 0.04	3.47 ± 0.55
Menhaden oil	1.22 ± 0.06[c]	56 ± 8[b]	0.62 ± 0.06[c]	0.58 ± 0.10[c]

[a]Values are mean ± SEM of 4-5 rats/group. Arachidonic acid concentrations were measured in the total lipids by gas chromatography, using 18:4 as an internal standard. PGE_2 concentrations were measured by radioimmunoassay. Values with superscripts in the experimental group fed the menhaden oil diet are significantly different from the corresponding values in the control group fed the corn oil diet, using Student's t-test. ([b]P<0.01, [c]P<0.001).

TABLE 3

Fatty Acid Composition of Total Phospholipids in SMSG of Rats Fed Diets Containing Ethyl Ester Concentrate (EEC) of n-3 Fatty Acids (Experiment 2)[a]

Fatty acid	5% Corn oil	4% EEC n-3 + 1% corn oil	5% EEC n-3
16:0 DMA	2.0 ± 0.7	3.1 ± 0.2	3.0 ± 0.2
16:0	19.2 ± 2.7	21.9 ± 1.8	19.0 ± 0.5
16:1n-7	1.1 ± 0	2.3 ± 0.1[c]	2.3 ± 0.1[c]
18:0 DMA	0.9 ± 0.1	0.4	0.6 ± 0.1
18:0	20.0 ± 2.1	18.3 ± 0.8	20.9 ± 1.2
18:1n-9	9.3 ± 0.6	10.0 ± 1.3	8.7 ± 0.4
18:1n-7	4.6 ± 0.1	4.1 ± 0.2	4.1 ± 0.1
18:2n-6	10.3 ± 0.2	6.9 ± 0.9[b]	0.7 ± 0.1[d,e]
20:3n-6	4.7 ± 0.6	1.1 ± 0.1[c]	0.6 ± 0.1[c]
20:4n-6	22.9 ± 0.2	7.5 ± 0.6[d]	7.6 ± 0.5[d]
20:5n-3	tr	13.8 ± 0.9[d]	16.5 ± 0.7[d]
22:4n-6	1.3 ± 0.1	tr	tr
22:5n-6	1.6 ± 0.1	tr	tr
22:5n-3	tr	2.6 ± 0.1[d]	3.2 ± 0.2[d]
22:6n-3	tr	5.5 ± 0.4[d]	7.3 ± 0.4[d]

[a]Values are area percent (mean ± SEM of 3 rats/group). FAME were analyzed by gas chromatography/mass spectrometry (GC/MS). Values with superscripts in the same row are significantly different from those in the control group (5% corn oil) using Student's t-test. [b]P<0.05, [c]P<0.01, [d]P<0.001. [e]Significantly different (P<0.001) from the corresponding value in the 4% EEC n-3 plus 1% corn oil group. DMA, dimethylacetal; tr, trace.

TABLE 4

Major Fatty Acids in Total Phospholipids in Gingiva of Rats Fed Diets Containing Ethyl Ester Concentrate (EEC) of n-3 Fatty Acids (Experiment 2)[a]

Fatty acid	5% Corn oil	4% EEC n-3 + 1% corn oil	5% EEC n-3
16:0	17.4 ± 2.3	18.6 ± 1.9	16.8 ± 2.1
16:1	1.9 ± 0.2	3.4 ± 0.4[b]	3.9 ± 0.3[c]
18:0	17.1 ± 1.1	15.6 ± 1.0	16.3 ± 0.8
18:1	16.1 ± 1.0	16.5 ± 1.5	19.3 ± 1.1
18:2n-6	13.4 ± 0.8	7.1 ± 0.5[c]	1.7 ± 0.1[c,d]
20:4n-6	14.7 ± 1.5	6.1 ± 0.4[c]	6.3 ± 0.5[c]
20:5n-3	tr	5.2 ± 0.6[c]	6.6 ± 0.3[c]
22:4n-6	2.2 ± 0.5	tr	tr
22:5n-3	tr	1.0 ± 0.3[e]	2.9 ± 0.7[e]
22:6n-3	tr	9.0 ± 1.9[b]	11.5 ± 1.2[c]

[a]Values are area percent (mean ± SEM of 4-5 rats/group). Only the major fatty acids are reported. There were several minor fatty acids in each group which made up the remainder of the total fatty acids. Values with superscripts in the same row are significantly different from the corresponding values in the control group (5% corn oil) using Student's t-test. [e]P<0.05, [b]P<0.01, [c]P<0.001. [d]Significantly different (P<0.001) from the corresponding value in the 4% EEC n-3 plus 1% corn oil group; tr, trace.

rats fed the corn oil-diet, these fatty acids were present only in small amounts (<1.0%). The levels of linoleic and arachidonic acid were significantly lower (P<0.001) in phospholipids of SMSG or gingiva of rats fed 10% menhaden oil than those fed the 10% corn oil diet. The concentrations of arachidonic acid in total lipids were 2-2.5 fold lower and the endogenous PGE_2 levels were 4-6 fold lower in SMSG and gingiva of rats fed the diet containing menhaden oil than those fed the corn oil diet (Table 2).

The fatty acid composition of total phospholipids in SMSG and gingiva of rats fed ethyl ester concentrate (EEC) of n-3 fatty acids is shown in Tables 3 and 4, respectively. When compared with the control group (5% corn oil), phospholipids from the two experimental groups contained higher levels of n-3 and lower levels of n-6 fatty acids. The levels of linoleic acid in phospholipids of rats fed diets containing 4% EEC plus 1% corn oil were 33% lower in SMSG and 47% lower in gingiva than the corresponding levels in the control group. In the group fed 5% EEC without corn oil, the linoleic acid levels were only 7% of the control group (5% corn oil) in the SMSG and 13% of the control group in the gingiva. Arachidonic acid levels in the two experimental groups were 33% of the control values in the SMSG and 42-43% in gingiva. Similar to the total phospholipids, lower levels of n-6 fatty acids (18:2 and 20:4) and higher levels of n-3 fatty acids (20:5, 22:5 and 22:6) were observed in the phosphatidylcholine, phosphatidylethanolamine, and the phosphatidylserine plus phosphatidylinositol fractions which were isolated from SMSG and gingiva of rats fed the two experimental diets containing EEC of n-3 fatty acids (data not shown).

The concentrations of arachidonic acid in total phospholipids and the stimulated *in vitro* production of LTC_4

TABLE 5

***In vitro* Production of Leukotriene C_4 and Endogenous Arachidonic Acid Levels in Total Phospholipids of SMSG and Gingiva of Rats Fed Diets Containing Ethyl Ester Concentrate (EEC) of n-3 Fatty Acids (Experiment 2)[a]**

Dietary fat	SMSG		Gingiva	
	Arachidonic acid	LTC_4	Arachidonic acid	LTC_4
	(mg/g)	(ng/g)	(mg/g)	(ng/g)
5% Corn oil	4.5 ± 0.5	28.0 ± 2.4	0.70 ± 0.11	2.0 ± 0.22
4% EEC n-3 plus 1% corn oil	1.3 ± 0[b]	17.4 ± 1.7[d]	0.28 ± 0.03[b]	1.1 ± 0.06[b]
5% EEC n-3	1.4 ± 0.1[b]	1.5 ± 0.3[c]	0.28 ± 0.03[b]	1.1 ± 0.21[d]

[a]Values are mean ± SEM of 3–5 rats in each group. LTC_4 production was measured by radioimmunoassay after 10 min incubation of the SMSG or gingival tissue at 37°C in the presence of 10 μM calcium ionophore, A23187. Arachidonic acid concentrations in total phospholipids were measured using 18:4 as an internal standard. Values with superscripts in the same column are significantly different from those in the control group (5% corn oil) using Student's t-test ([d]P<0.02, [b]P<0.01, [c]P<0.001).

were significantly lower in the SMSG and gingiva of rats fed 4% EEC plus 1% corn oil or 5% EEC of n-3 fatty acids than those fed 5% corn oil (Table 5). In gingiva, the LTC_4 production in the two experimental groups was the same, 55% of the control group. However, in the SMSG, the LTC_4 levels were several fold lower in the group fed 5% EEC than in the group fed 4% EEC plus 1% corn oil.

DISCUSSION

Although there are a number of studies on the effects of dietary n-3 fatty acids on eicosanoid levels in tissues, this is the first report on gingiva and salivary glands. Our results with gingiva and SMSG are consistent with the observations made on several other tissues that the levels of arachidonic acid in phospholipids are decreased by feeding diets rich in n-3 fatty acids (28–32). This may partly be due to the lower linoleic acid levels in the fish oil diets and partly due to the inhibitory effects of n-3 fatty acids on the metabolic conversion of linoleic acid to arachidonic acid. The n-3 fatty acids have been shown to inhibit the activity of Δ6 desaturase (28), a rate-limiting enzyme for the synthesis of arachidonic acid from linoleic acid (33,34). There is some evidence that the n-3 fatty acids also inhibit the rate of conversion of eicosatrienoic acid (20:3n-6) into arachidonic acid by inhibiting Δ5 desaturase activity (29).

The endogenous levels of PGE_2 in gingiva and the SMSG were also significantly lower in rats fed a menhaden oil-containing diet than a corn oil diet. Similar observations have been made in other tissues (35–38). Eicosapentaenoic acid and docosahexaenoic acid, the major n-3 fatty acids present in fish oils, are known to competitively inhibit the utilization of arachidonic acid by cyclooxygenase (12,39,40). The reduction observed in PGE_2 levels was about 4-fold in SMSG and 6-fold in the gingiva. The corresponding reduction in arachidonic acid levels was 2–2.5 fold. Thus, in both tissues there was a greater reduction in the endogenous PGE_2 levels than in the arachidonic acid levels. Similar observations were made with respect to the stimulated *in vitro* production of LTC_4 in the SMSG (Table 5). Although the experimental group fed 5% EEC of n-3 fatty acids had arachidonic acid concentrations which were about one-third of those in the control group fed 5% corn oil, the LTC_4 production was 19–20 fold lower in the experimental group than the control group. Similar results have been reported for other tissues (41). A reduction of 87% in the levels of the stimulated LTB_4 production was reported in neutrophils from essential fatty acid-deficient rats as compared with the control rats, even though the arachidonic acid content was reduced by only 34%.

The SMSG from rats fed 1% corn oil with 4% EEC of n-3 fatty acids did not show a reduction of the same magnitude as did the rats fed 5% EEC in the production of LTC_4. This may partly be due to the conversion of 18:2n-6 to 20:4n-6 during the incubation period (10 min at 37°C), thus resulting in an increase in the arachidonic acid pool. At this point, however, we do not have experimental evidence for this.

A number of investigators (42–49) have shown that nonsteroidal anti-inflammatory drugs which inhibit cyclooxygenase can retard the progression of periodontal disease in experimental animals and humans. In view of a significant reduction in the levels of endogenous PGE_2 and lower *in vitro* production of LTC_4 in gingiva of rats fed diets containing n-3 fatty acids, it is possible that the progression of periodontal disease may be retarded in humans ingesting supplements of n-3 fatty acids or those consuming large amounts of fish. Further investigations are needed to determine if the production of other arachidonic acid metabolites of the cyclooxygenase and lipoxygenase pathways in gingiva is also decreased by a diet rich in n-3 fatty acids. If so, this may provide a practical approach to decrease the production of the biochemical mediators of inflammation and thereby affect the course of periodontal disease. Because the feeding of a diet containing n-3 fatty acids was associated with significant reductions in the levels of PGE_2 and LTC_4 in SMSG, it is possible that gland function may also be altered. PGE_2 and LTC_4 have been shown to affect salivary secretion (14–17).

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Cholesterol Metabolism in the Rat Lactating Mammary Gland: The Role of Cholesteryl Ester Hydrolase

Kathleen M. Botham*
Department of Veterinary Basic Sciences, The Royal Veterinary College, London, NW1 OTU, United Kingdom

An acid cholesteryl ester hydrolase activity associated with a fraction containing mitochondria and lysosomes from rat lactating mammary glands was found to have a pH optimum of 5.0. Its sedimentation pattern was closely related to that of the lysosomal enzyme markers acid phosphatase and β-glucuronidase, suggesting that the activity is associated with the lysosomes. The enzyme was strongly inhibited by Cu^{2+}, but was inhibited little by other divalent metal ions. Acid cholesteryl ester hydrolase activity was almost completely abolished by *p*-hydroxymercuribenzoate, but this effect was reversed in the presence of an equimolar concentration of reduced glutathione (GSH), indicating that the enzyme requires free sulfhydryl groups for activity. These properties are similar to those of acid, lysosomal cholesteryl ester hydrolases found in other tissues. Acid cholesteryl ester hydrolase activity was 8–14 fold higher in mammary tissue from lactating as compared to virgin rats. Neutral cholesteryl ester hydrolase activities associated with the microsomal and cytosolic subcellular fractions were also increased in lactating glands, but to a lesser extent. In addition, a 2-fold increase in the activities of both the acid and microsomal neutral enzymes was seen during the first few days of lactation, while the cytosolic neutral activity remained constant. These results suggest that mammary gland cholesteryl ester hydrolases have a role in the regulation of cholesterol metabolism in mammary cells, and in the provision of cholesterol for secretion into milk. *Lipids 26,* **901–906 (1991).**

Cholesterol, an important component of milk fat, is secreted into milk during lactation in both esterified and unesterified form. Approximately 20–40% of milk cholesterol originates from *de novo* synthesis within the mammary gland, while the remaining 60–80% is derived from plasma lipoproteins (1–3). Cholesterol from both these sources, however, may be esterified and stored in the mammary gland as cholesteryl esters (4,5), which may in turn be hydrolyzed to unesterified cholesterol when required. In other tissues the immediate fate of lipoprotein cholesteryl esters after uptake is hydrolysis by an acid cholesteryl ester hydrolase located in the lysosomes (6). By contrast, intracellular stores of cholesteryl ester are hydrolyzed by enzymes with pH optima near neutral, located in the microsomal and soluble cell fractions (7,8). As both these types of cholesteryl ester hydrolase have regulatory roles in intracellular cholesterol metabolism in some tissues (6,7,9), similar enzymes in the mammary gland may play an important part in determining the cholesterol content of milk by regulating the amount available for secretion.

We have shown previously that the rat lactating mammary gland contains an acid cholesteryl ester hydrolase associated with the mitochondrial/lysosomal subcellular fraction, and two neutral activities found in the microsomal and cytosolic compartments (10). Tawil *et al.* (11) have also found cholesteryl ester hydrolases with pH optima near neutral in rat mammary tissue. Studies of the microsomal and cytosolic enzymes provide evidence for modulation of their activity by phosphorylation-dephosphorylation (12), suggesting that these enzymes are under hormonal control and have a regulatory role in mammary gland cholesterol metabolism.

In the current work, the properties of the acid cholesteryl ester hydrolase found in lactating glands in our earlier report (10) were investigated, and the activities of the acid and neutral enzymes in non-lactating tissue were compared to those of lactating tissue. In addition, changes in the activities of the three cholesteryl ester hydrolases, which occur during the early lactation period, were examined.

MATERIALS AND METHODS

Cholesteryl oleate, phosphatidylcholine (type XI-E), oleic acid, sodium taurocholate, *p*-hydroxymercuribenzoate (sodium salt), phenylmethylsulfonyl fluoride, glutathione (reduced form) (GSH), bovine serum albumin (essentially fatty acid free) and 4-morpholino-ethanesulfonic acid (MES) (free acid and sodium salt) were obtained from Sigma (Poole, Dorset, United Kingdom). Cholesteryl [1-^{14}C]oleate and [1-^{14}C]oleic acid were supplied by Amersham International (Aylesbury, Bucks., United Kingdom).

Animals. Abdominal and inguinal glands were excised from lactating Wistar rats 2–9 days post-partum, or from virgin rats 15–20 weeks old. The glands were cleaned of fat and connective tissue, finely minced with scissors and homogenized using a glass Teflon homogenizer in 5 vol of Tris-HCl buffer pH 7.2 (50 mM), containing 0.25 M sucrose (homogenizing buffer).

Preparation of subcellular fractions. The homogenate was centrifuged at 800 × *g* for 10 min (4°C) in a swing-out rotor and the floating fat layer was removed and discarded. The supernatant was then centrifuged at 12,000 × *g* for 10 min (4°C) using a fixed angle rotor. After careful removal of the floating fat layer and the supernatant, the pellet was washed with 5 mL homogenizing buffer and resuspended in MES buffer pH 5.0 (100 mM). This fraction containing the mitochondria and lysosomes was used in all experiments with the acid cholesteryl ester hydrolase, except where indicated otherwise. Microsomal and cytosolic subcellular fractions were prepared from the 12,000 × *g* supernatant as before (12). For partial separation of mitochondrial and lysosomal enzyme activities in some experiments, the supernatant from the first centrifugation (800 × *g*) was centrifuged sequentially at 2,500 × *g*, 8,000 × *g* and 12,000 × *g* (10 min, 4°C) using a fixed-angle rotor, and the resulting pellets were washed and resuspended as described above.

Assay of cholesteryl ester hydrolase. Cholesteryl ester hydrolase activities were determined by measuring the

*To whom correspondence should be addressed at Department of Veterinary Basic Sciences, The Royal Veterinary College, Royal College St., London, NW1 OTU, United Kingdom.

Abbreviations: CEH, cholesteryl ester hydrolase; EDTA, ethylenediaminetetraacetic acid; GSH, glutathione (reduced form); MES, 4-morpholino-ethanesulfonic acid.

release of [1-^{14}C]oleic acid from cholesteryl [1-^{14}C]oleate. The substrate was used as a mixed micelle with phosphatidylcholine and sodium taurocholate in molar ratios of 1:4:2 respectively, essentially as described by Hajjar *et al.* (13). Cholesteryl [1-^{14}C]oleate (185 KBq, 1.92 GBq/mmol) was added to a toluene solution containing phosphatidylcholine (57.6 μmol) and non-radioactive cholesteryl oleate (14.4 μmol) and the solvent was removed under a stream of nitrogen. Lipids were then resuspended in 5 mL Tris-HCl buffer pH 7.0 (50 mM) containing sodium taurocholate (28.8 μmol, 5.76 mM) and sonicated using a 0.5-inch horn for a total time of 30 min at 46°C. The resulting solution was centrifuged at 1,500 × *g* (10 min, room temperature) to remove metal fragments from the sonicator horn. Cholesteryl oleate/phosphatidylcholine vesicles were prepared in the same way, except that sodium taurocholate was omitted. Recovery of radioactivity in the substrate preparations was routinely >95%. The activity of acid cholesteryl ester hydrolase was assayed as follows: 150 μL MES buffer (pH 5.0, 100 mM) containing bovine serum albumin (essentially fatty acid free, 7.5 mg) was added to 200 μL of the mitochondrial/lysosomal subcellular fraction (approximately 0.2 mg protein) and incubated at 37°C for 10 min. The substrate (50 μL, 144 nmol cholesteryl oleate, 12.95 KBq/μmol) was then added to start the reaction. The final volume in the assay was 400 μL and the final concentrations of cholesteryl oleate, phosphatidylcholine and sodium taurocholate were 360 μM, 1440 μM and 720 μM respectively. After 15-min incubation at 37°C, the reaction was stopped with 1.5 mL chloroform/methanol/toluene (2:2.4:1, v/v/v) containing 0.29 mM oleic acid as carrier. Sodium hydroxide (50 μL, 1 M) was then added. The mixture was vortexed for 20 sec and finally centrifuged at 1,500 × *g* (25 min, 20°C) to separate the phases. The amount of sodium [1-^{14}C]oleate released into the upper phase was determined by liquid scintillation counting. Blank tubes containing substrate but no enzyme protein were included in each experiment. The efficiency of extraction of sodium [1-^{14}C]oleate was also estimated in each experiment, using mixed micelles prepared in the same way as the substrate except that [1-^{14}C]oleic acid was substituted for cholesteryl [1-^{14}C]oleate. Extraction efficiency was >85% and was unaffected by the amount of enzyme protein present. Cytosolic and microsomal neutral cholesteryl ester hydrolase activities were also assayed by this method using MES buffer (100 mM) at pH 6.6 and Tris-HCl (100 mM) at pH 7.0, respectively. Endogenous cholesteryl ester in the mitochondrial/lysosomal and microsomal fractions represented <2% of the total amount of substrate present in the assays. No cholesteryl ester was detected in the cytosolic fraction. Mixed micelles were used as substrate in all experiments except where indicated otherwise.

Other assays. The activities of glutamate dehydrogenase (14), acid phosphatase (15) and β-glucuronidase (16) were determined as described previously. Proteins were estimated by the method of Lowry *et al.* (17). Significance limits were calculated using Student's t-test or Pearson's correlation coefficient.

RESULTS

Previous studies have shown that rat lactating mammary glands contain a cholesteryl ester hydrolase activity with a pH optimum of about 5 associated with the subcellular fraction containing mitochondria and lysosomes (10). The effect of pH in the range 4–6.5 on the activity of this enzyme was tested to determine the pH optimum more precisely (Fig. 1), and a clear, sharp pH optimum at 5.0 was found. The rate of hydrolysis of cholesteryl ester at pH 5.0 was linear with both the time of incubation, up to approximately 30 min (Fig. 2, A), and with the amount of subcellular fraction protein added, up to about 0.2 mg (Fig. 2, B).

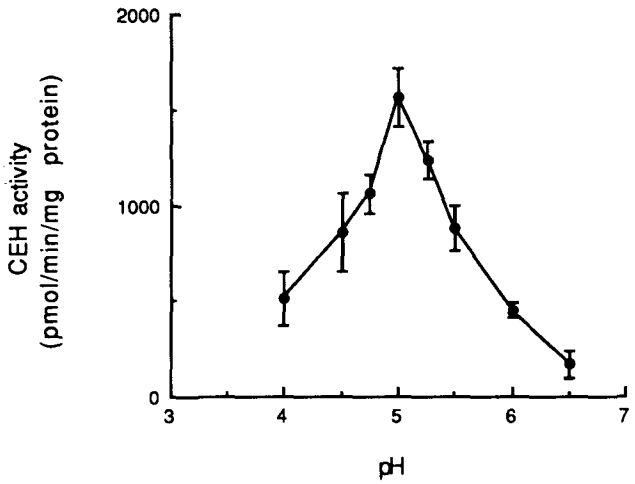


FIG. 1. The effect of pH on acid cholesteryl ester hydrolase activity. The activity of cholesteryl ester hydrolase in the subcellular fraction containing lysosomes and mitochondria prepared from rat lactating mammary tissue was assayed using sodium acetate (100 mM, pH 4.0–4.75) or MES (100 mM, pH 5.0–6.5) buffer. Each point is the mean from 3 experiments. Error bars show the standard error of the mean.

The low pH optimum and sedimentation pattern (10) of the acid cholesteryl ester hydrolase suggest that it may be a lysosomal enzyme. To test this possibility, three subcellular fractions containing mitochondria and lysosomes of increasing density were prepared from rat lactating mammary tissue and the activity of cholesteryl ester hydrolase in each fraction was compared to that of marker enzymes for mitochondria (glutamate dehydrogenase) and lysosomes (acid phosphatase and β-glucuronidase). The specific activities of the marker enzymes in each fraction relative to their specific activities in the whole mitochondrial pellet were consistent in preparations from four animals. Approximately 50% of the total activities (sum of the activities in the three fractions) of cholesteryl hydrolase (50.2 ± 2.1%), acid phosphatase (47.9 ± 1.5%) and β-glucuronidase (48.5 ± 15.4%) were associated with the pellet sedimenting at 8,000 × *g*. In contrast, only 27.0 ± 5.7% of the activity of glutamate dehydrogenase sedimented with this fraction. Over a number of experiments (data not shown), the percentage of the total activity of cholesteryl ester hydrolase associated with each fraction was much more strongly correlated with the percentage activities of acid phosphatase (Pearson's correlation coefficient, $r = 0.95$ $p<0.001$) and β-glucuronidase ($r = 0.95$ $p<0.001$) than with those of glutamate dehydrogenase ($r = 0.6$), suggesting

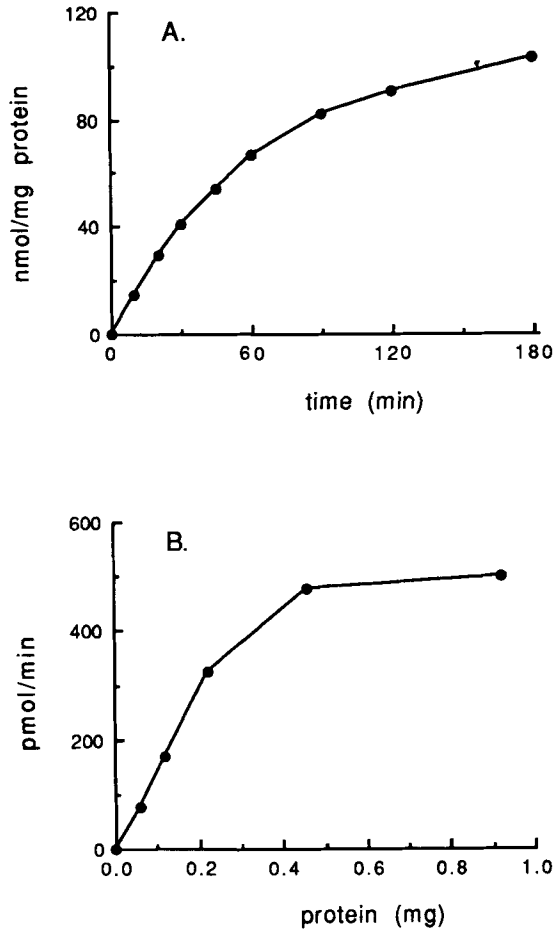


FIG. 2. The effect of time of incubation and enzyme protein concentration on acid cholesteryl ester hydrolase activity. The activity of cholesteryl ester hydrolase in the subcellular fraction containing lysosomes and mitochondria prepared from rat lactating mammary glands was assayed using the standard conditions described in Materials and Methods except that the time of incubation in the presence of the substrate (A), or the amount of subcellular fraction protein added (B), was varied as indicated. Each point is the mean from duplicate determinations and the experiment shown is typical of 4 performed in each case.

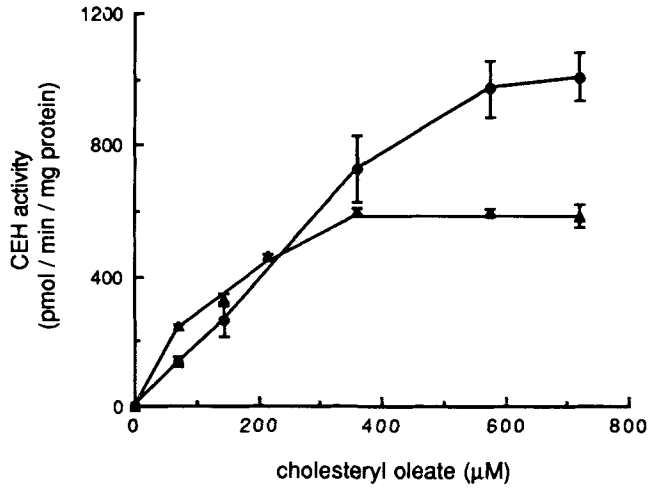


FIG. 3. The effect of substrate concentration on acid cholesteryl ester hydrolase activity. The activity of cholesteryl ester hydrolase in the subcellular fraction containing lysosomes and mitochondria prepared from rat lactating mammary tissue was determined using the concentrations of cholesteryl oleate indicated. Assays were carried out with the substrate in the form of mixed micelles (●) or cholesteryl oleate/phosphatidylcholine vesicles (molar ratio 1:4) in the presence of sodium taurocholate (720 μM) (▲). Each point is the mean from 3 experiments. Error bars show the standard error of the mean.

that the acid cholesteryl ester hydrolase is associated with the lysosomes in rat mammary tissue.

The activity of acid, lysosomal cholesteryl ester hydrolases in some tissues is influenced by the mode of presentation of the substrate (18). The highest activity is usually found when the cholesteryl ester is dispersed with phospholipid in micelles or vesicles with or without sodium taurocholate and/or digitonin (18,19). Previous experiments have shown that mammary gland acid cholesteryl ester hydrolase activity is decreased considerably when sodium taurocholate is omitted from the mixed micelle substrate, but can be substantially restored by separate addition of the bile salt (20). Addition of digitonin (50–250 μg/mL) to the assays did not significantly alter the observed activity in the presence or absence of sodium taurocholate (data not shown). In the experiment shown in Figure 3, the activity of acid cholesteryl ester hydrolase was assayed using different concentrations of cholesteryl oleate dispersed either as mixed micelles, or with phosphatidylcholine only (molar ratio 1:4) in the presence of sodium taurocholate (720 μM) added separately. The kinetics of the enzyme were markedly different with the two substrate presentation modes. These differences may be explained by the variation in the concentrations of sodium taurocholate which are added with the different cholesteryl oleate concentrations when the mixed micelle substrate is used. The detergent effect of the bile salt is likely to influence the size of the lipid/water interface between the enzyme and the substrate and thus modify the activity (18).

The effect of divalent metal ions (1 mM) on the activity of the acid cholesteryl ester hydrolase is shown in Table 1. The enzyme was strongly inhibited by Cu^{2+}, but was inhibited little by other divalent metal ions at the concentration used. Furthermore, ethylenediaminetetraacetic acid (EDTA) (1 mM) did not change the enzyme activity significantly (Table 2). The enzyme was inhibited by NaCl (10–100 mM) and NaF (10–50 mM) in a concentration-dependent way (Table 2). A concentration of 1 mM *p*-hydroxymercuribenzoate almost completely abolished the activity, while 1 mM GSH had only a small inhibitory effect. In the presence of equimolar concentrations of *p*-hydroxymercuribenzoate and GSH, however, activity of the enzyme was restored to the level found with GSH alone (Table 2). Finally, the serine esterase inhibitor phenylmethylsulfonyl fluoride (1 mM) caused a decrease in acid cholesteryl ester hydrolase activity of approximately 25% (Table 2).

Figure 4 shows the changes in the activities of the mam-

TABLE 1

The Effect of Divalent Metal Ions on Acid Cholesteryl Ester Hydrolase Activity[a]

Additions	Cholesteryl ester hydrolase activity (% control value)
None	100
$MgCl_2$	88.6 ± 2.2(5)
$MnCl_2$	83.3 ± 6.1(5)
$NiCl_2$	84.8 ± 5.2(5)
$CaCl_2$	74.7 ± 3.4(5)
$ZnCl_2$	80.9 ± 4.8(4)
$CuCl_2$	10.8 ± 1.5(4)

[a]The subcellular fraction containing lysosomes and mitochondria was prepared from rat lactating mammary tissue as described in Materials and Methods. Acid cholesteryl ester hydrolase activity was determined in the presence and absence of the metal ions indicated. The metal salts were added to the assays in the MES buffer prior to the preincubation period. Data are expressed as a percentage of the values found in the absence of added metal ions, and are the mean ± standard error of the mean from 4 or 5 experiments (indicated in brackets). Control values (pmol/min/mg protein) were 1054 ± 115 (4 experiments) and 1010 ± 99 (5 experiments).

TABLE 2

The Effect of Enzyme Inhibitors on Acid Cholesteryl Ester Hydrolase Activity[a]

Additions	Cholesteryl ester hydrolase activity (% control value)
None	100
NaF (10 mM)	46.0 ± 4.6(4)
NaF (50 mM)	16.1 ± 0.9(5)
NaCl (10 mM)	79.3 ± 4.1(4)
NaCl (100 mM)	52.8 ± 7.4(4)
EDTA (1 mM)	98.2 ± 2.5(5)
Phenylmethylsulfonyl fluoride (1 mM)	75.4 ± 1.3(4)
p-Hydroxymercuribenzoate (0.1 mM)	79.2 ± 2.1(5)
p-Hydroxymercuribenzoate (1 mM)	0.2 ± 0.1(5)
GSH (1 mM)	84.8 ± 1.5(4)
p-Hydroxymercuribenzoate (1 mM) + GSH (1 mM)	79.7 ± 3.2(4)

[a]The subcellular fraction containing mitochondria and lysosomes was prepared from rat lactating mammary tissue as described in Materials and Methods. Acid cholesteryl ester hydrolase activity was determined in the presence and absence of the compounds shown. Additions were made to the assays in the MES buffer prior to the preincubation period. Data are expressed as a percentage of the values found without additions, and are the mean ± standard error of the mean from 4 or 5 experiments (indicated in brackets). Control values (pmol/min/mg protein) were 1192 ± 87 (4 experiments) and 1132 ± 90 (5 experiments).

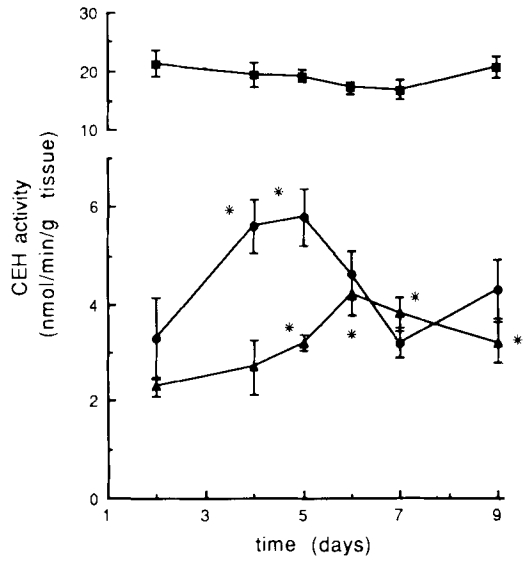


FIG. 4. The effect of time of lactation on acid and neutral cholesteryl ester hydrolase activities. The activities of acid and neutral cholesteryl ester hydrolases (CEH) in the subcellular fractions containing mitochondria and lysosomes (●), microsomes (▲) and cytosol (■) prepared from mammary glands taken from rats after the period of lactation shown were determined as described in Materials and Methods. Each point is the mean from at least 4 experiments. Error bars show the standard error of the mean. *Value is significantly different ($p<0.05$) from value at 2 days.

mary gland acid and neutral cholesteryl ester hydrolases which occur during the first 9 days of lactation. The results are expressed as nmol/min/g tissue so that their relative activities in whole tissue can be compared. The activity of the cytosolic neutral cholesteryl ester hydrolase was higher than that of the other two enzymes at all time points, and did not change significantly during the period studied. Acid cholesteryl ester hydrolase activity, however, increased significantly during the first few days of lactation, reaching a peak after 5 days, then declined to a value close to that seen at the start of the experiment after 7 days. The activity of the microsomal neutral enzyme also increased steadily during early lactation, reaching a maximum after 6 days. The relative specific activities of marker enzymes for lysosomes (acid phosphatase, 1.88 ± 0.15), microsomes (NADPH:cytochrome C reductase, 3.55 ± 0.21) and cytosol (lactate dehydrogenase, 1.71 ± 0.07) varied little in subcellular fractions of glands from seven rats between 2 and 9 days post-partum. No relationship between the relative specific activities of the enzymes and the number of days lactating was detected. Recovery of the marker enzymes in the appropriate fractions as a percentage of the total activity found in the homogenate was also consistent (± 5%). These results indicate that the changes in the activities of the three cholesteryl ester hydrolases during lactation are not caused by differences in purity or recovery of the individual preparations.

When subcellular fractions were prepared from mammary tissue from virgin rats, the activities of the three cholesteryl ester hydrolases under investigation were decreased considerably compared to the values obtained with lactating glands. The greatest difference, 8–14 fold, was seen in the activity of the acid cholesteryl ester hydrolase (non-lactating, 0.42 ± 0.11 nmol/min/g; peak lactating at 5 days, 5.8 ± 0.6 nmol/min/g) followed by the

microsomal enzyme, 3–5 fold (non-lactating, 0.75 ± 0.25; peak lactating at 6 days, 4.2 ± 0.4 nmol/min/g), while the cytosolic activity showed the smallest change, 2-fold (non-lactating, 9.3 ± 0.9; lactating at 2 days, 21.3 ± 2.3 nmol/min/g). The non-lactating values were obtained using tissue pooled from two or three rats because very much less mammary tissue is present in virgin rats. The data given are the mean ± standard error of the mean from three experiments using a total of eight animals.

DISCUSSION

Cholesteryl ester hydrolase activities with pH optima between 4.0 and 5.0 are associated with the lysosomes in a wide variety of tissue and cell types including liver (21,22), heart (23), aorta (24), fibroblasts (25,26), adrenal cortex (27) and adipose tissue (23). Our earlier experiments had suggested that a similar activity may be present in the rat lactating mammary gland (10). The results reported here confirm the presence in such tissue of an acid cholesteryl ester hydrolase with a pH optimum of 5.0 which sediments with lysosomal enzyme markers. Similar to the acid, lysosomal cholesteryl ester hydrolases in other tissues, the mammary gland enzyme is inhibited by Cu^{2+} (27–29), NaCl, and NaF (19,21,25). Furthermore, the inhibition of the mammary gland activity by *p*-hydroxymercuribenzoate, and its almost complete reversal by GSH (Table 2) indicates that the enzyme requires free sulfhydryl groups for activity, as has been found with similar enzymes in other cell types (21,22,24,25,30).

In a number of previous studies the activity of lysosomal cholesteryl ester hydrolase, even after extensive purification, could not be separated from that of lysosomal acid lipase, which is responsible for the hydrolysis of triglycerides, and it is believed that the same enzyme catalyzes both reactions (21,24,31). If this were the case with the mammary gland cholesteryl ester hydrolase, then the observed 25% inhibition by phenylmethylsulfonyl fluoride may indicate contamination of the preparation with other lipases, as lysosomal, acid lipases are not believed to be affected by serine esterase inhibitors. Imanaka *et al.* (32), however, have reported that the cholesteryl ester hydrolase activity of partially purified rabbit liver lysosomal lipase was inhibited by about 20% by 0.2 mM phenylmethylsulfonyl fluoride. The subcellular fraction used in the experiments described here may contain lipoprotein lipase, which is present in rat mammary tissue (33). This enzyme, however, has an alkaline pH optimum at about 8.5, and has virtually no activity below pH 6 (34). The milk of some species contains a bile salt-stimulated lipase (35), but this has not been detected in rat milk (36). Although the presence of other lipases in rat mammary tissue has not been reported, the possibility of some contamination by unknown lipases cannot be ruled out.

The lysosomal cholesteryl ester hydrolase has an important function in the regulation of cholesterol metabolism in some tissues (6,37). Increased flux of cholesterol from the plasma leads to decreased cholesterol synthesis and increased storage as esters. Feeding lactating rats a hypercholesterolemic diet has been shown to decrease cholesterol synthesis in the mammary gland (38), suggesting that cholesterol taken up from the plasma and processed by the acid cholesteryl ester hydrolase may also regulate cholesterol metabolism in mammary cells. In addition, neutral cholesteryl ester hydrolases in the adrenal cortex (7,9), liver (39) and adipose tissue (40) have been shown to be hormonally controlled by a mechanism involving phosphorylation-dephosphorylation. Thus they are believed to be involved in the regulation of the supply of unesterified cholesterol for metabolism in these tissue. Recent experiments in this laboratory (12) have suggested that the microsomal and cytosolic neutral cholesteryl ester hydrolases from rat mammary glands may be modulated by similar phosphorylation-dephosphorylation mechanisms. These enzymes, therefore, may play a part in regulating mammary gland cholesterol metabolism.

If the mammary gland cholesteryl ester hydrolases were important in the provision of unesterified cholesterol for secretion into milk, their activities would be expected to be higher in lactating as compared to non-lactating tissue. This was found to be the case for all three enzymes studied. The acid activity, however, showed the greatest increase (8–14 fold). A large proportion (60–80%) of milk cholesterol is derived from plasma lipoproteins (1–3), and the uptake of cholesterol from this source has been shown to be increased approximately 8-fold at the onset of lactation (41). Cholesteryl esters from chylomicrons have been shown to be internalized by mammary cells without prior hydrolysis (42). In addition, Raphael *et al.* (3) have reported that radioactivity from low density lipoprotein and very low density lipoprotein cholesteryl esters is transferred into milk. The results of the current work, therefore, suggest that the mammary gland acid cholesteryl ester hydrolase has a role in hydrolyzing lipoprotein cholesteryl ester to provide unesterified cholesterol for secretion into milk.

The experiments reported here demonstrate that the acid cholesteryl ester hydrolase activity found in the mammary gland has characteristics similar to those of acid, lysosomal cholesteryl ester hydrolases found in other tissues. In addition, the findings support the idea that the mammary gland acid and neutral cholesteryl ester hydrolases, like those found in other tissues, play a part in regulating intracellular cholesterol metabolism, and thus help control the availability of cholesterol for secretion into milk.

ACKNOWLEDGMENTS

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Effects of Perturbations in Hepatic Free and Esterified Cholesterol Pools on Bile Acid Synthesis, Cholesterol 7α-Hydroxylase, HMG-CoA Reductase, Acyl-CoA:Cholesterol Acyltransferase and Cytosolic Cholesteryl Ester Hydrolase

W.M. Grogan[a,*], M.L. Bailey[b], D.M. Heuman[b] and Z.R. Vlahcevic[b]

[a]Department of Biochemistry and Molecular Biophysics and [b]Division of Gastroenterology, McGuire Veterans Administration Hospital, Medical College of Virginia, Virginia Commonwealth University, Richmond, Virginia 23298-0614

Effects of expansion of the hepatic free cholesterol pool on bile acid and cholesterol metabolism and homeostasis were examined in rats fed cholesterol in high-fat diets or treated with oleyl-*p*-(n-decyl)-benzenesulfonate (ODS) or progesterone. Cholesterol feeding for 10–16 days, which increased free (33%) and esterified (6-fold) cholesterol, had no effect on cholate synthesis, total bile acid synthesis, or cholate turnover, whereas these activities were increased 60–80% by ODS and progesterone, which produced only small increases (19%) in free cholesterol. Cholesterol feeding reduced β-hydroxy-β-methylglutaryl (HMG)-CoA reductase (72%) and cholesteryl ester hydrolase (48%) and increased acyl-CoA:cholesterol acyltransferase (184%), whereas ODS and progesterone reversed these compensatory responses in cholesterol-fed rats. Cholesterol 7α-hydroxylase was changed no more than 22% by any treatment. A bolus of ODS elevated biliary cholesterol output 41% and shifted biliary bile acid synthesis and composition toward 12-deoxy bile acids. These effects were not seen in ODS-fed or progesterone-treated rats, in which cholesteryl ester stores were depleted. It is concluded that effects of free cholesterol on bile acid synthesis and biliary cholesterol are probably mediated by specific precursor or regulatory pools which can be independently regulated and which represent a relatively small fraction of hepatic free cholesterol.
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The role of hepatic free cholesterol in the regulation of bile acid synthesis is controversial. Bile acid synthesis, the major metabolic fate of cholesterol, is thought to be regulated by modulation of cholesterol 7α-hydroxylase (C7AH), a rate-limiting enzyme in this pathway (1), by a poorly understood feedback mechanism involving bile acid and sterol (2,3) and by availability of free cholesteryl substrate (4). Some studies have suggested that C7AH is not saturated by cholesterol levels present in microsomes (4–7). Moreover, cholesterol feeding, which increases bile acid synthesis in several species, may or may not increase C7AH activity (8–10). Other studies have suggested that microsomal free cholesterol levels are near saturation and, thus, not rate-limiting for C7AH under most conditions, that increased C7AH activity can be decoupled from increased cholesterol synthesis (11–14), and that increases in bile acid synthesis with cholesterol feeding are dependent on receptor-mediated uptake of cholesterol (15). Although some studies suggest that changes in size of the hepatic free cholesterol pool affect biliary cholesterol secretion (16,17), the effects of manipulation of the free cholesterol pool on bile acid synthesis have not been studied.

Compensatory mechanisms maintain intracellular free cholesterol levels within relatively narrow ranges, even when challenged by large influxes of dietary cholesterol (18–22). Best characterized of these mechanisms is gene level coordinate repression of serum lipoprotein receptors and HMG-CoA reductase (β-hydroxy-β-methylglutaryl-CoA reductase, HMGCR), the rate-limiting enzyme in cholesterol biosynthesis (23). Although detailed mechanisms are unclear, free cholesterol is also regulated by hepatic microsomal acyl-CoA:cholesterol acyltransferase (ACAT), the major cholesterol esterifying enzyme (24–26), cholesteryl ester hydrolases (CEH) (22,27–29) and C7AH, the initial rate-limiting enzyme in bile acid synthesis (1). HMGCR, ACAT, CEH and C7AH are apparently all subject to short-term regulation by protein kinases (28–32).

Precursor cholesterol for both bile acid synthesis and biliary cholesterol secretion seems to derive from intracellular pools of free cholesterol which can be distinguished under appropriate experimental conditions (16, 33–36). For example, free cholesterol derived from lipoprotein is apparently the preferred substrate for bile acid synthesis in man (33–35), whereas newly synthesized cholesterol seems to be the preferred substrate in rat (6,7,36). Biliary cholesterol seems to be derived preferentially from a free cholesterol pool in rapid equilibration with cholesteryl ester (16,17).

We have examined the effects of manipulation of hepatic free cholesterol pools on bile acid synthesis, biliary cholesterol secretion and the four key regulatory enzymes of cholesterol homeostasis, using oleyl-*p*-(n-decyl)-benzenesulfonate (ODS), a CEH activator (37), and progesterone, an ACAT inhibitor (16,17). In order to accentuate effects related to storage and mobilization of cholesteryl ester, liver cholesteryl ester stores have been mildly elevated by cholesterol feeding.

MATERIALS AND METHODS

Animals and diets. Adult male Sprague-Dawley rats (Charles River Breeding Laboratories, Cambridge, MA) were maintained on rat chow until weighing 200–250 g and then fed *ad libitum* 10 days on rat chow (Prolab RMH 3000, Agway Corp., Syracuse, NY) or rat chow supplemented with 10% (w/w) commercial vegetable fat (Crisco, Procter and Gamble, Cincinnati, OH) or 10% fat + 1%

*To whom correspondence should be addressed.

Abbreviations: ACAT, acyl-CoA:cholesterol acyltransferase; C7AH, cholesterol 7α-hydroxylase; CEH, cholesteryl ester hydrolase; HPLC, high-performance liquid chromatography; HMGCR, β-hydroxy-β-methylglutaryl-CoA reductase; ODS, oleyl-*p*-(n-decyl)-benzenesulfonate.

cholesterol (Sigma Chemical Co., St. Louis, MO). Fat was melted at 50°C and mixed with feed to achieve uniform distribution. Cholesterol was dissolved in molten fat prior to mixing with feed. Rats were regularly monitored for appearance, activity, feed and water consumption and bile, urine and fecal output. Rats used in these experiments had reached a weight of 250–350 g prior to surgery. Rats consuming less than 10 g feed or 15 mL water or producing less than 5 mL urine per day were eliminated from the study. Rats exhibiting a decline in bile flow due to catheter occlusion were excluded from data analysis.

Treatments. Oleyl-*p*-(n-decyl)-benzenesulfonate (ODS), synthesized as described previously by Quackenbush *et al.* (38), was administered either intraduodenally as a 40 μg bolus or as 0.01% of diet (w/w) dissolved in dietary fat prior to mixing of fat with chow. Treatment was normally begun 48 hr following surgery or 48 hr after addition of [26-^{14}C]-cholesterol, to permit recovery from surgery and equilibration of cholesterol pools. Whereas [^{3}H]cholate was normally given 24 hr after initiation of treatment, one group of 7 rats was given an ODS bolus 3–4 days after administration of radiolabel to permit monitoring of isotope dilution before and after treatment. Progesterone treated rats received intraduodenal boluses of 40 μg/kg progesterone (Sigma) dissolved in 500 μL corn oil. Bolus ODS or progesterone was injected in the duodenal return line of bile duct cannulated rats (see below) and bile flow was monitored for at least 30 min thereafter, with no diminution in flow rate.

Surgical techniques. Rats were provided with exteriorized enterohepatic circulation as described by Weis and Barth (39) and Kuipers *et al.* (40). A bile duct cannula was routed outside of the body, and joined to an intraduodenal cannula, for continuous reinfusion of bile. The simple overlapping junction of the two lines could be disconnected briefly for collection of bile or injection of an air bubble for flow rate determination. Details of the surgical procedure as carried out in this laboratory are described in Heuman *et al.* (41).

Bile acid synthesis and turnover by isotope dilution. As described by Heuman *et al.* (41), rats were given 1 μCi [2,4-^{3}H(N)]cholic acid (New England Nuclear-Dupont, Boston, MA; 25 Ci/mmol) or [24-^{14}C]cholic acid (40–60 mCi/mmol) 24 hr after surgery or initial treatment with ODS or progesterone. Twice each day, 20–40 mg bile was drained and weighed for measurement of mass and radioactivity of bile acids and cholesterol as described below. Cholate pool, intestinal absorption efficiency and fractional turnover were calculated from isotope dilution by the method of Linstedt (42). Bile salt secretion was calculated as the product of biliary bile salt concentration and bile flow rate, measured by timing movement of a bubble through a defined volume of cannula.

Bile acid synthesis by [26-^{14}C]cholesterol oxidation. At 24 hr after surgery, rats were given 10 μCi [26-^{14}C]cholesterol (New England Nuclear-Dupont; 53 mCi/mmol) in 400 μL corn oil as an intraduodenal bolus. Following a 48-hr waiting period to allow equilibration of radiolabel with cholesterol pools, rats were placed in CO_2 collection chambers at 6, 8 or 12 hr intervals, 2–4 times each day for 4 days. Details of procedure and collection apparatus are given in Duane *et al.* (43,44). Air was drawn through the sealed chamber under slightly negative pressure, into a series of 3 bubble traps containing monomethylamine, which traps >95% of expired CO_2. Rats were accommodated to chambers for approximately 15 min, prior to CO_2 collection for 30 min. ^{14}C was measured in aliquots of collection fluid by liquid scintillation counting, correcting for quenching by addition of internal standard. Bile acid synthesis was calculated as dpm expired $^{14}CO_2$/specific radioactivity of precursor pool. Specific radioactivity of the precursor pool was obtained two ways. Specific radioactivity of biliary cholesterol was calculated from concentrations of ^{14}C and cholesterol in bile samples and fitted to a log/linear decay curve by linear regression analysis, in order to calculate values for times at which bile samples were unavailable. Specific radioactivity of liver total and free cholesterol was also measured for 20 rats at termination of the experiment (see below). These values agreed with those obtained for biliary cholesterol from the final bile collection.

Biliary cholesterol determination. Bile samples were extracted with 25 mL $CHCl_3$/MeOH (2:1, v/v) according to the method of Folch *et al.* (45), as described previously (41). Following partitioning, the $CHCl_3$ phase was drawn off and brought to 25 mL with $CHCl_3$. A 5-mL aliquot was evaporated to dryness, redissolved in scintillation fluid and assayed for ^{14}C by liquid scintillation counting. A 10-mL aliquot was dried and assayed for cholesterol using a commercial kit, based on the cholesterol oxidase colorimetric determination (Sigma). Approximately 5% of samples were lost or discarded due to contamination. The MeOH/H_2O phase was saved for bile acid analysis.

Biliary bile acid determination. Biliary bile acid analysis was carried out as described previously (41). A 5-mL aliquot of MeOH/H_2O from bile extraction was assayed for ^{3}H by liquid scintillation counting. Taurine and glycine conjugates of cholate, deoxycholate, chenodeoxycholate, ursodeoxycholate and muricholate were measured in 1-mL aliquots by the high-performance liquid chromatography (HPLC) method of Nakayama and Nakagaki (46) as adapted by Heuman *et al.* (41).

Liver total and free cholesterol. Livers were extracted with $CHCl_3$/MeOH by the method of Folch *et al.* (45). Extract was dried under N_2 and redissolved in 25 mL $CHCl_3$. Radioactivity of total cholesterol was measured in 1-mL aliquots by liquid scintillation counting. Total cholesterol was determined in 1-mL aliquots as described by Klauda *et al.* (37). Free cholesterol was precipitated from duplicate 2-mL aliquots with digitonin as described by Klauda *et al.* (37). One precipitate was analyzed for cholesterol as described by Klauda *et al.* (37); the other was redissolved in 1 mL MeOH and assayed for radioactivity by liquid scintillation counting. Cholesteryl ester was calculated as the difference between total cholesterol and free cholesterol.

Enzyme assays. Neutral cytosolic CEH was measured at pH 7.4 in 104,000 × g supernatant essentially by the method of Deykin and Goodman (27). Cholesteryl oleate (Sigma), 100 nmol, containing 100,000 dpm [1,2,6,7-^{3}H(N)]cholesteryl oleate (New England Nuclear-Dupont; 83 Ci/mmol) was added in 100 μL acetone to 1 mL of cytosol containing 100 μg protein, and incubated for 30 min at 37°C. The reaction was terminated by extraction with $CHCl_3$/MeOH (2:1, v/v). Solvent was evaporated under N_2 and the residue was dissolved in $CHCl_3$. Unreacted cholesteryl oleate and free cholesterol product were separated by thin-layer chromatography, visualized with

I_2, scraped from the plate and assayed for radioactivity by liquid scintillation counting. Lysosomal acid CEH activity is not detected under conditions used for this assay (29).

Microsomal enzymes were measured by radiometric procedures used by Heuman *et al.* (47). Acyl-CoA:cholesterol acyltransferase was measured by the method of Erickson *et al.* (48). β-Hydroxy-β-methylglutaryl-CoA reductase was assayed by the procedure of Shefer *et al.* (1) as modified by Whitehead *et al.* (49). Cholesterol-7α-hydroxylase was assayed as described by Shefer *et al.* (50). Protein was determined by the method of Bradford (51). Whereas enzymes could only be assayed at termination, additional groups of 3–6 rats were sacrificed for measurement of enzyme activity, 1, 2, 3 and 4 days after administration of ODS (bolus).

RESULTS

Hepatic total and free cholesterol. As expected, cholesterol feeding increased hepatic total cholesterol levels 273% over fat-fed controls ($p<0.025$; see Table 1). Free cholesterol was increased by 33%, whereas the balance of increase was in cholesteryl ester. Administration of ODS in a single bolus produced no significant decrease in total cholesterol, but elevated free cholesterol by an additional 19% ($p<0.01$). Addition of 0.01% ODS to diet for 4 days lowered total hepatic cholesterol to the level of non-cholesterol-fed controls, whereas free cholesterol was much more variable and not significantly different from either cholesterol-fed or ODS (bolus) treated rats (Table 1). A similar result was obtained with progesterone, which reduced total cholesterol below non-cholesterol-fed control levels and gave final free cholesterol levels equivalent to cholesterol-fed controls. Both ODS-feeding and progesterone depleted cholesteryl ester to only 17–20% of total cholesterol, whereas cholesteryl ester was >50% of total cholesterol in all other groups. There were no significant differences in microsomal cholesterol levels at termination of the experiment.

Hepatic enzymes of cholesterol homeostasis. As shown in Table 2, cholesterol feeding reduced CEH and HMGCR activities 48% ($p<0.05$) and 72% ($p<0.001$), respectively, while elevating ACAT 184% ($p<0.001$) in comparison to controls. These effects were consistent with a compensatory response to increased influx of dietary cholesterol. In contrast, there was no significant effect on C7AH.

Administration of ODS as a bolus to cholesterol-fed rats increased CEH, HMGCR, and C7AH 160% ($p<0.001$), 250% ($p<0.001$) and 32% ($p<0.025$), respectively, and decreased ACAT 52% ($p<0.001$). Thus, ODS appeared to reverse the effects of cholesterol feeding on CEH, HMGCR and ACAT. However, only with CEH did the change more than compensate for effects of cholesterol feeding. Essentially the same results were obtained from rats (N = 3–6) sacrificed 1, 2, 3, 4, or 5 days post-treatment (data not shown).

ODS feeding and progesterone produced similar changes ($p<0.001$) in CEH, HMGCR and ACAT, but had no significant effect on C7AH (Table 2). Interestingly, the vehicle fat for dietary cholesterol by itself produced significant increases in ACAT ($p<0.005$) and C7AH ($p<0.001$), in comparison to rats fed unmodified rat chow. Thus, with one or more diets, each potential regulatory enzyme of cholesterol homeostasis and bile acid synthesis varied independently with respect to the other three enzymes.

Bile synthesis and cholate turnover. Figure 1 shows a typical isotope decay curve for seven rats which were monitored both before and after administration of ODS bolus. Decay of [^{14}C]cholate specific radioactivity was log-linear with respect to time both before and within 48 hr after ODS treatment but exhibited a sharp change in slope at 24–48 hr post-treatment, consistent with rapid equilibration to a new steady state with a higher turnover rate. [^{3}H]Cholate given 48 hr after ODS treatment (Fig. 1, 2nd isotope) produced a decay curve parallel to that of [^{14}C]cholate. In contrast, cholesterol-fed controls exhibited log-linear decay throughout the test period. As shown in Table 3, cholate pool size did not change with any treatment, which serves as further evidence that this

TABLE 1

Effects of Cholesterol Feeding, ODS and Progesterone on Hepatic Free, Esterified and Microsomal Cholesterol[a]

Diet/treatment	Free cholesterol	Cholesterol ester	Microsomal cholesterol
	(mg/g liver)	(mg/g liver)	(mg/mg protein)
Chow	1.1 ± 0.0	1.2 ± 0.1	25 ± 2
10% fat	1.2 ± 0.1	1.4 ± 0.1	26 ± 3
10% fat, 1% cholesterol	1.6 ± 0.1[b]	8.1 ± 2.0[c]	29 ± 2
10% fat, 1% cholesterol, ODS bolus	1.9 ± 0.1[d]	5.5 ± 0.9	26 ± 2
10% fat, 1% cholesterol, 0.01% ODS-fed	1.9 ± 0.2	0.4 ± 0.2[e]	32 ± 2
10% fat, 1% cholesterol, progesterone bolus	1.6 ± 0.2	0.4 ± 0.1[e]	29 ± 3

[a]Rats were fed chow, chow + 10% fat or chow + 10% fat + 1% cholesterol for 10 days. Following surgery as described in Materials and Methods, chow, fat and cholesterol groups were continued on respective diets for an additional 6 days before measurement of free and total cholesterol. Other groups were treated as cholesterol-fed rats, except for treatments. ODS bolus and progesterone groups received a single bolus of ODS or progesterone, respectively, 2 days after surgery. ODS was added to diet of ODS-fed, beginning 2 days after surgery. Esterified cholesterol was calculated by difference. Values are mean ± SEM (N = 4–12).

[b]Different from 10% fat control, $p<0.025$.

[c]Different from 10% fat control, $p<0.01$.

[d]Different from 1% cholesterol control, $p<0.01$.

[e]Different from 1% cholesterol control, $p<0.005$.

TABLE 2

Effects of Cholesterol Feeding, ODS and Progesterone on Key Enzymes of Cholesterol and Bile Acid Metabolism in the Liver (nmol/hr/mg protein)[a]

Diet/treatment	CEH	ACAT	HMGCR	C7AH
Chow	20 ± 2	9 ± 1	3.8 ± 0.4	0.8 ± 0.02
10% fat	26 ± 5	14 ± 1[b]	2.9 ± 0.3	1.1 ± 0.09
10% fat, 1% cholesterol	13 ± 2[c]	39 ± 4[d]	0.8 ± 0.2[d]	0.9 ± 0.07
10% fat, 1% cholesterol, ODS bolus	34 ± 4[e]	19 ± 2[e]	2.8 ± 0.5[e]	1.2 ± 0.08[f]
10% fat, 1% cholesterol, 0.01% ODS-fed	28 ± 2[e]	16 ± 1[e]	2.5 ± 0.3[e]	0.9 ± 0.07
10% fat, 1% cholesterol, progesterone bolus	29 ± 7	27 ± 1[g]	1.6 ± 0.2[g]	0.7 ± 0.04

[a] See Table 1 for description of diets and treatments. CEH, cholesteryl ester hydrolase; ACAT, acyl-CoA:cholesterol acyltransferase; HMGCR, HMG-CoA reductase; C7AH, cholesterol 7α-hydroxylase. Values are mean ± SEM (N = 4-22).

[b] Different from chow control, $p<0.005$.

[c] Different from 10% fat control, $p<0.05$.

[d] Different from 10% fat control, $p<0.001$.

[e] Different from 1% cholesterol control, $p<0.001$.

[f] Different from 1% cholesterol control, $p<0.025$.

[g] Different from 1% cholesterol control, $p<0.01$.

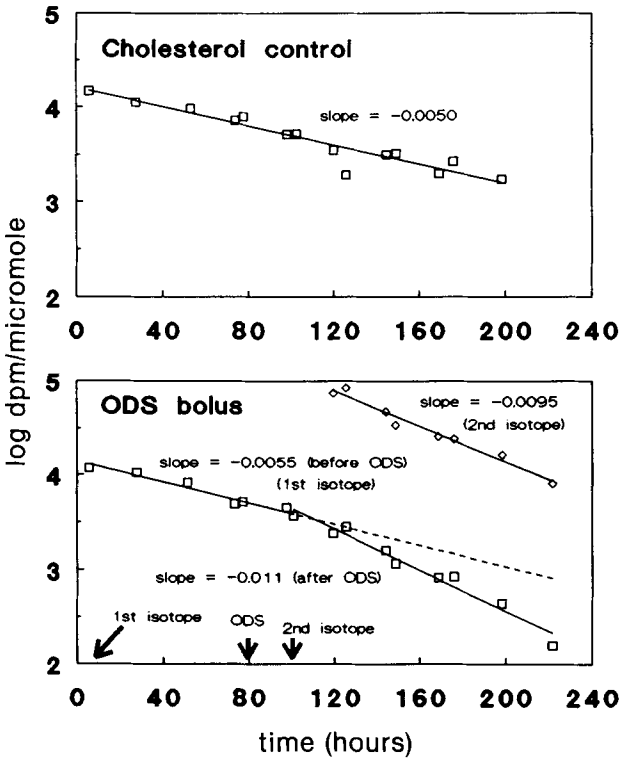


FIG. 1. Typical isotope dilution plot for radiolabeled cholate showing log-linear decay of specific radioactivity before and after treatment with ODS. As indicated by arrows [^{14}C]cholate (1st isotope) was given 1 day after surgery; a single bolus of ODS was given 3 days later; [^{3}H]cholate (second isotope) was given 1 day after ODS. Bile samples were taken at times indicated and analyzed for cholate and radioactivity (double isotope counting) as described in Materials and Methods. Solid line is fitted to data points shown by linear regression analysis. Dotted line is extrapolation of decay before ODS. A total of 7 rats were treated in this way with similar results. Upper panel shows simultaneous control.

pool was in steady state during linear phases of decay. Inasmuch as mean before and after ODS values were virtually identical to those obtained from all cholesterol-fed or ODS treated rats, respectively, isotope dilution and biliary composition data from these studies, in which rats were used as their own controls, are not reported separately.

As shown in Table 4, neither cholate synthesis, determined by isotope dilution, nor total bile acid synthesis, measured by [26-^{14}C]cholesterol oxidation, was significantly affected by either fat feeding or cholesterol feeding. In contrast, ODS, whether given as a bolus or fed, and progesterone increased cholate and bile acid synthesis 64-83%. However, only in the case of ODS bolus was the increase accompanied by a significant increase in C7AH (Table 2).

As shown in Table 3, cholate pool size was not significantly affected by any diet or treatment. Cholesterol feeding did not significantly alter fractional turnover (half-life) of cholate, but did produce a trend toward lower ($p<0.2$) absorption efficiency and pool size. In contrast, ODS, given as a bolus or fed, and progesterone reduced half-life 33% ($p<0.01$). This increased turnover was apparently due to a small but highly significant decline in absorption efficiency (Table 3), *i.e.*, the fraction of radiolabel from cholate reabsorbed on each passage through the enterohepatic circulation.

Biliary cholesterol secretion. As shown in Figure 2, biliary cholesterol output was not significantly changed by fat or cholesterol feeding. Following ODS bolus, which did not deplete liver cholesteryl ester stores (Table 1), biliary cholesterol exhibited a significant 41% increase ($p<0.025$). In contrast, ODS feeding and progesterone, which depleted cholesteryl ester stores, increased output 7% and 35%, respectively, but neither increase was statistically significant. Output was highly variable in individual rats over four days of collection but was not correlated with time after initiation of treatments (data not shown). Whereas bile flow rate was not changed significantly by any treatment (1.85 ± 0.10 mL/hr for cholesterol-fed,

TABLE 3

Parameters of Cholic Acid Turnover as a Function of Fat and Cholesterol Feeding and Treatment with ODS or Progesterone[a]

Diet/treatment (N)	Half-life	Absorption efficiency	Cholate pool
	(days)	(%)	(μmol/100 g)
Chow (6)	2.8 ± 0.2	95.8 ± 0.5	86 ± 6
10% fat (9)	2.8 ± 0.5	95.9 ± 0.3	87 ± 12
10% fat, 1% cholesterol (16)	2.4 ± 0.2	95.2 ± 0.4	72 ± 8
10% fat, 1% cholesterol, ODS bolus (11)	1.6 ± 0.2[b]	92.9 ± 0.9[c]	88 ± 23
10% fat, 1% cholesterol, 0.01% ODS-fed (7)	1.6 ± 0.2[b]	92.3 ± 0.9[d]	88 ± 11
10% fat, 1% cholesterol, progesterone bolus (9)	1.6 ± 0.2[d]	91.6 ± 2.0	82 ± 16

[a]Rats were fed indicated diets for 10 days, prior to cannulation of bile duct. Treatments were begun 48 hr after surgery and 24 hr after administration of radiolabeled cholate. Bile samples were collected twice daily for 4-5 days and turnover parameters were determined by isotope dilution as described in Materials and Methods. Values are mean ± SEM for individual rats.

[b]Different from cholesterol control, $p<0.005$.

[c]Different from cholesterol control, $p<0.05$.

[d]Different from cholesterol control, $p<0.01$.

TABLE 4

Effects of Cholesterol Feeding, ODS and Progesterone on Cholate Synthesis and Total Bile Acid Synthesis (μmol/hr/100 g rat)[a]

Diet/treatment	Cholate	Total bile acid
Chow	0.9 ± 0.1	1.4 ± 0.2
10% fat	0.9 ± 0.1	1.3 ± 0.1
10% fat, 1% cholesterol	0.9 ± 0.1	1.4 ± 0.2
10% fat, 1% cholesterol, ODS bolus	1.5 ± 0.2 ($p<0.05$)	2.6 ± 0.2 ($p<0.001$)
10% fat, 1% cholesterol, 0.01% ODS-fed	1.6 ± 0.2 ($p<0.005$)	2.1 ± 0.2 ($p<0.025$)
10% fat, 1% cholesterol, progesterone bolus	1.7 ± 0.4 ($p<0.1$)	2.2 ± 0.3 ($p<0.025$)

[a]Diets and treatments are as described in Table 1. Cholate synthesis was determined by isotope dilution and total bile acid synthesis, by [26-^{14}C]cholesterol oxidation, measured 2-3 times daily for 4-5 days after initiation of treatment, as described in Materials and Methods. Values are mean ± SEM of average outputs from individual rats (N = 6-16 rats). P values are given for significance of differences between treated and cholesterol-fed.

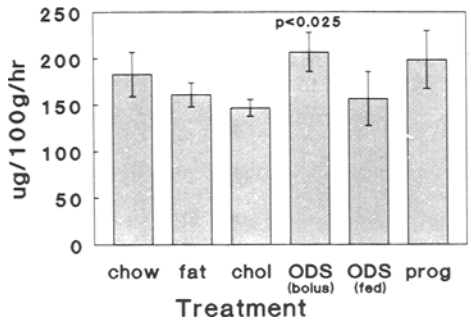


FIG. 2. Effects of cholesterol feeding, ODS and progesterone on biliary cholesterol output. Diets and treatments are as described in Figure 1. Cholesterol was measured in bile samples collected twice daily for 4-5 days after initiation of treatment. Values are mean ± SEM of average output from individual rats (N = 7-15 rats). P value is given for significance of difference between ODS bolus and cholesterol-fed.

1.88 ± 0.13, for ODS bolus), biliary cholesterol concentration paralleled output.

Biliary bile acid output. As shown in Table 5, decline in biliary cholate output and increase in output of chenodeoxycholate and its metabolites (12-deoxy bile acids) with fat and cholesterol feeding resulted in a highly significant increase in the ratio 12-deoxy bile acids/cholate ($p<0.005$). ODS bolus further increased 12-deoxy bile acids to a value 67% higher than chow controls ($p<0.01$) and 17% higher than cholesterol-fed. The ratio also increased still further but not significantly. In contrast, ODS feeding and progesterone, which depleted cholesteryl ester stores (Table 1), essentially reversed the effects of cholesterol loading, increasing cholate and decreasing 12-deoxy bile acids, with a resultant decrease in ratio ($p<0.005$). No significant differences occurred in total bile acid output (Table 5) or concentration among any of the groups, consistent with decreased absorption efficiency.

DISCUSSION

Previous studies have suggested that hepatic free cholesterol levels, which are tightly regulated over a wide range of dietary influx (18-22,37), may play a role in regulation of bile acid synthesis (5-7) and biliary cholesterol secretion (16,17,34,35). We have examined effects of cholesterol

TABLE 5

Effects of Cholesterol Feeding, ODS and Progesterone on Biliary Bile Acid Output (μg/hr/100 g rat)[a]

Group/treatment	Cholate	12-Deoxy bile acids	Total bile acids	12-Deoxy bile acids/cholate
Chow	23 ± 3	20 ± 3	55 ± 8	0.8 ± 0.06
10% fat	23 ± 2	23 ± 2	55 ± 4	1.0 ± 0.07
10% fat, 1% cholesterol	20 ± 2	28 ± 2[b]	57 ± 4	1.6 ± 0.13[c]
10% fat, 1% cholesterol, ODS bolus	21 ± 2	33 ± 3[d]	65 ± 4	1.7 ± 0.20
10% fat, 1% cholesterol, 0.01% ODS-fed	22 ± 1	24 ± 2[e]	54 ± 4	1.1 ± 0.06[e]
10% fat, 1% cholesterol, progesterone bolus	26 ± 2[f]	25 ± 2[g]	60 ± 4	1.0 ± 0.10[h]

[a]Diets and treatments are as described in Figure 1. Taurine and glycine conjugates of biliary bile acids were measured in bile samples collected twice daily for 4-5 days after initiation of treatment. 12-Deoxy bile acids include conjugates of chenodeoxycholate and its metabolites. Values are mean ± SEM of averages from individual rats (N = 7-16 rats).

[b]Different from Chow value, $p<0.05$.

[c]Different from fat-fed value, $p<0.005$.

[d]Different from Chow value, $p<0.05$.

[e]Different from ODS bolus, $p<0.025$.

[f]Different from cholesterol-fed, $p<0.025$.

[g]Different from ODS bolus, $p<0.05$.

[h]Different from ODS bolus, $p<0.05$.

feeding, ODS and progesterone, all of which are known to perturb cholesterol homeostasis and elevate free cholesterol levels in liver (16,17,37), on key enzymes of cholesterol metabolism (CEH, ACAT, HMGCR and C7AH), biliary cholesterol, and bile acid synthesis and turnover. Earlier studies by us suggested that stimulation of bile acid turnover by ODS, though seen without cholesterol feeding, is enhanced in cholesterol-fed rats and that any effects of ODS or progesterone which are mediated by mobilization of cholesteryl ester should be enhanced by cholesterol loading (16,37,52). Thus, cholesterol was added to diets of all treated groups.

Role of free cholesterol in bile acid synthesis and cholesterol homeostasis. Cholesterol feeding, ODS and progesterone produced substantial changes in bile acid synthesis, cholate turnover, biliary cholesterol secretion, bile acid composition, and key regulatory enzymes of cholesterol homeostasis. However, none of these effects correlated well with the measured hepatic pool of free cholesterol. In fact, elevations of free cholesterol were modest and statistically significant only with cholesterol-feeding and ODS-bolus (Table 1). Thus, it is unlikely that the observed effects can be ascribed to increases in free cholesterol unless those increases are highly localized. In fact, metabolically non-equivalent intracellular pools of free cholesterol can be demonstrated under appropriate experimental conditions (16,33-35,53). In rat, one such pool appears to be in rapid equilibrium with cholesteryl esters (16), which, in the current study, varied 20-fold among the various groups and were virtually depleted by ODS-feeding and progesterone (Table 1). It follows that the size of this free cholesterol pool would be affected by relative rates of esterification/hydrolysis and limited by the supply of cholesteryl esters.

With cholesterol feeding, metabolic compensatory responses are apparent in suppressed CEH, suppressed HMGCR and activated ACAT (Table 2). Accompanying this metabolic state are accumulation of cholesteryl esters and a substantial increase in 12-deoxy bile acids/cholate (Table 5). There is considerable evidence for existence of multiple pathways for bile acid synthesis, drawing on different precursor pools (53-55). The current data might be seen as evidence that newly absorbed cholesterol comprises a pool that is preferentially metabolized to chenodeoxycholate rather than cholate. However, results of ODS and progesterone treatment suggest a somewhat more complex interpretation.

Administration of a single ODS bolus abolished the compensatory response to cholesterol feeding, stimulating CEH and HMGCR and suppressing ACAT (Table 2), despite a further increase in free cholesterol. C7AH activity increased, but not in proportion to increases in bile acid synthesis (Table 4). Increases in biliary cholesterol secretion (Fig. 2) and bile acid synthesis in absence of a normal compensatory response to increased free cholesterol or commensurate increase in C7AH suggest augmentation of specific precursor pools, which do not induce strong compensatory responses, perhaps free cholesterol released locally from cholesteryl ester. Whereas 12-deoxy bile acids/cholate (Table 5) and total bile acid synthesis, but not cholate synthesis, are highest with the ODS bolus, a free cholesterol pool, already enlarged by cholesterol feeding and providing substrate preferentially for chenodeoxycholate synthesis, may be further increased. This view is supported by Klauda *et al.* (37), who found that ODS elevated chenodeoxycholate/cholate in non-cholesterol-fed rats.

With ODS feeding, a similar pattern of reversal of effects of cholesterol feeding was seen, with respect to three of the enzymes, CEH, ACAT and HMGCR. Bile acid synthesis was also substantially elevated, but in absence of any elevation of C7AH. Although hepatic free cholesterol remained elevated, it was not significantly higher than cholesterol-fed controls, despite enzyme levels favoring elevation. This was probably due to depletion of cholesteryl ester stores (Table 1). With depletion of cholesteryl ester, biliary cholesterol reached a minimum (Fig. 2) and 12-deoxy bile acids/cholate dropped to levels of fat-fed

controls (Table 5), despite elevated HMGCR (Table 2) and free cholesterol (Table 1). This suggests that free cholesterol derived from hydrolysis of cholesteryl ester may be preferentially used for chenodeoxycholate synthesis and biliary cholesterol. Stone *et al.* (16) have proposed such a precursor pool for biliary cholesterol, on the basis of short-term effects of progesterone on microsomal cholesterol and biliary cholesterol secretion. This view was also supported by current results with progesterone.

Like ODS feeding, progesterone abolished the compensatory responses of CEH, HMGCR and ACAT to cholesterol feeding, although free cholesterol levels were the same as cholesterol-fed. Bile acid synthesis was elevated by progesterone, without elevated C7AH. Biliary cholesterol output was not significantly different from that of any other group and 12-deoxy-bile acids/cholate was significantly lower than that of cholesterol-fed or ODS-bolus. Here, too, the cholesteryl ester pool was greatly reduced, limiting any free cholesterol pool dependent on hydrolysis for maintenance. Inasmuch as cholesterol synthesis is not impaired and cholesterol dietary intake should not be impaired by a single progesterone bolus, these results also suggest that precursor pools for chenodeoxycholate and biliary cholesterol secretion derive preferentially from hydrolysis of cholesteryl ester.

Implications for regulation of bile acid synthesis. As microsomal C7AH did not correlate with *in vivo* bile acid synthesis, these studies lend support for the view that the free cholesterol substrate pool may limit rates of bile acid synthesis (4–7). It is apparent that the conditions of this study prevented the increase in bile acid synthesis normally seen with cholesterol feeding (8–10). This is perhaps due to limitation of a critical substrate or regulatory free cholesterol pool by an exaggerated compensatory response, of one or more of the key regulatory enzymes, to cholesterol feeding (Table 2). In this regard, the 10% vehicle fat added to all test diets elevated ACAT 56% over Chow control (Table 2), demonstrating a significant effect on the regulatory environment and calling for further study of the role of dietary fat in regulation of bile acid synthesis. Supporting this view is the recent report from this laboratory showing induction of C7AH in cholesterol-fed rats on low-fat diets (2). Even in the absence of increased bile acid synthesis, compensatory responses of CEH, HMGCR and ACAT to cholesterol feeding persisted (Table 2). Moreover, the increased ratio, 12-deoxy bile acids/cholate, reported by other investigators (21,56), also persisted (Table 5). Thus, several of the effects associated with cholesterol feeding were apparently uncoupled from increased bile acid synthesis. However, cholesterol-fed rats showed a trend ($p<0.2$) toward lower cholate absorption efficiency (Table 3), which, upon longer-term feeding, might have led to a measurable compensatory increase in synthesis.

Although bile acid synthesis did not correlate well with free cholesterol levels in this study, it did increase with metabolic conditions favoring increases in free cholesterol levels, *i.e.*, elevated CEH and HMGCR, and suppressed ACAT. Thus, reversal by ODS and progesterone of the normal homeostatic compensatory responses to increased free cholesterol may result in augmentation of a precursor or regulatory pool which is more accessible to C7AH than the larger intracellular free cholesterol pool.

Elevated output of 12-deoxy bile acids was seen with elevated cholesteryl ester (cholesterol-fed, ODS bolus) but not with depleted cholesteryl ester stores (ODS-fed, progesterone) (Table 5). Moreover, cholate synthesis accounted for essentially all increase in bile acid synthesis in treated groups with depleted cholesteryl ester, suggesting that chenodeoxycholate synthesis may be limited by cholesteryl ester stores.

Implications for mode of action of ODS. Earlier studies on mode of action of ODS and its analogs suggested that these compounds lower serum and liver cholesterol in hypercholesterolemic rats by inhibiting intestinal cholesterol absorption but not bile acid absorption (37,52). Although the mechanism for inhibition of cholesterol absorption was unclear, an analog increased CEH activity in intestinal mucosa. Whereas synthesis of cholesteryl ester is an essential step in cholesterol transport, *i.e.*, chylomicron formation, this enhanced hydrolysis may be responsible for decreased absorption. Hypocholesterolemic effect persisted when the analog was administered intravenously or remained in the liver for as long as three weeks after termination of treatment. Moreover, these compounds increased cholate and chenodeoxycholate turnover and [26-^{14}C]cholesterol oxidation, *in vivo*. Coinciding with these effects was a 2–3 fold increase in hepatic neutral CEH and both serum and liver free cholesterol. Thus, activation of CEH may be a common mechanism for metabolic effects in liver and absorption effects in intestinal mucosa.

The current studies lend support to a parenteral mode of action for ODS, inasmuch as action persisted for several days after administration of a bolus, and effects on bile acid turnover were delayed for at least 24 hr (Fig. 1). Moreover, effects of ODS on compensatory responses by liver enzymes to cholesterol feeding were not appreciably different between ODS-bolus and ODS-fed. Differences in biliary cholesterol secretion and bile acid composition with ODS as well as progesterone seem to have been related to size of the cholesteryl ester storage pool as well as influx of dietary cholesterol. Thus, it seems likely that the mode of action of ODS is related to a shift in equilibration between hepatic free cholesterol and cholesteryl ester and perhaps augmentation of precursor pools for biliary cholesterol and bile acid synthesis. However, the pattern of changes in enzyme activity clearly suggests disruption of compensatory responses to influx of dietary cholesterol, which include decreased HMGCR and CEH and increased ACAT. It is difficult to suggest a mechanism for this disruption, which seems to persist, even with elevated levels of free cholesterol, and, apparently, with or without impairment of intestinal absorption. However, Pandak *et al.* (57,58) have reported decreased CEH, HMGCR and C7AH and increased ACAT in response to mevinolin or AY9944, both inhibitors of cholesterol synthesis. Both these and the current studies suggest coordinate regulation of these enzymes, which has been confounded to produce paradoxical patterns of regulation under the respective experimental conditions.

A somewhat unexpected outcome of this study is the similarity in detail between effects of ODS and progesterone on bile acid synthesis and turnover and cholesterol homeostasis. As already noted, earlier studies (52) suggested that inhibition of cholesterol absorption plays a prominent role in the hypocholesterolemic effect of ODS, whereas effects of progesterone are thought to

be mediated at the transcriptional level. However, in view of progesterone's inhibitory effect on ACAT and stimulatory effect on CEH, seen by us and others (16,59), ODS and progesterone may share a common mode of action, based on a shift in steady state equilibration point between free cholesterol and cholesteryl ester, to which other effects could also be ascribed. Stone *et al.* (16) have suggested such a mode of action for progesterone. Although their primary mechanisms of action need not be the same, interaction of ODS with steroid receptors is not out of the question, especially in view of the substantial metabolic effects of ODS. While highly speculative, such a mechanism of action might play a role in disruption of compensatory response to cholesterol feeding, which is apparently mediated by a steroid repressor (60,61).

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Effect of Dietary Fat on Rat Liver Microsomal and Mitochondrial/Lysosomal Dolichol, Phospholipid and Cholesterol

Åsa Jakobsson-Borin[*,a] **Örjan Tollbom**[b] and **Gustav Dallner**[a,c]

[a]Department of Biochemistry, Arrhenius Laboratories, University of Stockholm, S-106 91 Stockholm, [b]Department of Cell Biology, Faculty of Health Sciences, University of Linköping, S-581 85 Linköping and [c]Division for Basic Research in Dementia, Clinical Research Center at Huddinge Hospital, Karolinska Institutet, S-141 86 Huddinge, Sweden

The influence of different fat diets on liver phospholipid, cholesterol and dolichol was studied. Rats were separated into four groups and fed standard laboratory chow (control), a diet containing linolenic acid, a coconut oil diet, or a corn oil-containing diet. After five weeks, microsomes and mitochondrial/lysosomal fractions were prepared from the liver, and lipid compositions were analyzed. No changes in phospholipid content were observed. In control animals, the fatty acid compositions of phosphatidylcholine and phosphatidylethanolamine in the two subfractions were similar. However, these two phospholipids showed different fatty acid patterns, which were altered independently upon dietary treatment. The dietary treatments resulted, in most cases, in decreased cholesterol and dolichol contents and, especially in microsomes, in a decreased level of esterification of both lipids. The fatty acid compositions of cholesteryl esters in the two subfractions showed significant differences and cholesterol was esterified to a large extent with linolenic acid when this fatty acid was supplied in the diet. The same dietary treatment exerted different effects on the cholesterol localized in the two different intracellular compartments. This difference was most pronounced in rats fed the corn oil-containing diet; microsomal cholesteryl esters exhibited increased saturation, whereas cholesteryl esters in the mitochondrial/lysosomal fraction displayed decreased saturation. Dolichyl esters in the two cellular compartments had different fatty acyl compositions, with a considerably higher degree of saturation in microsomes. The various diets influenced the nature of the fatty acid moieties present in the isolated fractions and the effects on the two subfractions were opposite. The diet containing linolenic acid decreased the degree of saturation in microsomal dolichyl esters and increased the degree of saturation in the mitochondrial/lysosomal fraction. The results demonstrate that the fatty acid compositions of both dolichyl and cholesteryl esters display organelle specificity. Both the content of these lipids and their fatty acid compositions are greatly influenced by dietary conditions, and the esterification processes at different cellular locations exhibit independent regulation, regardless of the fatty acid content of the diet.

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The lipid compositions of biological membranes are quite stable and dietary or experimental pathological conditions are usually thought to cause only limited changes in membrane phospholipid content and composition. The effect of phospholipids on cellular properties, including membrane architecture, fluidity, stability and enzyme activation, explains why large changes in composition are not compatible with cell survival (1). However, a number of investigations in recent years have shown that the fatty acid compositions of phospholipids can be manipulated by dietary treatment. Most of the *in vivo* studies examined either the liver or the brain of the rat (2–7). In these experiments the effect of standard diet was compared with fat-free diets and diets containing lard or various types of oils.

The effects described for total or individual phospholipids and for homogenates or isolated subfractions vary somewhat, in part because of differences in the experimental conditions employed. The modifications described are quite moderate and, depending on the type of diet employed, 5–10% increases or decreases in certain monosaturated or polyunsaturated fatty acids (FA) are observed. In most experiments the relative amount of saturated FA did not change significantly. In those experiments where larger changes in the fatty acid pattern were observed after only a short dietary regime, the FA composition of the total lipid extract was analyzed (8,9). These changes are likely to be present primarily in triglycerides and not in phospholipids.

Modifications in the FA compositions of phospholipids need not be large to result in changes in membrane and enzymatic functions. Furthermore, these changes may influence the structure, composition and amount of components other than phospholipids. Such modifications have important effects on protein configurations and distributions, thereby influencing a number of membrane-associated functions.

In the present experiments young developing rats were subjected to dietary treatment in an attempt to change the phospholipid compositions of major liver organelles. The aim was to determine to what extent these modifications are accompanied by changes in cholesterol and dolichol contents, as well as in the extent of esterification of these two lipids. In addition, the possible influence of dietary conditions on the fatty acid compositions of cholesteryl and dolichyl esters was determined.

MATERIALS AND METHODS

Chemicals and diet. Ergosterol was purchased from Steraloids Inc. (Wilton, NH). Dolichol-23 was isolated from the bovine pituitary gland (10) and the cholesteryl ester standard was synthesized using [^{14}C]cholestereol (Radiochemical Centre, Amersham, England) and hepta-

*To whom correspondence should be addressed.

Abbreviations: CM, chloroform/methanol; CMW, chloroform/methanol/water; FA, fatty acid; FFA, free fattty acid; GC, gas chromatography; HDL, high-density lipoprotein; HPLC, high-performance liquid chromatography; LDL, low-density lipoprotein; PC, phosphatidylcholine; PE, phosphatidylethanolamine; TLC, thin-layer chromatography.

decanoyl chloride (11) (Sigma, St. Louis, MO). Borontrifluoride (in methanol) and linolenic acid were purchased from Merck (Darmstadt, Germany) and Kodak (Rochester, NY), respectively. The "fat-free" test diet was purchased from U.S. Biochemical Corporation (Cleveland, OH).

Animals. Male Sprague-Dawley rats weighing 60 g at the start of the experiment were used. Control rats received the basal diet consisting of 24% protein, 49% carbohydrate and 5.4% total fat, including 0.2% cholesterol. For the preparation of specific diets, a "fat-free" test diet was mixed either with 20% linolenic acid, 20% coconut oil or 20% corn oil by weight and then formed into pellets. The fat-free diet consisted of 17% cellufil, 59% sucrose, 21% casein, 4% salt mixture and vitamins. Rats received the diets *ad libitum* for five weeks.

At the end of the dietary period, the rats were decapitated and their livers homogenized. The microsomal and mitochondrial/lysosomal fractions were prepared as described earlier (12). The mitochondrial fraction isolated by differential centrifugation contains the majority of the lysosomes (13). The combined mitochondrial/lysosomal fraction is suitable for assaying lysosomal polyprenol content, because polyisoprenols are present only in very low concentrations in mitochondria. Protein was measured using the Biuret procedure with bovine serum albumin as the standard (14).

Lipid extraction and separation. For extraction of lipids, one mL of the isolated subfractions or 1 mL of dietary chow dissolved in 0.25 M sucrose was mixed with 21 mL chloroform/methanol (CM, 14:7, v/v). After 30 min at 37°C, the solvent was removed and the proteins reextracted with 9 mL CM (2:1, v/v). All solvent mixtures contained 0.15% butylated hydroxytoluene as antioxidant. The two extraction mixtures were combined and filtered, and the internal standards ergosterol, [^{14}C]cholesteryl heptadecanoate and dolichol-23 were added. The extract was partitioned by the addition of 6.5 mL of water, and the chloroform phase was washed twice with theoretical upper phase chloroform/methanol/water (CMW, 3:48:47, v/v/v). After evaporation of the solvent under N_2, the lipids were dissolved in 4 mL chloroform and placed on an aminopropyl column (Supelco, Bellefonte, PA). Neutral lipids were removed with 4 mL chloroform/2-propanol (2:1, v/v) and the free fatty acids were eluted with 4 mL 2% acetic acid in diethyl ether. Phospholipids were eluted with 8 mL methanol.

The mixture containing neutral lipids was evaporated and the lipids were dissolved in 100 μL chloroform. Methanol (4 mL) was added and the mixture was placed onto a C-18 reversed phase column (Supelco). Cholesterol and cholesteryl esters were eluted with 21 mL methanol and dolichol and dolichyl esters were removed with 8 mL n-hexane. The methanol eluate was evaporated and the lipids were dissolved in n-hexane and transferred to a silica gel column [Silica gel 60 (Merck), packed in a Pasteur pipette]. Cholesteryl esters were eluted with 8 mL 2% diethyl ether in n-hexane, followed by the elution of cholesterol with 8 mL chloroform.

The mixture containing free FA was evaporated under N_2. The residue was dissolved in CM (2:1, v/v) and placed onto silica thin-layer chromatography (TLC) plates (Merck), which were then developed with petroleum ether/diethyl ether/acetic acid (90:10:2, v/v/v). After visualization with Rhodamine 6G, the fatty acid-containing spot was scraped off and extracted with CM (2:1, v/v) for gas chromatographic (GC) analysis.

Phospholipids were resolved using silica thin-layer chromatographic (TLC) plates with a concentrating zone (Merck). The phospholipids were eluted first with chloroform/diethyl ether/acetic acid (70:30:2, v/v/v) and thereafter with chloroform/methanol/H_2O (70:30:4, v/v/v) in the same direction. The lipid spots containing phosphatidylcholine (PC) and phosphatidylethanolamine (PE) were visualized with Rhodamine 6G and removed for the determination of phosphorus (15). A schematic representation of the course of lipid separation is presented in Figure 1.

High-performance liquid chromatography (HPLC). HP Hypersil ODS (3 μm) was the HPLC column used for reversed phase separation. For the isolation and quantitation of dolichol and dolichyl esters, a convex gradient was applied from the initial methanol/2-propanol/water (60:40:5, v/v/v) in pump system A to 60% *n*-hexane/2-propanol (70:30, v/v) in pump system B at a flow rate of 2 mL per min with a program time of 20 min. The absorbance of the eluate at 210 nm was monitored. In this system the individual dolichols were eluted first, followed by the dolichyl esters. Since dolichol is present as a series of polyisoprenes with different UV spectra, the total amount of dolichol was calculated as described earlier (16).

Cholesterol was separated using a linear gradient with methanol/water (9:1, v/v) in pump system A to methanol/2-propanol (4:1, v/v) in pump system B at a flow rate of 1.5 mL per min. The isolated cholesteryl ester fractin was hydrolyzed in 0.8 mL tetrahydrofuran with 0.2 mL 0.66 M KOH in methanol, and cholesterol was quantitated by HPLC.

Gas chromatography. Phospholipids, dolichyl esters,

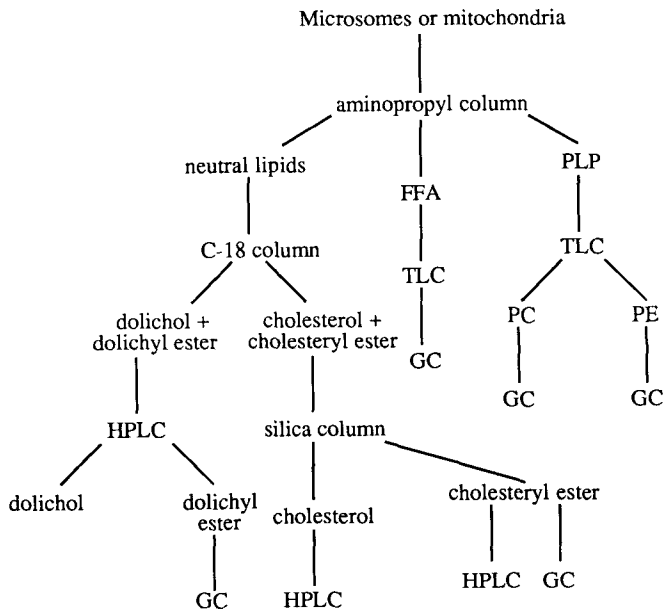


FIG. 1. Schematic illustration of the course of lipid separation and quantitation.

TABLE 1

Fatty Acid Compositions of the Different Dietary Components[a]

FA	Control diet	Linolenic acid	Coconut oil	Corn oil
	(% of total)			
14:0	1.7	0.1	48.3	0.1
16:0	21.3	5.2	23.9	10.9
18:0	4.1	2.3	6.8	
18:1	16.0	22.3	16.6	31.3
18:2	46.2	17.2	4.4	56.6
18:3	5.9	52.9		0.7
20:0	2.2			0.3
22:6	2.6			0.1
Total				
Saturated	27.1	7.6	79.0	11.0
Monounsaturated	16.0	22.3	16.6	31.3
Polyunsaturated	56.8	70.1	4.4	57.7

[a]For determination of the FA compositions, the control laboratory chow, linolenic acid mixture, coconut oil and corn oil samples were extracted and the fatty acid methyl esters were quantitated using GC.

free fatty acids and cholesteryl esters were dissolved in a borontrifluoride/methanol mixture for hydrolysis and methylation. The tubes were sealed under N_2 and kept in a boiling water bath for 15–45 min. The methylated fatty acids were extracted three times with *n*-hexane/diethyl ether (1:1, v/v). The analysis was performed by capillary gas chromatography using cyanosiloxane 60 as the stationary phase (17).

RESULTS

Diet. Lipids in the standard laboratory chow (control diet) were extracted and the total fatty acid composition was determined (Table 1). The control diet contained a relatively low level of saturated fatty acids, almost 50% 18:2 and low levels of other polyunsaturated fatty acids. Linolenic acid, a commercial product, consisted of 53% 18:3 and 47% 18:1 and 18:2. Half the fatty acids in coconut oil were myristic acid and the other two major components were 16:0 and 18:1. Only about 11% of the FA in corn oil were saturated; the remainder consisted of 18:1 and 18:2.

Phospholipids. Lipids were analyzed in the isolated microsomes and mitochondrial/lysosomal fractions. The various diets did not affect the protein content of these fractions (not shown in Table). The two major phospholipids in the isolated subfractions were PC and PE. The total and relative amounts of these phospholipids did not change under the various dietary conditions.

The distributions of saturated, monounsaturated and polyunsaturated fatty acids in microsomal PC and PE were similar, but substantial differences in the detailed compositions of these lipids were observed (Table 2). The ratio of 16:0/18:0 was approximately 0.9 in microsomal PC, while the corresponding value for PE was 0.5. In PE, the 18:2 content was less than in PC, whereas the content of 22:6 was twice as much as that of PC. When 18:3 was supplied in the diet, the degree of saturation increased as well as the relative amounts of 18:1 and 18:2. These increases were compensated for by a 53% reduction in the content of arachidonic acid. Coconut oil in the diet led to increased levels of saturated and monounsaturated FA and to lowered amounts of polyunsaturated FA. The corn oil-containing diet resulted in a moderate decrease in 16:0 and a 25% elevation in the arachidonic acid level. The diet containing 18:3 increased the levels of 18:0 and 22:6 and decreased that of 20:4 in microsomal PE. In rats given the coconut oil diet, the levels of saturated FA were elevated, while those of 18:2 and 22:6 decreased.

The fatty acid compositions of PC and PE in the mitochondrial/lysosomal fraction were very similar to those in their microsomal counterparts (Table 3). Consequently, substantial differences between these two lipids in the mitochondria/lysosomes were also present. Changes similar to those which occurred in microsomal PC and PE were observed in the PC and PE of mitochondria/lysosomes after various dietary treatments.

Cholesterol. Microsomal cholesterol was found primarily in free form, with only about 9% being esterified with a fatty acid (Table 4). Significant decreases in the content

TABLE 2

Fatty Acid Compositions of Microsomal PC and PE[a]

	PC				PE			
FA	Control	Linolenic acid	Coconut oil	Corn oil	Control	Linolenic acid	Coconut oil	Corn oil
	(percent of total)							
16:0	20.6 ± 0.9	27.2 ± 1.1	23.6 ± 0.8	17.7 ± 2.7	14.5 ± 0.7	15.8 ± 0.8	17.7 ± 0.5	15 ± 0.5
16:1	0.6 ± 0.1	0.6 ± 0.1	1.5 ± 0.1	0.5 ± 0.1	0.2 ± 0.1	0.2 ± 0.02	0.7 ± 0.2	0.4 ± 0.1
18:0	23.3 ± 1.2	21.4 ± 1.7	26.8 ± 1.7	23.2 ± 1.0	28.4 ± 0.6	35.4 ± 1.0	32.1 ± 0.8	28.6 ± 0.3
18:1	5.8 ± 0.1	9.1 ± 0.2	12.8 ± 0.9	5.6 ± 0.5	4.0 ± 0.2	4.6 ± 0.3	5.3 ± 0.6	4.4 ± 0.5
18:2	15.2 ± 0.6	20.3 ± 0.7	9.1 ± 1.8	12.5 ± 0.1	10.1 ± 0.7	9.8 ± 0.8	3.5 ± 1.0	7.0 ± 0.12
18:3	0.1 ± 0.0	1.1 ± 0.2	0.2 ± 003		0.1 ± 0.1	0.8 ± 0.2	0.05 ± 0.07	
20:4	26.4 ± 0.9	12.3 ± 0.5	20.5 ± 2.6	32.9 ± 1.5	25.8 ± 0.9	11.8 ± 0.3	27.0 ± 1.8	28.7 ± 0.1
22:6	8.0 ± 0.6	8.0 ± 0.6	5.5 ± 0.6	7.6 ± 0.8	16.9 ± 1.1	21.6 ± 0.6	13.7 ± 1.1	15.8 ± 0.5
Total								
Saturated	43.9	48.7	50.4	40.9	42.9	51.2	49.9	43.6
Monounsaturated	6.4	9.7	14.3	6.1	4.2	4.8	6.0	4.8
Polyunsaturated	49.7	41.6	35.3	53.0	52.9	44.0	44.2	51.6

[a]PC and PE were isolated from the microsomal lipid extract by TLC and their fatty acid contents were determined by GC. The values are the means ± S.D. for seven animals.

TABLE 3

Fatty Acid Compositions of Mitochondrial/Lysosomal PC and PE[a]

FA	PC				PE			
	Control	Linolenic acid	Coconut oil	Corn oil	Control	Linolenic acid	Coconut oil	Corn oil
	(percent of total)							
16:0	19.5 ± 2.0	25.4 ± 1.5	19.9 ± 2.2	17.7 ± 2.6	14.0 ± 2.4	12.0 ± 1.2	11.6 ± 1.9	12.9 ± 1.9
16:1	0.5 ± 0.2	1.0 ± 0.2	1.4 ± 0.4	0.4 ± 0.1	0.7 ± 0.4	0.5 ± 0.3	0.6 ± 0.1	0.6 ± 0.2
18:0	25.8 ± 0.9	25.4 ± 3.0	28.8 ± 0.4	25.0 ± 1.4	31.4 ± 3.0	38.7 ± 1.8	36.2 ± 2.1	31.9 ± 1.4
18:1	6.5 ± 0.5	10.2 ± 0.3	14.4 ± 0.8	6.0 ± 1.0	3.6 ± 1.1	3.9 ± 0.4	4.5 ± 0.7	3.3 ± 0.4
18:2	13.4 ± 0.2	17.9 ± 0.3	7.3 ± 1.7	11.9 ± 0.8	7.0 ± 0.7	8.3 ± 0.9	2.3 ± 0.7	5.0 ± 0.4
18:3		1.0 ± 0.2				0.5 ± 0.1		
20:4	25.7 ± 2.0	10.8 ± 0.7	21.9 ± 3.8	31.7 ± 1.6	25.4 ± 1.3	12.0 ± 0.6	29.7 ± 1.1	29.6 ± 1.4
22:6	8.6 ± 0.5	8.2 ± 1.3	6.8 ± 0.8	7.2 ± 0.7	17.9 ± 1.0	24.1 ± 2.1	15.1 ± 1.4	16.7 ± 1.6
Total								
Saturated	45.3	50.8	48.7	42.7	45.4	50.7	47.8	44.8
Monounsaturated	7.0	11.2	15.8	6.4	4.3	4.4	5.1	3.9
Polyunsaturated	47.7	37.9	35.3	50.8	50.3	44.9	47.1	51.3

[a]Mitochondrial PC and PE were separated by TLC and their fatty acid compositions were analyzed by GC. The values are the means ± S.D. for seven animals.

TABLE 4

Cholesterol and Cholesteryl Ester Contents in Microsomes and Mitochondria/Lysosomes[a]

Diet	Cholesterol	Cholesteryl FA	% Esterified
	(nmol/mg protein)		
Microsomes			
Control	67.0 ± 3.0	6.3 ± 0.9	9
Linolenic acid	70.6 ± 2.6	8.7 ± 0.6	11
Coconut oil	52.9 ± 3.3	3.1 ± 0.1	6
Corn oil	51.4 ± 2.4	2.1 ± 0.1	4
Mitochondria			
Control	16.5 ± 1.4	1.3 ± 0.4	7
Linolenic acid	17.1 ± 2.4	0.5 ± 0.1	3
Coconut oil	17.7 ± 2.9	1.4 ± 0.2	8
Corn oil	14.8 ± 2.7	0.5 ± 0.1	3

[a]Cholesterol and cholesteryl FA were quantitated as described in Materials and Methods. The values are the means ± S.D. for six animals.

of microsomal cholesterol and in the degree of esterification were found after feeding rats the coconut oil or corn oil diet. When the corn oil-containing diet was fed to rats, the cholesterol content decreased moderately in the mitochondrial/lysosomal fraction; the other two diets increased the cholesterol content moderately. Only a small portion of cholesterol was esterified.

The fatty acid compositions of cholesterol in microsomes and mitochondria/lysosomes were very different (Table 5). While the degree of saturation in microsomes was 25%, this value in mitochondria/lysosomes was 51%, and the relative amount of palmitoleic acid was four times greater. The amount of 18:1 was much less in mitochondria/lysosomes and polyunsaturated FA constituted 13% of the total, whereas the corresponding value for microsomes was 42%. Microsomal cholesterol was, to a high degree, esterified with 18:3 in rats fed the diet containing linolenic acid. Dietary linolenic acid increased the degree of saturation, doubled the relative amount of 16:1, and decreased the content of 18:2 + 20:4 + 22:6 from 41% to 13%. The coconut oil diet elevated both the relative amounts of saturated and monounsaturated FA and decreased the levels of polyunsaturated FA. Surprisingly, the corn oil diet increased the relative amounts of saturated FA and 16:1 in microsomal cholesterol and reduced the level of polyunsaturated FA. When rats were given a diet enriched in 18:3, the content of 16:0 decreased, while the levels of 18:2 and 18:3 increased in mitochondrial/lysosomal cholesteryl esters. This diet also caused a decrease in total saturation and an elevation in total polyunsaturated FA. Larger changes occurred in the microsomal fraction than in the mitochondrial/lysosomal fraction when rats were fed the coconut oil diet. In rats fed the corn oil diet, changes in mitochondrial/lysosomal cholesteryl esters were similar to those which occurred in phospholipids; the levels of saturated FA decreased and those of polyunsaturated FA increased.

Dolichol. The three diets, particularly the one containing 18:3, significantly decreased dolichol and dolichyl ester levels in both the microsomal and mitochondrial/lysosomal fractions (Table 6). Furthermore, the degree of esterification decreased in microsomes of rats fed the 18:3 or the coconut oil diet. These diets, however, did not affect the degree of esterification in the mitochondrial/lysosomal fraction. Dolichyl esters in microsomes from control rats have a FA composition which is quite different from that of the other lipids (Table 7). One-third of the total FA is 18:0 and one-fourth is 16:0. The only polyunsaturated FA present, besides trace amounts of 18:3, is 6% of 18:2. In the mitochondrial/lysosomal dolichyl ester fraction the relative amount of 18:0 is 50% less than in microsomes, and one-third of the total is polyunsaturated FA. Rats fed the 18:3 diet had decreased levels of 18:0 in microsomes and increased levels of 16:0 in the mitochondrial/lysosomal fraction. The relative amounts of both monounsaturated and polyunsaturated FA increased in microsomes, but decreased in mitochondrial/lysosomal dolichyl esters. The total relative levels of saturated and unsaturated FA in microsomes of rats fed the coconut oil diet were not affected, but that of monounsaturated FA doubled in the mitochondrial/lysosomal frac-

TABLE 5

Fatty Acid Compositions of Cholesteryl Esters in Microsomes and Mitochondria[a]

FA	PC				PE			
	Control	Linolenic acid	Coconut oil	Corn oil	Control	Linolenic acid	Coconut oil	Corn oil
	(percent of total)							
16:0	18.2 ± 1.0	20.3 ± 1.3	22.4 ± 2.6	22.0 ± 5.0	34.6 ± 1.2	27.4 ± 1.4	33.2 ± 2.5	28.5 ± 1.6
16:1	3.8 ± 0.7	7.9 ± 1.5	11.4 ± 2.7	9.9 ± 3.2	17.1 ± 1.6	11.2 ± 2.1	11.8 ± 1.6	15.9 ± 2.4
18:0	6.8 ± 0.7	9.3 ± 0.9	16.3 ± 2.1	11.3 ± 1.2	16.9 ± 0.8	15.2 ± 1.9	17.0 ± 1.6	15.7 ± 1.7
18:1	29.5 ± 2.7	31.8 ± 0.4	36.5 ± 4.6	24.3 ± 3.1	18.5 ± 1.0	24.2 ± 1.5	26.1 ± 1.8	19.0 ± 1.8
18:2	25.8 ± 1.3	11.0 ± 0.6	5.8 ± 0.7	21.3 ± 6.1	10.0 ± 2.0	12.2 ± 1.6	6.3 ± 1.9	14.9 ± 2.2
18:3	1.4 ± 1.3	17.3 ± 1.5	2.0 ± 1.5			7.7 ± 0.8		
20:4	10.2 ± 2.5	1.7 ± 0.2	5.6 ± 0.9	11.2 ± 1.6	2.9 ± 0.8	2.1 ± 2.2	5.6 ± 3.0	3.7 ± 3.1
22:6	4.3 ± 1.2	0.7 ± 0.1						2.3 ± 1.5
Total								
Saturated	25.0	29.6	38.7	33.3	51.5	42.6	50.2	44.2
Monounsaturated	33.3	39.7	47.9	34.2	35.6	35.4	37.9	34.9
Polyunsaturated	41.7	30.7	13.4	32.5	12.9	22.0	11.9	20.9

[a]The lipid extracts were subjected to silica gel chromatography and the cholesteryl ester fraction was hydrolyzed and used for the isolation of fatty acids. The values are the means ± S.D. for six animals.

TABLE 6

Dolichol and Dolichyl Ester Contents in Microsomes and Mitochondria/Lysosomes[a]

Diet	Dolichol	Dolichyl FA	% Esterified
	(nmol/mg protein)		
Microsomes			
Control	0.08 ± 0.01	0.07 ± 0.01	47
Linolenic acid	0.04 ± 0.005	0.01 ± 0.003	21
Coconut oil	0.06 ± 0.01	0.03 ± 0.005	33
Corn oil	0.05 ± 0.005	0.04 ± 0.005	46
Mitochondria			
Control	0.24 ± 0.02	0.40 ± 0.04	63
Linolenic acid	0.9 ± 0.01	0.14 ± 0.02	61
Coconut oil	0.13 ± 0.02	0.24 ± 0.02	64
Corn oil	0.14 ± 0.02	0.26 ± 0.02	65

[a]Dolichol and dolichyl esters were isolated by aminopropyl and C-18 column chromatography, followed by quantitation on reversed phase HPLC. The values are the means ± S.D. for six animals.

tion. Dolichol esters in both fractions were only slightly changed in rats fed corn oil, but in microsomes the level of 16:0 increased greatly and the level of 18:0 decreased. The isoprenoid patterns of dolichol and dolichyl esters were similar in microsomes and in the mitochondrial/lysosomal fraction, and no modification in these patterns was observed after the various dietary treatments.

DISCUSSION

Most of the dolichol, and part of the cholesterol, in liver and blood are esterified with a fatty acid. Esterification changes the properties of these lipids considerably, but the exact functional role(s) of the esterified derivative is not known. It is often claimed that association with fatty acids is necessary for storage, but no direct evidence is presently available (18).

A number of data indicate that fatty acids may serve as targeting signals, directing lipids to appropriate cellular locations (17). Uptake of cholesterol from the intestine (19) and secretion of low-density lipoprotein (LDL) to the blood (20) require esterification. In addition, the extensive esterification associated with high-density lipoprotein (HDL) may also regulate the distribution of cholesterol in the blood (21). Most dolichol is esterified in the endoplasmic reticulum by a specific transferase (10), and subsequent hydrolysis of the esters may occur both in lysosomes and plasma membranes (22). A number of experiments have demonstrated that intracellular targeting of dolichol requires esterification.

The type of phospholipid and the FA composition of these lipids are important for both the distribution and for the biosynthesis of cholesterol and dolichol. It has been demonstrated that cholesterol is associated preferentially with PC (23), while dolichol favors PE (24). Increasing concentrations of dolichol and dolichyl esters affect model membranes containing PE with unsaturated FA, causing destabilization, increased fluidity of phospholipid FA, and increased permeability (25,26). The nature of phospholipids and their FA compositions directly regulate enzymes of cholesterol biosynthesis (27,28) and of dolichol biosynthesis (29–31) and thereby regulate the levels of these two lipids. Cholesterol, dolichol and ubiquinone have a common initial biosynthetic pathway terminating with farnesyl-PP. It has been established that these three lipids have a common regulatory step at the level of the hydroxymethylglutaryl-CoA reductase (32), and an increase or decrease in biosynthesis of one of these lipids has well-characterized effects on the biosynthesis of the other two lipids.

A number of studies have shown that dietary changes can significantly influence the fatty acid compositions of phospholipids in the liver, as well as in other tissues (2,5,6). In our study, the effects of four diets (control, 18:3, coconut oil and corn oil) on microsomal and mitochondrial/lysosomal fractions were examined. We have found changes in the FA compositions of phospholipids and of two esterified products of the mevalonate pathway, but the mechanisms for these dietary changes are obviously different. *De novo* synthesis of phospholipids, cholesterol

TABLE 7

Fatty Acid Compositions of Microsomal PC and PE[a]

FA	Microsomes				Mitochondria			
	Control	Linolenic acid	Coconut oil	Corn oil	Control	Linolenic acid	Coconut oil	Corn oil
	(percent of total)							
16:0	22.2 ± 3.7	26.7 ± 3.5	40.4 ± 12.7	37.4 ± 7.2	19.5 ± 4.2	33.4 ± 9.1	26.4 ± 2.7	23.0 ± 3.0
16:1	5.6 ± 5.4	26.9 ± 3.7	16.7 ± 12.1	12.1 ± 7.6	12.1 ± 2.4	9.2 ± 5.3	17.1 ± 3.6	18.6 ± 3.3
18:0	50.8 ± 3.4	26.1 ± 4.6	33.5 ± 6.9	24.8 ± 4.0	24.8 ± 4.0	24.3 ± 8.9	14.8 ± 3.1	15.4 ± 1.6
18:1	15.1 ± 4.1	10.4 ± 3.2	4.5 ± 6.9	17.3 ± 6.9	13.7 ± 2.3	14.6 ± 4.4	21.4 ± 3.0	16.2 ± 2.2
18:2	5.7 ± 1.6	7.0 ± 0.9	4.7 ± 6.5	3.2 ± 2.9	12.8 ± 4.7	11.7 ± 5.4	10.5 ± 1.1	15.2 ± 2.8
18:3	0.6 ± 1.4	0.4 ± 0.8			2.0 ± 3.9	2.6 ± 2.9		
20:4		2.5 ± 2.3		5.2 ± 5.2	13.6 ± 2.6	4.2 ± 2.4	9.8 ± 2.1	11.6 ± 4.0
22:6					1.5 ± 1.8			
Total								
Saturated	73.0	52.8	73.9	62.2	44.3	57.7	41.2	38.4
Monounsaturated	20.7	37.3	21.2	29.4	25.8	23.8	38.5	34.8
Polyunsaturated	6.3	9.9	4.7	8.4	29.9	18.5	20.3	26.8

[a]The total dolichyl ester fractions were isolated by HPLC and used to prepare fatty acid methyl esters for GC. The values are the means ± S.D. for six animals.

and dolichol is localized primarily in the endoplasmic reticulum, from where these lipids are transported both to the mitochondria and lysosomes to serve as membrane components or metabolic substrates (33). Modification of FA moieties in phospholipids can be achieved by *de novo* synthesis of new species, but most of FA are exchanged through transacylation reactions (34,35). No such exchange reactions occur with cholesterol or dolichol in liver, and modification of their FA patterns thus involves exclusively esterification of the free lipid.

The FA compositions of PC and PE in the microsomes and in the mitochondrial/lysosomal fraction were identical. However, these two lipids are modified independently during dietary treatment.

The dietary treatments employed here, particularly with coconut and corn oils, clearly depress the biosynthesis of products of the mevalonate pathway. The independent regulation of cholesterol esterification, in comparison with phospholipids and dolichol, is apparent from its high degree of unsaturation and the uptake of 18:3 from the diet. Furthermore, the degree of polyunsaturation decreased in microsomes of rats fed the coconut oil or corn oil diet, while mitochondrial/lysosomal cholesteryl esters were unchanged or enriched in polyunsaturated FA.

An independent regulation of the esterification process for dolichol is also apparent. Microsomes from control rats possess dolichyl esters with as much as 70% saturation, while the mitochondrial/lysosomal lipids show only about 40% saturation. The level of saturation decreased in microsomes of rats given the 18:3 diet, but increased in the mitochondrial/lysosomal fraction.

Our results show that the metabolic turnover of the fatty acyl moieties of various lipids in hepatocytes are regulated independently. The levels of phospholipids are constant, while those of cholesterol and dolichol are variable. The decreased levels of cholesterol and dolichol indicate inhibition of the mevalonate pathway. Decreased synthesis of these two lipids is probably compensated for, to some extent, by a decreased excretion of cholesterol and dolichol from the cell, thereby maintaining a basic cellular level of these lipids necessary for basic functions. Characteristically, phospholipid fatty acid compositions are species specific, but not organelle specific (36), while cholesterol and dolichol in the different organelles represent different pools which are regulated independently.

Both the properties of the fatty acyl transferases of the different lipids and their regulation are quite different. The large and opposite changes in the distribution of lipid esters in the subfractions suggest that in our experiments, modifications in FA compositions are not caused by an excess or deficiency of specific fatty acids in the substrate pool. The dietary treatments had a direct influence on the enzymes of the biosynthetic pathways.

Intracellular transport of newly synthesized cholesteryl and dolichyl esters occurs rapidly and effectively *via* the cytoplasm, independently of protein transport, and specific protein carriers for this purpose have not been identified (22,37,38). For these reasons, transport mechanisms other than those involving exclusive protein carriers should exist. It has been proposed repeatedly and also is supported by experimental findings that FA participate in the lipid transport process. The observations that the FA compositions of cholesteryl and dolichyl esters are different in various cellular organelles and that the FA present in the diet do not directly determine the FA composition of these two mevalonate pathway lipids favor the proposal that individual FA in the esterified forms function as targeting signals during the movement from the endoplasmic reticulum to specific cellular membranes.

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Formation of Dityrosine and Other Fluorescent Amino Acids by Reaction of Amino Acids with Lipid Hydroperoxides

Kiyomi Kikugawa*, Tetsuta Kato and **Akira Hayasaka**
Tokyo College of Pharmacy, Tokyo 192-03, Japan

Formation of fluorescence by the reaction of various amino acids with lipid hydroperoxides, *i.e.*, linoleic acid 13-monohydroperoxide, methyl linoleate 13-monohydroperoxide and phosphatidylcholine hydroperoxide, in the presence of methemoglobin was investigated. Two types of fluorescence were produced: fluorescent dityrosine (3,3′-dityrosine) from tyrosine, and unidentified fluorophores with α- and ε-amino groups of various amino acids. While the former was stable after treatment with borohydride, the latter fluorophores were readily destroyed. The rate of dityrosine formation was rapid, and the yield of dityrosine was dependent on the concentrations of tyrosine and the lipid hydroperoxides. Butylated hydroxytoluene and tocopherol inhibited the formation of dityrosine, but did not affect the formation of fluorophores on the amino groups. Dityrosine appears to be formed by radical reaction of the lipid hydroperoxides, while the other fluorophores seem to be created by non-radical mechanisms.
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Fluorescent lipofuscin pigments accumulate with age in tissues and cells (1). Pigment formation has been thought to be due to lipid peroxidation, as incubation of tissues or cells under aerobic conditions is known to produce fluorescence (2). Several mechanistic studies on fluorescence formation during lipid peroxidation have been undertaken (3-5). Peroxidation of lipids yields various reactive species including lipid peroxyl radicals, alkoxyl radicals, lipid hydroperoxides, and aldehydes (6). Among the products identified monofunctional aldehydes have been considered as possible candidates that contribute to the development of fluorescence (4,5,7-12). It is known that more reactive lipid peroxyl and alkoxyl radical species cause damage on proteins (13-15), but no evidence of their contribution to the formation of fluorescence has been brought forward.

Lipid hydroperoxides (ROOH) undergo heterolytic or homolytic cleavage to produce peroxyl radicals (ROO•) or alkoxyl radicals (RO•) when they react with metals or metal complexes (16-19). In the present study, we investigated the formation of fluorescence by reaction of various amino acids with lipid hydroperoxides in the presence of methemoglobin. This paper describes two possible reaction pathways to fluorescence formation: a radical pathway which yields fluorescent dityrosine (3,3′-dityrosine) and a non-radical pathway which produces fluorophores by reaction with amino groups of amino acids.

MATERIALS AND METHODS

Materials. Amino acids, L-arginine (Arg), L-histidine (His), L-lysine (Lys) hydrochloride, L-methionine (Met) and L-phenylalanine (Phe) were obtained from Ajinomoto Company, Ltd. (Tokyo, Japan). Glycine (Gly), L-tryptophan (Trp) and L-tyrosine (Tyr) were obtained from Wako Pure Chemical Industries, Ltd. (Osaka, Japan). L-Cysteine (Cys) was the product of Kanto Chemical Company, Ltd. (Tokyo, Japan). Carbobenzoxy (Z)-amino acids, Z-Arg, Z-L-aspartic acid (Z-Asp), Z-L-isoleucine (Z-Ileu), Z-Phe, Z-L-proline (Z-Pro), Z-L-serine (Z-Ser), Z-Trp and Z-L-valine (Z-Val) were obtained from Wako Pure Chemical Industries. Z-His was from Sigma Chemical Company (St. Louis, MO). Nα-Z-Lys was from Fluka Chemie AG, Buchs, Switzerland. Z-Tyr was from Peptide Institute, Inc. (Osaka, Japan).

Commercial reagent-grade hydrogen peroxide (31% in water) used was estimated to be 9.11 M by iodometric titration. dl-α-Tocopherol was a product of Eisai Company, Ltd. (Tokyo, Japan). Butylated hydroxytoluene (BHT) was obtained from Nikki Universal Company (Tokyo, Japan). Horseradish peroxidase (HRP) [EC 1.11.1.7], methemoglobin (MetHb) from human, and superoxide dismutase Type I from bovine blood [EC 1.15.1.1] were obtained from Sigma. The concentration of MetHb was determined by use of a molecular extinction coefficient of MetHb subunit at 630 nm, 4010 (20), and expressed on a basis of MetHb tetramer.

Analysis. Ultraviolet absorption spectra were taken with a Hitachi (Tokyo, Japan) U-2000 double beam spectrophotometer. Fluorescence spectra were measured on a Hitachi 650-40 fluorescence photospectrometer. Fluorescence intensity at maximum excitation and emission relative to the intensity of 0.1 μM quinine sulfate in 0.1 N sulfuric acid was determined. Chemiluminescence intensity was counted on a Lumicounter 1000 machine (Nition Rikakikai Seisakusho, Ltd., Tokyo, Japan). High-performance liquid chromatography (HPLC) was carried out on a Shimadzu (Osaka, Japan) LC-2, a Hitachi 655A-11 or a Hitachi L-6000 liquid chromatograph equipped with an Inertsil ODS column (4.6 mm i.d. × 25 cm) (Gasukuro Kogyo Company, Ltd., Tokyo, Japan) or a YMC A-303 ODS column (4.6 mm i.d. × 25 cm) (Yamamura Research Laboratories, Kyoto, Japan). Elution solvents employed were: Solvent A, 0.5% acetic acid/methanol (29:1, v/v); Solvent B, 0.1 M ammonium formate/methanol (6:4, v/v); Solvent C, 0.1 M ammonium formate/methanol (1:1, v/v); and Solvent D, 0.1 M ammonium formate/methanol (4:6, v/v). Ultraviolet absorbing peaks were detected by a Shimadzu SPD-6A or a Hitachi 638-41 spectrophotometric detector. Fluorescent peaks were detected using a Shimadzu RF-530 fluorescence spectromonitor.

Peroxide values were determined according to the method of Wheeler (21).

Linoleic acid 13-monohydroperoxide (LOOH) and its

*To whom correspondence should be addressed at Tokyo College of Pharmacy, 1432-1 Horinouchi, Hachioji, Tokyo 192-03, Japan.

Abbreviations: BHT, butylated hydroxytoluene; HPLC, high-performance liquid chromatography; HRP, horseradish peroxidase; LOOH, linoleic acid 13-monohydroperoxide; MetHb, methemoglonin; MLOOH, methyl linoleate 13-monohydroperoxide; PC, phosphatidylcholine; PCOOH, phosphatidylcholine hydroperoxide; ROOH, lipid hydroperoxide; Z, carbobenzoxy.

methyl ester (MLOOH). Linoleic acid 13-monohydroperoxide (LOOH) was prepared according to the method of Gardner (22) using linoleic acid (Nippon Oil and Fats Company, Ltd., Tokyo, Japan) and lipoxygenase Type I from soybean [EC 1.13.11.12] (Sigma). Briefly, the solution of linoleic acid in borate buffer (pH 10) containing Tween 20 was purged with oxygen gas in the presence of lipoxygenase. The chloroform/methanol extract of the acidified reaction mixture was applied to a column of silicic acid, which was eluted with diethyl ether/*n*-hexane mixture. The ultraviolet absorption spectrum of each fraction was carefully monitored, and the fractions containing LOOH with a maximum absorption at 234 nm were collected. The purity of the hydroperoxide was 95–105% when estimated on the basis of its extinction coefficient at 234 nm, 24500 (23), and 97–106% when estimated on the basis of its theoretical peroxide value, 6400 meq/kg.

Methyl linoleate 13-monohydroperoxide (MLOOH) was prepared as follows. LOOH (500 mg) was dissolved in 20 mL of diethyl ether, and then an excess of diazomethane in diethyl ether was added. The mixture was kept at room temperature for 5 min and evaporated *in vacuo* to dryness. The methylated product was applied to a column (2 cm i.d. × 25 cm) of silica gel, and the column was eluted with diethyl ether/*n*-hexane (15:85, v/v). The peak fractions detected at 234 nm were collected and evaporated *in vacuo* to dryness. MLOOH was obtained in a yield of 320 mg. The product showed a single spot at R_f 0.1 on a silica gel thin-layer chromatogram developed with diethyl ether/*n*-hexane (15:85, v/v), when detected by potassium iodide. The purity of the product was 94.3% based on its theoretical peroxide value, 6140 meq/kg.

Phosphatidylcholine hydroperoxide (PCOOH). Egg yolk phosphatidylcholine (PC) (Coatsome NC-10 from Nippon Oil and Fats Company) was photoxidized in the presence of methylene blue according to the method of Miyazawa *et al.* (24). The reaction mixture was subjected to preparative reverse phase thin-layer chromatography, using DC-Fertigplatten RP-8F_{254}S (E. Merck, Darmstadt, Germany) and methanol/chloroform/water (10:1.5:0.5, v/v/v) as developing solvent. Phosphatidylcholine (PC) appeared at R_f 0.21 when detected by iodine vapor; the product appeared at R_f 0.27 when detected with iodine vapor, potassium iodide and ultraviolet light. The product was extracted with chloroform/methanol (1:1, v/v), and the extract was evaporated to dryness (73 mg). The product showed an absorption maximum at 233 nm in ethanol, and a peroxide value of 2600–3200 meq/kg.

Dityrosine. Dityrosine (3,3′-dityrosine) was prepared according to a previously reported method (25). A mixture of 5 mM Tyr, 5.25 mM hydrogen peroxide and 3 mg HRP in 0.2 M borate buffer (pH 9.5) (200 mL) was incubated at 37°C for 2 hr. Dityrosine was separated from small amounts of Tyr, and its trimer through a column of cellulose phosphate P-10 (Whatman Inc., Clifton, NJ). About 20 mg of dityrosine was obtained as a brownish colored powder. HPLC analysis of the preparation, using an Inertsil ODS column and Solvent A at a flow rate of 0.8 mL/min, showed a single ultraviolet and fluorescent peak at a retention time of 13.5 min. Ultraviolet absorption and fluorescence spectra (Fig. 1) of the preparation were identical with those reported (25–27). The concentration of dityrosine was determined based on its molar extinction coefficient at 284 nm (0.02 N HCl), 5400 (26).

Loss of Z-amino acids by LOOH/MetHb. A mixture of 1.0 mM Z-amino acid and LOOH (10 mM)/MetHb (1.3 μM) in 0.1 M phosphate buffer (pH 7.5) was incubated at 37°C for 24 hr. The Z-amino acid that remained unchanged was determined by HPLC of the reaction mixture using a YMC A-303 ODS column and Solvent B (for Z-Ser, Arg, Asp, Lys and His), Solvent C (for Z-Tyr) and Solvent D (for Z-Trp) at a flow rate of 0.8 mL/min. The peak due to Z-amino acid was detected at 260 nm, and its peak height was compared with that of the standard Z-amino acid solution.

Formation of fluorescence in reaction of amino acids (Z-amino acids) with lipid hydroperoxide/MetHb. A mixture of amino acid (Z-amino acid) and LOOH (MLOOH or PCOOH)/MetHb in 0.1 M phosphate buffer (pH 7.0, 7.5) or 0.2 M borate buffer (pH 9.5) was incubated at 37°C for 24 hr. The reaction mixture was then treated with 88 mM sodium borohydride at 0°C for 4 hr. Fluorescence spectra and relative intensities of the mixture were measured before and after the treatment with borohydride.

Formation of dityrosine and loss of Tyr in reaction of Tyr with LOOH/MetHb or hydrogen peroxide/HRP. A mixture of Tyr and LOOH/MetHb or hydrogen peroxide/HRP in the buffer was incubated at 37°C for the indicated period. The amount of dityrosine produced was determined by direct fluorescence measurement and HPLC analysis of the reaction mixture. Fluorescence intensity of the reaction mixture was measured after dilution into the same buffer, and the amount of dityrosine was obtained by comparison of the intensity with that of the standard dityrosine in the same buffer. HPLC analysis was done using an Inertsil ODS column and Solvent A at a flow rate of 0.8 mL/min. The peak due to dityrosine was detected at a retention time of 13.5 min by fluorescence at 300 (excitation) and 400 nm (emission), and by absorption at 280 nm. The amount of dityrosine was determined by comparing the peak heights estimated by both fluorescence and absorption with those obtained from the standard dityrosine solution. The amount of Tyr that remained unchanged was determined similarly. The height of the peak due to Tyr detected at 280 nm and of that which appeared at a retention time of 10.5 min were compared with that of a standard Tyr solution.

RESULTS

Formation of fluorescence in the reaction of amino acids and lipid hydroperoxides in the presence of MetHb. Lipid hydroperoxides (ROOH) are cleaved into radical species, lipid peroxyl radicals (ROO•) or alkoxyl radicals (RO•), by interaction with ferric complexes (16–19). In the present investigation, these lipid radicals were generated when hydroperoxides of linoleic acid (LOOH), methyl linoleate (MLOOH) and phosphatidylcholine (PCOOH) interacted with MetHb.

The decrease in Z-amino acids (1mM) caused by the reaction of an excess amount of LOOH (10 mM)/MetHb (1.3 μM) in phosphate buffer (pH 7.5) was investigated (Table 1). Loss of Nα-Z-Lys, Z-Tyr, Z-His and Z-Trp was significant but relatively small. The result, however, indicated that heterocyclic, amino and phenolic groups in amino acids were more susceptible to LOOH/

TABLE 1

Loss of Z-amino Acids Caused by Reaction with LOOH/MetHb[a]

Z-Amino acid	% Loss	
	2 hr	24 hr
Z-Ser	nd[b]	0
Z-Arg	nd[b]	2.9
Z-Asp	nd[b]	0
Nα-Z-Lys[c]	9.7	12.9
Z-Tyr[c]	3.6	5.7
Z-His[c]	13.0	nd[b]
Z-Trp[c]	9.1	17.6

[a]A mixture of 1.0 mM Z-amino acid and LOOH (10 mM)/MetHb (1.3 μM) in 0.1 M phosphate buffer (pH 7.5) was incubated at 37°C, and % loss of Z-amino acid was determined by HPLC analysis (See Materials and Methods).

[b]nd, not determined.

[c]Control experiments of the Z-amino acid alone and with MetHb (1.3 μM) did not show any significant loss after 24 hr.

MetHb than alkyl groups, which is consistent with earlier observations (14).

Formation of fluorescence in the reaction of amino acids or Z-amino acids with lesser amounts of lipid hydroperoxides/MetHb was investigated (Table 2). In the reaction of amino acids with LOOH/MetHb in phosphate buffer (pH 7.5), the reaction mixtures of Gly, Arg, Lys, Phe and His produced fluorescence with relative intensities slightly higher than those of the controls without the amino acids. They showed spectra with excitation maxima at 353–360 nm and emission maxima at 430–435 nm. The reaction mixture of Tyr produced fluorescence whose relative intensity was much higher than that of the control. The mixture showed a spectrum with an excitation maximum at 320 nm and an emission maximum at 410 nm. In similar reactions with Z-amino acids, only Nα-Z-Lys and Z-Tyr produced fluorescence, and no marked fluorescence was produced from other Z-amino acids. Hence, the amino groups in amino acids and the phenolic function in Tyr may be responsible for fluorescence development.

The formation of fluorescence was more significant in the reactions of amino acids and Z-amino acids with LOOH/MetHb in borate buffer (pH 9.5). While the fluorescence spectra of the reaction mixtures of Gly, Arg, Lys, Phe, His and Nα-Z-Lys were similar to those observed with the reaction mixtures in phosphate buffer, the maximum wavelengths of fluorescence from the reaction mixtures of Tyr and Z-Tyr were shorter than those observed with the reaction mixtures in phosphate buffer. This spectral difference of the reaction mixtures of Tyr and Z-Tyr in borate buffer may be due to the effect of borate on the fluorescence. While fluorescence from amino acids except for Tyr, and from Nα-Z-Lys was readily lost upon borohydride treatment, fluorescence from Tyr and Z-Tyr remained stable.

The reactions of Nα-Z-Lys and Z-Tyr with MLOOH/MetHb or PCOOH/MetHb in borate buffer (pH 9.5) gave marked fluorescence with characteristics similar to those obtained from the reaction of LOOH/MetHb.

Hence, two types of fluorescence were produced in the reaction of amino acids with the lipid hydroperoxides: the fluorescence on α- and ε- amino groups of the amino acids which was readily destroyed by borohydride treatment and the fluorescence on Tyr which was resistant to the treatment.

Formation of dityrosine in reaction of Tyr with LOOH in the presence of MetHb. Fluorescent dityrosine (3,3′-dityrosine) is a known oxidation product of Tyr (25–27). Standard dityrosine, prepared by oxidation of Tyr with hydrogen peroxide/HRP (25), showed absorption maxima at 285 (in acid) and 315 nm (in alkali). It exhibited the characteristic fluorescence spectra shown in Figure 1. Fluorescence spectra and intensities were influenced by pH and solvent. Fluorescence in acidic media showed maxima at 285 (excitation) and 410 nm (emission) and a relative molar intensity of 0.05. Fluorescence in alkaline medium showed maxima at 320 (excitation) and 410 nm (emission) and relative molar intensities of about 0.40. Fluorescence in borate buffer (pH 9.5) showed maxima at 298 (excitation) and 384 nm (emission) and a relative intensity of 0.10. The spectral difference in borate buffer may be due to the formation of some type of complex between dityrosine and borate (26).

The fluorescence data of the reaction mixtures of Tyr and Z-Tyr with lipid hydroperoxides/MetHb in phosphate and borate buffers listed in Table 2 were similar to those of standard dityrosine in the buffers (Fig. 1). As standard dityrosine is resistant to borohydride treatment, the stability of fluorescence of the reaction mixtures of Tyr and Z-Tyr upon borohydride treatment (Table 2) supported the idea that the reactions produced dityrosine and Z-dityrosine, respectively. HPLC of the reaction mixture of Tyr and an excess amount of LOOH/MetHb in borate buffer revealed a single fluorescent peak at a retention time of 13.5 min (Fig. 2A), which coincided with that of dityrosine formed by the reaction of Tyr with hydrogen peroxide/HRP (Fig. 2B). Ultraviolet absorption and fluorescence spectra of the peak fraction in various solvents were the same as those of standard dityrosine. Hence, the fluorophore formed in the reaction of Tyr with LOOH/MetHb was dityrosine.

The yield of dityrosine and the loss of Tyr in the reaction of Tyr with LOOH/MetHb under various conditions was estimated (Table 3). In the reaction of 5 mM Tyr with an excess amount of LOOH/MetHb at pH 9.5, the yield of dityrosine amounted to 20% accompanied by about 30% loss of Tyr. A similar reaction of Tyr with hydrogen peroxide/HRP yielded a higher amount of dityrosine (50%) accompanied by an extensive loss of Tyr (about 95%). The yield of dityrosine in the reaction of 1.35 mM Tyr with LOOH/MetHb at pH 7.5 increased as the concentration of LOOH/MetHb increased, but the yield was only 1.5% even at the high concentration of LOOH (10 mM)/MetHb (1.3 μM). In a similar reaction with hydrogen peroxide/HRP, the yield of dityrosine was maximal (8.4%) at the lower concentration of hydrogen peroxide (1 mM)/HRP (10 μg/mL) and decreased at the higher concentrations.

The time course of the formation of dityrosine and the loss of Tyr in the reaction of 1.4 mM Tyr with an excess amount of LOOH/MetHb at pH 7.5 were followed (Fig. 3). The formation of dityrosine rapidly reached a maximum after 2 min, whereas the loss of Tyr continued over 24 hr. In a similar reaction with hydrogen peroxide/HRP, the formation of dityrosine was gradual and reached a maximum after 2 hr, whereas the loss of Tyr continued over 24 hr. The results indicate that the formation of dityrosine in the reaction of Tyr with LOOH/MetHb de-

TABLE 2

Formation of Fluorescence in the Reaction of Amino Acids with Lipid Hydroperoxides, and the Effect of Borohydride Treatment on the Fluorescence

Reaction mixture[a]			Fluorescence[b]					
			Reaction mixture			Reaction mixture after borohydride treatment[c]		
Hydroperoxide/MetHb concentration	Amino acid (Z-amino acid) concentration	pH	Ex max nm	Em max nm	Relative intensity	Ex max nm	Em max nm	Relative intensity
	None[d]		358	434	9			
	Gly		353	430	22			
	Met		358	430	13			
LOOH/MetHb	Cys 10 mM	7.5	341	428	2			
2.3 mM/5.7 μM	Arg		353	430	17			
	Lys		358	432	25			
	Phe		359	435	14			
	Tyr		320	410	104			
	His		360	432	15			
	None		359	437	12			
	Z-Val		360	435	11			
LOOH/MetHb	Z-Arg 5 mM	7.0	363	448	7			
2 mM/7 μM	Nα-Z-Lys		360	435	20			
	Z-Phe		360	436	12			
	Z-Tyr		295	410	149			
	None		355	432	24	349	427	29
	Gly		352	430	53	341	420	29
LOOH/MetHb	Arg 10 mM	9.5	355	432	37	345	425	26
5 mM/14 μM	Lys		353	430	59	348	425	33
	Phe		356	430	46	350	428	28
	Tyr		298	388	399	298	386	385
	His		367	423	57	356	428	29
	None		354	430	27	347	425	26
	Z-Arg		355	430	20	347	426	21
LOOH/MetHb	Nα-Z-Lys 10 mM	9.5	351	428	69	346	422	36
5 mM/14 μM	Z-Phe		353	430	26	347	424	25
	Z-Tyr		297	384	591	298	384	595
	Z-His		354	430	20	349	427	23
MLOOH/MetHb	None		358	437	5	358	440	10
5 mM/14 μM	Nα-Z-Lys 10 mM	9.5	355	432	66	354	432	14
	Z-Tyr		298	385	639	298	385	664
PCOOH/MetHb	None		360	442	8	342	425	12
8 μeq/mL^{-1}/14 μM	Nα-Z-Lys 10 mM	9.5	356	435	26	350	430	12
	Z-Tyr		298	385	165	297	383	173

[a]A mixture of amino acid (Z-amino acid) at the indicated concentration and hydroperoxide/MetHb at the indicated concentration in 0.1 M phosphate buffer (pH 7.5 or 7.0) or 0.2 M borate buffer (pH 9.5) was incubated at 37°C for 24 hr. Fluorescence spectra and intensities of the reaction mixture before and after borohydride treatment were measured. Control experiment of amino acid (Z-amino acid) alone, or a mixture of amino acid (Z-amino acid) with MetHb (14 μM) did not produce any detectable fluorescence under the conditions.

[b]The relative fluorescence intensities underlined indicate increased fluorescence as compared with the value obtained without the amino acid (Z-amino acid) [none].

[c]The reaction mixture was treated with borohydride as described in Materials and Methods.

[d]Control experiment without amino acid (Z-amino acid) [none] produced fluorescence probably due to the globin moiety of MetHb.

pended on the concentrations of the substrate and of the hydroperoxide, and that the rate of dityrosine formation was rapid.

Contribution of radical species to dityrosine formation. Effect of radical scavengers on fluorescence formation in the reaction of Lys and Tyr with LOOH/MetHb at pH 9.5 was investigated (Table 4). Sodium azide that scavenges singlet oxygen (28), superoxide dismutase that destroys superoxide (29), methanol and mannitol that scavenge hydroxyl radicals (30), and dl-α-tocopherol and BHT as chain breaking antioxidants (31) were added to the reaction mixtures. The formation of fluorescence from Lys did not appear to be prevented by any of these scavengers. In contrast, the formation of dityrosine was dramatically inhibited by dl-α-tocopherol and BHT. Dityrosine formation in the reaction of Tyr with hydrogen peroxide/HRP was similarly inhibited by the antioxidants. The formation of dityrosine from Tyr appears to be caused by the radical species generated from LOOH/MetHb.

Tyr phenoxy radical, which emits chemiluminescence,

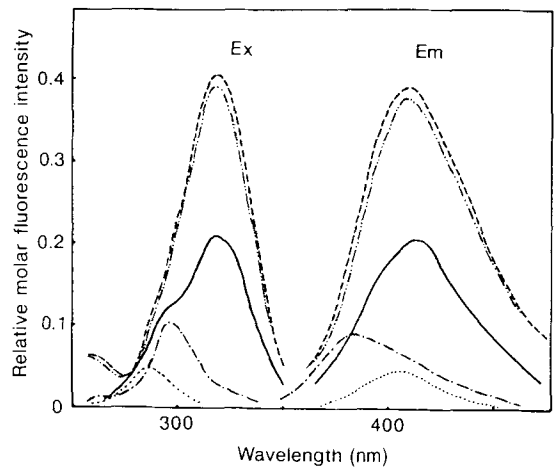


FIG. 1. Fluorescence spectra of dityrosine and its molar fluorescence intensities relative to quinine sulfate, in 0.02 N HCl (.....), 0.1 M phosphate buffer (pH 7.0) (——), 0.2 M carbonate buffer (pH 9.5) (– – –), 0.2 M borate buffer (pH 9.5) (—·—), and 0.02 N NaOH (—··—).

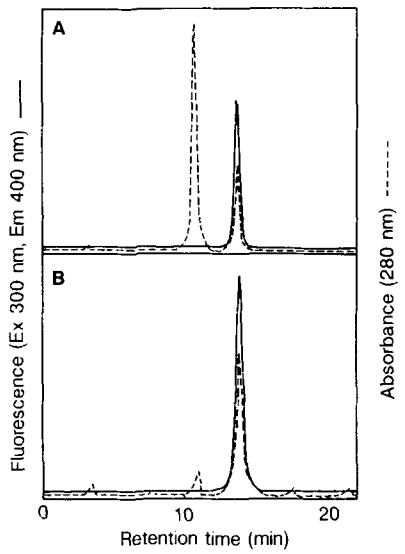


FIG. 2. HPLC of the reaction mixtures of 5.0 mM Tyr and LOOH (16 mM)/MetHb (54 μM) (A) and hydrogen peroxide (5 mM)/HRP (15 μg/mL) (B) in 0.2 M borate buffer (pH 9.5) incubated at 37°C for 2 hr. HPLC was performed using an Inertsil ODS column and 0.5% acetic acid/methanol (29:1, v/v) at a flow rate of 0.8 mL/min.

has been detected in the reaction of Tyr with hydrogen peroxide/HRP (32). The reaction mixtures of Tyr and LOOH/MetHb at pH 7.5 and pH 9.5 emitted intense chemiluminescence (Fig. 4A). Emission of chemiluminescence was maximal at 3 min of the reaction and ceased after 40 min. Similar reaction mixtures of Tyr with hydrogen peroxide/HRP emitted chemiluminescence (Fig. 4B), and the intensities gradually increased during 40 min. The results suggest that the radical species from LOOH abstracted a hydrogen radical from Tyr to give a Tyr phenoxy radical, which in turn condensed to dityrosine.

DISCUSSION

Fluorescence formation in aged tissues has received considerable attention as it is regarded an index for aging (1-5). There is considerable evidence that lipid peroxidation of tissues and cells can lead to the development of fluorescence. Lipid peroxidation generally proceeds through the formation of lipid hydroperoxides (ROOH) as primary products, which are subsequently cleaved into peroxyl (ROO•) and alkoxyl radicals (RO•). The latter are then decomposed to aldehydes (6).

Various aldehyde species can produce fluorescence by interaction with amino acids and proteins (3-5). Malonaldehyde had long been considered to be responsible for fluorescence formation (3), although it is only of minor consequence because its levels in peroxidized tissues are typically much lower than generally expected. Also the fluorescence characteristics produced from malonaldehyde are quite different from those produced from peroxidized lipids (4,5). Aldehyde species other than malonaldehyde are now being considered as more likely candidates for fluorescence formation (4,5,7-12).

TABLE 3

Formation of Dityrosine and Loss of Tyr in Reaction of Tyr with LOOH/MetHb and Hydrogen Peroxide/HRP

Reaction mixture[a]					Dityrosine μM			
						By HPLC analysis[b]		
Tyr mM	LOOH/MetHb mM/μM	Hydrogen peroxide/HRP mM/μg/mL	pH	Reaction time hr	By fluorescence intensity[b]	Fluorescence detection	Ultraviolet detection	Tyr mM By HPLC analysis[b]
5.0	16/54		9.5	2	540	470	460	3.36
5.0		5/15	9.5	2	1390	1290	1310	0.22
1.35	1/0.13		7.5	24		1		
1.35	5/0.65		7.5	24		4		
1.35	10/1.3		7.5	24		10.5		
1.35		1/10	7.5	24		58		
1.35		5/50	7.5	24		31		
1.35		10/100	7.5	24		31		

[a]A mixture of Tyr and LOOH/MetHb or hydrogen peroxide/HRP in 0.1 M phosphate buffer (pH 7.5) or 0.2 M borate buffer (pH 9.5) was incubated at 37°C for the indicated period.

[b]See Materials and Methods.

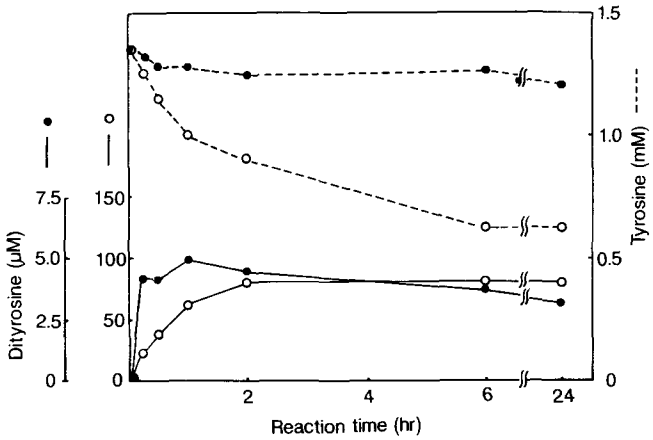


FIG. 3. Time course of the formation of dityrosine (—) and the loss of Tyr (---) in the reaction of 1.35 mM Tyr with LOOH (10 mM)/MetHb (1.3 μM) (●) and hydrogen peroxide (1 mM)/HRP (10 μg/mL) (○) in 0.1 M phosphate buffer (pH 7.5) at 37°C. Dityrosine and Tyr were determined by HPLC as described in Materials and Methods.

In the present investigation, an alternative mechanism for fluorescence formation during lipid peroxidation emerged. The reactive lipid peroxyl (ROO·) or alkoxyl (RO·) radicals generated from lipid hydroperoxides (ROOH) produced by reaction with amino acids, two types of fluorescence. One was due to fluorescent dityrosine from Tyr, the other was due to fluorescence derived from α- and ε-amino groups of the amino acids (Scheme 1). The first appears to be produced by a radical reaction. The peroxyl and alkoxyl radicals seem to abstract a hydrogen radical from Tyr to produce the Tyr phenoxy radical which in turn condenses to fluorescent dityrosine. This mechanism is consistent with the facts that antioxidants prevent the formation of dityrosine, and that chemiluminescence due to the phenoxy radical (32) is emitted during the reaction. The second type of fluorescence seems to be produced by a non-radical reaction. Since the fluorescence spectra of the fluorophores that are produced on the amino groups are similar to those obtained by reaction of amino acids with LOOH in the absence of ferric complexes (33), lipid hydroperoxides as such or some types of their non-radical degradation products seem to be involved in the formation of fluorophores on the amino groups.

TABLE 4

Effect of Scavengers on Fluorescence Formation in the Reaction of Lys and Tyr with LOOH/MetHb and Hydrogen Peroxide/HRP[a]

	LOOH/MetHb		Hydrogen peroxide/HRP
Scavenger added	Fluorescence from Lys[b]	Dityrosine from Tyr[c]	Dityrosine from Tyr[c]
None	100	100	100
Sodium azide 10 mM	88	101	114
Superoxide dismutase 0.1 mg/mL	104	109	109
Methanol 4%	96	114	69
Mannitol 100 mM	94	98	109
dl-α-Tocopherol 100 μM[d]	80	24	1
BHT 100 μM[d]	113	0	0

[a]Mixtures of 1.0 mM Lys or Tyr, and LOOH (1.0 mM)/MetHb (1.5 μM) or hydrogen peroxide (1.0 mM)/HRP (3 μg/mL) in 0.2 M borate buffer (pH 9.5) was incubated at 37°C for 24 hr in the presence of scavenger.

[b]Fluorescence intensity at 350 (excitation) and 430 nm (emission) was compared.

[c]The amount of dityrosine determined by HPLC analysis (fluorescence) was compared.

[d]Acetonitrile (30%) was incorporated into the reaction mixture to dissolve the agents.

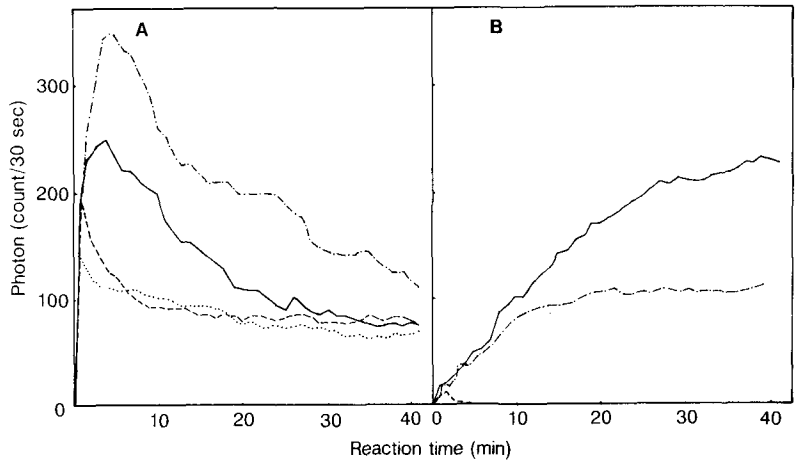
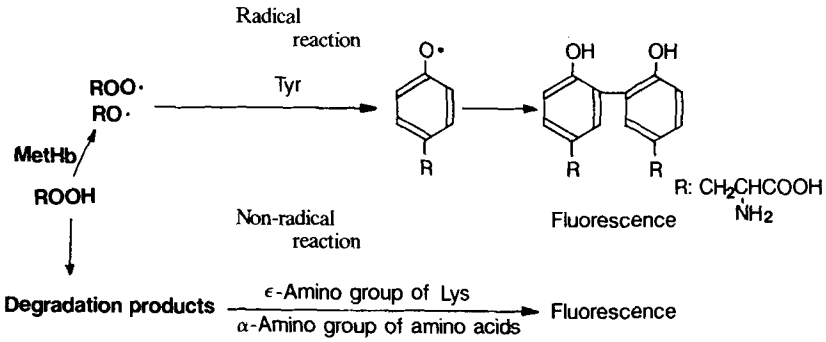


FIG. 4. Chemiluminescence intensities of the reaction mixtures of 1.35 mM Tyr and LOOH (10 mM)/MetHb (1.3 μM) (A) and hydrogen peroxide (1 mM)/HRP (10 μg/mL) (B) in 0.1 M phosphate buffer (pH 7.5) (—) and in 0.2 M borate buffer (pH 9.5) (——) incubated at 37°C. The controls without Tyr at pH 7.5 (– – –) and at pH 9.5 (·····) were similarly counted.



SCHEME 1

The fluorescence spectra of dityrosine and of the fluorophores produced on the amino groups are different. The wavelengths of maximum excitation and emission of dityrosine are shorter than those of the fluorophores on the amino groups. Furthermore, both can be distinguished by borohydride treatment. Dityrosine is resistant to borohydride treatment, whereas the fluorophores on the amino groups are not.

The present findings on the formation of dityrosine by lipid peroxyl or alkoxyl radicals appear important also from another point of view. Although dityrosine was first recognized some 30 years ago (34), there is renewed interest in its occurrence in natural proteins (26,35-44). Although dityrosine can be produced by reaction with hydrogen peroxide/HRP or with ovoperoxidase (25,26, 34,42), by radiolysis (45), by ultraviolet irradiation (27), by reaction with tetranitromethane (46), ozone (47), periodate (48), or by reaction with bromine radicals (49), most of these reactions do not seem to take place in *in vivo* systems. In a previous paper (50), we described the formation of fluorescence and intermolecular cross-links in α-crystallin by reaction with lipid hydroperoxides, and suggested the formation of fluorescent dityrosine and other fluorophores in the protein. The present findings suggest that lipid peroxidation contributes to dityrosine formation in tissues. Induction of lipid peroxidation in tissues generates lipid peroxyl and alkoxyl radicals, which could play an important role in the formation of dityrosine in natural proteins.

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Changes in Lipids of Pigeon "Milk" in the First Week of Its Secretion

Sreerama Shetty and **S.N. Hegde***
Division of Applied Zoology, Department of Biosciences, Mangalore University, Mangala Gangotri, 574 199, India

Pigeon "milk" (PM) collected from the crop of 1- to 5-day-old squabs was analyzed to examine whether there were changes in lipid composition during the first week of secretion. The high PM fat content (9–11%) remained fairly constant in the first 5 days of secretion. The mean percentage of neutral lipids, glycolipids and phospholipids was 80, 12 and 8%, respectively. Unlike the content of neutral lipids, glycolipid and phospholipid levels increased significantly between day 1 and day 5 of secretion. Triglycerides, the major neutral lipids, decreased by 24% between day 1 and day 5, while free sterols, monoglycerides and hydrocarbons increased by 8%, 11% and 2.5%, respectively, during the same period; diglycerides and sterol esters, however, remained unchanged. The ratio of saturated to unsaturated fatty acids was 0.27 and it remained unchanged. Medium-chain (C_{10}, C_{12} and C_{14}) and odd-chain (C_{15} and C_{17}) fatty acid contents were low. Fatty acids longer than C_{20} were absent. Palmitic acid, the major saturated fatty acid, increased by 42% from day 1 to day 5, whereas stearic acid decreased by 48% during the same period. Oleic acid, the predominant unsaturated fatty acid, also decreased from 51 to 45% between the first and fifth day of PM secretion. Polyunsaturated acids (18:2, 18:3 and 20:4) accounted for 26% and 30% of the total fatty acids on day 1 and day 5, respectively. Although lipid changes in the crop of squabs prior to collection of samples cannot totally be ruled out, the nature of lipid changes is likely to reflect cellular breakdown that precedes PM secretion by parent pigeons.
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Pigeon "milk" (PM) is an avian crop secretion exuded by pigeons and doves of the family Columbidae (1). The altricial birds called squabs are fed by regurgitation with PM by female and male parents in the first three post-hatching weeks (1,2). However, after day 5 of secretion, PM becomes mixed with increasing quantities of grains which makes it more difficult to obtain PM in satisfactorily pure form.

Earlier PM analyses suggested that PM contains about 8% lipid, 14% protein, 0.05% glycogen (3,4), and 2.6% minerals (5). More recently it has been reported that PM lipids consist primarily of triglycerides (81.2%) and phospholipids (12.7%) (6,7). As the squabs increase their body weight 8-fold in the first week, and because fat is the principal energy source (60%) in the PM diet (Shetty, S., and Hegde, S.N., unpublished data), it was of interest to determine whether the lipid composition would change during this period. The occurrence of certain types of glycolipids of PM is also reported here for the first time.

*To whom correspondence should be addressed.

Abbreviations: DG, diglyceride(s); FID, flame ionization detector; PM, pigeon "milk"; TG, triglyceride(s); TLC, thin-layer chromatography.

MATERIALS AND METHODS

Rock or house pigeons (*Columba livia*) that were bred and maintained on a mixed-grain cereal based diet (3.5% lipids), supplemented with minerals and vitamins, were used. PM was collected as a semi-solid material once a day through small, surgically made slits on the crop of the squabs as described by Pase *et al.* (8). The slits were closed by double sutures after extraction of the milk (a maximum of 4–6 g of milk could be collected at a time). Samples contaminated by grains were sorted by hand. PM samples obtained on different days of secretion were separately pooled and stored in the cold (−20°C). Total lipids were extracted from small samples with a mixture of chloroform/methanol (2:1, v/v) and washed with water to remove non-lipid contaminants following the method of Folch *et al.* (9). Non-lipid contaminants were also removed by passing the extracts through Sephadex G-25 (Pharmacia Fine Chemicals, Uppsala, Sweden) columns (10). Lipids were concentrated on a rotary flash evaporator under vacuum. The lipid content was estimated spectrophotometrically based on organic carbon using stearic acid as standard (11). Total lipids in a minimum volume of chloroform were separated into neutral lipids, glycolipids and phospholipids by eluting with chloroform, acetone and methanol, respectively, using silicic acid columns (12). The lipid classes were estimated on the basis of organic carbon as mentioned above.

Neutral lipids loaded onto heat-activated silicic acid columns were fractionated into hydrocarbons, steryl esters, triacylglycerols, free sterols, diacylglycerols and monoacylglycerols by successive elution with hexane, 15% benzene, 5% diethyl ether, 15% diethyl ether, 30% diethyl ether (all in hexane) and finally with pure diethyl ether (13). Glycolipids were separated into acylsterol glycosides, monogalactosyl diglycerides, sterol glycosides and digalactosyl diglycerides by eluting with a mixture of chloroform/acetone (9:1, v/v, 8:2, v/v, 7:2, v/v, and 2:8, v/v, respectively) (14). Phospholipids were separated into phosphatidic acid plus cardiolipin, phosphatidylethanolamine plus phosphatidylserine, phosphatidylinositol plus phosphatidylcholine, and sphingomyelin plus lysophosphatidylcholine using chloroform/methanol 95:5, v/v, 80:2, v/v, 50:50, v/v and pure methanol, respectively (15). Neutral lipid and phospholipid fractions were identified on thin-layer chromatography (TLC) plates by comparison with standards (Sigma Chemical Company, St. Louis, MO) using petroleum hydrocarbon (b.p. 60–80°C)/diethyl ether/acetic acid (80:20:1, v/v/v) and chloroform/methanol/water (65:25:4, v/v/v) as solvent systems, respectively. However, in respect to glycolipids, only the homogeneity was checked by TLC employing chloroform/methanol/water (65:25:4, v/v/v) as solvent system. For estimation of fatty acids, the total lipids were saponified with alcoholic potassium hydroxide and methylated with diazomethane (16). Fatty acid methyl esters were analyzed using a gas chromatograph (Schimadzu Gc-9A, Kyoto, Japan) fitted with flame ionization detector (FID) and recorder C-R3A.

TABLE 1

Changes in the Lipids of Pigeon "Milk" in the First Five Days of Its Secretion

Day of secretion	Total lipid (g/100 g wet wt)	Percentage of 3 major lipid classes		
		Neutral lipids	Glycolipids	Phospholipids
1	9.24 ± 0.71[a]	84.55 ± 6.75	9.50 ± 1.72	5.95 ± 1.14
2	10.40 ± 0.65	81.94 ± 6.33	10.51 ± 1.53	7.55 ± 1.32
3	10.83 ± 0.89	80.65 ± 8.14	13.22 ± 1.63	6.13 ± 1.80
4	11.23 ± 0.70	77.98 ± 5.20	14.06 ± 4.36	7.95 ± 1.72
5	11.36 ± 0.98	72.53 ± 3.98	15.05 ± 4.35	12.42 ± 2.81
P[b]	NS[c]	NS[c]	<0.05	<0.05

[a]Values are mean ± SE of 8 determinations on samples pooled from 10 collections.
[b]Significance of difference between day 1 and day 5 sample.
[c]NS, not significant.

Separation was achieved on 3 m × 2 mm columns of 10% EGSS-Y on Chromosorb W (100–200 mesh, acid washed) operated isothermally at 190°C. Different components were identified with reference to known standards (Sigma) by plotting the logarithm of their retention times against carbon numbers. The data were statistically processed by applying Student's t-test.

RESULTS

Changes in different classes of PM lipids in the first five days of secretion are shown in Table 1. PM has about 9–11% total lipid which did not undergo significant change between day 1 and day 5. Total lipid content determined upon removal of non-lipid contaminants by Folch washing was not different from that obtained by passing chloroform/methanol extracts through Sephadex-G columns. Hence the former procedure was adopted in the present work. Neutral lipids were in the range of 73–85% with a mean value of about 80%; their level decreased slightly but not significantly in the first week. Glycolipids were in the range of 10–15% (mean 12%) and increased significantly ($P<0.05$) between day 1 and day 5. Similarly phospholipids accounted for about 6–12% (mean 8%) and increased significantly ($P<0.05$) in the first week of secretion. Among neutral lipids of PM, the level of major triglyceride fractions decreased from 59% to 35% ($P<0.001$) whereas that of free sterols, monoglycerides and hydrocarbons increased from 16% to 24% ($P<0.01$), 8–19% ($P<0.001$) and 1.5–4% ($P<0.01$), respectively. Steryl esters (2.3%) and diglycerides (14%) remained constant, however (Fig. 1).

As illustrated in Figure 2, the digalactosyl diglycerides and acylsterol glycosides decreased from 33 to 25% ($P<0.01$) and 28 to 23% ($P<0.05$), respectively, while monogalactosyl diglycerides increased from 11 to 26% ($P<0.001$) of glycolipids between day 1 and day 5. In contrast, sterol glycosides (25–27%) did not change significantly during this period. Changes in the phospholipids are shown in Figure 3. Phosphatidylcholine (plus phosphatidylinositol) and phosphatidylethanolamine (plus phosphatidylserine) decreased from 41 to 35% ($P<0.02$) and 27 to 22% ($P<0.05$) of phospholipids between day 1 and day 5, respectively. Whereas sphingomyelin plus lysophosphatidylcholine increased significantly from 17 to 27% ($P<0.01$), phosphatidic acid plus cardiolipin

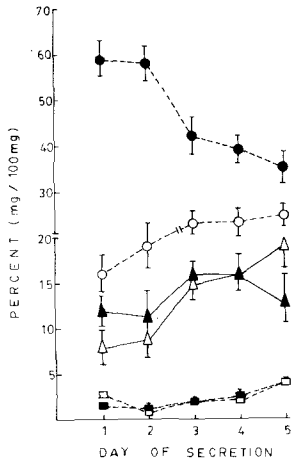


FIG. 1. Changes in the relative proportion of neutral lipid classes in pigeon milk during the first five days of secretion. Symbols: ●--●, triglycerides; ○--○, free sterols; ▲—▲, diglycerides; △—△, monoglycerides; □--□, steryl esters; ■--■, hydrocarbons. Vertical bars indicate SE of 4 determinations.

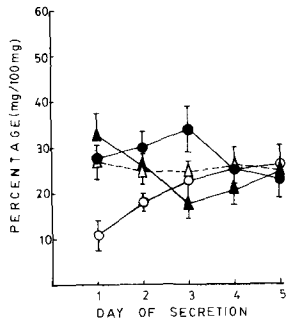


FIG. 2. Changes in the relative proportion of glycolipid classes in pigeon milk during the first five days of secretion. Symbols: ▲—▲, digalactosyl DGS; ●—●, acylsterol glycoside; △--△, Sterol glycoside; ○—○, monogalactosyl DGS. Vertical bars indicate SE of 4 determinations.

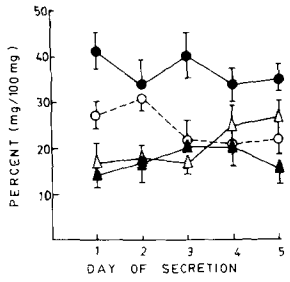


FIG. 3. Changes in the relative proportion of phospholipid classes in pigeon milk during the first five days of secretion. Symbols: ●—●, phosphatidylcholine (plus phosphatidylinositol); ○--○, phosphatidylethanolamine; △—△, sphingomyelin plus lysophosphatidylcholine; ▲—▲, phosphatidic acid plus cardiolipin. Vertical bars indicate SE of 4 determinations.

TABLE 2

Fatty Acid Composition (% total) of Pigeon "Milk" Total Lipids[a]

	Day of secretion				
Fatty acid	1	2	3	4	5
10:0	0.2	0.3	0.6	0.9	0.7
12:0	0.1	0.1	0.2	0.6	0.9
14:0	0.9	0.6	0.9	1.1	0.9
15:0	0.6	0.1	0.5	0.3	0.3
16:0	11.0	12.9	15.4	14.8	15.7
16:1	1.9	2.6	3.1	2.1	2.1
17:0	0.1	0.1	Tr	Tr	Tr
18:0	8.3	7.2	7.3	5.0	4.4
18:1	50.9	50.1	46.8	44.8	44.9
18:2	22.6	22.1	22.4	28.4	28.1
18:3	3.3	1.6	2.5	2.0	2.0
20:0	Tr	Tr	Tr	Tr	Tr
20:4	Tr	2.3	Tr	Tr	Tr

[a]Mean percentage values of two determinations made on samples pooled from 10 collections. Tr, trace.

(14-15%) remained almost constant between day 1 and day 5.

Due to small sample size, the fatty acid changes could not be tested statistically. However, the apparent trends indicated that between day 1 and day 5, oleic, stearic and linolenic acids decreased, from 51 to 45%, from 8 to 4%, and from 3 to 2%, respectively (Table 2). In contrast, linoleic and palmitic acids appeared to increase from 23 to 28% and from 11 to 16%, respectively; palmitoleic acid (2%) remained almost constant.

DISCUSSION

It should be pointed out that the parent pigeons do not store PM at any time in their crop cavity and hence the samples had to be extracted (once a day, normally between 11:00-12:00) from the crop of squabs. The time elapsed from parental feeding to surgical collection was about 1-2 hr. During this period it is possible that PM undergoes certain changes in the squab crop due to microbial action as both PM and parent pigeon crop mucosa were found to harbor lactobacilli and streptococci (17). Endogenous lipolytic activity was unlikely because neither the crop and saliva of parents nor those of squabs is a source of lipase (1). Likewise, retrograde flow of digesta containing pancreatic lipase was also ruled out because of the presence of a gizzard and a long proventriculus between crop and duodenum of squabs (1). Although our definition of pigeon "milk" is restricted to crop contents of freshly fed squabs, the samples, as used in the present study and for reasons outlined above, were assumed to represent nearly true parental secretions. Secondly, beyond day 5 of secretion the small quantity of PM gets mixed up with large quantities of parent's food with the result that the milk/grain ratio of 20 on day 1 is reduced to 0.5 on day 5 (Shetty, S., and Hegde, S.N., unpublished data). It was for this reason that the analyses were restricted to the first 5 days.

Lactation by mammals is believed to have evolved primarily to maximize growth and viability of the young. PM bears similarity with mammalian milk inasmuch as it provides virtually all nutrients during the initial phase of postnatal growth; however, unlike mammalian milk which has been analyzed from a large number of species [see review by Jenness and Sloan (18) and Oftedal (19)], relatively little is known about PM. The high lipid content (9-11%) together with moderate amounts of dry matter (26%) appear to contribute much to the high energy value (25 KJ/g) of PM (Shetty, S., and Hegde, S.N., unpublished data).

The postnatal increase in body weight of squabs was 8-, 18- and 22-fold in the first, second and third week, respectively (Shetty, S., and Hegde, S.N., unpublished data). This growth rate surpasses even that of the fastest growing seal pups (20). The carbohydrate content of PM was only 1.5%, and free lactose was absent. Another feature of PM is that despite the significant decrease in protein content, fat levels did not change in the first week of secretion (Shetty, S., and Hegde, S.N., unpublished data).

Although there was a decrease in triglycerides (TG), neutral lipid content of PM remained unchanged because of the increase in the proportion of free sterols and monoglycerides. As PM was extracted from the squabs, some, if not all of the partial glycerides could be the products of TG lipolysis by PM bacteria (17). The free sterol content, which was higher than that of esterified sterols, increased from day 1 to day 5. This could possibly be due to increased cellular turnover in the parent crop gland. The polar lipid content of PM was higher than that of mammalian milk (21). As polar lipids are generally associated with cell membranes, an increase in their proportion also suggests increased cellular breakdown in the crop. Although PM lipids have been studied previously (6,7), the presence of glycolipids was not reported; the latter increased from day 1 to day 5 with carbohydrate contents of 24-37% (Shetty, S., and Hegde, S.N., unpublished data). Their source could be either bacteria (17) or plausibly the sloughed-off cells of parent crop mucosa of which PM is composed. PM glycolipids probably serve as precursors of structural components of cells and tissues in fast developing squabs. Phosphatidylcholine (plus trace amount of phosphatidylinositol) and phosphatidylethanolamine were the major phospholipid fractions of PM. Like glycolipids, phospholipids of PM also increased in the first week (Table 1). Although their content was greater than

that of mammalian milk (19,21), it was comparable to that of mammalian saliva (22). An increase in phospholipid content in the first 5 days of secretion was again indicative of higher tissue turnover in parent pigeons during this period.

The saturated fatty acid proportion of 21-25% in PM though comparable to that of the northern fur seal (*Collorhinus ursinus*) milk (27.3%), was lower than that of the milk of sow (35.7%), human (55.7%) and cow (57.5%) (23). Medium-chain fatty acids (C_{10}, C_{12} and C_{14}) were in low proportions unlike those found in the milk of ruminants, elephants and rabbits (24). C_{15} and C_{17} acids were the only odd-chain fatty acids detected and their content was also much less than that in mammalian milk. Oleic acid (18:1) and linoleic (18:2) acid contents of PM were higher than those of most mammalian milk. Fatty acids with a carbon chain longer than 20 were absent in PM. About 80% of the fatty acids of PM were unsaturated which compared well with fur seal milk (23). The levels of linoleic and linolenic acids were much higher in PM than human colostrum (25). This property may contribute to the fast postnatal development of squabs because of the importance of the metabolites of linoleic and linolenic acids in cell membranes of tissues, such as brain and retina, and as precursors of eicosanoids.

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Free Sterols, Steryl Esters, and Lipid Phosphorus in Needles of Scot's Pine (*Pinus sylvestris* L.)

Christine Fischer* and **Wolfgang Höll**
Institut für Botanik und Mikrobiologie, Technische Universität München, München, Germany

Free sterols, steryl esters, and lipid phosphorus were measured in new (current year) needles of Scot's pine during an annual cycle, and also in one-, two-, and three-year-old needles collected shortly after bud break. Sterols were identified and quantified by capillary gas chromatography and gas chromatography/mass spectrometry. Steryl esters were hydrolyzed enzymatically. Newly emerged needles contained highest amounts of free sterols and lipid phosphorus, probably reflecting increased membrane and organelle production, but low levels of steryl esters. Mature needles contained approximately equal amounts of free and esterified sterols. The molar phospholipid/free sterol ratio was 3:1 at all the time periods studied. A dramatic increase of steryl esters was observed in the one-, two-, and three-year-old needles at times when new needles emerged. The individual free and esterified sterols were sitosterol, campesterol (presumably together with its C-24 epimer), and cholesterol at approximately 88, 10, and 2%, respectively. Isofucosterol, an intermediate in sitosterol biosynthesis, was present almost exclusively in newly emerged needles. Esterified sterols contained only trace amounts of isofucosterol. Shifts in favor of cholesterol and 24ξ-methyl cholesterol occurred in the steryl esters during needle differentiation, and saturation grade of esterified fatty acids decreased. In mature needles, the composition of free sterols and steryl esters remained constant throughout the year.
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The importance of sterols on membrane stability, fluidity and permeability is well known (1-6). In addition, there appear to be developmentally regulated functions for sterols to control ontogenetic changes like growth or reproduction (7,8). The different sterols presumably reflect different roles (9).

Pines and all other trees examined belong to the "main line" plants, in that they have the characteristic type of dominant Δ^5-sterols (9). Sitosterol, campesterol (presumably with its C-24 epimer dihydrobrassicasterol), 5α-sitostanol, and traces of cholesterol have been detected in the wood and the bark of different species of the genus *Pinus* (10-13). The levels of free and esterified sterols increase towards the center of the trunk (14,15). Only traces of steryl glycosides and acylated steryl glycosides are detectable in Scot's pine wood (16).

Because the amounts and composition of tree sterols are well known, comparatively little information is available about the fluctuation of sterols during ontogenetic changes like bud break or heartwood formation (17,18). The present paper describes the annual fluctuations of free sterols, steryl esters, and lipid phosphorus in developing needles of Scot's pine, and also in one-, two-, and three-year-old needles collected shortly after bud break.

*To whom correspondence should be addressed at the Institut für Botanik und Mikrobiologie, Technische Universität München, Arcisstr. 21, 8000 München 2, Germany.

Abbreviations: FAME, fatty acid methyl ester; FID, flame ionization detector; GC/MS, gas chromatography/mass spectrometry; PTV, Programmed Temperature Vaporizer; TLC, thin-layer chromatography.

MATERIALS AND METHODS

Needle material. Newly emerged needles of seven (30-year-old) Scot's pine trees were collected monthly in a forest near Munich, Germany. The collection dates in 1988 were: February 2, March 23, April 24, May 24, June 24, July 22, August 23, September 23, October 25, November 24, and December 23. In 1989, needles were collected on January 26, May 19, and June 26. On May 19, 89 newly emerged, one-, two-, and three-year-old needles were collected. Immediately after collection, the needles were frozen in dry ice, lyophilized and homogenized with liquid nitrogen using a micro dismembrator (Braun, Melsungen, Germany).

Extraction of free sterols. The needle powder (20 mg) was extracted for 20 min with 1 mL chloroform/methanol (2:1, v/v) containing 10 μM 5α-cholestan-3β-ol as an internal standard and washed twice with 0.5 mL chloroform/methanol (2:1, v/v). The combined extracts were dried under a stream of nitrogen.

Isolation, purification and enzymatic hydrolysis of steryl esters. The needle powder (20 mg) was extracted with 1 mL chloroform/methanol (2:1, v/v) for 30 min, and washed twice with 0.5 mL of the chloroform/methanol mixture. In addition, every third sample was extracted with 1 mL chloroform/methanol (2:1, v/v) containing 20 μM cholesteryl oleate as an internal standard. The combined extracts were dried under nitrogen and redissolved in 50 μL chloroform/methanol (2:1, v/v) for thin-layer chromatography (TLC). Samples were applied quantitatively to TLC plates (0.2 mm Silica gel 60 on aluminum). Authentic cholesteryl palmitate served as marker. Plates were developed twice in petroleum hydrocarbon (b.p. 40-60°C)/diethyl ether/acetic acid (90:10:1, v/v/v) (19). Spots carrying the cholesteryl palmitate standard were made visible by spraying with sulfuric acid/acetic acid (1:1, v/v) and by heating for 3 min at 160°C (20). Unsprayed plate areas corresponding to cholesteryl palmitate were scraped off and eluted with chloroform/methanol (2:1, v/v) as described above.

For enzymatic hydrolysis, 200 μL potassium phosphate buffer (0.05 M, pH 8.0) containing sodium taurocholate (7.8 mM) and Triton X-100 (0.1%) were added to the dry samples. After vigorous mixing for 30 seconds (Vortex mixer), specimens were treated with lipoprotein lipase (7.5 U/mL, E. Merck, Darmstadt, Germany) and incubated for 17 hr at 37°C (21). For gas chromatography, 20 nmol 5α-cholestan-3β-ol and 40 nmol pentadecanoic acid were added as external standards to each sample. Samples were then taken to dryness again.

Derivatization and gas chromatography of sterols.

Bis(trimethylsilyl)trifluoroacetamide (0.1 mL) in anhydrous pyridine (1:5, v/v) was added to the samples. The solutions were heated for 15 min at 80°C. Before injection into the gas chromatograph, samples were diluted with chloroform to a final concentration of approximately 50 ng sterol derivatives per 1 μL (injection volume).

A DANI 8500 Capillary Gas Chromatograph (Monza, Italy) was equipped with a Programmed Temperature Vaporizer (PTV) injection system, a flame ionization detector (FID) and a 25-m fused silica capillary column coated with DB-1 (J&W Scientific, Folsom, CA) with an internal diameter of 0.25 mm and a film thickness of 0.25 μm. The chromatographic conditions used were as follows: Carrier gas, helium at 2 mL/min; detector gases, hydrogen at 56 mL/min and air at 180 mL/min; make-up gas, nitrogen at 40 mL/min. Splitless injection mode with solvent split was used; the split ratio was 1:20. The column temperature was programmed with a 2-min hold at 200°C, followed by heating at 15°C/min to 280°C and a final 10-min hold at 280°C. The temperature of the PTV was 60°C for 6 seconds, followed by a very rapid heating (900°C/min) to 300°C. This final temperature was held for 15 min. The detector temperature was 300°C. Sterol derivatives were quantified using a Shimadzu C-R3A integrator (Kyoto, Japan).

Derivatization and gas chromatography of fatty acids. For synthesis of fatty acid methyl esters (FAME), steryl ester samples (after hydrolysis) were treated with 0.1 mL of boron trifluoride (14% in methanol) for 30 min at 80°C. After adding 1 mL of water, the mixtures were extracted three times with 1 mL of diethyl ether. The combined extracts were washed two times with 1 mL of water and then taken to dryness under a stream of nitrogen. Before gas chromatography, samples were diluted with chloroform to a final concentration of 50 ng FAME/μL.

FAME were determined on a fused silica 30-m DB-225 (J&W) with an internal diameter of 0.25 mm and a film thickness of 0.25 μm. The column temperature program was a 2-min hold at 150°C, followed by heating at 10°C/min at 210°C. This final temperature was held for 10 min. The carrier gas was helium at 3 mL/min. All other conditions were as described above, with the exception of the final PTV-temperature and the detector temperature at 230°C.

Lipid phosphorus. TLC areas corresponding to phospholipids were scraped off, and phosphorus was determined according to Chen *et al.* (22).

Gas chromatography/mass spectrometry (GC/MS). GC/MS was performed with a Finnigan model M112S (Sunnyvale, CA) operated in the electron impact mode. The ion source was operated at 70eV and with a 700 μA ionization current. The ion source temperature was approximately 200°C, and the interface temperature was about 250°C. The gas chromatograph was a Carlo Erba model 4160 (Milano, Italy). Carrier gas and oven temperature program were as described above.

Experimental design and statistics. Experiments were designed as single factor experiments with repeated measures. Populations were checked for Gauss distribution using the Kolmogoroff-Smirnoff test. Homogeneity of variance was checked using the F_{max} test. Means were tested for significance using a single factor analysis of variance for repeated measures. For multiple comparisons, the LSR test was performed. Regression lines were ascertained using the least square method. Pearson's correlation coefficient was tested for significance using the *t*-test according to Fisher.

Experimental errors of three different measurements using the same sample (expressed as coefficients of variation) were 2–4% for free sterols, 4–6% for steryl esters, and 10–15% for lipid phosphorus, respectively. Tree-to-tree variations were quite large and are specified in the legends to the Figures.

RESULTS

Total amounts. Figure 1 shows that newly emerged needles (May) contained much higher amounts of free sterols than do older needles (differences are significant on the 99.9% level). In subsequent months, the amounts of free sterols decreased and remained more or less constant until the end of the year. Compared to the preceding months, February and March needles contained significantly higher amounts (95% level) of free sterols. No significant changes were detectable in the total amounts of free sterols in one-, two-, and three-year-old needles collected shortly after bud break (Table 1).

When compared with differentiated needles, newly emerged needles contained small amounts of steryl esters (Fig. 1). During the summer the concentration of steryl esters increased and then remained constant till bud break at the end of April. In May, the now one-year-old needles showed very high amounts of steryl esters (differences were significant on the 99.9% level). Four weeks later, steryl esters decreased again, but the needles showed still significantly higher amounts (95% level) than in most of the months before bud break. This increase in the esterified sterols during bud break was also true for needles older than one year (Table 1). The amounts of steryl esters in May increased as needle age increased.

The seasonal fluctuation of lipid phosphorus in needles of Scot's pine during their first year of life is shown in Figure 2. Newly emerged needles contained the highest amounts of lipid phosphorus. Needles collected in October and November showed higher amounts than needles collected in February and March (95% level). No differences were observed between one-, two-, and three-year-old needles collected in May (Table 1).

Composition of sterols and steryl esters. 24ξ-Ethyl cholesterol, probably sitosterol (configuration at C-24 was

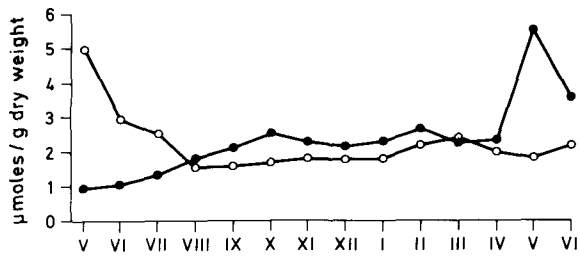


FIG. 1. Seasonal changes of free sterols (○) and steryl esters (●) in newly emerged needles of Scot's pine (n=7). Tree-to-tree variations, expressed as 95% confidence interval, were 6–20% of the mean for free sterols, and 11–38% for steryl esters. Bud break occurred at the end of April and needle maturation was in August. Roman numbers indicate months of collection. For significance of differences between means see the Results section.

TABLE 1

Free Sterols, Steryl Esters, and Lipid Phosphorus in Newly Emerged, One-, Two-, and Three-Year-Old Needles of Scot's Pine Collected Shortly After Bud Break

	Years							
	0		1		2		3	
Free sterols								
Total amounts[a]	4.75[b]	17[c]	1.85	7	1.86	9	1.52	45
Composition[d]								
Cholesterol	3.9	45	1.0	36	1.5	28	1.2	25
24ξ-Methyl cholesterol	10.0	10	10.5	12	8.6	14	7.7	27
Sitosterol	64.1	4	88.5	2	89.9	1	91.1	3
Isofucosterol	22.0	3	nd[e]		nd		nd	
Steryl esters								
Total amounts[a]	1.99	15	5.55	26	7.83	25	9.90	46
Composition[d]								
Cholesterol	4.0	27	1.0	60	1.0	80	1.2	50
24ξ-Methyl cholesterol	8.5	10	5.4	17	5.5	11	4.7	23
Sitosterol	86.8	3	92.5	1	92.0	1	92.7	2
Isofucosterol	0.7	56	1.1	48	1.5	73	1.4	92
16:0	24.8	15	8.7	14	8.5	16	7.1	23
18:0	6.0	22	5.1	23	4.9	24	2.9	45
18:1ω9	20.0	14	5.5	20	5.6	25	6.9	19
18:2ω6	20.2	14	26.7	10	25.3	10	28.5	18
$18:2\Delta^{5.9}$	9.5	18	15.5	8	14.8	8	15.4	16
18:3ω3	3.6	36	8.9	11	9.5	10	11.6	45
$18:4\Delta^{5.9.12.15}$	0.3	97	3.3	30	3.4	18	3.9	46
20:0	7.0	43	0.8	112	0.6	67	0.6	32
20:1ω9	3.2	62	0.3	95	1.5	89	1.6	51
20:2ω6	2.5	60	1.1	82	1.5	73	1.1	45
20:3ω3	0.9	52	7.0	46	6.0	55	1.9	42
$20:3\Delta^{5.11.14}$	2.0	20	17.1	9	18.4	11	18.5	22
Lipid Phosphorus[a]	19.2	19	5.7	28	6.9	22	5.5	49

[a] μmoles/g Dry weight.
[b] Means (n=7).
[c] Coefficient of variation (%).
[d] Percent of total amounts.
[e] Not detectable.

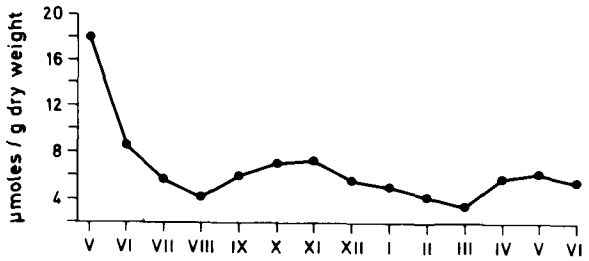


FIG. 2. Seasonal changes of lipid phosphorus in newly emerged needles of Scot's pine (n=7). Tree-to-tree variation, expressed as 95% confidence interval, was 9–45% of the mean. See Figure 1 for further explanations.

not determined), is the principal free sterol throughout the investigated period (Fig. 3). Campesterol (presumably with its C-24 epimer) was about 10% in all months investigated. Cholesterol was present only in minor amounts. In newly emerged needles, isofucosterol accounted for 25% of the total free sterols. Needles collected in subsequent months contained only trace amounts of isofucosterol.

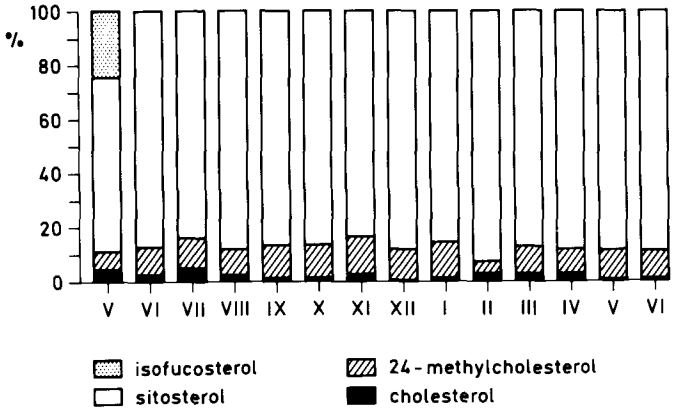


FIG. 3. Seasonal changes in the individual free sterols (relative amounts) in newly emerged needles of Scot's pine (n=7). Tree-to-tree variations, given as 95% confidence interval, were: cholesterol, 19–100%; 24-methyl cholesterol, 8–95%; sitosterol, 1–12%; and isofucosterol, 6%. See Figure 1 for further explanations.

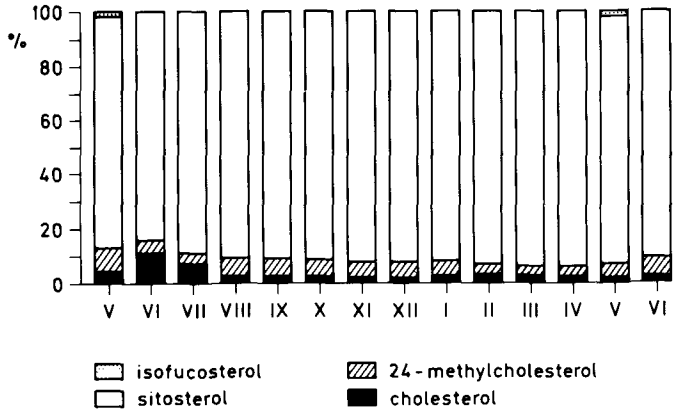


FIG. 4. Seasonal changes in the individual esterified sterols (relative amounts) in newly emerged needles of Scot's pine (n=7). Tree-to-tree variations, given as 95% confidence interval, were: cholesterol, 26–74%; 24-methyl cholesterol, 8–42%; sitosterol, 1–5%; isofucosterol, 50–100%. See Figure 1 for further explanations.

The steryl composition of the steryl ester fraction (Fig. 4) reflected the composition of the free sterol fraction. Sitosterol accounted for 85–95% of the steryl ester fraction throughout the year. The relative amounts of 24ξ-methyl cholesteryl esters and cholesteryl esters were 4–7% and 2–10%, respectively. Only trace amounts of isofucosteryl esters were detectable. Significant differences (mostly 99.9% level) in the steryl ester composition occurred between newly emerged and differentiated needles. Young needles contained relatively large amounts of 24ξ-methyl cholesteryl (in the first four weeks) and cholesteryl esters (in the first three months).

The major constituent fatty acids in the steryl ester fraction (Table 1) were palmitic (16:0), oleic (18:1ω9), and linoleic (18:2ω6) acid, together with two nonmethylene-interrupted polyunsaturated acids, $18:2\Delta^{5.9}$ and $20:3\text{-}\Delta^{5.11.14}$. Myristic (14:0), stearic (18:0), linolenic (18:3ω3), octadecatetraenoic ($18:4\Delta^{5.9.12.15}$), arachidic (20:0), eicosenoic (20:1ω9), eicosadienoic (20:2ω6), and eicosatrienoic (20:3ω3) acids were detected in minor amounts. Traces

TABLE 2

Seasonal Changes in the Individual Steryl Ester Fatty Acids (Relative Amounts) in Newly Emerged Needles of Scot's Pine

Month	16:0		18:1ω9		18:2ω6		18:2Δ5.9		18:3ω3		18:4 Δ5.9.12.15		20:0		20:1ω9		20:3ω3		20:3 Δ5.11.14		Others[a]	
5	22.6[b]	11[c]	18.3	12	20.9	11	11.7	18	3.2	12	1.2	25	5.1	35	3.2	91	1.9	105	2.1	48	9.8	12
6	13.0	23	12.6	28	27.6	17	12.2	28	4.9	10	1.5	47	3.0	27	2.9	15	2.4	79	9.5	12	10.4	106
7	10.9	19	7.6	18	29.0	19	11.5	23	6.7	13	2.0	30	2.1	57	2.3	87	3.1	59	14.6	13	10.2	99
8	13.1	18	10.7	42	26.0	8	11.5	40	9.5	16	2.5	36	1.7	53	0.6	72	2.9	68	16.5	17	5.0	29
9	10.4	18	9.6	41	23.0	7	13.4	23	7.9	26	3.3	27	1.3	77	0.5	12	4.0	44	20.7	21	5.9	70
10	9.5	19	6.9	32	21.3	13	15.3	27	8.0	16	4.7	34	1.3	78	0.7	18	6.1	58	21.2	20	5.0	68
11	11.2	16	9.4	44	19.9	10	15.9	22	7.5	23	4.6	28	1.1	73	0.6	31	6.4	54	18.6	17	4.8	93
12	13.0	17	8.8	29	18.1	10	14.9	17	9.4	19	5.4	44	2.0	75	0.5	81	5.1	49	16.2	19	6.6	15
1	11.9	11	9.5	39	18.6	11	16.2	15	8.0	19	5.4	37	1.4	57	nd[d]		6.0	66	16.3	10	6.7	91
2	9.2	27	7.1	11	20.7	10	20.3	23	7.6	28	5.0	26	1.2	42	0.7	57	3.6	55	16.2	24	8.4	63
3	8.9	20	8.5	13	20.1	11	20.7	13	6.4	17	5.5	9	1.2	58	1.2	58	3.9	45	14.9	11	8.7	78
4	8.2	12	7.2	19	24.2	8	20.7	14	6.3	17	4.4	20	1.3	31	1.5	126	4.0	50	14.5	9	7.7	18
5	8.7	14	5.5	20	26.7	10	15.5	8	8.9	11	3.3	24	0.8	98	0.3	91	7.0	46	17.1	9	6.2	86
6	9.5	14	5.6	29	25.3	7	11.9	7	9.5	12	2.1	19	1.6	83	1.1	82	4.4	54	16.4	14	12.6	132

[a] 18:0, 14:0, 12:0, and Different unsaturated C_{16} fatty acids. Differences between individual months are not significant (95% level).

[b] Means (n=7).

[c] Coefficients of variation (%).

[d] Not detectable.

(below 0.5%) of lauric acid (12:0) and various C_{16} polyunsaturated acids were present, but are not included in Table 1. Myristic, stearic, and eicosadienoic esters did not show any seasonal fluctuations (Table 2). The youngest needles (May) contained highest amounts of palmitic, oleic, arachidic, and eicosenoic acid and lowest amounts of linolenic, octadecatrienoic, and the two eicosatrienoic acids (99.9% level). The relative amounts of linoleic ester were higher in summer needles than in winter needles; those of the 18:2Δ5.9 acid esters were high in winter, but low in summer needles (99% level).

One-, two-, and three-year-old needles collected shortly after bud break were very similar in their free sterol and steryl ester compositions (Table 1).

DISCUSSION

Our results show that needles newly emerged in May contained high amounts of free sterols (Fig. 1) and lipid phosphorus (Fig. 2). Lipid phosphorus was associated with phosphatidylcholine, phosphatidylethanolamine, phosphatidylserine (traces), and probably phosphatidylinositol (unpublished data). Differentiated needles of conifers synthesize cell walls, resin ducts, cuticle, etc., associated with organelles, such as Golgi vesicles. Thus, the high amounts of free sterols and phospholipids probably indicate a high density of biomembranes which are typical for young and growing plant tissue (2). During maturation, the free sterol content decreased, which is in accordance with what has been observed for apple trees (17), but contrasts with what has been reported for herbaceous plants (23).

In Scot's pine, however, high amounts of sterols do not necessarily indicate the presence of biomembranes. Towards the center of the trunk, the free sterol/phospholipid ratio increases and the dead heartwood contains considerable amounts of free sterols, but no phospholipids (14,15). The free sterol/phospholipid ratio of needles remained constant (1:3) at all time periods studied. This may indicate that needle sterols are predominantly membrane constituents, while heartwood sterols have different functions. It has been suggested that they are part of a defense mechanism against pathogens (24,25).

In apple buds, an increase in free sterols and a decrease in steryl esters (possibly due to hydrolysis) was shown to occur at the end of dormancy and during spring (17). High levels of sterols in developing Scot's pine needles may also result from steryl ester hydrolysis in buds before bud break. Total amounts of steryl esters were low in newly emerged needles of Scot's pine (Fig. 1). In the subsequent months, they increased and remained constant from August to April of the following year. With the exception of the one-, two-, and three-year-old needles collected in May, there was a remarkable correlation between the amounts of free and esterified sterols ($r=0.708$; $P<0.01$; Fig. 5)—the higher the concentration of free sterols, the lower the concentration of esterified sterols.

The significance of steryl ester changes in needles of Scot's pine is difficult to assess because of the lack of information about the physiological role of these molecules in plants. The relationship between free and

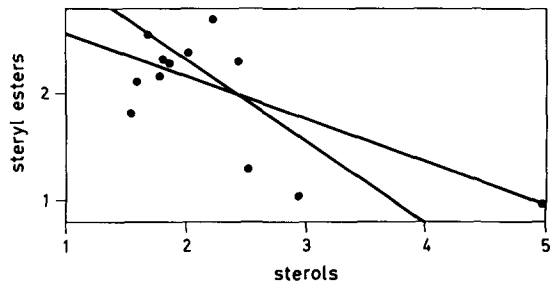


FIG. 5. Relationship between free sterols and steryl esters (μmoles/g dry weight) in Scot's pine needles during their first year. Both regression lines are given (YSE = 2.99 − 0.44XS and XS = 4.56 − 1.15 YSE).

esterified sterols in young pine needles may indicate that steryl esters serve as a store for sterols, as it is suggested for yeast steryl esters (26). It seems unlikely that the dramatic increase of steryl esters in the older pine needles during bud break (Table 1) is due to senescence.

When steryl esters increase in the older needles, stored or newly synthesized energy rich products, such as triglycerides and carbohydrates, are mobilized and translocated into the emerging twigs (27-29). Steryl esters may play a role in the mobilization of food reserves in mature needles, *e.g.*, in regulating lipolytic enzymes. It is an open question as to whether newly emerging needles obtain sterols from older needles, in addition to carbohydrates and triglycerides. Steryl esters may be involved in intercellular sterol transport, as it occurs in mammals and is postulated to occur in plants (2,30,31).

In accordance with earlier reports (10-13), sitosterol represented by far the largest amounts both in the free and esterified form. The relative amounts of 24ξ-methyl cholesterol (campesterol and dihydrobrassicasterol) were somewhat higher in the free sterols than in the steryl esters. In contrast, more cholesterol was found in esterified form. We did not detect 5α-sitostanol in Scot's pine needles, although this stanol is present in *Pinus sylvestris* wood in considerable amounts (10,15). Tissue specialization affecting the presence or absence of stanols has been reported from two species of *Larix* (9)—24ξ-methylstanol was present only in the wood, not in the bark. Nes (9) suggested that reduction of the Δ^5-bond plays some important role in wood. Stigmasterol, another common "main line" plant sterol, could not be detected in Scot's pine needles and is also absent in pine wood and bark (10-13,15).

Isofucosterol has not been reported in *Pinus* species before. In newly emerged needles, isofucosterol contributed about 25% of total free sterols. Only trace amounts of this sterol could be detected in needles collected in the subsequent months. In the steryl ester fraction, isofucosterol is negligible. 24(Z)-Ethylidene sterols, such as isofucosterol, are precursors of 24α-sterol products in higher plants (32-34). Since isofucosterol occurred almost exclusively in newly emerged needles, one can conclude that free sterols are formed *de novo* and are only (if at all) partly translocated from other parts (*e.g.*, older needles, wood) of the tree. The absence of isofucosterol in the steryl ester fraction indicates that reduction of the Δ24(28) double bond takes place prior to esterification in sitosteryl ester biosynthesis.

The composition of individual steryl ester fatty acids is complex and does not differ from the fatty acid composition of total needle lipids reported by Jamieson and Reid (35). In addition to the common methylene-interrupted fatty acids, four unusual, nonmethylene-interrupted polyunsaturated acids with Δ^5 unsaturation were present in Scot's pine needles. Δ^5 Acids have been found in all conifer leaf lipids examined (35).

The composition of individual free sterols did not (with the exception of newly emerging needles; Fig. 3) change during the course of the year. This contrasts with the results of studies on plants other than conifers; in tobacco (23), sorghum (7), and maize (30), the level of Δ^{22}-sterols like stigmasterol increased with plant age. Without using techniques such as ^1H nuclear magnetic resonance (NMR) or ^{13}C NMR, no information is available about

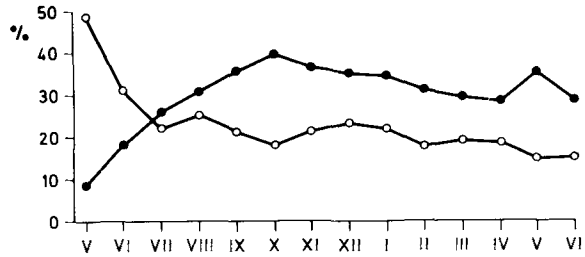


FIG. 6. Seasonal changes of steryl ester fatty acids differing in their degree of unsaturation (relative amounts) in newly emerged needles of Scot's pine (n=7). 16:0+20:0+18:1ω9+20:1ω9 (○); 18:3ω3+18:4$\Delta^{5.9.12.15}$+20:3ω3+20:3$\Delta^{5.11.14}$ (●). See Figure 1 for further explanations.

changes in the level of C-24 epimers during needle maturation. In *Cucurbita maxima*, an ontogenetic shift occurred from approximately equal amounts of 24α- and 24β-ethyl sterols to a predominance of the former during seedling development (36).

There was a shift in favor of 24ξ-methyl cholesterol and cholesterol in steryl ester composition during needle maturation (Fig. 4), but more pronounced changes occurred in the esterified fatty acids (Table 2). The proportion of polyunsaturated acids with three or four double bonds increased rapidly. This increase was accompanied by a rapid decrease of saturated or monounsaturated steryl ester fatty acids ($r=-0.825$; $P<0.001$; Fig. 6). Similar changes of fatty acids differing in their degree of unsaturation occurred on the leaf lipids of *Larix* during the early part of the growing season (35). The changes of the two 18:2-isomers in the steryl esters (Table 2) seemed to depend on needle development rather than on season. The relative amount of linoleic ester increased ($r=0.850$, $P<0.01$), and that of the 18:2$\Delta^{5.9}$ acid ester decreased ($r=-0.734$, $P<0.01$) with rising temperature. In other lipid fractions of Scot's pine needles, the seasonal changes of linoleic acid differed from those found in the steryl ester fraction; in the triglycerides, linoleic acid increased in winter and decreased in summer needles ($r=-0.670$, $P<0.01$; unpublished data).

The total amounts and the fatty acid composition of steryl esters extracted from *P. sylvestris* sapwood and heartwood did not change during the course of the year (18). Changes within the steryl esters described here for Scot's pine needles, however, are striking, and could reflect the physiology of needle differentiation.

ACKNOWLEDGMENTS

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METHOD

Selected Ion Monitoring Gas Chromatography/Mass Spectrometry of 1,2-Diacylglycerol *tert*-Butyldimethylsilyl Ethers Derived from Glycerophospholipids

Toshiaki Ohshima* and **Chiaki Koizumi**
Department of Food Science and Technology, The Tokyo University of Fisheries, Tokyo, Japan

Selected ion monitoring was used in conjunction with gas chromatography/mass spectrometry to analyze *tert*-butyldimethylsilyl ethers (*tert*-BDMS) of 1,2-diacyl-*sn*-glycerols derived from naturally occurring glycerophospholipids, including those of *Escherichia coli*, soybean, egg yolks and porcine liver. First, the fatty acid composition of the unknown glycerophospholipid was determined by gas-liquid chromatography (GLC) and, based on that, the fatty acids (mostly >0.5 wt%) were selected for monitoring the characteristic fragmentation ions produced from the fatty acid residues of the corresponding *tert*-BDMS derivatives of 1,2-diacyl-*sn*-glycerols. Next, the *tert*-BDMS derivatives were separated by GLC on a 65% methylphenylsilicone gum wall-coated open-tubular (WCOT) column according to the degree of unsaturation and the chain length of the fatty acid residues, and then directly introduced into the ion source of the mass spectrometer. The selected fragmentation ions, $[RCO+74]^+$ representative of the fatty acid residues, and $[M-57]^+$ indicative of the molecular weight of the derivatives, were monitored simultaneously. It thus became possible to determine the molecular species of the *tert*-BDMS derivatives by measuring a specific combination of two $[RCO+74]^+$ and an $[M-57]^+$ ion with the same retention time on the selected ion monitoring (SIM) profile. High background noise caused by volatilization of stationary phase at high temperature was largely overcome by selected ion monitoring. However, the fragmentation ion peaks produced from *tert*-BDMS derivatives of highly unsaturated glycerophospholipids showed a distorted SIM profile, which was attributed to interaction between the *tert*-BDMS derivatives and the methylphenylsilicone phase of the column. Use of a WCOT column with a more polar liquid phase is therefore recommended for the analysis of highly unsaturated molecular species.
***Lipids* 26, 940-947 (1991).**

In the past decade, wall-coated open-tubular (WCOT) column gas-liquid chromatography (GLC) has become a well accepted tool for the analysis of lipids. Earlier, apolar phases such as OV-1 and SE-30 were used in WCOT columns for the separation of triacylglycerols and related compounds based on the number of total carbon atoms present in the molecule (1-7). The subsequent development of "polar" phases such as methylphenylsilicone gum (equivalent to OV-17) with high temperature stability (up to 370°C) allowed for the separation of triacylglycerols not only according to chain length, but also according to the number of ethylenic bonds in the fatty acid residues (4,8-12). More polar cyanopropyl-phenylsilicone phases, such as SP 2330, have been applied to the resolution of trimethylsilylated and *tert*-butyldimethylsilylated 1,2-diradyl glycerols derived from glycerophospholipids (13-18). To accomplish assignments of peak components with a degree of certainty, ordinary mass spectrometry coupled with gas chromatography (GC/MS) on apolar phase WCOT columns have successfully been used (6,19, 20). Our preliminary studies with polar phase columns showed that high background noise caused by volatilization of stationary phase from the column, especially at high temperatures, interfered with the feeble signals arising from the samples. When the injected sample size was increased to improve the ratio of sample signal over background noise, sample-overload on the WCOT column occurred, and peak resolution suffered. Although the application of GC/MS on apolar columns can provide useful information (21,22), it requires time-consuming preliminary separations, such as by argentation thin-layer chromatography; furthermore, GLC retention times need to be assigned based on those of authentic standards. Selected ion monitoring (SIM) in GC/MS generally provides specificity and sensitivity for the selected ions which are characteristic of the constituent compounds. We recently separated vegetable oil triacylglycerols (23) and *tert*-BDMS of 1,2-diacyl-*sn*-glycerols derived from glycerophospholipids of marine origin (24-26) by GLC on a 65% methylphenylsilicone phase WCOT column, and demonstrated the applicability of SIM in GC/MS to the assignment of components separated by GLC on such a polar column. In the present study, selected ion monitoring in GC/MS was applied to the analysis of *tert*-BDMS of 1,2-diacyl-*sn*-glycerols derived from various naturally occurring glycerophospholipids.

MATERIALS AND METHODS

Glycerophospholipids. 1,2-Diacyl choline glycerophospholipids (CPL) from soybean, 1,2-diacyl ethanolamine glycerophospholipids (EPL) from *Escherichia coli*, and authentic 1-palmitoyl-2-stearoyl, 1-palmitoyl-2-oleoyl, 1-palmitoyl-2-linoleoyl, 1-palmitoyl-2-arachidonoyl, 1,2-dilinolenoyl and 1-stearoyl-2-linoleoyl CPL were purchased from Avanti Polar Lipids, Inc. (Pelham, AL). Turkey egg yolk CPL (60% purity) and porcine liver EPL (98% purity) were obtained from Sigma Chemical Company (St. Louis, MO). Hen egg yolk EPL and authentic 1,2-distearoyl CPL

*To whom correspondence should be addressed at Dept. of Food Science and Technology, The Tokyo University of Fisheries, Konan 4, Minato-ku, Tokyo 108, Japan.

Abbreviations: *tert*-BDMS, *tert*-butyldimethylsilyl ether; CPL, choline glycerophospholipid; EPL, ethanolamine glycerophospholipid; FID, flame ionization detector; GC/MS, gas chromatography/mass spectrometry; GLC, gas-liquid chromatography; SIM, selected ion monitoring; TC/DB, total acyl carbons *vs.* double bonds; WCOT; wall-coated open-tubular; Δ, cycropropanoic fatty acid, *e.g.*, 17:0Δ represents 17:0 fatty acid with a cyclopropanoid ring.

were purchased from Serdary Research Laboratories Inc. (London, Ontario, Canada).

Lipid derivatization. A 10-mg portion of CPL or EPL was mixed with 2 mL of 0.1 M Tris-HCl buffer (pH 7.2), containing 5 mM $CaCl_2$ and 1 mL diethyl ether/ethanol (98:2, v/v), and 400 units (0.1 mL in the above buffer) of phospholipase C of *Bacillus cereus* (Boehringer Mannheim, Germany) was added. The mixture was shaken vigorously for 2 hr at 37°C (27). The 1,2-diacylglycerol thus obtained was isolated from the reaction mixture by preparative thin-layer chromatography (TLC) on Silica Gel G (E. Merck, Darmstadt, Germany) using petroleum hydrocarbon (b.p. 30–60°C)/diethyl ether/acetic acid (70:30:1, v/v/v) as developing solvent. The 1,2-diacylglycerol was recovered from the silicic acid with diethyl ether, the extract was transferred into a reaction vial, and the solvent was evaporated under a nitrogen stream. An aliquot of *tert*-butyldimethylchlorosilane/imidazole reagent (Gasukuro Kogyo, Tokyo, Japan) was added to the reaction vial and the mixture was incubated at 80°C for 1 hr to obtain *tert*-butyldimethylsilyl ether (*tert*-BDMS) of 1,2-diacyl-*sn*-glycerol (28,29). The product was subsequently purified by preparative TLC on Silica Gel G using petroleum hydrocarbon/diethyl ether/acetic acid (90:10:1, v/v/v) as the first developing solvent and toluene as the second solvent in the same direction.

For the analysis of the fatty acid composition of the glycerophospholipids, an aliquot of CPL or EPL was mixed in a reaction vial with 3 mL 14% BF_3 in methanol (Wako Pure Chemical, Osaka, Japan) and heated at 100°C for 1 hr to obtain the corresponding fatty acid methyl esters (30).

Lipid analyses. The *tert*-BDMS derivatives of 1,2-diacyl-*sn*-glycerols were analyzed by GLC using a Shimadzu GC 12A instrument (Shimadzu Seisakusho, Kyoto, Japan), equipped with a flame ionization detector (FID), a MP65HT fused silica WCOT column (65% methylphenylsilicone, 0.1 μm film thickness, 0.25 mm i.d. × 25 m; Quadrex, New Haven, CT), and a Shimadzu movable on-column injector. The injector temperature was held at 370°C. The column oven was programmed from 230 to 350°C at 5°C/min. Helium was used as carrier gas at a constant inlet pressure of 1.7 Kg/cm^2. Usually 1–2 μL of a 0.02% hexane solution of a *tert*-BDMS derivative was injected.

Selected ion monitoring in the mass spectrometric analysis of *tert*-BDMS derivatives of 1,2-diacyl-*sn*-glycerols was done on a Shimadzu QP 1000 quadrupole mass spectrometer fitted with an electron impact ionization (EI) source to which the outlet of the MP65HT column (0.1 μm film-thickness, 0.25 mm i.d. × 15 m) was directly connected. Mass spectra were acquired at 0.4 second intervals using 3 KV accelerating energy, 70 eV electron beam energy, and a source temperature of 310°C.

The fatty acid compositions of CPL and EPL were determined by GLC using a Shimadzu GC 12A instrument equipped with an FID and a Supelcowax-10 fused silica WCOT column (0.25 μm film-thickness, 0.25 mm i.d. × 30 m, Supelco Japan, Tokyo) at 195°C with helium as carrier gas.

The molecular species are designated, for example, as 16:0-18:0, which describes the palmitoyl-stearoyl *tert*-BDMS derivative. It should be noted that the designation does not specify the positional distribution of the component fatty acids on the glycerol moiety.

RESULTS AND DISCUSSION

Selected ion monitoring of tert-BDMS derivatives prepared from authentic phospholipids. It is well established that the characteristic fragmentation ions produced by EI mass spectrometry of *tert*-BDMS of 1,2-diacyl-*sn*-glycerols include $[R+148]^+$, $[R+130]^+$, $[RCO+74]^+$, $[RCO]^+$ and $[R]^+$, indicative of the acyl chains, and $[M-57]^+$, $[M-RO]^+$ and $[M-RCOO]^+$ indicative of the molecular weight of the compounds (28,29). Observed ion intensities depend on the number of carbons and ethylenic bonds in the fatty acyl chains linked to the *sn*-1 and *sn*-2 positions of the glycerol backbone (31). Our preliminary studies indicated that neither $[RCO+74]^+$ nor $[M-57]^+$ overlapped with any other ions. Therefore, we chose $[RCO+74]^+$ and $[M-57]^+$ for selected ion monitoring. The calculated *m/z* of the selected fragmentation ions produced from typical *tert*-BDMS of 1,2-diacyl-*sn*-glycerols are summarized in Table 1.

TABLE 1

Calculated *m/z* of Fragmentation Ions from Typical Molecular Species of *tert*-Butyldimethylsilyl Ethers of 1,2-Diacyl-*sn*-glycerols Derived from Glycerophospholipids

TC/DB[a]	Molecular species of *tert*-BDMS diacylglycerol	*m/z* of Selected fragment ion	
		$[RCO+74]^+$	$[M-57]^+$
28:0	14:0-14:0	285	569
30:0	14:0-16:0	285, 313	597
	15:0-15:0	299	597
30:1	14:0-16:1	285, 311	595
31:0Δ[b]	14:0-17:0Δ	285, 325	609
32:0	16:0-16:0	313	625
32:1	16:0-16:1	313, 311	623
33:0	15:0-18:0	299, 341	639
	16:0-17:0	313, 327	639
33:0Δ	14:0-19:0Δ	285, 353	637
	16:0-17:0Δ	313, 325	637
33:1Δ	16:1-17:0Δ	311, 325	635
34:0	16:0-18:0	313, 341	653
34:0ΔΔ	17:0Δ-17:0Δ	325	649
34:2	16:0-18:2	313, 337	649
	17:1-17:1	325	649
34:3	16:0-18:3	313, 335	647
35:0	17:0-18:0	327, 341	667
35:0ΔΔ	17:0Δ-18:0Δ	325, 341	665
35:1	17:0-18:1	327, 339	665
35:1Δ	17:0Δ-18:1	325, 339	663
35:2	17:0-18:2	327, 337	663
36:0	18:0-18:0	341, 341	681
36:0ΔΔ	17:0Δ-19:0Δ	325, 353	677
36:2	18:0-18:2	341, 337	677
	18:1-18:1	339, 339	677
36:3	18:1-18:2	339, 337	675
36:4	16:0-20:4	313, 361	673
	18:2-18:2	337, 337	673
36:5	18:2-18:3	337, 335	671
36:6	18:3-18:3	335, 335	669
38:3	18:0-20:3	341, 363	703
38:4	18:0-20:4	341, 361	701
	18:1-20:3	339, 363	701
38:5	18:1-20:4	339, 361	699
38:6	16:0-22:6	313, 385	697
40:4	18:0-22:4	341, 389	729
40:6	18:0-22:6	341, 385	725

[a] TC, the number of total acyl carbons; DB, the number of double bonds.

[b] Δ Designates cyclopropanoic fatty acid.

Typical SIM profiles of the molecular species derived from authentic 1,2-diacyl glycerophospholipids are shown in Figure 1 (a) and (b). Prior to SIM of *tert*-BDMS of 1,2-diacyl-*sn*-glycerols derived from unknown glycerophospholipids, a preliminary analysis of the constituent fatty acids would be needed to determine the ions to be monitored. However, this was not necessary for the authentic phospholipids shown here (Fig. 1). At 8.2 min after injection, the unresolved peaks Nos. 1 and 2 were detected when the fragmentation ion at *m/z* 313 was selectively monitored. When the ions at *m/z* 341 and *m/z* 653 were monitored, two additional ion peaks, each having the same retention time as ion peak No. 1 shown on the trace of *m/z* 313, appeared on the SIM profile. The characteristic combination of these selected ion peaks is indicative of the molecular species 16:0-18:0, as predicted from the specific combinations of fragmentation ions listed in Table 1. For peak No. 2, from the two ion peaks with the same retention time at *m/z* 339 due to $[RCO+74]^+$ arising from an oleic acid (18:1) residue and at *m/z* 651 due to $[M-57]^+$ indicative of a total carbon/double bond ratio (TC/DB) of 34:1, a molecular species of 16:0-18:1 is suggested. For peak No. 6, a molecular species of 16:0-20:4 is indicated, because the fragmentation ions at *m/z* 313 due to the palmitic acid (16:0) residue, at *m/z* 361 due to the arachidonic acid (20:4) residue, and at *m/z* 673 indicative of $[M-57]^+$ of the molecular species of 36:4, were detected at the same retention time. The relative ion intensity of only 19 at *m/z* 361 was noticeably lower than that of 1770 at *m/z* 313, although both fragmentation ions were originally present at the same abundance in the molecule. The ion peak at *m/z* 673 due to $[M-57]^+$ of 36:4 was detected, as well as the peak at *m/z* 675, but the intensity of *m/z* 673 was twice as high as that of *m/z* 675; this is probably due to the more complex fragmentation patterns of polyunsaturated fatty acid residues (31). The lower stability of unsaturated molecular species of triacylglycerols in EI mass spectrometry previously has been pointed out (23). We conclude from the above results that not only the combination of the characteristic fragmentation ions shown in Table 1, but also the intensity of the ions should be considered to substantiate the assignment of peaks that reflect the molecular species.

Molecular species of tert-*BDMS of 1,2-diacyl-*sn*-glycerols from* E. coli *glycerophospholipids.* The ethanolamine glycerophospholipids of *E. coli* were taken as an example of a naturally occurring phospholipid. A typical gas chromatogram of the *tert*-BDMS of the 1,2-diacyl-*sn*-glycerols derived from EPL of *E. coli* is shown in Figure 2. In order to determine the $[RCO+74]^+$ ions suitable for selective ion monitoring in GC/MS, the fatty acid composition of *E. coli* EPL was determined by GLC/FID; the results obtained are shown in Table 2. Based on these data, the more prominent (mostly >0.5 wt%) fatty acid residues were chosen and their characteristic fragmentation ions were selectively monitored, as shown in Figure 3. The WCOT columns used in GLC/FID (Fig. 2) and in GC/MS (Fig. 3) had the same liquid phase, although the column lengths were different. Therefore, the elution orders of the *tert*-BDMS of 1,2-diacylglycerols in both GLC/FID and

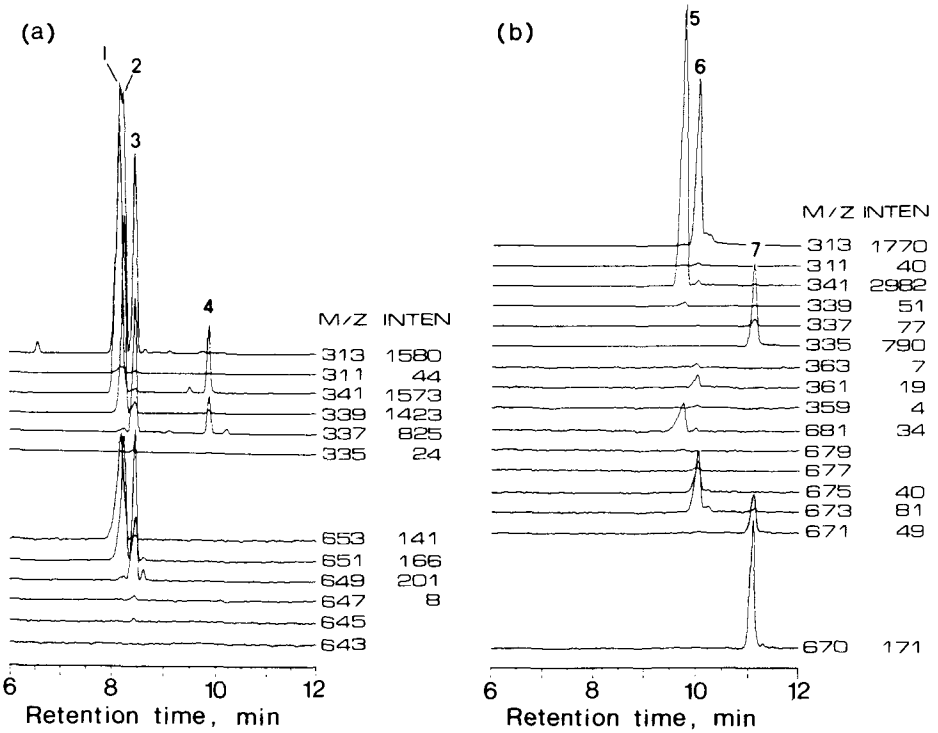


FIG. 1. Selected ion monitoring profiles arising from a mixture of *tert*-butyldimethysilyl ethers of 1-palmitoyl-2-stearoyl, 1-palmitoyl-2-oleoyl, 1-palmitoyl-2-linoleoyl, 1-stearoyl-2-linoleoyl, 1,2-distearoyl, 1-palmitoyl-2-linoleoyl, 1-stearoyl-2-linoleoyl, 1,2-distearoyl, 1-palmitoyl-2-arachidonoyl and 1,2-dilinolenoyl-*sn*-glycerols derived from authentic standards of glycerophospholipids. (a): 1, 16:0-18:0; 2, 16:0-18:1; 3, 16:0-18:2; 4, 18:0-18:2; and (b): 5, 18:0-18:0; 6, 16:0-20:4; 7, 18:3-18:3. Column, MP65HT (0.1 μm film-thickness, 0.25 mm i.d. × 15 m) at 230–350°C programmed at 5°C/min; ion source, 70 eV at 310°C.

TABLE 2

Fatty Acid Compositions (wt%) of Glycerophospholipids of Various Origins[a]

Fatty acid	*E. coli* EPL	Soybean CPL	Turkey egg yolk CPL	Hen egg yolk EPL	Porcine liver EPL
14:0	2.13 ± 0.02	0.12 ± 0.00	0.23 ± 0.01	—	—
14:1n-7	0.15 ± 0.00	0.63 ± 0.09	—	—	—
Pristanic	—	—	—	—	0.86 ± 0.01
15:0*anteiso*	—	0.10 ± 0.03	—	—	—
15:0	—	0.09 ± 0.02	0.09 ± 0.00	—	—
16:0	47.97 ± 0.13	17.53 ± 0.29	29.50 ± 0.88	17.06 ± 0.02	4.92 ± 0.04
16:1n-9	5.09 ± 0.02	—	—	—	—
16:1n-7	3.72 ± 0.02	—	2.20 ± 0.08	—	0.16 ± 0.01
16:1n-5	0.20 ± 0.00	—	0.04 ± 0.00	—	—
16:1*trans*	0.13 ± 0.00	—	—	—	—
Phytanic	0.50 ± 0.01	—	—	—	—
16:2n-7	0.87 ± 0.02	—	0.05 ± 0.00	—	0.18 ± 0.00
17:0	0.85 ± 0.02	0.16 ± 0.01	0.19 ± 0.00	0.30 ± 0.00	0.68 ± 0.00
16:3n-4	—	—	0.10 ± 0.00	—	—
17:1n-8	1.16 ± 0.02	—	—	—	0.13 ± 0.00
17:0Δ	8.61 ± 0.02	—	—	—	—
16:4n-3	—	—	0.17 ± 0.00	—	1.91 ± 0.02
17:2n-8	—	—	—	0.41 ± 0.00	—
17:2n-5	—	—	—	—	0.22 ± 0.00
16:4n-1	—	—	—	—	0.15 ± 0.00
18:0	0.71 ± 0.00	3.98 ± 0.02	14.92 ± 0.22	26.98 ± 0.02	37.19 ± 0.13
18:1n-9	1.66 ± 0.01	10.54 ± 0.03	25.15 ± 0.25	18.48 ± 0.03	6.00 ± 0.22
18:1n-7	9.26 ± 0.05	1.59 ± 0.02	1.36 ± 0.02	0.94 ± 0.02	1.02 ± 0.01
18:1n-5	—	—	0.11 ± 0.01	—	0.22 ± 0.01
19:0Δ	7.82 ± 0.04	—	—	—	—
18:2n-6	0.14 ± 0.01	59.82 ± 0.09	16.32 ± 0.15	13.32 ± 0.00	7.52 ± 0.03
18:3n-3	0.59 ± 0.01	4.68 ± 0.02	0.11 ± 0.00	—	0.17 ± 0.00
18:3b[b]	3.31 ± 0.02	—	—	—	—
18:3c[b]	1.21 ± 0.02	—	—	—	—
20:1n-9	0.31 ± 0.01	—	0.10 ± 0.01	0.13 ± 0.01	0.10 ± 0.00
20:1n-7	—	0.10 ± 0.00	—	0.14 ± 0.00	0.14 ± 0.00
20:2n-6	0.04 ± 0.00	—	—	0.22 ± 0.00	0.18 ± 0.00
20:2h[b]	1.83 ± 0.02	—	—	—	—
20:4n-6	—	—	4.27 ± 0.13	13.47 ± 0.02	29.19 ± 0.10
20:5n-3	—	—	—	—	0.42 ± 0.05
21:5n-3	—	—	—	0.16 ± 0.00	—
22:4n-6	—	—	—	0.62 ± 0.01	2.11 ± 0.02
22:5n-3	—	—	0.13 ± 0.00	0.21 ± 0.01	2.17 ± 0.03
22:6n-3	—	—	1.57 ± 0.01	2.22 ± 0.01	1.41 ± 0.02
24:1n-9	—	—	—	—	0.21 ± 0.02

[a]Data are presented as mean ± S.D. (n=3).

[b]Positional isomers (ref. 37).

GC/MS could be expected to be similar. The procedure for peak assignments was as follows:

Peak No. 1 in Figure 3 posesses three characteristic fragmentation ions at *m/z* 285, *m/z* 313 and *m/z* 597. These are due to the $[RCO+74]^+$ from the myristic acid (14:0) residue, $[RCO+74]^+$ from the palmitic acid (16:0) residue, and $[M-57]^+$ of the molecular species with a TC/DB ratio of 30:0, respectively. Consequently, the species corresponding to peak No. 1 was identified as 14:0-16:0.

For peak No. 2, the combination of the fragmentation ion peaks at *m/z* 285 due to the myristic acid residue and at *m/z* 311 due to palmitoleic acid (16:1) suggested that the peak component may be 14:0-16:1; however the ion intensity at *m/z* 595 due to $[M-57]^+$ of the molecular species 30:1 was feeble.

The fragmentation ion at *m/z* 285 of peak No. 3 was considered to be due to a myristic acid residue. It was difficult to confirm the precursors of the corresponding fragmentation ion *m/z* 325 based on mass spectrometric data only because the EI mass spectra of cyclopropanoic fatty acids and of the corresponding normal-chain fatty acids are generally quite similar. There are two possibilities in regard to the origin of the fragmentation ion at *m/z* 325; $[RCO+74]^+$ could represent a 17:1 residue or a cyclopropanoic fatty acid (17:0Δ) residue. EPL of *E. coli* contains 17:0Δ as one of the prominent constituent fatty acids as shown in Table 2. The fatty acid composition confirms that most of the fragmentation ions at *m/z* 325 were therefore derived from 17:0Δ cyclopropanoic acids. In general, cyclopropanoic fatty acids are relatively resistant to electron impact ionization (32), and therefore their fragmentation becomes similar to that of normal fatty acids (32). In the present case, 17:0Δ acid is esterified to the glycerol moiety, and the esterified 17:0Δ would be

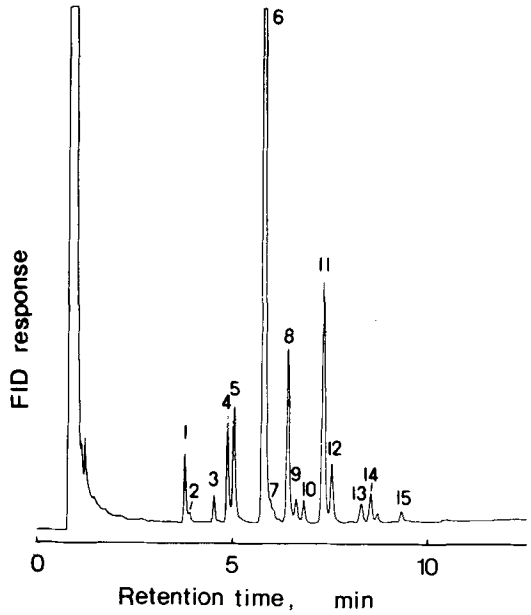


FIG. 2. FID open-tubular gas chromatogram of *tert*-butyldimethylsilyl ether of 1,2-diacyl-*sn*-glycerol derived from *Escherichia coli* ethanolamine glycerophospholipids. Column, MP65HT (0.1 μm film-thickness, 0.25 mm i.d. × 25 m) at 230–350°C programmed at 5°C/min; injector, a solventless moveable on-column injector at 370°C; carrier gas, helium at 1.75 Kg/cm^2.

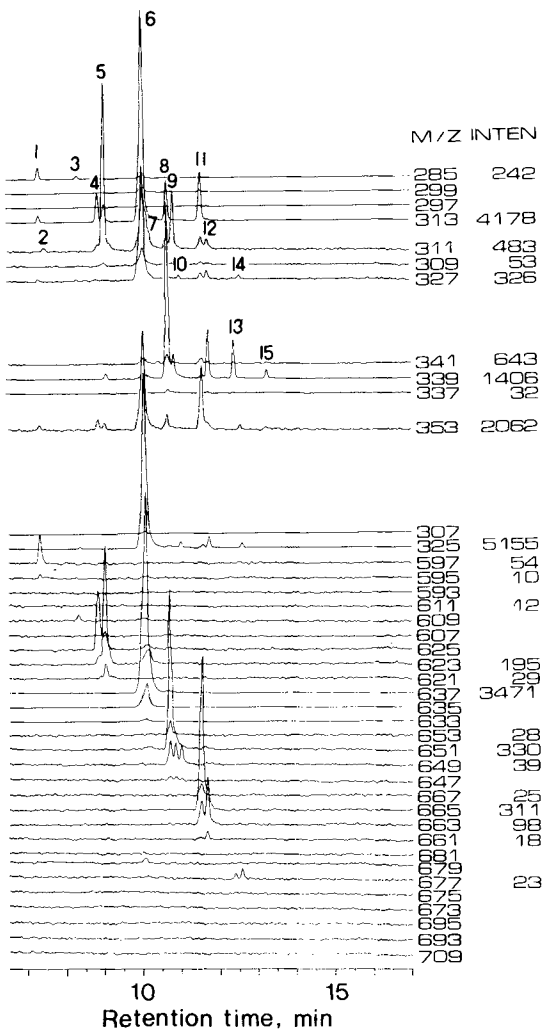


FIG. 3. Selected ion monitoring profiles arising from *tert*-butyldimethysilyl ether of 1,2-diacyl-*sn*-glycerols derived from *Escherichia coli* ethanolamine glycerophospholipids. The conditions are as in Figure 1. See the text for peak assignments.

expected to be more stable against EI ionization than the corresponding free fatty acids or methyl esters. Consequently, the molecular species of peak No. 3 was identified as 14:0-17:0Δ with the ions at *m/z* 325 and *m/z* 609 being mainly due to the 17:0Δ acid residue and to a molecular species of 31:0Δ, respectively. A small amount of 14:0-17:1 would also expected to exist in peak No. 3.

The dominant molecular species of peak No. 4 was identified as 16:0-16:0, since *m/z* 313 due to $[RCO+74]^+$ of the palmitic acid residues and *m/z* 625 due to $[M-57]^+$ of molecular species were selectively monitored.

Peak No. 5 consists mainly of 16:0-16:1, since *m/z* 311 due to the palmitoleic acid residue and *m/z* 313 due to the palmitic acid residue were observed with *m/z* 623 due to the molecular species 32:1. A fragmentation in peak at *m/z* 353 with weak intensity showed that 13:0-19:0Δ was indicated as a minor component of peak No. 5.

Peak No. 6 was a prominent component of *E. coli* EPL as estimated from the FID gas chromatogram in Figure 3. The fragmentation ions detected were relatively complicated. The fatty acid composition of the EPL shown in Table 2 clearly shows that the fragmentation ion at *m/z* 325 was mainly due to the 17:0Δ acid residue. Similarly, the ion at *m/z* 353 was due to a cyclopropanoic 19:0Δ acid residue. Consequently, there were four molecular species in peak No. 6—16:0-17:0Δ and 16:0-17:1 based on ions *m/z* 313, *m/z* 325 and *m/z* 637, 16:1-17:0 based on ions *m/z* 311, *m/z* 327 and *m/z* 637, and 14:0-19:0Δ based on ions *m/z* 285, *m/z* 353 and *m/z* 637. From the intensity of the ions, we estimated that 16:0-17:0Δ was the most prominent molecular species in peak No. 6.

Peak assignments of Nos. 7, 8 and 9 were relatively easy. Peak No. 7 was assigned to the molecular species 16:1-17:0Δ and 16:1-17:1. Peaks Nos. 8 and 9 were 16:0-18:1 and 16:1-18:1, respectively.

It was difficult to identify all components contained in peak No. 10 in detail, since the combinations of fragmentation ions at *m/z* 325 due to 17:0Δ and/or 17:1 and *m/z* 649 due to 34:1Δ and/or 34:2 indicate the following possibilities—17:1-17:1, 17:0Δ-17:0Δ and 17:1-17:0Δ. Based on the fatty acid composition given in Table 2, 17:0Δ-17:0Δ would be the most prominent component of peak No. 10.

The characteristic fragmentation ions of peak No. 11 suggested that 16:0-19:0Δ would be a prominent molecular species; 18:0-17:0Δ also was included in the peak as a minor component.

Peak Nos. 12, 13, 14 and 15 were easily identified as 18:1-17:0Δ and 18:1-17:1, 18:1-18:1, 17:0Δ-19:0Δ and 17:1-19:0Δ, and 18:1-19:0Δ, respectively.

Molecular species of glycerophospholipids with one cyclopropanoic fatty acid residue, such as 16:0-17:0Δ and 16:0-19:0Δ, have been reported in *E. coli* phosphatidylglycerol (33) and EPL (34). The molecular species composition of glycerophospholipids are known to be affected by

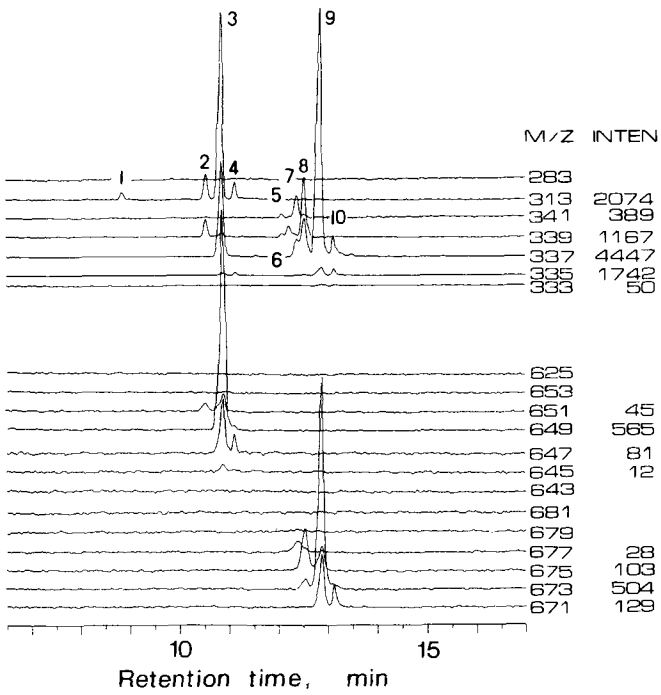


FIG. 4. Selected ion monitoring profiles arising from *tert*-butyldimethysilyl ether of 1,2-diacyl-*sn*-glycerols derived from soybean choline glycerophospholipids. 1, 16:0-16:0; 2, 16:0-18:1; 3, 16:0-18:2; 4, 16:0-18:3, 5, 18:0-18:0; 6, 18:1-18:0; 7, 18:0-18:2; 8, 18:1-18:2; 9, 18:2-18:2; 10, 18:2-18:3. The conditions were those given in Figure 1.

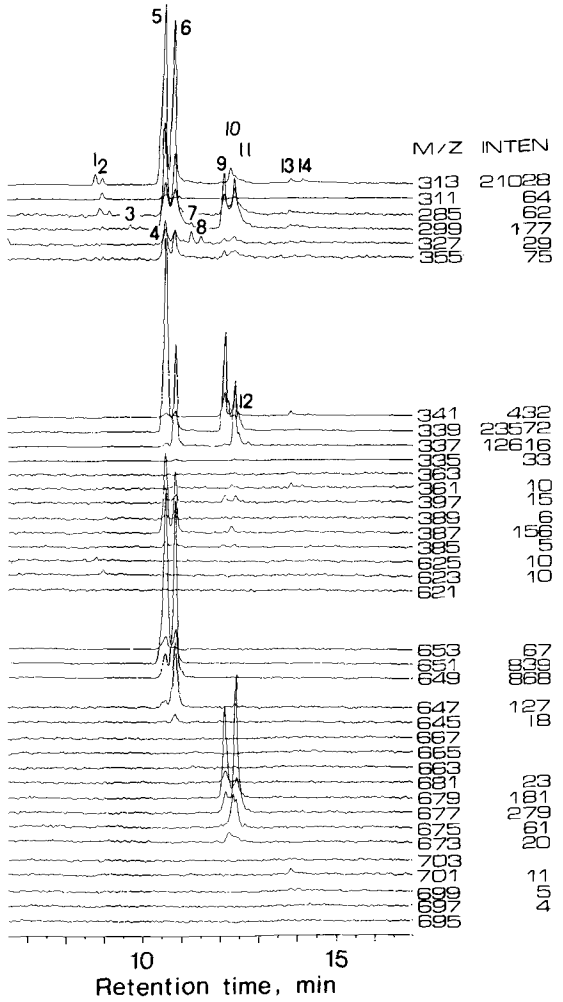


FIG. 5. Selected ion monitoring profiles arising from *tert*-butyldimethysilyl ether of 1,2-diacyl-*sn*-glycerols derived from turkey egg yolk choline glycerophospholipids. 1, 16:0-16:0, 2, 16:0-16:1; 3, 15:0-18:1; 4, 16:0-18:0; 5, 16:0-18:1; 6, 16:0-18:2; 7, 17:0-18:1; 8, 17:0-18:2; 9, 18:0-18:1n-9; 10, 18:0-18:1n-7; 11, 18:0-18:2; 12, 18:1-18:2; 13, 18:0-20:4; 14, 18:1-20:4. The conditions were those given in Figure 1.

changes in environmental conditions (33,34). However, molecular species containing cyclopropanoic fatty acid residues on both the *sn*-1 and the *sn*-2 positions of the glycerol moiety, such as 17:0Δ-17:0Δ and 17:0Δ-19:0Δ, as we determined in the present study, are novel findings in the *E. coli* EPL. Under our experimental conditions, a molecular species having a cyclopropanoic fatty acid residue gives the same retention time in GLC as a species having fatty acid with one ethylenic double bond.

Further applications. The SIM profiles of *tert*-BDMS derivatives of 1,2-diacyl-*sn*-glycerols prepared from glycerophospholipids of soybean, turkey egg yolk, hen egg yolk, and porcine liver are shown in Figures 4, 5, 6 and 7, respectively. The results of previous fatty acid analyses are shown in Table 2. Applications of the present techniques demonstrated that complete separation between highly unsaturated molecular species was difficult on the liquid phase used, as has been pointed out elsewhere (12,23). The derivatives from soybean CPL, possessing up to three ethylenic bonds per fatty acid residue, were separated well under the present experimental conditions. However, hen egg yolk EPL (Fig. 6), rich in 20:4n-6 (Table 2), showed poorer resolution than did turkey egg yolk CPL (Fig. 5). The results shown in Figure 7 are consistent with the above description: porcine liver EPL with higher levels of polyunsaturated fatty acids (PUFA), probably from an animal fed a fish meal diet (35), showed relatively poor resolutions between highly unsaturated molecular species such as 18:0-20:4 (peak No. 10) and 16:0-22:6 (peak No. 11). This could be attributed to a partial polymerization of the PUFA residues on the WCOT column in the high temperature range (12), or be due to geometric isomer artifacts produced at higher elution temperature (36).

Usually the SIM approach has been restricted to the quantitative analysis of known materials. In this study, however, SIM was applied to unknown materials. Reliable quantification can be accomplished by the combination of GLC/FID and GC/MS-SIM. One of the strong points of the present technique is that peak assignment is possible despite high background levels caused by column bleeding, which appears unavoidable with polar phase columns. A comparison of the retention times between authentic standards and samples, which are indispensable in apolar phase WCOT column GLC, is not always required in the present technique. Once the fatty acid composition of an unknown glycerophospholipid is known, then exact peak assignments become possible. However, severe distortions of peaks corresponding to highly unsaturated molecular species can occur under present conditions. This means that the method has somewhat limited practicality for such samples. Therefore, even more

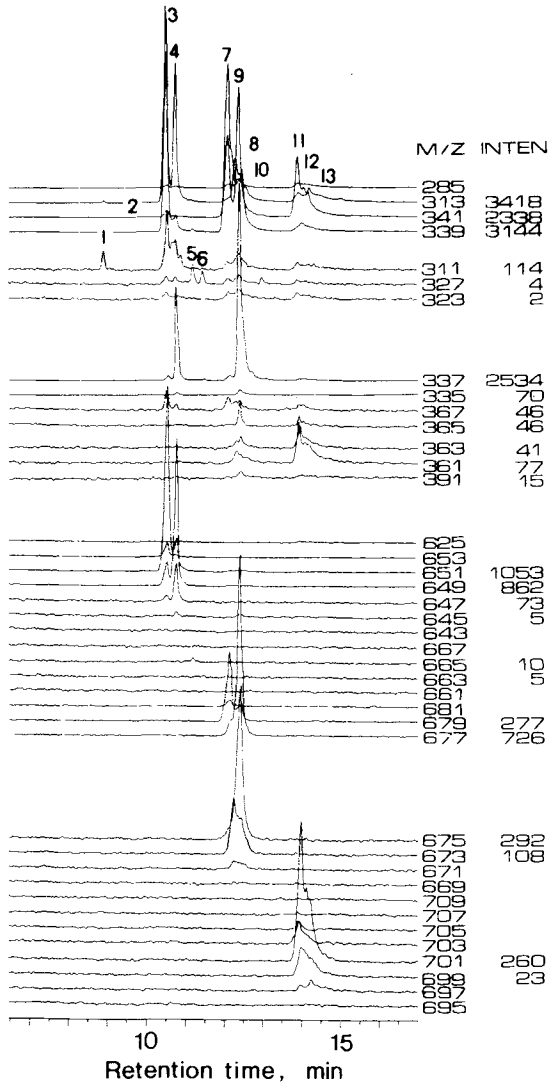


FIG. 6. Selected ion monitoring profiles arising from *tert*-butyldimethysilyl ether of 1,2-diacyl-*sn*-glycerols derived from hen egg yolk ethanolamine glycerophospholipids. 1, 16:0-16:0; 2, 15:0-18:0; 3, 16:0-18:1; 4, 16:0-18:2; 5, 17:0-18:0; 6, 17:0-18:1; 7, 18:0-18:1n-9; 8, 18:0-18:1n-7; 9, 18:1n-9:18:2; 10, 18:1n-7-18:2; 11, 18:1-20:4; 12, 18:1-20:3; 13, 18:1-20:4. The conditions were those given in Figure 1.

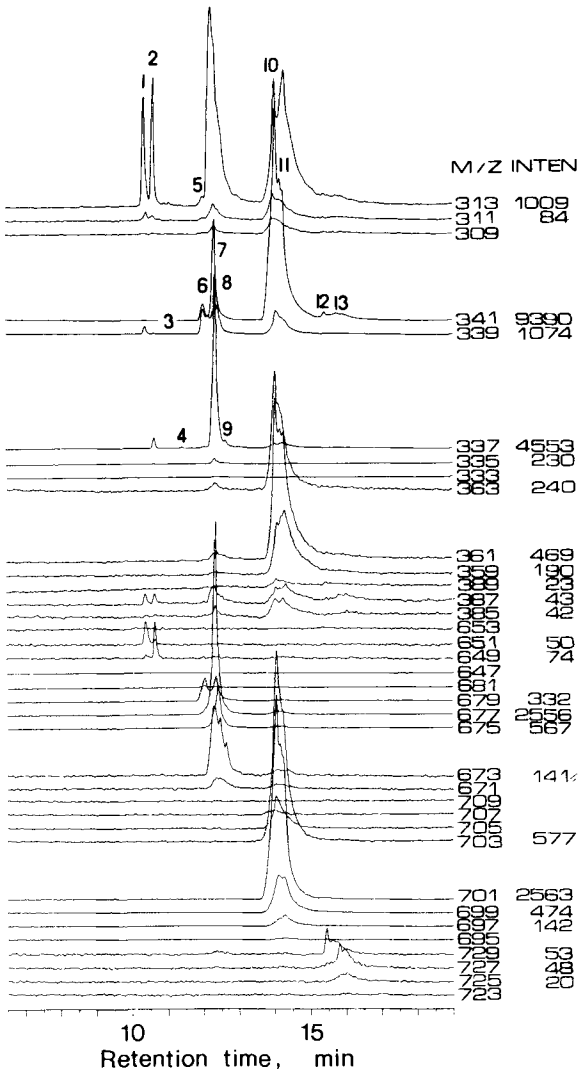


FIG. 7. Selected ion monitoring profiles arising from *tert*-butyldimethysilyl ether of 1,2-diacyl-*sn*-glycerols derived from porcine liver ethanolamine glycerophospholipids. 1, 16:0-18:1; 2, 16:0-18:2; 3, 17:0-18:1; 4, 17:0-18:2; 5, 18:0-18:1n-9; 6, 18:0-18:1n-7; 7, 18:0-18:2; 8, 18:1-18:2; 9, 18:2-18:2; 10, 18:0-20:4; 11, 16:0-22:6; 12, 18:0-22:4; 13, 18:0-22:6. The conditions are as in Figure 1.

polar columns to improve resolution of highly unsaturated molecular species are needed. Further applications of the technique to other lipid classes separated on more polar columns would be expected.

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Double-Bond Localization in Heneicosapentaenoic Acid by a Gas Chromatography/Mass Spectrometry (GC/MS) Method

Kohei Yamamoto[a,1], **Akira Shibahara**[b,*], **Takao Nakayama**[a,1] and **Goro Kajimoto**[b,2]
[a]General Testing Research Institute of Japan Oil Stuff Inspectors' Corporation, Kobe 658, and [b]Department of Nutrition, Kobe-Gakuin University, Kobe 651-21, Japan

Double-bond locations in heneicosapentaenoic acid from eel lipids were determined by a method involving gas chromatography/mass spectrometry (GC/MS). The methyl esters of the pentaenoate fraction were first partially reduced with hydrazine. The resulting *cis*-monoenoates were then methylthiolated, and the resultant adducts were analyzed by GC/MS. The key fragmentation ions generated by the cleavage between the methylthio-substituted carbons were used to ascertain the original double-bond positions in the native fatty acid esters. Based on mass spectral evidence, the acid was identified as all-*cis*-6,9,12,15,18-heneicosapentaenoic acid.
***Lipids 26,* 948–950 (1991).**

We have recently reported a convenient method for determining the double-bond positions in polyenoic fatty acids using gas chromatography/mass spectrometry (GC/MS) (1). The method involves partial reduction of methyl polyenoate(s) with hydrazine, methylthiolation of the resulting monoenoates, and GC/MS analysis of the resultant adducts. With this method, it is only necessary to prior isolate the methyl polyenoate(s) from the other methyl esters. This is accomplished by conventional fractionation based on the degree of unsaturation before partial reduction with hydrazine. In the present paper, we describe the application of this approach to the determination of the double-bond positions in heneicosapentaenoic acid (21:5), which was recently detected in trace amounts in eel lipids using capillary gas chromatography (GC).

Fatty acids with odd-numbered carbon chains have been recognized as normal but minor components of many natural lipids (2). Mayzaud and Ackman (3) focused on an odd-chain polyenoic acid, 21:5, in marine lipids and identified the acid in seal oil as all-*cis*-21:5(Δ6,9,12,15,18). These researchers (3) also cited and discussed the previous data which their group had obtained concerning the occurrence of 21:5 in several marine organisms (3; and references therein). Other structures of 21:5 that had been suggested previously include 21:5n-2 in herring oil (4) and 21:5n-5 in *Euglena gracilis* lipids (5).

MATERIALS AND METHODS

All chemicals were analytical reagent grade, and solvents were distilled before use.

The edible parts of eels *(Anguilla japonica),* grown in Lake Hamana (in Shizuoka prefecture), were homogenized with chloroform/methanol (2:1, v/v) (6). The lipids obtained were converted to fatty acid methyl esters (FAME) by methanolysis (0.5 N KOH/methanol) and successive methylation (BF_3/methanol) (6). The resulting FAME were purified by silica gel column chromatography (6).

An aliquot (*ca.* 0.2 g) of the purified FAME was dissolved in 50 mL of methanol containing 5 g of urea, stirred for 20 min at 50°C, and allowed to stand for 60 min at −20°C. The non-urea-complexing FAME mixture was filtered while below 0°C. After adding water, the filtrate was extracted twice with hexane. The recovered fraction, rich in polyenoates, was then fractionated by argentation thin-layer chromatography (AgTLC) (6) with hexane/diethyl ether (50:50, v/v), and the pentaenoate band corresponding to authentic all-*cis*-20:5(Δ5,8,11,14,17) methyl ester was recovered.

The pentaenoate fraction (*ca.* 5 mg) was dissolved in 1.2 mL of an 8.3% (v/v) methanolic hydrazine hydrate solution, containing 0.1 mL of 30% H_2O_2, and was stirred for 90 min at 50°C. After acidification with 6 N HCl, the resultant products were extracted with hexane and separated by AgTLC with hexane/diethyl ether (80:20, v/v) as developing solvent. The monoenoate band corresponding to authentic methyl *cis*-monoenoates was scraped off and extracted with diethyl ether. The extract was then washed several times with a saturated NaCl solution to remove trace amounts of contaminating $AgNO_3$, which would be strongly inhibitory to the formation of dimethyl disulfide (DMDS) adducts. The separations were monitored by capillary GC (7), capillary GC/MS (7), and packed-column GC/MS using the chemical-ionization mode (100 eV) with isobutane as a reactant gas.

The purified monoenoate fraction was methylthiolated according to our earlier procedure (6,8) with the following modifications. The fraction (*ca.* 0.5 mg) was incubated with 0.3 mL of DMDS containing 4 mg of I_2 for 30 min at 35°C. After adding 1 mL of hexane/diethyl ether (1:1, v/v) with stirring, a 10% (w/v) aqueous $Na_2S_2O_3$ solution was added with vigorous vortexing until the color of I_2 disappeared. The mixture was allowed to stand for a few minutes, and then an aliquot of the upper phase containing the resultant adducts was withdrawn and injected directly into a packed-column GC/MS system. The GC/MS system was operated in the electron impact ionization mode (20 eV) under conditions similar to those reported earlier (6).

Other analytical procedures were the same as those used in our previous work (6).

RESULTS AND DISCUSSION

Figure 1 shows a capillary gas chromatogram of the pentaenoate fraction prepared from eel lipids. Authentic

[1]Mailing address: General Testing Research Institute of Japan Oil Stuff Inspectors' Corporation, Mikagetsuka-machi, Higashinada-ku, Kobe 658, Japan.

*To whom correspondence should be addressed at Department of Nutrition, Kobe-Gakuin University, Arise, Ikawadani-cho, Nishi-ku, Kobe 651-21, Japan.

[2]Mailing address: Department of Nutrition, Kobe-Gakuin University, Arise, Ikawadani-cho, Nishi-ku, Kobe 651-21, Japan.

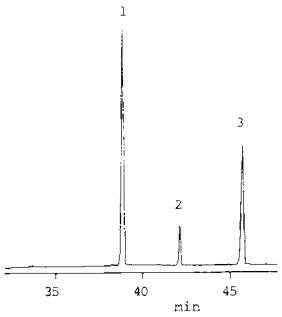


FIG. 1. Capillary gas chromatogram of fatty acid methyl esters of the pentaenoate fraction prepared from eel lipids. Peak identifications are discussed in the text. Peak 1, 20:5; 2, 21:5; 3, 22:5. Column, fused silica ULBON HR-SS-10 (50 m × 0.25 mm i.d., chemically bonded type); column temperature, isothermal at 100°C for 5 min, programmed at 4°C/min to 210°C and held for an additional 20 min; carrier gas, helium at 18.2 cm/s.

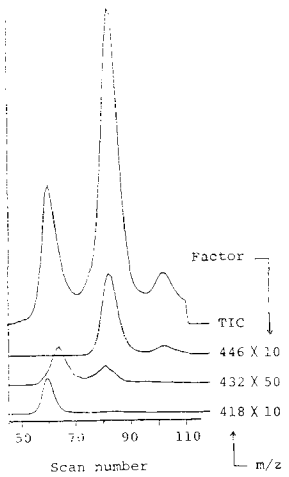


FIG. 2. Mass chromatogram of dimethyl disulfide adducts of methyl monoenoates formed by partial hydrazine reduction of the pentaenoate fraction. The ions at *m/z* 418, 432 and 446 are the molecular ions of the adducts of 20:1, 21:1 and 22:1 methyl esters, respectively. The predominant adducts of major 20:1 methyl esters are cut off from the analytical line by using an automated pre-cut valve to prevent ion source contamination. Column, 1 m × 3 mm i.d. glass column packed with 2% OV-101 on 100–120 mesh Chromosorb WHP; column temperature, 250°C; carrier gas, helium at 40 mL/min; ionization energy, 20 eV; scan range, 0–450 a.m.u. at 5.1 s/cycle.

all-*cis*-20:5(Δ5,8,11,14,17) methyl ester eluted at the same position as peak 1. For peaks 1, 2 and 3 in Figure 1, GC/MS analysis, using the chemical-ionization mode, showed quasi-molecular ions (M + 1) at *m/z* 317 (for peak 1), 331 (for peak 2) and 345 (for peak 3); the ions indicated the presence of 20:5, 21:5 and 22:5 methyl esters. In the other fractions, none of the peaks coincided with peak 2 and no quasi-molecular ion at *m/z* 331 was detected by capillary GC and GC/MS using chemical-ionization.

The infrared and ultraviolet spectra (data not included) of the pentaenoate fraction showed no absorption in the 960–970 cm^{-1} region or in the 230–235 nm region, which indicated that the fraction contained neither *trans* double bonds nor conjugated double bonds. The unsaturated ester corresponding to peak 2 in Figure 1 had the same mobility in AgTLC as did authentic all-*cis*-20:5(Δ5,8,11,14,17) methyl ester (see Materials and Methods). These results suggested that the three esters (peaks 1, 2 and 3 in Fig. 1) had similar double-bond configurations and methylene-interrupted arrangements.

After complete hydrogenation of an aliquot of the pentaenoate fraction in the presence of platinum black (6), three peaks were detected in capillary GC. The retention times of the peaks agreed with those of authentic straight chain 20:0, 21:0 and 22:0 methyl esters, respectively. Capillary GC/MS analysis of the hydrogenated products gave the same mass spectra as did the authentic standards. Hence, the unsaturated ester corresponding to peak 2 in Figure 1 was determined to be 21:5 methyl ester with a straight fatty acid chain.

The pentaenoate fraction was then partially reduced with hydrazine. From the reduced products, a *cis*-monoenoate fraction was recovered by AgTLC. No band for *trans*-monoenoate was observed. After methylthiolation, the DMDS adducts of the monoenoates were analyzed by packed-column GC/MS. Figure 2 shows the mass chromatogram, in which the ions at *m/z* 418, 432 and 446 are the molecular ions of the adducts of 20:1, 21:1 and 22:1 methyl esters, respectively.

Two peaks were registered at scan numbers 63 and 79 on the mass chromatogram of the molecular ion at *m/z* 432. In general, DMDS adducts of monoenoic positional isomers having an identical chain length are partially separated even on a short packed-column; this is due to the position of the carbons to which two methylthio groups are attached. As shown in Figure 2, the DMDS adducts (*m/z* 446) of 22:1 methyl esters (originating from 22:5 methyl ester) eluted as two peaks, and the DMDS adducts (*m/z* 418) of 20:1 methyl esters (originating from 20:5 methyl ester) eluted in the same pattern. In this mass chromatogram, the predominant DMDS adducts of major 20:1 methyl esters, which would be expected to elute before scan number 50, were cut off by using an automated pre-cut valve to prevent ion source contamination.

Figure 3 shows the mass spectra used for confirming the original double-bond positions in the 21:5 methyl ester and illustrates the fragmentation patterns of the individual adducts. The electron impact ionization of the adducts produced the recognizable molecular ion at *m/z* 432 and the three representative key fragmentation ions. Of the three definitive fragmentation ions, two were generated by the cleavage between the methylthio-substituted carbons at the original double-bond position in the molecule, and the remaining ion was derived by loss of methanol from the ion containing the ester group (6–8).

In spectrum A, the presence of DMDS adducts of 21:1(Δ6), 21:1(Δ9) and 21:1(Δ12) methyl esters was confirmed based on the detection of sets of the key fragmentation ions at *m/z* 257, 175 and 143, at *m/z* 215, 217 and 185, and at *m/z* 173, 259 and 227, respectively (*cf.* structures in Fig. 3). The presence of DMDS adducts of 21:1(Δ15) methyl ester (spectrum B) and 21:1(Δ18) methyl ester (spectrum C) was confirmed in the same manner based on mass spectral evidence (*cf.* structures in Fig. 3). The peaks of the unmarked ions in spectrum B were those overlapping in spectrum A; the peaks of the unmarked ions in spectrum C were due to the fragmentation of DMDS adducts of 22:1 methyl esters which co-eluted in the mass chromatogram (*cf.* Fig. 2). These results suggested that the original double-bond positions in these

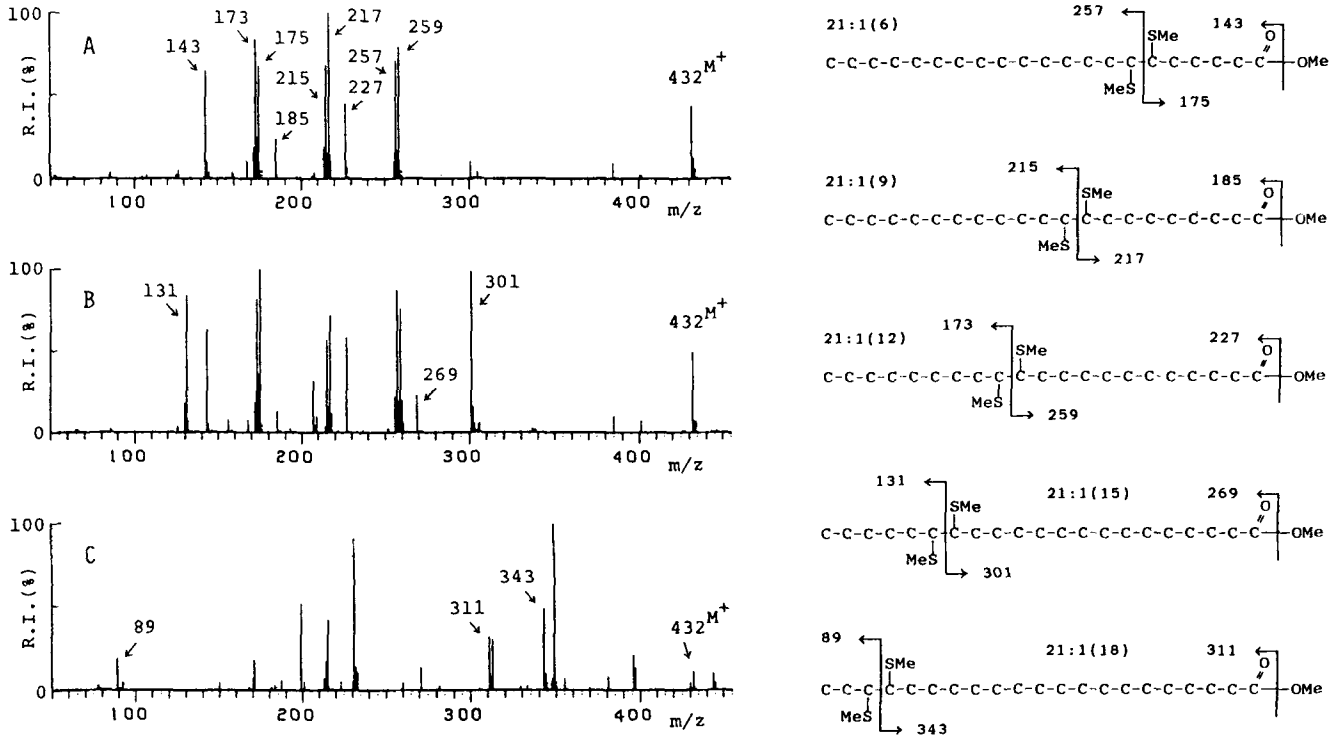


FIG. 3. Mass spectra of dimethyl disulfide adducts of 21:1 methyl esters. Spectra A, B and C are measured at scan numbers 63, 65 and 79, respectively, in the mass chromatogram shown in Figure 2. The schemes illustrate the fragmentation patterns of the individual adducts.

21:1 methyl esters were at the C-6, C-9, C-12, C-15 and C-18 carbons, respectively.

The five types of 21:1 methyl esters recovered from the *cis*-monoenoate fraction (see above) were formed from the 21:5 methyl ester by partial reduction with hydrazine, which is known to reduce double bonds without geometrical or positional isomerization of the remaining double bonds (9). Consequently, the 21:5 could be identified as all-*cis*-21:5(Δ6,9,12,15,18).

The structure of this methylene-interrupted all-*cis*-21:5n-3 in the eel lipids agrees with that reported previously in seal oil (3). No other double-bond positional isomers could be detected in the 21:5 fraction, and no other odd-chain polyenoic acids having more than five double bonds could be detected in these lipids. The proportion of 21:5n-3 relative to total fatty acids is very low in the eel lipids (below 0.5%) as it is in other marine lipids (below 1%) (3; and references therein). Possible explanations for the occurrence of 21:5n-3 in marine lipids have been discussed by Mayzaud and Ackman (3). The acid might be formed from 22:5n-3 by an α-oxidation pathway or from a hydrocarbon (3,6,9,12,15,18-heneicosahexaene and so forth) by an ω-oxidation route. However, the pathways of 21:5n-3 formation and also the reasons for 21:5n-3 accumulation have not yet been determined. The occurrence of 21:5n-3 in the lipids of cultivated eels might have resulted from a partial marine fish meal diet.

In the present study, we have employed our recently devised method (1) to locate the double-bond positions in trace amounts of 21:5n-3. This convenient method is very useful for confirming the structures of polyenoic fatty acids. The original data on the method for polyenoic acid analysis (*i.e.*, its reliability and applicability) are summarized in our recent paper (1).

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Identification of Natural Diacylglycerols as the 3,5-Dinitrophenylurethanes by Chiral Phase Liquid Chromatography with Mass Spectrometry

Y. Itabashi[1], **L. Marai** and **A. Kuksis***
Banting and Best Department of Medical Research, University of Toronto, C.H. Best Institute, Toronto, Canada M5G 1L6

This study reports a facile identification of the molecular species of enantiomeric diacylglycerols by combining chiral phase high-performance liquid chromatography with positive chemical ionization mass spectrometry. For this purpose the 3,5-dinitrophenylurethane (DNPU) derivatives of *sn*-1,2(2,3)-diacylglycerols are separated on an R(+)-naphthylethylamine polymer column (25 cm × 0.46 cm ID) using an isocratic solvent system made up of hexane/dichloroethane/acetonitrile (85:10:5, by vol) or isooctane/*tert*-butyl methyl ether/acetonitrile/isopropanol (80:10:5:5, by vol). About 1% of the column effluent (1 mL/min) was admitted to a quadrupole mass spectrometer (Hewlett-Packard, Palo Alto, CA) *via* direct liquid inlet interface, and positive chemical ionization spectra were recorded over the range of 200–900 mass units. The DNPU derivatives of diacylglycerols yield characteristic $[M-DNPU]^+$ and $[RCO+74]^+$ ions for each diacylglycerol species from which the molecular weight and exact pairing of fatty acids can be unequivocally obtained. The characteristic ions appear to be generated in nearly correct mass proportions as indicated by preliminary quantitative comparisons.
***Lipids* 26, 951–956 (1991).**

The chiral phase high-performance liquid chromatography (HPLC) described by Takagi and Itabashi (1) provides a rapid and complete resolution as the 3,5-dinitrophenylurethane (DNPU) derivatives of enantiomeric diacylglycerols with identical fatty acids, but separation of enantiomers containing different fatty acids results in overlap or interdigitation of many molecular species, and identification of individual *sn*-1,2- and *sn*-2,3-enantiomers becomes difficult or impossible. Furthermore, the chiral stationary phases thus far developed fail to provide a complete separation of molecular species within either enantiomer class.

We have recently overcome this difficulty by collecting separately the enantiomeric fractions by chiral phase HPLC, regenerating the diacylglycerols without isomerization, converting them to the TMS ethers, and identifying the molecular species of the diacylglycerols by polar capillary gas-liquid chromatography (GLC) (2). Although this method yields excellent results, the procedure is laborious and could lead to isomerization and loss of sample.

In the present study we have combined chiral phase HPLC with mass spectrometry (LC/MS) for the identification and quantitation of molecular species overlapping between enantiomers and within each enantiomer class. For this purpose we have employed positive chemical ionization mass spectrometry (CIMS), which provides significant $[RCO+74]^+$ and $[M-DNPU]^+$ fragments necessary for unequivocal identification of diacylglycerol DNPU derivatives in the effluents of chiral phase HPLC columns. Under the LC/MS conditions, significant intensities are also obtained for the negative ions corresponding to $[M]^-$, $[DG+Cl]^-$, $[DNPU]^-$, and $[DNPU+Cl]^-$, which do not provide additional structural information, although they increase the sensitivity of detection of the diacylglycerol derivatives in the LC/MS effluent (3). A preliminary report has appeared (4).

MATERIALS AND METHODS

rac-1,2-Dioleoylglycerol was prepared by Grignard degradation from standard trioleoylglycerol (Sigma Chemical Co., St. Louis, MO). Cocoa butter, corn oil and a hydrogenated low molecular weight fraction of butteroil were those used by our laboratory in previous studies (3,5,6). In each instance, the triacylglycerols were isolated by normal phase thin-layer chromatography (TLC) using a neutral solvent system (6). The mixed *sn*-1,2(2,3)-diacylglycerols were prepared from the purified triacylglycerols by Grignard degradation and purified by boric acid TLC (7), which also removed any X-1,3-diacylglycerols. The dinitrophenylurethanes were prepared by reacting the purified diacylglycerols with 3,5-dinitrophenylisocyanate in dry toluene in the presence of pyridine, as described by Itabashi and Takagi (1).

Chiral phase HPLC. The chiral phase HPLC analyses were performed on a Hewlett-Packard Model 1084 liquid chromatograph (Palo Alto, CA) equipped with a column (25 cm × 4.6 mm ID) containing R-(+)-naphthylethylamine polymer as the liquid phase (YMC-Pack A-KO3, YMC Inc., Tokyo, Japan) (8). For combined LC/MS

[1] Present Address: Department of Chemistry, Faculty of Fisheries, Hokkaido University, Hakodate, Japan.

*To whom correspondence should be addressed at BBDMR, University of Toronto, 112 College Street, Toronto, Canada M5G 1L6.

Abbreviations: CIMS, chemical ionization mass spectrometry; DNPU, dinitrophenylurethanes; GLC, gas-liquid chromatography; HPLC, high-performance liquid chromatography; LC/MS, liquid chromatography/mass spectrometry; TLC, thin-layer chromatography.

The abbreviations 14:0, 16:1, 18:2, etc. represent normal chain fatty acids of 14, 16, 18, etc. acyl carbons and 0, 1, 2, etc. double bonds, respectively; 16:0-18:1, etc. represent diacylglycerols containing 16:0 and 18:1 fatty acids of unspecified positional distribution; *sn* indicates stereospecific numbering of glycerol carbons; *sn*-1,2-diacylglycerols and *sn*-2,3-diacylglycerols are enantiomeric diacylglycerols of unspecified fatty acid composition; *rac*-1,2-diacylglycerols are racemic diacylglycerols representing equal amounts of *sn*-1,2- and *sn*-2,3- enantiomers; *sn*-1,2(2,3)-diacylglycerols are a mixture of *sn*-1,2- and *sn*-2,3-diacylglycerols of unspecified proportion of enantiomers and unspecified fatty acid composition and positional distribution; X-1,3-diacylglycerols are diacylglycerols of unspecified fatty acid composition and reverse isomer content.

analyses the original solvent system was modified to contain either hexane/dichloroethane/acetonitrile (85:10:5, by vol) or isooctane/*tert*-butyl methyl ether/acetonitrile/isopropanol (80:10:5:5, by vol). The columns were operated at a flow rate of 1.0 mL/min or less and at 25°C oven temperature. About 30–50 μg of the DNPU derivative dissolved in the same solvent as the mobile phase was admitted to the column using an automatic sample injector.

LC/MS. For LC/MS, about 1% of the HPLC effluent was admitted to a Hewlett-Packard Model 5985B quadrupole mass spectrometer *via* a direct liquid inlet interface (9). Positive chemical ionization mass spectra were taken every 5 s over the entire chromatogram in the mass range 200–900, and the data were recorded by a computer. Chloride attachment negative chemical ionization spectra of the dinitrophenylurethanes were recorded over a similar mass range. The HPLC solvent served as the reagent gas and the source of chloride for chloride attachment negative chemical ionization (3). The stored data were recalled and analyzed with the help of a Hewlett-Packard data system (Model HP-1000E) and a graphics terminal (Model HP 2648A) as previously described (9,10).

RESULTS AND DISCUSSION

Figure 1 shows the chiral phase LC/MS profile of the DNPU derivatives of *rac*-1,2-dioleoylglycerols derived from trioleoylglycerol. Previous work has established the earlier elution of the *sn*-1,2-enantiomer (8). The single ion plots show the presence of the $[RCO+74]^+$ (*m/z* 339) and the $[M-DNPU]^+$ (*m/z* 603) fragments in both enantiomeric peaks. The total mass spectra recorded in Figure 2 indicate the absence of any other significant positive ions, and are similar for both enantiomers. An identical LC/MS spectrum was obtained for the DNPU derivative of X-1,3-dioleoylglycerol, which emerges ahead of the *sn*-1,2-isomer (results not shown). Interestingly, the $[M-RCOO]^+$ ion is not obtained under the LC/MS conditions. The *sn*-1,2- and *sn*-2,3-dioleoylglycerols were derived from the trioleoylglycerol in the anticipated 1:1 mass proportion as indicated by the peak area ratios.

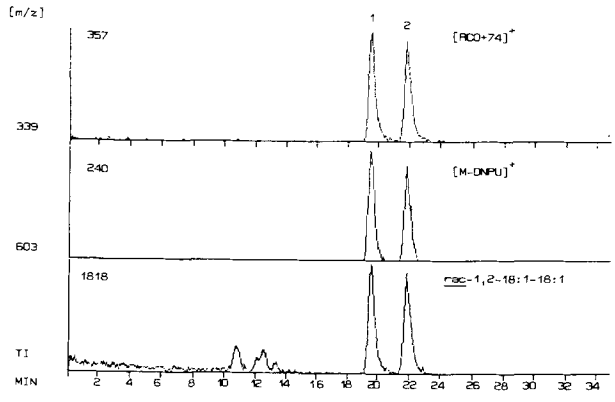


FIG. 1. Chiral phase LC/MS profile of the DNPU derivatives of *rac*-1,2-dioleoylglycerol. TI, total ion current; *m/z* 339, $[RCO+74]^+$ (oleic acid); *m/z* 603, $[M-DNPU]^+$ (dioleoylglycerol). HPLC solvent system: isooctane/*tert*-butyl methyl ether/acetonitrile/isopropanol (80:10:5:5, by vol). Other LC/MS conditions are as given in Materials and Methods.

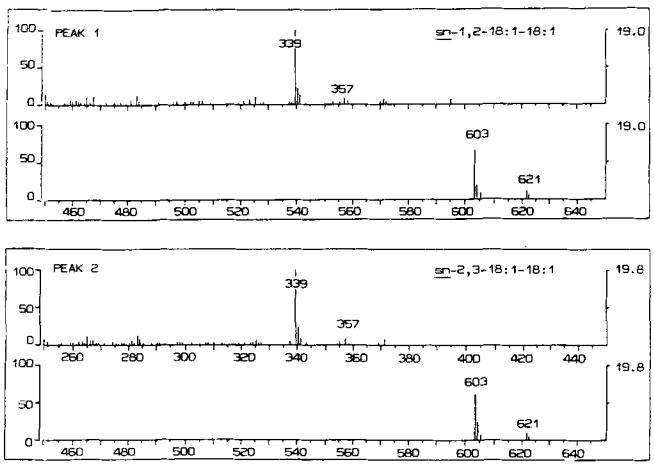


FIG. 2. Full mass spectra of Peaks 1 and 2 (Fig. 1) as recorded over the range of 250–650 mass units. LC/MS conditions as in Figure 1.

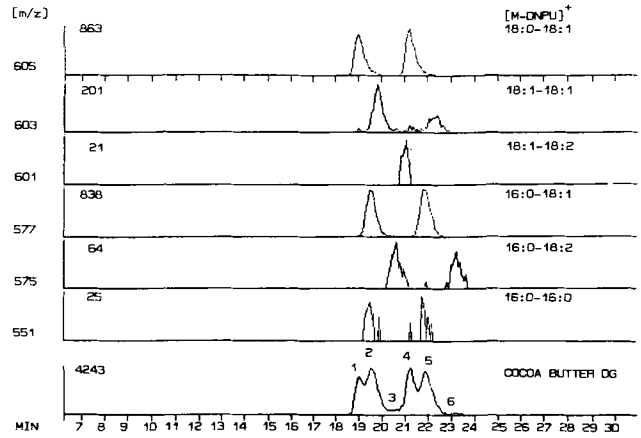


FIG. 3. Chiral phase LC/MS profile of the DNPU derivatives of *sn*-1,2(2,3)-diacylglycerols derived from cocoa butter triacylglycerols by Grignard degradation. TI, total ion current; *m/z* 551, 575, 577, 601, 603, 605 represent $[M-DNPU]^+$ of 16:0-16:0, 16:0-18:2, 16:0-18:1, 18:1-18:2, 18:1-18:1 and 18:0-18:1 diacylglycerols, respectively. LC/MS conditions as in Figure 1.

Figure 3 shows the chiral phase LC/MS profile of the DNPU derivatives of *sn*-1,2(2,3)-diacylglycerols derived from cocoa butter triacylglycerols by Grignard degradation. In this instance the diacylglycerols are resolved into the *sn*-1,2- and *sn*-2,3-enantiomers, each of which represents several molecular species of diacylglycerols, as demonstrated by the single ion ($[M-DNPU]^+$) plots recorded in Figure 3. On the basis of these ions it is possible to recognize 18:0-18:1 (*m/z* 605), 16:0-18:1 (*m/z* 577) and 18:1-18:1 (*m/z* 603) as the major components and 16:0-18:2 (*m/z* 575), 16:0-16:0 (*m/z* 551) and 18:1-18:2 (*m/z* 601) as minor components. (Corresponding $[RCO+74]^+$ ions not shown). Figure 4 shows the full mass spectra of Peaks 1 and 4 (Fig. 3). Peak 1 contains 18:0-18:1 as the major species as indicated by the $[RCO+74]^+$ ions at *m/z* 339 and 341 and the $[M-DNPU]^+$ ion at *m/z* 605. Peak 4 is identical to Peak 1 in composition, as it contains the corresponding *sn*-2,3-enantiomer. Since the cocoa butter triacylglycerols are asymmetric, the proportions of the

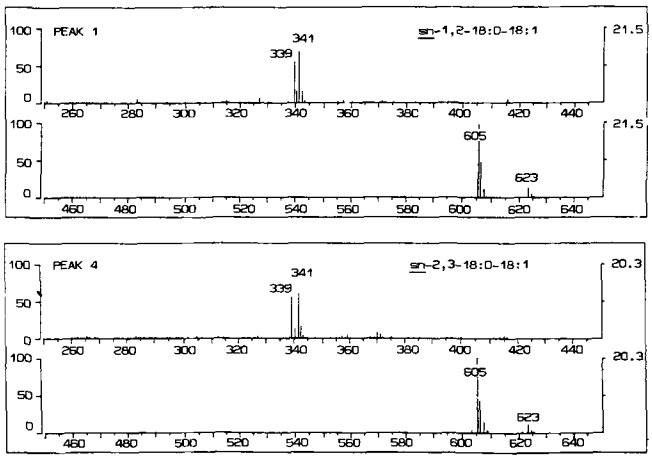


FIG. 4. Full mass spectra of Peaks 1 and 4 (Fig. 3) as recorded in the range of 250–650 mass units. LC/MS conditions as in Figure 1.

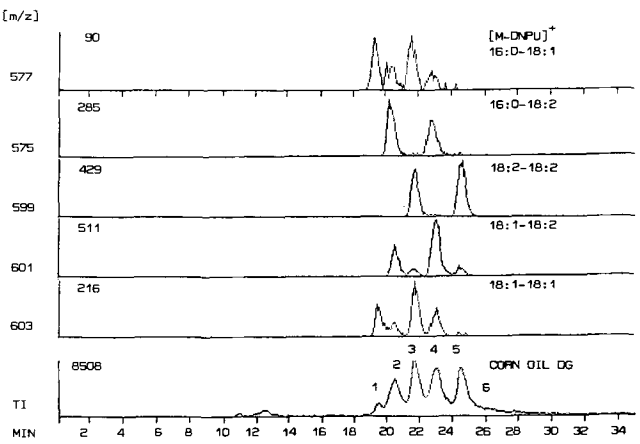


FIG. 5. Chiral phase LC/MS of the DNPU derivatives of *sn*-1,2(2,3)-diacylglycerols derived from corn oil triacylglycerols by Grignard degradation. TI, total ion current; *m/z* 575, 577, 599, 601 and 603 represent $[M-DNPU]^+$ of 16:0-18:2, 16:0-18:1, 18:2-18:2, 18:1-18:2 and 18:1-18:1, respectively. LC/MS conditions as in Figure 1.

two enantiomers in the two peaks are different. A complete identification of all the molecular species is given in Table 1 along with the characteristic fragment ions. The proportions of the individual components in any overlapping diacylglycerol species were obtained from the uncorrected ratios of the characteristic $[RCO+74]^+$ ions as previously described for the $[MH-RCOOH]^+$ ions of the silyl ethers of diacylglycerols (9,10). It should be noted that Peak 4 also contained the minor *sn*-1,2-18:1-18:2 species, which overlapped with the *sn*-2,3-diacylglycerols. The relative proportions of the *sn*-1,2- and *sn*-2,3-diacylglycerols given by the uncorrected peak areas and the estimates from overlapping species correspond closely to the peak area ratios determined earlier (2) by polar capillary GLC, following removal of the DNPU groups from the enantiomeric *sn*-1,2- and *sn*-2,3-diacylglycerols recovered from the chiral column. The slight preference observed for the association of the mono- and diunsaturated fatty acids with the *sn*-1,2-enantiomers would be anticipated from the earlier stereospecific analyses of Brockerhoff and Yurkowski (11).

Figure 5 shows the chiral phase LC/MS profile of the DNPU derivatives of *sn*-1,2(2,3)-diacylglycerols derived from corn oil triacylglycerols. In this instance there is

TABLE 1

Major Ions in the Positive LC/CIMS Spectra of DNPU of *sn*-1,2- and *sn*-2,3-Diacylglycerols from Cocoa Butter Triacylglycerols

HPLC peak	$[RCO+74]^+$ *m/z*	$[M-DNPU]^+$ *m/z*	Molecular species[a]	Quantitation LC/MS[b]	Quantitation GLC[c]
1	341	607	*sn*-1,2-18:0-18:0		0.1
	313,341	579	*sn*-1,2-16:0-18:0	0.2	0.3
	341,339	605	*sn*-1,2-18:0-18:1	19.7	20.9
2	313	551	*sn*-1,2-16:0-16:0	0.4	0.2
	313,339	577	*sn*-1,2-16:0-18:1	21.7	19.6
	341,337	603	*sn*-1,2-18:0-18:2	2.0	1.5
	339	603	*sn*-1,2-18:1-18:1	2.5	4.4
3	313,337	575	*sn*-1,2-16:0-18:2	1.5	2.2
4	339,337	601	*sn*-1,2-18:1-18:2	0.3	0.2
	341	607	*sn*-2,3-18:0-18:0		0.2
	313, 341	579	*sn*-2,3-16:0-18:0	0.4	0.3
	341,339	605	*sn*-2,3-18:0-18:1	22.6	22.0
5	313	551	*sn*-2,3-16:0-16:0	0.3	0.3
	313,339	577	*sn*-2,3-16:0-18:1	23.7	21.6
	341,337	603	*sn*-2,3-18:0-18:2	2.5	1.6
	339	603	*sn*-2,3-18:1-18:1	1.0	1.2
6	313,337	575	*sn*-2,3-16:0-18:2	1.2	1.8

[a] The diacylglycerol species are identified only by enantiomeric configuration and molecular association of fatty acids; positional distribution of fatty acids is not specified. Note late elution of *sn*-1,2 enantiomer containing the 18:1-18:2 pair of fatty acids.

[b] Uncalibrated $[M-DNPU]^+$.

[c] Combined chiral phase HPLC and polar capillary GLC (2). The GLC analysis revealed the presence of 0.3% *sn*-1,2-20:1-18:1 and 1.4% *sn*-2,3-20:1-18:1.

considerable overlap and interdigitation of the *sn*-1,2- and *sn*-2,3-diacylglycerols, as demonstrated by the single ion ([M−DNPU]$^+$) plots recorded in Figure 5. On the basis of these ions it is possible to recognize 18:1-18:2 (*m/z* 601), 18:2-18:2 (*m/z* 599), 16:0-18:2 (*m/z* 575) and 18:1-18:1 (*m/z* 603) as major components, and 16:0-18:1 (*m/z* 577) as a minor component. (Corresponding [RCO+74]$^+$ ions not shown). Figure 6 shows the complete mass spectra of Peaks 2 and 4 (Fig. 5). Peak 2 contains two major molecular species: 16:0-18:2, as indicated by the [RCO+74]$^+$ ions at *m/z* 313 and 337, and an [M−DNPU]$^+$ ion at *m/z* 575, and 18:1-18:2, as indicated by the [RCO+74]$^+$ ions at *m/z* 339 and 337, and an [M−DNPU]$^+$ ion at *m/z* 601. The diacylglycerol types of ions were observed to appear as 18 mass units higher fragment ions due to the addition of water. The composition of Peak 4 is identical to that of Peak 2, except for differences in the proportions of the molecular species. A complete identification of all the molecular species is given in Table 2, along with the characteristic fragment ions. The relative proportions of the individual *sn*-1,2- and *sn*-2,3-diacylglycerols given by the uncorrected peak areas correspond rather closely to the peak area ratios determined earlier by combined chiral phase HPLC and polar capillary GLC (2).

Figure 7 shows the chiral phase LC/MS profile of the DNPU derivatives of the *sn*-2,3-diacylglycerols derived from a hydrogenated short chain triacylglycerol fraction of a butteroil distillate. The triacylglycerols in this distillate had been shown to contain butyric acid in the *sn*-3 position (12). Following Grignard degradation the butyric

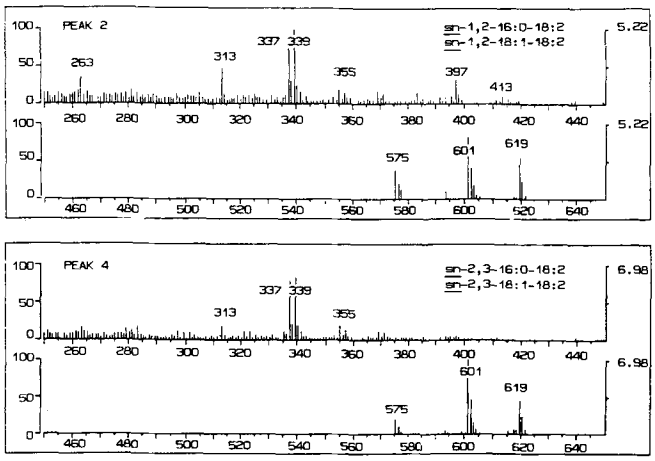


FIG. 6. Full mass spectra of Peaks 2 and 4 (Fig. 5) as recorded in the range of 250–650 mass units. LC/MS conditions as in Figure 1.

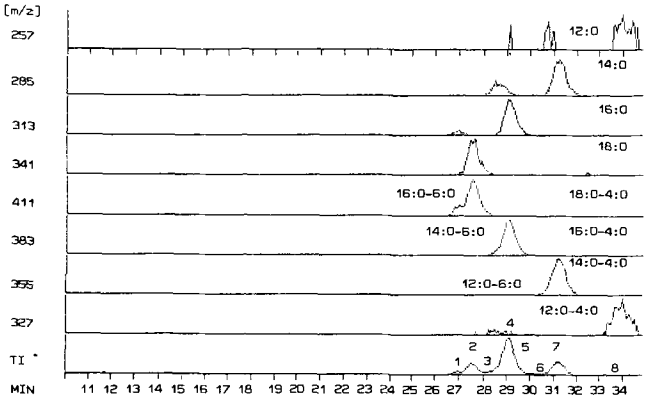


FIG. 7. Chiral phase LC/MS of the DNPU derivatives of *sn*-2,3-diacylglycerols derived from a hydrogenated short chain triacylglycerol fraction of butteroil by Grignard degradation. TI, total ion current; *m/z* 257, 285, 313, and 341 represent [RCO+74]$^+$ of lauric, myristic, palmitic and stearic acids, respectively; while *m/z* 327, 355, 383 and 411 represent [M−DNPU]$^+$ of 12:0-4:0, 14:0-4:0, 16:0-4:0 and 18:0-4:0, respectively. LC/MS conditions as in Figure 1.

TABLE 2

Major Ions in the Positive LC/CIMS of DNPU Derivatives of *sn*-1,2- and *sn*-2,3-Diacylglycerols from Corn Oil Triacylglycerols

HPLC peak	[RCO+74]$^+$ *m/z*	[M−DNPU]$^+$ *m/z*	Molecular species[a]	Quantitation LC/MS[b]	GLC[c]
1	313,339	577	*sn*-1,2-16:0-18:1	2.8	3.4
	339	603	*sn*-1,2-18:1-18:1	4.3	4.6
	341,337	603	*sn*-1,2-18:0-18:2	1.0	1.2
2	313,337	575	*sn*-1,2-16:0-18:2	10.5	11.0
	339,337	601	*sn*-1,2-18:1-18:2	10.1	12.7
3	337	599	*sn*-1,2-18:2-18:2	14.4	17.1
	313,339	577	*sn*-2,3-16:0-18:1	3.3	2.4
	339	603	*sn*-2,3-18:1-18:1	5.9	4.0
	341,337	603	*sn*-2,3-18:0-18:2	2.0	0.9
4	313,337	575	*sn*-2,3-16:0-18:2	8.2	5.9
	339,337	601	*sn*-2,3-18:1-18:2	18.5	16.3
5	337	599	*sn*-2,3-18:2-18:2	19.0	20.5

[a] Molecular species identified as in Table 1.

[b] Uncalibrated [M-DNPU]$^+$.

[c] Combined chiral phase HPLC and polar capillary GLC (2). The minor 18:0-18:1 enantiomers identified by HPLC/GLC were not estimated by LC/MS.

TABLE 3

Major Ions in the Positive LC/CIMS of DNPU Derivatives of *sn*-2,3-Diacylglycerols from a Hydrogented Short Chain Triacylglycerol Fraction of a Butteroil Distillate

HPLC peaks	$[RCO+74]^+$ *m/z*	$[M-DNPU]^+$ *m/z*	Molecular species[a]	Quantitation LC/MS[b]	UV[c]
1	(173),313	411	16:0-6:0	1.9	4.9
2	(145),341	411	18:0-4:0	13.9	19.7
3	(173),285 (145),327	383 397	14:0-6:0 17:0-4:0	4.6	7.0
4,5	(145),313	383	16:0-4:0	51.4	42.7
6	(145),299	369	15:0-4:0	3.2	3.2
7	(173),257 (145),285	355 355	12:0-6:0 14:0-4:0	22.9	19.5
8	(145),257	327	12:0-4:0	2.0	3.0

[a]Molecular species identified as in Table 1.
[b]Uncalibrated $[M-DNPU]^+$.
[c]UV response of HPLC detector at 254 nm.

acid-containing diacylglycerols yielded a separate TLC band which migrated more slowly than the longer chain diacylglycerols. Preparation of the DNPU derivatives and chiral phase HPLC revealed only the presence of the *sn*-2,3-diacylglycerols, as established by parallel analyses of standards. The composition of the molecular species of these diacylglycerols was determined by LC/MS. The single ion plots indicate the elution times of the major components identified as 18:0-4:0 (*m/z* 411), 16:0-4:0 (*m/z* 383), and 14:0-4:0 (*m/z* 355) on the basis of the $[RCO+74]^+$ and $[M-DNPU]^+$ fragments. (Corresponding $[RCO+74]^+$ ions for butyric acid not shown). Figure 8 shows the full mass spectra for Peaks 2 and 7 (Fig. 7), which contain single diacylglycerol species, as demonstrated by the ion plots recorded in Figure 7. On the basis of these ions it is possible to recognize the $[RCO+74]^+$ corresponding to stearic acid (*m/z* 341) and the $[M-DNPU]^+$ corresponding to 18:0-4:0 (*m/z* 411) in Peak 2. Although the $[RCO+74]^+$ corresponding to butyric acid (*m/z* 161) is not seen, the detection of the long chain acid along with the correct molecular weight and chiral phase HPLC retention time is sufficient to identify the molecular species in Peak 2. Likewise, the $[RCO+74]^+$ corresponding to myristic acid (*m/z* 285) and the $[M-DNPU]^+$ corresponding to 14:0-4:0 (*m/z* 355), along with the anticipated HPLC retention time, establish the identity of the diacylglycerol in Peak 7. In both instances a minor peak was seen for the $[M-DNPU+18]^+$ ion.

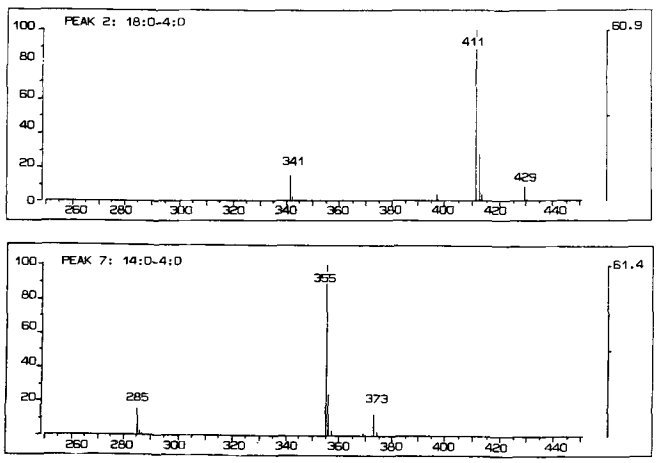


FIG. 8. Full mass spectra of Peaks 2 and 7 (Fig. 7) as recorded in the range of 250–500 mass units. LC/MS conditions as in Figure 1.

Table 3 lists the molecular species of diacylglycerols identified by chiral phase LC/MS in the short chain triacylglycerols of the butteroil distillate. The uncalibrated quantitative LC/MS response differs significantly from the UV signal of the HPLC detector and requires correction.

In conclusion, chiral phase LC/MS with positive chemical ionization provides an effective method for the unequivocal identification of molecular species of enantiomeric diacylglycerols from natural sources. The method establishes the order of elution of the species, which is the same for the *sn*-1,2 and the *sn*-2,3 isomers. The higher molecular weight and more unsaturated species are eluted last. Accurate quantitation, however, may require calibration of the LC/MS response.

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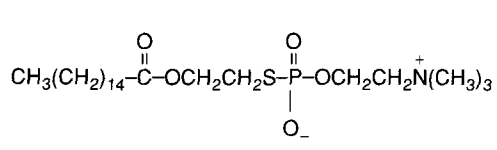
COMMUNICATION

Inhibition of the *Clostridium perfringens* Phospholipase C Hydrolysis of a Thiophosphate Analog of Lysophosphatidylcholine by Micelle-Bound Ammonium and Sulfonium Cations

Paul R. Young,* William R. Snyder and **Rosemary F. McMahon**
Department of Chemistry, University of Illinois at Chicago, Chicago, Illinois 60680

Cetyltrimethylammonium and *n*-octadecyldimethylsulfonium bromides inhibit the *Clostridium perfringens* phospholipase C-catalyzed hydrolysis of 1-*S*-phosphocholine-2-*O*-hexadecanoyl-1-mercapto-2-ethanol (1) at pH 7.5, 37°C, μ = 0.15 with KCl. Mixed micelles containing 1 and either inhibitor are substrates for the enzyme and the fraction of activity remaining is a monotonic, but non-linear function of the mole fraction of inhibitor. Simple saturation kinetics are observed as the concentration of 1 is increased in mixed micelles containing a constant mole fraction of inhibitor. Inhibition constants for cetyltrimethylammonium and *n*-octadecyldimethylsulfonium bromides are 0.66 ± 0.04 and 0.25 ± 0.02 mM, respectively. The data suggest that the significant inhibition previously observed for soluble alkyldisulfonium salts (K_{50} for dodecamethylene-*bis*(dimethylsulfonium) bromide, 27 μM) is dependent upon bifunctional cationic interactions rather than hydrophobic binding.
***Lipids* 26, 957-959 (1991).**

Phospholipase C (PLC, phosphatidylcholine choline phosphohydrolase, EC 3.1.4.3) catalyzes the hydrolysis of the glycerophosphate ester bond in phospholipids. The PLC enzymes from *Bacillus cereus* and *Clostridium perfringens* have been widely studied and reviewed (1-4). Because of the central role of phospholipases C in inflammation, there has been considerable interest in the regulation and inhibition of phospholipase C activity (5). We have recently reported (6) that the hydrolysis of 1-*S*-phosphocholine-2-*O*-hexadecanoyl-1-mercapto-2-ethanol (1) Scheme 1 (8,9), catalyzed by *C. perfringens* phospholipase C, follows simple saturation kinetics which are consistent with an ordered, rapid equilibrium mechanism in which Ca^{2+} binds to the enzyme followed by 1. Further, the hydrolysis of 1 was found to be inhibited by alkyldiamines, alkyldiammonium and alkyldisulfonium salts by a simple non-competitive mechanism in which inhibitor binds reversibly to all enzyme forms (6; Young, P.R., Snyder, W.R. and McMahon, R.F., submitted for publication). The alkyldisulfonium salts (*i.e.*, dodecamethylene-*bis*(dimethylsulfonium) bromide, K_{50} = 27 μM) are among the most active soluble phospholipase C inhibitors thus far reported. In the present study, we examined the inhibition of the hydrolysis of 1 by insoluble, micelle-bound, alkylammonium and alkylsulfonium salts. Unlike the soluble sulfonium salts examined previously, *n*-octadecyldimethylsulfonium bromide is only slightly more effective in inhibiting the enzyme than a comparable trimethylammonium salt.



SCHEME 1

MATERIALS AND METHODS

Materials. All chemicals used were readily available commercial products and were used without further purification unless otherwise noted. 1-*S*-Phosphocholine-2-*O*-hexadecanoyl-1-mercapto-2-ethanol (1) was prepared by the method described by Cox *et al.* (7), as modified by Snyder (8). *n*-Octadecyldimethylsulfonium bromide was prepared by reaction of *n*-octadecyl mercaptan with a large excess of CH_3I in methanol containing $NaHCO_3$ at room temperature, for three days (10). The resulting oil was chromatographed over Dowex-1 chloride in methanol and was characterized by nuclear magnetic resonance (NMR) spectroscopy; elemental analyses were found to be within standard limits. Phospholipase C (*C. perfringens*, 300 units/mg protein) was from Sigma Chemical Co. (St. Louis, MO) and was used as supplied.

Kinetics. The critical micelle concentration (CMC) of 1 was determined to be 20 μM at 37°C and an ionic strength of 0.15 (maintained with KCl) (6). Kinetic determinations were performed at 37°C, μ = 0.15 (KCl), [S] > 20 μM, pH 7.5 [10 mM 3-(*N*-morpholino)propanesulfonic acid (MOPS)] and dithio-*bis*(2-nitrobenzoic acid) (DTNB) = 500 μM, using a Hitachi (Hitachi, Ltd., Tokyo, Japan) 100-40 spectrophotometer equipped with an automatic cell changer and a thermostated cell compartment, as described previously (6). Substrate micelles, prepared by mixing stock solutions of 1, benzalkonium chloride and inhibitors, produced clear solutions and did not require sonication. All kinetic determinations were run in duplicate, and kinetic parameters were determined by non-linear least squares fit of the data to the appropriate equation. No induction period was observed during initial rate studies. The initial rate of hydrolysis of 1 was found to be a simple linear function of enzyme concentration over the concentration range [E] = 12-96 μg/mL, based on enzyme solid, as supplied.

*To whom correspondence should be addressed at the Department of Chemistry, University of Illinois at Chicago, P.O. Box 4348, Chicago, IL 60680.

Abbreviations: BAC, benzalkonium chloride; CMC, critical micelle concentration; CTAB, cetyltrimethylammonium bromide; DTNB, dithio-*bis*(2-nitrobenzoic acid); MOPS, 3-(*N*-morpholino)propanesulfonic acid; NMR, nuclear magnetic resonance; ODSB, *n*-octadecyldimethylsulfonium bromide; PLC, phospholipase C; TNB, dianion of thio-(2-nitrobenzoic acid).

RESULTS AND DISCUSSION

The hydrolysis of the thiolester linkage in **1** by *C. perfringens* phospholipase C results in the release of phosphocholine and one equivalent of free thiol (7,9) (Scheme 2). In the presence of 5,5′-dithio-*bis*(2-nitrobenzoic acid) (DTNB), the thiol produced reacts to release one mole of 5-thio-2-nitrobenzoate (TNB) and form mixed disulfide. This intermediate can react with a second mole of free thiol, releasing another mole of TNB and forming the disulfide shown in Scheme 2. The initial velocity of DTNB reduction, monitored at 412 nm in the presence of 70 μM **1** (37°C, μ = 0.15 and [Ca^{2+}] = 50 mM) followed simple saturation kinetics with increasing DTNB concentration, with an apparent half-maximum at [DTNB] = 20 μM; kinetic determinations were routinely performed at [DTNB] = 500 μM where the reaction rate is independent of [DTNB] (6).

Initial velocities for the enzyme-catalyzed hydrolysis of **1** have been found to follow simple saturation kinetics under the conditions cited above, and generate linear double reciprocal plots (6). Linear mixed-type slope-intercept effects are observed for the hydrolysis of **1** with variable [Ca^{2+}] at fixed concentrations of **1**, and a simple slope effect is observed as the concentration of **1** is varied at several fixed concentrations of Ca^{2+}, consistent with a simple ordered rapid equilibrium mechanism in which Ca^{2+} binds to the enzyme first followed by the substrate (6).

Because of the simplicity of this system, we have previously examined the inhibition of the *C. perfringens* phospholipase C-catalyzed hydrolysis of **1** by a series of alkyldiamines (*i.e.*, 1,12-diaminododecane), by natural polyamines and by alkyldiammonium and alkyldisulfonium salts (6). The inhibition by the *bis*(dimethylsulfonium) compounds was especially striking with half-maximum inhibition occurring at 222 and 27 μM for the decyl- and dodecyl- compounds, respectively (Young, P.R., Snyder, W.R. and McMahon, R.F., submitted for publication). Further, there was a significant difference in inhibition constant between analogous ammonium and sulfonium compounds, the sulfonium compounds being over 300-fold more effective and ranking as some of the most powerful soluble phospholipase C inhibitors known. For alkyldiamines, previous work (6) has shown that the intervening alkyl chain bound to the enzyme in a manner that was non-competitive with substrate, and that the dissociation constant for the E|[S]|[Ca^{2+}]|[I] complex was a simple function of alkyl chain length with ≈ 74% of the available binding energy being utilized toward the stabilization of the inhibitory complex (6, 11-13). To examine the possibility that a simple alkyl sulfonium salt may bind to the enzyme in a similar manner and allow full utilization of hydrophobic binding potential towards inhibition, we have examined the inhibition of the hydrolysis of **1** by cetyltrimethylammonium bromide (CTAB) and by *n*-octadecyldimethylsulfonium bromide (ODSB) incorporated into mixed micelles containing substrate. Such micelles are substrates for the enzyme and the fraction of activity remaining is a monotonic, but non-linear function of the mole fraction of inhibitor (Fig. 1). For CTAB, the rate of the reaction decreases linearly to approximately 40% mole fraction inhibitor. The fact that the data are *below* the dashed line connecting 100% and zero activity, means that inhibition by CTAB is being observed, not simply substrate dilution within the

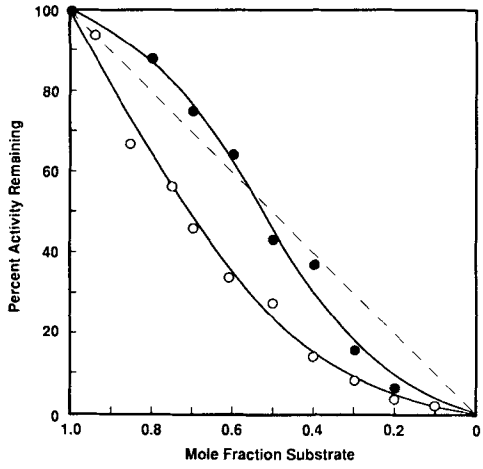
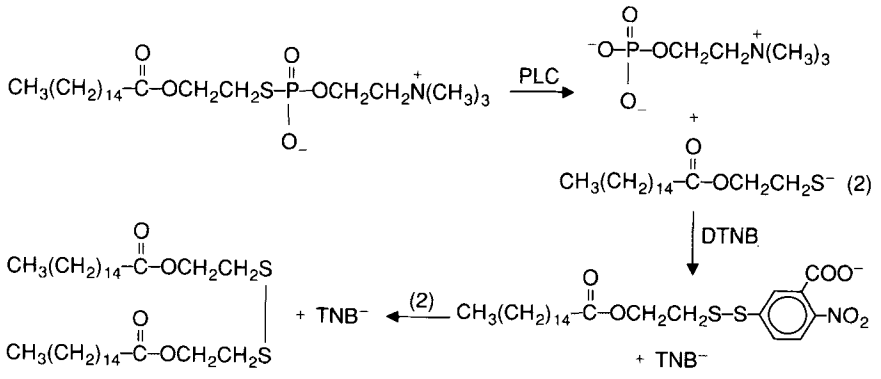


FIG. 1. The effect of the mole fraction of inhibitor present within micelles of 1-*S*-phosphocholine-2-*O*-hexadecanoyl-1-mercapto-2-ethanol (1), on the hydrolysis of 1 by *C. perfringens* phospholipase C, for cetyltrimethylammonium bromide (○) and *n*-octadecyldimethylsulfonium bromide (●). Assays were under the following conditions; pH 7.5, 37°C, μ = 0.15 (KCl), [Ca^{2+}] = 50 mM, [1] = 50 μM, 5,5′-dithio*bis*(2-nitrobenzoic acid) [DTNB] = 500 μM, and the reduction of DTNB was monitored at 412 nm.



SCHEME 2

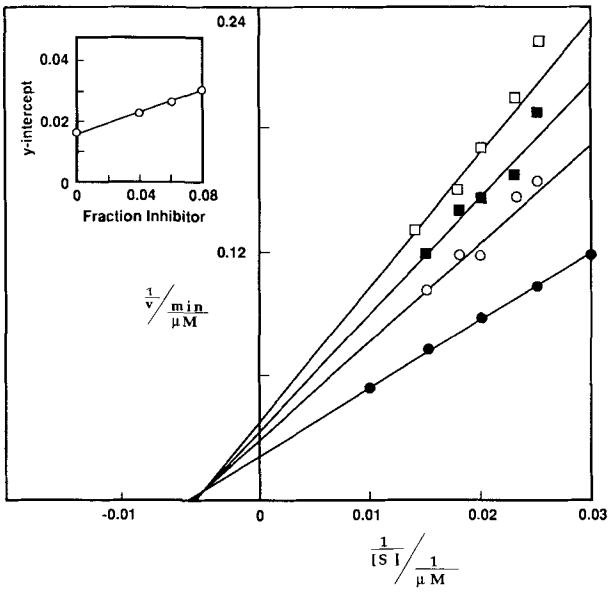


FIG. 2. Double reciprocal plots of 1/v *vs* 1/[S] for the hydrolysis of 1-*S*-phosphocholine-2-*O*-hexadecanoyl-1-mercapto-2-ethanol catalyzed by *C. perfringens* phospholipase C at the following fixed mole fractions of *n*-octadecyldimethylsulfonium bromide; (●), zero; (○), 0.04; (■), 0.06; (□), 0.08. Assays were under the following conditions; pH 7.5, 37°C, μ = 0.15 (KCl), [Ca^{2+}] = 50 mM, 5,5'-dithiobis(2-nitrobenzoic acid) [DTNB] = 500 μM, and the reduction of DTNB was monitored at 412 nm. Benzalkonium chloride (BAC) constituted 70% of the ternary micelles and was varied such that [1] + [BAC] was constant. *Inset*: Replot of (y-intercept) *vs* fraction *n*-octadecyldimethylsulfonium bromide.

mixed micelle. For ODSB, low mole fractions of the "inhibitor" stimulate enzyme activity, as demonstrated by the slight positive deviations above the 0-100% line. This effect is readily reproducible in duplicate experiments, and might result from effects of the sulfonium salt on gross micelle structure. The fact that the sulfonium compound is actually an inhibitor of the enzyme is shown clearly in Figure 2 where the mole fraction of inhibitor in the complex is maintained as a constant by substituting benzalkonium chloride (BAC) for 1 in a three-component mixed micelle. At the mole fraction of BAC used in these experiments (0.70), BAC has no effect on the rate of hydrolysis of 1 and simply functions as an inert diluent. Using this approach, simple saturation kinetics are observed at low concentrations of 1 as demonstrated in Figure 2 and the analogous plot for CTAB. In these plots, the common intersection point which is observed gives the approximate dissociation constant for the E|[Inhibitor] complex (14). Inhibition constants for CTAB and OSDB (obtained from negative x-intercept replots) are 0.66 ± 0.04 and 0.25 ± 0.02 mM, respectively. Although these constants are for binary BAC/inhibitor micelles, it is clear that the large difference in inhibition constant previously observed between structurally similar ammonium and sulfonium compounds (Young, P.R., Snyder, W.R. and McMahon, R.F., submitted for publication) is not observed in the present case. If the origin of the previously observed differences lies in the ability of sulfonium salts to form tight contact ion-pairs or tetracoordinate sulfuranes (15) with anionic groups on the enzyme surface, the present data suggest that the formation of these complexes is dependent upon bifunctional cationic interactions rather than hydrophobic binding to the enzyme surface.

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An Aldehyde-Containing Galactolipid from the Red Alga *Gracilariopsis lemaneiformis*

Zhi D. Jiang and **William H. Gerwick***
College of Pharmacy, Oregon State University, Corvallis, Oregon 97331

An unprecedented aldehyde-containing digalactosyldiacylglycerol (DGDAG) has been isolated from the red alga *Gracilariopsis lemaneiformis* and structurally defined. Its structure was determined as 1-*O*-[5-hydroxy-12-oxo-dodeca-6(E),8(E),10(E)-trienoyl]-2-*O*-palmitoyl-3-*O*-[-β-D-galactopyranosyl-6,1-α-D-galactopyranosyl]-glycerol by nuclear magnetic resonance (NMR) spectroscopy, mass spectrometry and degradation to simple fragments. The identification of this aldehyde-containing DGDAG from *G. lemaneiformis*, in addition to our previous findings of unusual galactolipids, further indicates that this alga has unique lipoxygenase capacities.
***Lipids* 26, 960-963 (1991).**

Galactolipids are major constituents of the chloroplast membrane in higher plants (1). The biosynthesis of galactolipids occurs *via* two separate metabolic routes, termed the eukaryotic and prokaryotic pathways, which occur at different locations in the cell and result in the incorporation of different acyl groups in the resultant complex lipid species (2). Our previous work has shown that the red alga *Gracilariopsis lemaneiformis* is both a rich source of oxylipins (3,9) as well as of unusual oxygenated galactolipids (4). The continuation of our investigation of these lipid products from *G. lemaneiformis* has led us to isolate another unusual galactolipid which possesses an aldehyde-containing fatty acid as an acyl substituent.

EXPERIMENTAL PROCEDURES

General. Nuclear magnetic resonance (NMR) spectra were recorded on Bruker (Karlsruhe, Germany) AM 400 and AC 300 spectrometers. All shifts are reported relative to an internal tetramethylsilane (TMS) standard. Mass spectra were recorded on a Kratos (Manchester, England) MS 50TC. Infrared spectra were obtained on a Nicolet (Madison, WI) 510 spectrometer. Merck (Darmstadt, Germany) aluminum-backed thin-layer chromatography (TLC) sheets were used for TLC, and all solvents were distilled prior to use.

Collection, extraction and isolation. Gracilariopsis lemaneiformis was collected from exposed intertidal pools at Cape Perpetua on the Oregon coast on May 17, 1988. A preserved specimen of this alga is deposited in the herbarium at the Department of Botany, University of California, Berkeley, CA. The seaweed was immediately frozen with CO_2 (solid) and stored frozen until used. The freshly defrosted alga (350 g dry wt) was homogenized in $CHCl_3$/MeOH (2:1, v/v), steeped for 30 min, and then filtered. Solvent was removed *in vacuo* to give 3.15 g of a dark green oil.

The organic extract was acetylated overnight at room temperature with excess acetic anhydride in pyridine (1:1, v/v). The excess reagents were removed *in vacuo* to yield *ca.* 3.5 g of a residue. This was fractionated by vacuum silica gel chromatography using a gradient of EtOAc in isooctane. The materials eluting with EtOAc/isooctane (1:1, v/v) were further purified by normal phase high-performance liquid chromatography (HPLC) [Alltech RSIL 10μ, 50 cm × 10 mm, EtOAc/isooctane (1:1, v/v)] and reverse phase HPLC [Lichrosorb RP-18 7μ, 25 cm × 10 mm, MeOH/H_2O (9:1, v/v)] to give 5.5 mg of compound 2.

Compound 2. A colorless oil, $[\alpha]_D^{23}$ + 52.3° (c = 0.81, MeOH); ultraviolet (UV) (MeOH) λ_{max} = 306 nm (ε = 4.9 × 10^4); infrared (IR) (neat) 3024.5, 3021.7, 2924.2, 2853.2, 1746.1, 1678.5, 1370.2, 1222.8, 1158.2, 1059.7, 1021.7, 665.0 cm^{-1}; FAB MS (positive ion) *m/z* (rel. intensity) 1196 (M^+, 0.5), 1137 (M^+−AcO, 4.6), 931 (6.7), 619 (4.6), 561 (2.7), 501 (2.0), 411 (1.2), 331 (100), 289 (8.6), 229 (8.6); High resolution fast atom bombardment mass spectrometry (FAB MS) (positive ion) obs. 1196.56050 ($C_{59}H_{88}O_{25}$, 0.92 mmu dev.); ^1H NMR (benzene-d_6, 400 MHz) δ 9.40 (1H, *d*,*J* = 7.8 Hz), 6.48 (1H, *ddd*,*J* = 3.5, 11.3, 14.8 Hz), 6.19 (1H, *m*), 6.13 (1H, *m*), 6.02 (1H, *dd*,*J* = 7.9, 15.4 Hz), 5.8 (3H, *m*), 5.7 (3H, *m*), 5.5 (2H, *m*), 5.4 (1H, *m*), 5.2 (2H, *m*), 4.57 (1H, *dd*,*J* = 2.6, 12.2 Hz), 4.4 (3H, *m*), 4.3 (2H, *m*), 4.1 (1H, *m*), 3.83 (1H, *dd*, 1H, *J* = 6.5, 10.1 Hz), 3.70 (1H, *dd*,*J* = 5.8, 10.8 Hz), 3.40 (1H, *t*,*J* = 5.9 Hz), 3.24 (1H, *dd*,*J* = 5.3, 10.2 Hz), 2.40 (1H, *m*), 2.32 (1H, *m*), 2.21 (2H, *t*,*J* = 6.9 Hz), 2.01 (3H, *s*), 1.97 (3H, *s*), 1.83 (3H, *s*), 1.81 (3H, *s*), 1.80 (3H, *s*), 1.77 (3H, *s*), 1.7 (4H, *m*), 1.67 (3H, *s*), 1.62 (3H, *s*), 1.3-1.5 (26H, *m*), 0.97 (3H, *t*,*J* = 6.9 Hz); for ^1H and ^{13}C NMR data in $CDCl_3$ see Table 1.

Acid hydrolysis of compound 2. Compound 2 (7 mg, partially obtained from a subsequent recollection of the alga) was dissolved in 2 mL of MeOH and 1 mL of 6% H_2SO_4 and refluxed for 3 hr. The mixture was diluted with 2 mL of H_2O, MeOH was removed *in vacuo*, and the residue was extracted with CH_2Cl_2 (3 × 1 mL). The extract was treated with CH_2N_2 in diethyl ether (30 min, RT) and then separated by preparative TLC (EtOAc/hexane, 1:1, v/v) to give compound 3 as a colorless oil (0.8 mg, 55% yield); UV (MeOH) λ_{max} = 310 nm (ε = 2.1 × 10^4); ^1H NMR ($CDCl_3$) δ 9.58 (1H, *d*, 8.0 Hz), 7.14 (1H, *dd*, 15.3, 11.1 Hz), 6.68 (1H, *dd*, 14.8, 10.8 Hz), 6.45 (1H, *dd*, 14.9, 11.1 Hz), 6.34 (1H, *dd*, 15.3, 10.8 Hz), 6.18 (1H, *dd*, 15.3, 8.1 Hz), 5.84 (1H, *dd*, 15.8, 8.1 Hz), 3.67 (3H, *s*), 3.65 (1H, *m*), 3.29 (3H, *s*), 2.34 (2H, *t*, 7.1 Hz), 1.55-1.75 (4H, *m*); electron impact mass spectra (EI MS) (rel. intensity) obs M^+ *m/z* 252 (40.7), 184 (9.5), 151 (32.8), 145 (10.1), 129 (16.7), 110 (16.6), 105 (13.3), 91 (68.4), 85 (19.5). The aqueous solution after CH_2Cl_2 extraction was neutralized with $Ba(OH)_2$. Precipitated $BaSO_4$ was removed by centrifugation, and the H_2O was concentrated *in vacuo* to a

*To whom correspondence should be addressed.

Abbreviations: COSY, chemical shift correlation spectroscopy; DGDAG, digalactosyldiacylglycerol; HETCOR, heteronuclear chemical shift correlation spectroscopy; 12*R*,13*S*-diHETE, 12*R*,13*S*-dihydroxyeicosatetraenoic acid; NMR, nuclear magnetic resonance; TMS, trimethylsilane; HPLC, high-performance liquid chromatography; HR FAB MS, high-resolution fast atom bombardment mass spectrometry; EI MS, electron impact mass spectrometry.

TABLE 1

^{1}H and ^{13}C NMR Data for Aldehyde DGDAG Derivative 2 from *Gracilariopsis lemaneiformis*[a]

Carbon no.	^{1}H NMR			^{13}C NMR
	ppm	m	*J* (Hz)	ppm
Galactose 1				
1‴	4.48	*d*	7.9	101.76
2‴	5.20	*m*		69.07
3‴	5.01	*dd*	10.5, 3.2	71.37
4‴	5.42	*br d*	3.0	67.92
5‴	3.85	*m*		71.99
6‴	3.77	*dd*	9.8, 5.4	65.87
	3.43	*dd*	9.8, 7.2	
Galactose 2				
1⁗	4.95	*d*	3.7	96.81
2⁗	5.12	*dd*	10.0, 3.6	68.30
3⁗	5.29	*dd*	10.0, 3.3	68.57
4⁗	5.42	*br d*	3.0	68.04
5⁗	4.16	*m*		67.41
6⁗	4.18	*m*		61.87
	4.12	*m*		
Glycerol				
1	4.32	*dd*	11.2, 3.0	63.06
	4.14	*m*		
2	5.18	*m*		70.14
3	3.99	*dd*	10.8, 4.6	67.77
	3.64	*dd*	10.8, 5.4	
Acyl group at *sn*-1				
1′				172.40
2′	2.33	*m*		33.50
3′	1.69	*m*		20.66
4′	1.67	*m*		33.75
5′	5.32	*m*		73.27
6′	5.89	*dd*	15.0, 6.6	136.71
7′	6.38	*dd*	15.0, 10.2	131.69
8′	6.62	*dd*	14.7, 10.2	140.24
9′	6.48	*dd*	14.7, 10.9	131.25
10′	7.10	*dd*	15.2, 10.9	149.92
11′	6.17	*dd*	15.2, 8.0	132.34
12′	9.57	*d*	8.0	192.08
Acyl group at *sn*-2				
1″				172.76
2″	2.31	*m*		34.40
3″	1.61	*m*		25.20
4″-15″[b]	1.2-1.4	*m*		32.29, 30.15
				30.09, 29.96
				29.77, 29.73
				29.52, 23.06
16″	0.88	*s*		14.31
Acetates[c]				
	1.96, 1.91			170.04, 20.71, 20.66
	1.78, 1.76			169.80, 20.48, 20.35
	1.75, 1.72			169.42, 20.29, 20.18
	1.62, 1.57			169.02, 19.96, 19.87

[a] ^{1}H NMR spectrum was recorded in $CDCl_3$ at 300.13 MHz, the ^{13}C NMR spectrum in benzene-d_6 at 100.61 MHz with TMS as an internal chemical shift reference; ^{13}C NMR chemical shifts were assigned by a ^{1}H-^{13}C heteronuclear chemical shift correlation experiment.

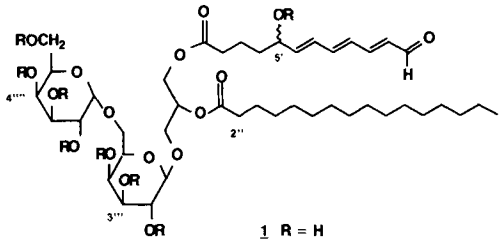
[b] Signals overlapped.

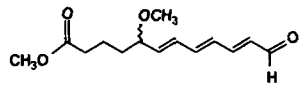
[c] Not assigned.

volume of 1 mL. The solution showed a positive optical rotation (+ 0.055, c = 0.69, 33% sugar yield). Paper chromatography (H_2O/butanol/HOAc, 5:4:1, v/v/v) of the residue gave a band which matched a galactose standard after being made visible with aniline phthalate (5).

RESULTS AND DISCUSSION

The lipid extract of an early summer collection of the temperate alga *Gracilariopsis lemaneiformis* was acetylated to improve the stability and chromatographic characteristics of its polar metabolites. Vacuum silica gel chromatography yielded a fraction which contained a yellow acid-charring compound even more polar than previously reported galactolipids from this alga (4). Further purification of this fraction by HPLC yielded pure compound 2 as *ca.* 0.2% of the extract (Fig. 1).





3

FIG. 1. Formulae.

Compound 2, $[\alpha]_D^{23}$ + 52.3° (c = 0.81, MeOH), gave a strong UV absorption at 306 nm. A molecular formula of $C_{59}H_{88}O_{25}$ was generated by positive ion HR FAB MS, and therefore, compound 2 possessed 16 degrees of unsaturation. Eight of these could be easily assigned to 8 acetate carbonyl functionalities which were readily observed by ^{1}H NMR and ^{13}C NMR analysis. Seven of these eight acetates were localized to an acetylated digalactosyl substituent (see Table 1), which accounted for an additional two degrees of unsaturation.

The digalactosyldiacylglycerol (DGDAG) nature of compound 2, including the sugar linkages and relative stereochemistry, was revealed by comparison of ^{1}H NMR and ^{13}C NMR data with those of previously identified galactolipids from *G. lemaneiformis* (4). Further, in similarity to MS data obtained for other DGDAG (4), the positive ion FAB MS of compound 2 showed key fragment ions at *m/z* 931 (loss of *sn*-1 substituent, $C_{14}H_{17}O_5$) and *m/z* 941 (loss of *sn*-2 substituent, $C_{16}H_{31}O_2$) which indicated that palmitic acid was one of the acyl substituents. The possibility that this was present as an *iso* or *anteiso* acid was ruled out by observation of a single high field methyl group in the ^{1}H NMR spectrum of 2.

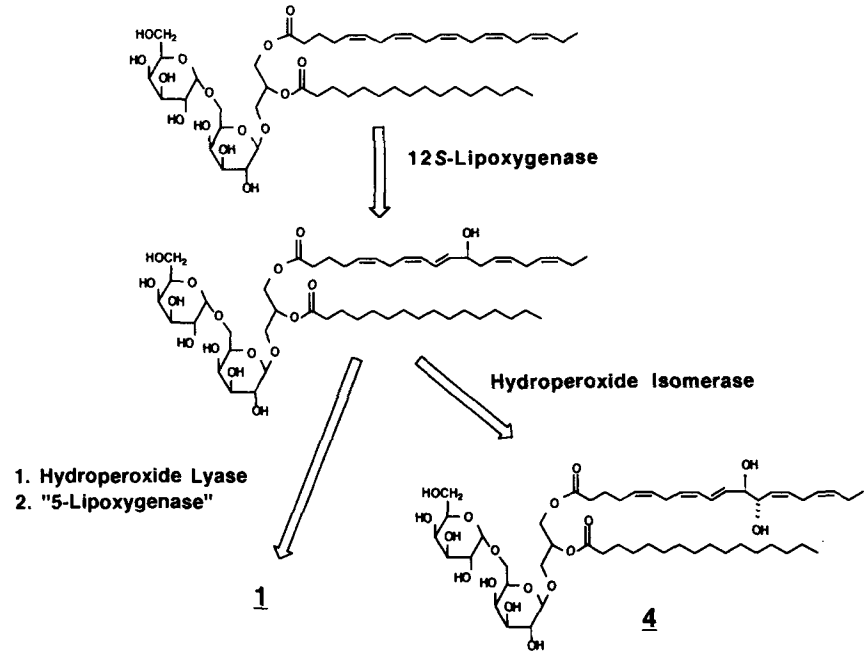


FIG. 2. Proposed biogenesis of *Gracilariopsis lemaneiformis* metabolites 1 and 4 (4) showing the intermediacy of a 12-hydroperoxide species.

Further, the ^{1}H NMR spectrum of compound 2 showed that the second acyl substituent contained an aldehyde at δ 9.57 which was connected to an all *trans* triene by ^{1}H-^{1}H COSY and coupling constants analysis (Table 1). The last remaining secondary acetate was positioned to the carboxyl side of this conjugated trienal. However, due to overlap in the high field region of the ^{1}H NMR for 2, it was not possible to unequivocally determine the number of methylene groups between this acetoxy functionality and the carboxylic ester. Hence, acid hydrolysis of compound 2 was carried out to give, following extraction, methylation and purification, this unusual acyl substituent as a pure compound (3). The EI MS of the derivatized fragment gave a molecular ion at *m/z* 252 indicating a formula of $C_{14}H_{20}O_4$. A methoxyl group could now be placed adjacent to the triene functionality based on ^{1}H NMR analysis, a fact confirmed by a major fragment ion at *m/z* 151 resulting from cleavage of the C(4)-C(5) bond. Apparently, the acetate ester was also removed by acid hydrolysis, and the resulting allylic alcohol was sufficiently activated to be methylated with CH_2N_2. In this isolated fragment, methylene groups at C(2), C(3) and C(4) could be confidently assigned, and hence, defined 3 as methyl 5-methoxy-12-oxo-dodeca-6(E),8(E),10(E)-trienoate.

In the ^{1}H NMR spectrum of compound 2 in benzene-d_6, the *sn*-2 C(2)″ protons of the palmitoyl group appeared as a second order ABX_2 pattern. This is likely the result of sterically hindered rotation of the *sn*-2 acyl chain, as previously seen in related galactolipids (4). Further, the aqueous fraction resulting from acid hydrolysis of compound 2 contained only galactose as judged by TLC and showed a positive optical rotation which indicated their D-configurations. Assembly of the above components yielded the natural product structure as 1-*O*-[5-hydroxy-12-oxo-dodeca-6(E),8(E),10(E)-trienoyl]-2-*O*-palmitoyl-3-*O*-[-β-D-galactopyranosyl-6,1-α-D-galactopyranosyl]-glycerol (1).

The acyl substituent at *sn*-1 in metabolite 1 is interesting because oxidized acyl substituents are extremely rare in galactolipids and because the positions of oxygenation and unsaturation in the chain suggest a unique biogenesis. Specifically, the aldehyde at *sn*-1 C(12)′ is likely generated by action of hydroperoxide lyase (6,7)[1] on a 12-lipoxygenase introduced hydroperoxide (8,9) in a 20 carbon polyunsaturated fatty acid substituent (either arachidonoyl or eicosapentaenoyl (4)). Oxidation at C(5)′ may be arising through separate action of an unknown 5-lipoxygenase (Fig. 2). It is interesting to note, in analogy to the biosynthesis which we have recently shown for free 12*R*,13*S*-dihydroxy-5*Z*,8*Z*,11*E*,14*Z*-eicosatetraenoic acid in this same alga (9), that the previously reported *G. lemaneiformis* DGDAG (*e.g.* 4) which contain the ω3 analog of this unusual acyl substituent (4) as a component, likely also arise through a 12-hydroperoxide intermediate. Consequently, this hypothesis predicts that natural products 1 and 4 could both be metabolites of a common intermediate (Fig. 1).

[1]The hydroperoxide lyase product 12-oxo-5*Z*,8*Z*,10*E*-dodecatrienoic acid was recently characterized as a product of arachidonic acid added to a cell free preparation of *G. lemaneiformis* (10). Further, the 100,000 × *g* supernatant from fresh tissue homogenates of *G. lemaneiformis* metabolizes, in a time-dependent manner, exogenous arachidonic acid to materials which absorb in the ultraviolet (UV) at λ_{max} = 237 (conjugated diene, major absorbance) and λ_{max} = 282 (conjugated dienal, minor absorbance) (Wise, M., and Gerwick, W.H., unpublished data). The latter provides further evidence for the occurrence of hydroperoxide lyase in this alga.

ACKNOWLEDGMENTS

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Platelet-Activating Factor (PAF): An Introduction[1]

Shoshichi Nojima
Faculty of Pharmaceutical Sciences, Teikyo University, Kanagawa, Japan

Acidic lipid mediators, such as prostaglandins and leukotrienes, play important physiological roles and are generally regarded as autacoids or local hormones. One of the most potent autacoids, platelet-activating factor (PAF), belongs to a more recently discovered class of mediators with a broad range of biological activities. Research by three independent groups led to the discovery of PAF as the first example of a complex phospholipid, a minute amount of which exhibits potent bioactions. Earlier studies on ether-linked glycerophospholipids provided the basis for establishing the chemical structure of PAF as a 1-*O*-alkyl-2-acetyl-*sn*-glycero-3-phosphocholine. Two significant biological activities, platelet activation and hypotensive activity, of this unique lipid factor were independently discovered and further defined by the groups of prominent scientists who opened up this interesting research field.

In 1922, the first structural determination of an ether-linked lipid was made by Tsujimoto and Toyama (1), who isolated three types of alkyl glycerol ethers from the unsaponifiable lipids of liver oil of ray, shark and rat fish. The first ether-linked glycerophospholipids were isolated in 1958 by Carter and his associates (2), who identified 1-*O*-octadecylglycerophosphoethanolamine derived from egg yolk phosphatides.

It is now known that significant amounts of alkylacylglycerophosphocholine, a precursor of PAF, occur in a variety of animal tissues, cells and microorganisms, including various blood cells such as macrophages, platelets and neutrophils. In 1972, Benveniste, Henson and Cochrane (3) reported the leukocyte-dependent aggregation of rabbit platelets. When leukocytes were prepared from IgE-sensitized rabbits and treated with the specific antigen, platelets were strongly aggregated. The investigators reasoned that, upon stimulation, IgE-sensitized basophils degranulated and released a soluble factor that caused the platelet aggregation. Benveniste *et al.* (3) called this soluble factor platelet-activating factor and suggested a possible role of PAF in acute allergic reactions.

In 1979, Demopoulos, Pinckard and Hanahan (14) found that a synthetic glycerophospholipid, 1-*O*-alkyl-2-acetyl-*sn*-glycero-3-phosphocholine (AAGPC) had the same physicochemical and biological properties as native PAF. AAGPC elicited the secretion of serotonin from rabbit platelets. They also showed that both AAGPC and PAF were converted to inactive forms through base-catalyzed methanolysis and restored to functional forms by reacetylation. In 1980, Hanahan and his group (5) purified native PAF from activated basophils and characterized it extensively. The authors concluded that naturally occurring PAF is indeed AAGPC.

Long before the discovery of "PAF" through the cultivation of activated basophils, Muirhead, Jones and Stirman (6) found in 1960 that two lipid fractions of renomedullary tissue with different polarity exerted a hypotensive effect. Since these fractions protected animals from the development of experimental hypertension, they were called antihypertensive polar renomedullary lipid (APRL) and antihypertensive neutral renomedullary lipid (ANRL). In 1979, Blank, Snyder, Byers, Brooks and Muirhead (7) reported that the structure of APRL is 1-*O*-alkyl-2-acetyl-*sn*-glycero-3-phosphocholine. They showed that semisynthetic PAF exhibited potent antihypertensive action in experimentally hypertensive rats with the same effectiveness as did natural APRL.

PAF is a very potent autacoid; the ED_{50} value for guinea pig platelet activation is about 3×10^{-10} M. An intravenous injection into rat of about 60 ng/body PAF causes severe hypotension. In addition to activation of platelets and induction of hypotension, PAF has a wide spectrum of biological activities. PAF induces contraction of smooth muscle cells, cardiac effects and increase of vascular permeability. It also activates neutrophils, and induces monocyte aggregation, eosinophil chemotaxis, macrophage activation, an increase in liver glycogenolysis, and many other effects. It is now widely accepted that PAF-sensitive cells and tissues are activated upon binding of PAF to a specific binding site, which is probably a PAF receptor present on the cell surface.

PAF is generated, upon appropriate stimulation, by basophils, monocytes-macrophages, neutrophils, platelets and endothelial cells. Several organs, including lung and kidney, have been shown to produce PAF. So far, the assay of the amount of PAF present in biological materials has been done by bioassay or gas chromatography-mass spectrometry. A new radioimmunoassay method recently has been developed, enabling us to quantify the amount of PAF in a more reliable and sensitive manner than before.

The precursor of PAF, alkylacylglycerophosphocholine, is stored in the cell membrane and, upon stimulation of the cell, is immediately utilized to generate PAF. So far two routes of PAF biosynthesis have been demonstrated. In the "remodelling pathway", lysoPAF is formed by deacylation of alkylacylglycerophosphocholine. Subsequent acetylation of lysoPAF is catalyzed by a PAF acetyltransferase, which is assumed to be activated by stimulus-coupled phosphorylation. In the "*de novo* pathway", alkylacetylglycerol serves as a substrate for a cholinephosphotransferase. Degradation and inactivation of PAF is mainly catalyzed by PAF acetylhydrolase (known to be present in both plasma and various tissues) to yield lysoPAF. In target cells, lysoPAF is often converted further to alkylacylglycerophosphocholine by an arachidonoyl-specific transferase. It has therefore been argued that the synthesis and degradation of PAF is coupled with arachidonic acid metabolism.

Although the precise role of PAF *in vivo* is still not fully understood, PAF has been implicated in a number of pathological reactions, such as gram-negative septic shock and asthma. Several anti-PAF drugs (receptor antagonists) which are expected to be utilized as either

[1]Presented at the Third International Conference on Platelet-Activating Factor and Structurally Related Alkyl Ether Lipids, Tokyo, Japan, May 1989.

Abbreviations: AAGPC, 1-*O*-alkyl-2-acetyl-*sn*-glycero-3-phosphocholine; ANRL, antihypertensive neutral renomedullary lipid; APRL, antihypertensive polar renomedullary lipid; PAF, platelet-activating factor.

therapeutic or prophylactic medications against these PAF-related disorders have been developed.

Recent research on PAF bioaction has led to a better understanding of the roles of PAF in many physiological and pathological reactions. Further studies are obviously required to determine the precise *in vivo* involvement of PAF in inflammation, allergy-asthma, nephritis, reproductive processes, hypotension, neuropathology and other biological processes in which PAF is though to be involved.

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Studies on the Selectivity of Enzymes Involved in Platelet-Activating Factor Formation in Stimulated Cells[1]

H. van den Bosch[a,*], A. Sturk[b], J.W. ten Cate[b] and A.J. Aarsman[a]
[a]Centre for Biomembranes and Lipid Enzymology, University of Utrecht, Padualaan 8, 3584 CH Utrecht, and [b]Department of Hematology, Division of Hemostasis and Thrombosis, Academic Medical Centre, 1105 AZ Amsterdam, The Netherlands

The present studies were undertaken to obtain further insight into the selectivities of the enzymes, i.e., phospholipase A_2 and acetyltransferase, involved in platelet-activating factor (PAF) production upon stimulation of human polymorphonuclear leukocytes (PMN) and platelets. After appropriate stimulation of the cells in the presence of [^{3}H]acetate the total PAF and analogs, i.e., 1-alkyl-2-acetyl-, 1-alkenyl-2-acetyl-, and 1-acyl-2-acetyl-glycero-3-phosphocholine were isolated by high performance liquid chromatography. The isolated mixture was subjected to treatment with phospholipase A_1 to differentiate acetate incorporation into 1-ether linked and 1-ester linked species. The ratio of acetate incorporation into 1-ether linked vs 1-ester linked PAF analogs amounted to 13.8 ± 1.0 and 1.3 ± 0.1 for PMN and platelets, respectively. When compared to the ratio of 1-ether linked and 1-ester linked species in the diradylglycerophosphocholine precursors in each cell type, i.e., 1.13 for PMN and 0.22 for platelets, these data suggested a pronounced selectivity for the phospholipase A_2 and/or acetyltransferase in the process of PAF production. When the experiments were repeated with cells that had been pretreated with phenylmethanesulfonylfluoride (PMSF) to block the acetylhydrolase, the most dramatic effects were observed on acetate incorporation into 1-acyl-2-acetyl-glycero-3-phosphocholine, which increased much more than that into 1-alk(en)yl-2-acetyl-glycero-3-phosphocholine. Under these conditions, the ratio of acetate incorporation into 1-ether linked vs 1-ester linked PAF analogs became 1.4 ± 0.2 and 0.17 ± 0.02 for PMN and platelets, respectively. These values are very close to the 1-ether linked vs 1-ester linked species in the diradylglycerophosphocholine precursors for PAF in the respective cell type. These data suggested that the selectivities of phospholipase A_2 and/or acetyl transferase for ether-linked species, as observed in non-PMSF treated cells, are only apparent and caused by rapid degradation of the 1-acyl analog either before or after acetylation. In line with this interpretation, we demonstrated that 1-acyl-2-acetyl-GPC can be deacylated to water-soluble acetyl-GPC and GPC by sonicated PMN and platelets and that this deacylation is completely blocked in sonicates from PMSF-pretreated cells. In addition, evidence is presented which indicates that the enzyme responsible for deacylation may be a lysophospholipase.
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The synthesis of platelet-activating factor (PAF) upon appropriate stimulation of a large variety of cell types involves a deacylation of the cellular PAF precursor, i.e., 1-*O*-alkyl-2-acyl-GPC, to lysoPAF by a phospholipase A_2 and subsequent acetylation of lysoPAF to PAF by an acetyltransferase (1). Studies on the selectivity of these enzymes, both in intact cells and in cell free systems, have indicated that both are not completely discriminatory against the 1-ester linked analogs of the substrates. Using cell free systems, the Ca^{2+}-dependent cytosolic phospholipase A_2 from human neutrophils (2) and human platelets (3) appeared to use 1-acyl- and 1-alkyl-2-arachidonoyl-GPC equally well. The increased activity of this enzyme in cytosol prepared from A23187-stimulated cells suggests that this phospholipase A_2 may be involved in PAF production upon cell stimulation, but there is no further proof for this. Likewise, the membrane-bound acetyltransferase shows increased activity after cell stimulation (4-7), and the enzyme from spleen microsomes utilized both 1-alkyl- and 1-acyl-lyso-GPC, although the former at a two-fold higher rate (8,9). As suggested by the specificities of the phospholipase A_2 and the acetyltransferase, PAF synthesis in rabbit (10) and human (7) platelets and in rabbit (11,12) and human (11) neutrophils concomitantly produced the 1-acyl-2-acetyl-GPC analog to various extents.

PAF is inactivated through catabolism by acetylhydrolase. This enzyme is localized in the cytosol of cells (13), shows an increased activity upon stimulation of some cells (4) and also metabolizes both 1-alkyl- and 1-acyl-2-acetyl-GPC (13). Acetylhydrolase is inhibited by diisopropylfluorophosphate (13) and phenylmethanesulfonylfluoride (PMSF) (14) and treatment of platelets with PMSF resulted in an increased PAF recovery upon thrombin stimulation (15). However, acetate incorporation into PAF analogs was not differentiated into incorporation into 1-alkyl-and 1-acyl-2-acetyl-GPC. In attempts to increase PAF recovery from stimulated human PMN and platelets by pretreatment of the cells with PMSF, we noticed to our surprise that acetate incorporation in 1-acyl-2-acetyl-GPC increased much more dramatically than that in PAF. This observation led to a careful analysis of the relative amounts of 1-alk(en)yl-2-acetyl-GPC and 1-acyl-2-acetyl-GPC in non-PMSF-treated and PMSF-treated cells. The results easily can be interpreted by assuming that under cellular conditions neither the phospholipase A_2 nor the acetyltransferase involved in PAF production shows much selectivity for 1-ether linked *vs* 1-ester linked substrates, and that the 1-ester linked species in control cells, but not in PMSF-treated cells, are rapidly

[1]Based on a paper presented at the Third International Conference on Platelet-Activating Factor and Structurally Related Alkyl Ether Lipids, Tokyo, Japan, May 1989.
*To whom correspondence should be addressed.

Abbreviations: PMN, polymorphonuclear leukocytes; PAF, platelet-activating factor; 1-alk(en)yl-2-acetyl-GPC, total 1-ether linked 2-acetyl-GPC, i.e., sum of 1-alkyl- and 1-alkenyl- species, PMSF, phenylmethanesulfonylfluoride; acetyltransferase, acetyl-CoA:lysoPAF acetyltransferase (EC 2.3.1.67); acetylhydrolase, PAF-acetylhydrolase (EC 3.1.1.47); GP[^{14}C]C, glycero-3-phospho[^{14}C]choline; HPLC, high performance liquid chromatography.

degraded by PMSF-sensitive deacylases. This hypothesis will be further elaborated upon in the Results and Discussion section.

EXPERIMENTAL

The source of chemicals and the detailed experimental procedures have been described elsewhere (16), and only a brief description will be given here.

Cell isolation. Gelfiltered platelets and PMN-enriched leukocytes were isolated from acid-citrate-dextrose anticoagulated human blood, as described previously (17).

PMSF-treatment and cell stimulation. Platelets ($1-3 \times 10^8$ cells/mL) and leukocytes ($2-3 \times 10^7$ cells/mL) were incubated 15 min at 37°C with 2 mM PMSF (15) added from a 2 M stock solution in dimethylsulfoxide. Control cells only received the solvent. Excess PMSF was removed by centrifugation and washing of the cells. Leukocytes (0.4×10^7 cells) and platelets (6×10^8 cells) were stimulated at 37°C with 10 μM A23187 and 2.5 U/mL bovine thrombin, respectively. Just prior to stimulation, 10 μCi/mL of [^{3}H]acetate (sodium salt, spec. activity 3.4 Ci/mmol) was added.

Isolation and characterization of PAF and analogs. Cell stimulation was stopped by addition of chloroform and methanol, and lipids were extracted according to Bligh and Dyer (18). The chloroform layer was dried, and lipids were dissolved in hexane/isopropanol (50:50, v/v) for HPLC separation essentially as described by Blank and Snyder (19). Fractions were dried, the residues dissolved in 0.2 mL ethanol and aliquots were used for radioactivity determination, biological measurement of PAF activity using a rabbit platelet aggregation assay (17) and for phospholipase A_1 treatment to differentiate between acetate incorporation into ether-linked and ester-linked PAF and analogs. The overall recovery of the PAF isolation procedure was 65% (range 58–68%, n = 8) as determined from simultaneously tested samples receiving [^{3}H]PAF at the time of lipid extraction of cells stimulated in the absence of [^{3}H]acetate, and values were corrected for this recovery. PAF was characterized as previously described (17).

Differentiation of [^{3}H]acetate incorporation into 1-alk(en)yl-2-acetyl-GPC and 1-acyl-2-acetyl-GPC was done by treatment of the total PAF analog mixture as isolated from the HPLC column with *Rhizopus arrhizus* lipase with phospholipase A_1 activity as described (16,20). The percentage [^{3}H]acetate incorporation into analogs was established by Bligh and Dyer extraction (18) and measurement of the radioactivity in the chloroform layer (1-alk(en)yl-2-acetyl-GPC) and the methanol/water layer (1-hydroxyl-2-acetyl-GPC derived from 1-acyl-2-acetyl-GPC). Control experiments with 1-acyl-2-acetyl-GP[^{14}C]C indicated nearly quantitative (mean 97%, range 96.5–97.5%, n = 4) conversion of the 1-acyl-2-acetyl-GPC. By contrast, 1-[^{3}H]alkyl-2-acetyl-GPC was completely resistant to phospholipase A_1 treatment since little lysoPAF (mean 2.3%, range 1.2–3.0%, n = 4) was formed by this procedure, indicating that the phospholipase A_1 was not contaminated by phospholipase A_2.

Preparation and metabolism of 1-acyl-2-acetyl-GP[^{14}C]C. The title compound was prepared by phospholipase A_2 degradation of dipalmitoyl-GP[^{14}C]C and subsequent reacetylation of the isolated lyso-compound by established procedures (21). The purified compound was mixed with 1-[^{3}H]alkyl-2-acetyl-GPC and incubated with cell sonicates prepared from control and PMSF-treated leukocytes and gelfiltered platelets.

RESULTS AND DISCUSSION

Figure 1 shows a time course of PAF production in control PMN after stimulation with Ca^{2+}-ionophore, measured as biological activity and *via* [^{3}H]acetate incorporation. As can be seen, acetate incorporation into the total mixture of PAF and analogs is essen-

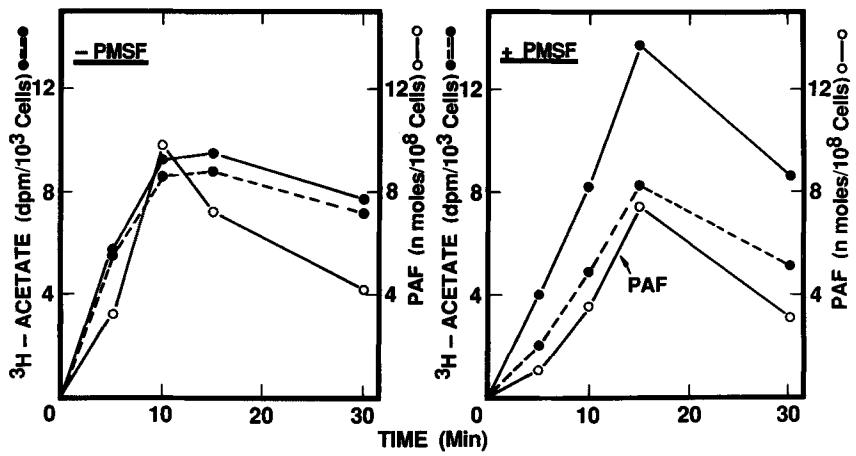


FIG. 1. PAF production in A23187-stimulated PMN. PAF production was measured as biological activity (open symbols) and via [^{3}H]acetate incorporation (closed symbols). In the latter case, the solid line represents incorporation into total PAF and analogs, i.e., 1-alk(en)yl- plus 1-acyl-2-acetyl-GPC, whereas the dashed line indicates incorporation into 1-alk(en)yl-2-acetyl-GPC remaining after phospholipase A_1 treatment of the total mixture.

tially confined to the 1-ether linked species (closed symbols, dashed line). Acetate incorporation into total analogs, including 1-acyl-2-acetyl-GPC, is only slightly higher. These data suggest that the total process of PAF production in stimulated PMN is somehow specific for ether linked species.

The experiments were repeated with PMSF-pretreated cells to block the acetylhydrolase to increase the yield of PAF. Surprisingly, PAF production, both when measured as biological activity and via acetate incorporation into 1-alk(en)yl-2-acetyl-GPC, did not increase as compared to the non-PMSF treated controls. Rather, what increased was the total [^{3}H]acetate incorporation due to an extra incorporation in the 1-acyl-2-acetyl-GPC. The latter became labeled to extents approaching those observed for 1-alk(en)yl-2-acetyl-GPC (Fig. 1, right panel).

The above phenomenon was even more pronounced in human platelets (Fig. 2). Upon stimulation with thrombin, very little PAF is produced in control cells and no attempts were made in this experiment to differentiate acetate incorporation into ether linked and ester linked species of PAF and analogs. Notably, PMSF-treatment resulted in dramatically increased total [^{3}H]acetate incorporation. Some of this additional incorporation was PAF, presumably due to inhibition of acetylhydrolase by PMSF, but the vast majority appeared to be present in the acyl analog of PAF as indicated by the large difference between the solid and the dashed line through the closed symbols (Fig. 2, right panel).

We considered it unlikely that PMSF would stimulate a biosynthetic reaction, i.e., acetate incorporation into 1-acyl-2-acetyl-GPC. First, it did not stimulate acetate incorporation into 1-alkyl-2-acetyl-GPC in PMN (Fig. 1), but only into 1-acyl-2-acetyl-GPC. Second, PMSF reacts with serine residues in the active site of enzymes, and by doing so acts as an inhibitor rather than as a stimulator of enzymes. To reconcile the accumulation of 1-acyl-2-acetyl-GPC in PMSF-treated cells with this general mode of action of PMSF, we hypothesized that it would prevent a normally occurring breakdown of this 1-acyl-2-acetyl-GPC. A further suggestion for this hypothesis was obtained when we realized that the relatively much higher accumulation of the 1-acyl analog, as compared to the 1-alkyl analog, in platelets likely reflected the fact that platelet diradylglycerophosphocholine (diradylGPC) consists for a much larger proportion of diacyl species than PMN diradylGPC. Thus, if this hypothesis were correct, the [^{3}H]acetate incorporation data may have an important message on the specificities of the enzymes involved in PAF synthesis as summarized in Figure 3. The ratio of 1-ether linked and 1-ester linked species in total PMN diradylGPC amounts to 1.13. This ether/ester ratio in the total PAF analogs accumulating after stimulation of the cells became 13.8, suggesting at least a ten-fold preference for either phospholipase A_2 or acetyltransferase for ether linked species. However, in cells pretreated with PMSF this ratio became 1.4, a value very close to that of the diradylGPC precursor for PAF and PAF analog synthesis. Similarly, the ratio of 1-ether linked and 1-ester linked PAF and analogs in PMSF-treated platelets showed a value of 0.17, i.e., much lower than the ratio of 1.4 in PMN, but very close to the ratio of 0.22 in the total platelet diradylGPC. Again, the ether/ester ratio of 1.3 in PAF and analogs accumulating in non-PMSF-treated platelets was much higher, suggesting a six-fold preference for ether linked species in PAF production in platelets. However, we interpret the data given in Figure 3 as indicating that neither the phospholipase A_2 nor the acetyltransferase under cellular conditions exhibits much specificity for ether linked species. LysoPAF and 1-acyl-glycerophosphocholine are apparently formed in a ratio that is close to that in the precursor diradylGPC of each cell type, whereafter the acyl analog is rapidly degraded, either before or after the acetylation step, to give the suggestion of selectivity for ether linked species. This normally occurring degradation of the acyl analogs (in non-PMSF-treated cells) is prevented by PMSF treatment and, due to the lack of selectivity of phospholipase A_2 and acetyltransferase, the ether/ester ratio in PAF analogs approaches that in the precursor diradylGPC of the respective cells.

To investigate whether the cells contain enzymes capable of specifically degrading 1-acyl-2-acetyl-GPC,

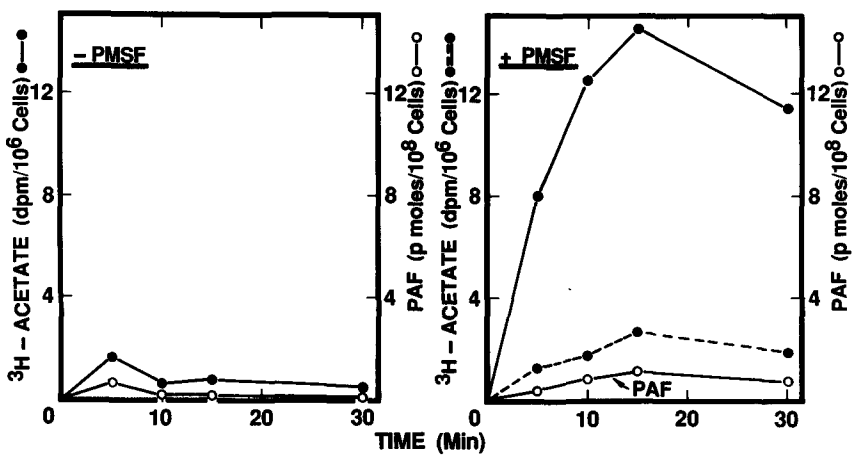


FIG. 2. PAF production in thrombin-stimulated platelets. Symbols as in Figure 1.

experiments were done with sonicates prepared from control and PMSF-pretreated PMN and platelets. These cell-free systems were incubated with a mixture of 1-[^{3}H]alkyl-2-acetyl-GPC and 1-acyl-2-acetyl-GP[^{14}C]C for different time periods as indicated in Figure 4. The incubations were stopped by a Bligh and Dyer lipid extraction and radioactivity in the chloroform and methanol/water layers were analyzed. As can be seen in Figure 4, the [^{14}C]choline-labeled acyl analog is time-dependently converted into a water-soluble component, both by PMN and platelet sonicates, and this process is completely blocked in sonicates from PMSF-pretreated cells. Two types of experiments were done to obtain further insight into the pathway for the degradation of 1-acyl-2-acetyl-GPC. First, the chloroform layers of the incubations as described in Figure 4 were analyzed by HPLC. As can be seen in Figure 5, both the ^{3}H- and the ^{14}C-radioactivity gave a single coinciding peak. The ^{3}H/^{14}C-ratio in this peak from 60 min incubations performed with sonicates from PMSF-treated PMN (Fig. 5, right panel) was identical to that in zero-time controls (Fig. 5, left panel). However, the peak of PAF and lysoPAF analogs, which were not resolved in this experiment, obtained from 60 min in-

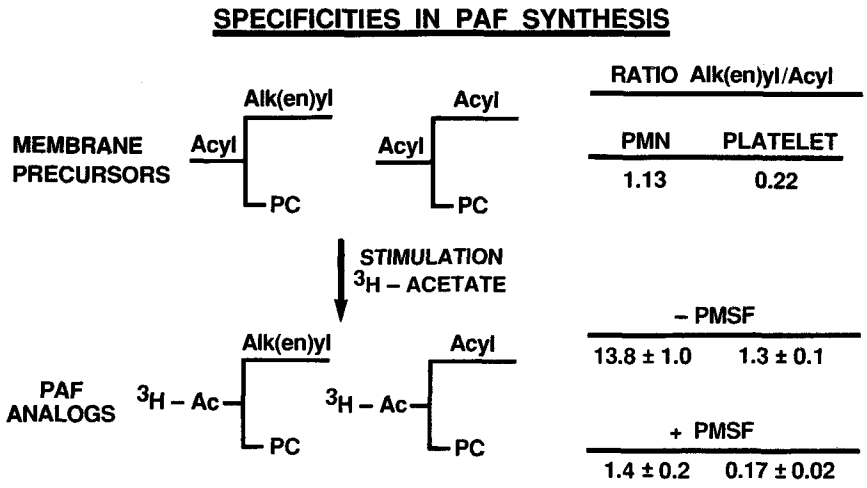


FIG. 3. Enzyme specificities in PAF synthesis. The ratio of 1-ether linked (alk(en)yl) to 1-ester linked (acyl) species in PMN and platelet diradylGPC precursors for PAF and PAF analog synthesis was calculated from the compositional data given in references 22 and 23, respectively. The ratio in total PAF and analogs accumulating in control (-PMSF) and PMSF-treated cells was determined (n=5) as described under Experimental.

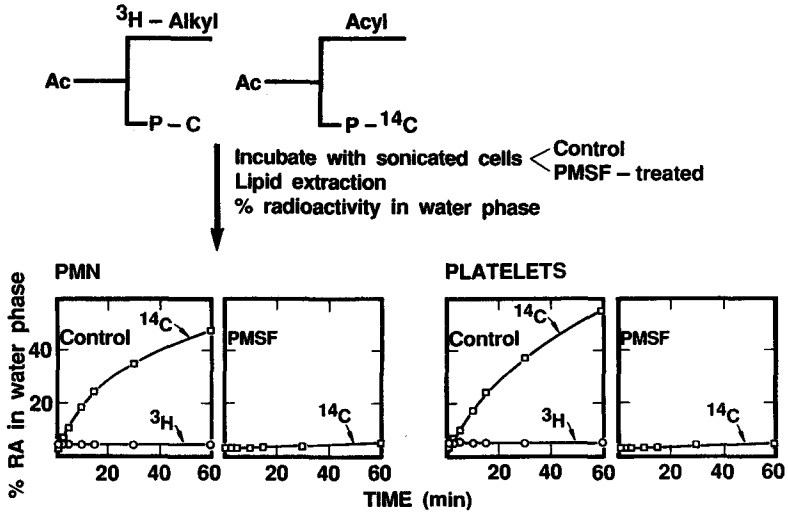


FIG. 4. PMSF prevents degradation of 1-acyl-2-acetyl-GPC. Cell sonicates were incubated with trace amounts of 1-[^{3}H]alkyl-2-acetyl-GPC (0.03 pmoles) and 1-acyl-2-acetyl-GP[^{14}C]C (25 pmoles), each containing 3000 dpm of radioactivity and representing less than 0.1% of the endogenous substrates. After incubation for the indicated time periods the reactions were stopped by lipid extraction and aliquots of the chloroform (Fig. 5) and methanol/water layer were analyzed for radioactivity.

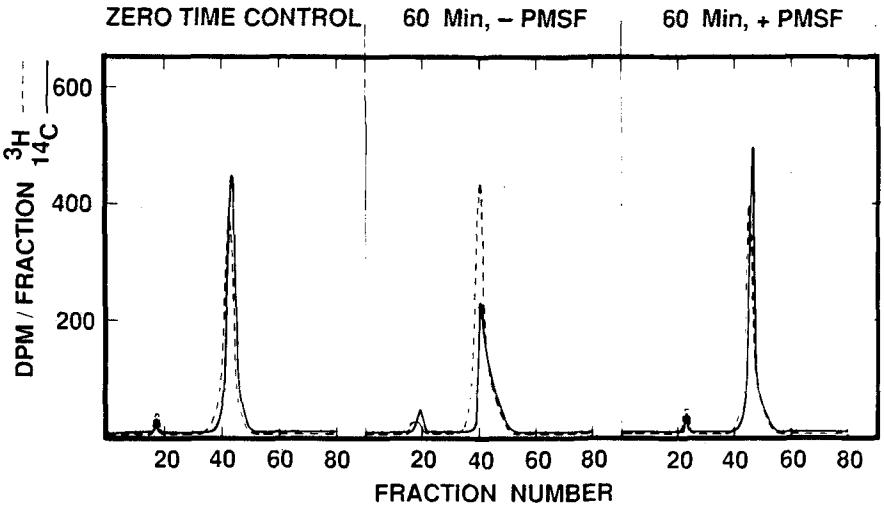


FIG. 5. HPLC of lipid extracts. The chloroform layer of incubations as described in the legend of Figure 4 was subjected to HPLC analysis as described under Experimental. Left: zero-time control; Middle: 60 min incubation with sonicate from non-PMSF-treated PMN; Right: 60 min incubation with sonicate from PMSF-treated PMN.

cubations performed with non-PMSF-treated PMN (Fig. 5, middle panel) had lost a considerable amount of the ^{14}C-label in full agreement with the appearance of about 50% of the ^{14}C-label in the methanol/H_2O layer (Fig. 4). Very similar patterns were observed for the chloroform layers obtained from incubations with platelet sonicates (data not shown). Most importantly, the fact that single ^{3}H-radioactivity peaks are observed indicates that 1-[^{3}H]alkyl-2-acetyl-GPC is not degraded by phospholipase C or D-type enzymes under the conditions of the experiments. This also suggests that the 1-acyl-2-acetyl-GP[^{14}C]C is not metabolized by such enzymes and that the appearance of water-soluble ^{14}C-radioactivity (Fig. 4) is not due to [^{14}C]phosphocholine or [^{14}C]choline formation. This was confirmed in the second type of experiment to identify the pathway of 1-acyl-2-acetyl-GPC degradation, i.e., identification of the water-soluble degradation product. The results (Table 1) clearly indicate the formation of GPC, along with a component that was tentatively identified as acetyl-GPC (*vide infra*). Collectively, the results demonstrate that control PMN and platelets contain enzyme(s) capable of deacylating the acyl analog of PAF, and this process is blocked in PMSF-treated cells. These findings provide additional support for the notion that the selectivity of phospholipase A_2 and acetyltransferase for ether linked species in PAF production is only apparent and explainable by degradation of the simultaneously produced acyl analogs, either 1-acyl-2-lyso-GPC or 1-acyl-2-acetyl-GPC. Of course, any other biochemical pathway that selectively removes the 1-acyl-2-lyso-GPC prior to acetylation could also contribute to the apparent specificity for ether linked species in PAF production. In this respect, it is interesting to note that the acyl-CoA-dependent acylation of 1-radyl-2-lyso-GPC generally proceeds several-fold faster with the 1-acyl analog than with the 1-ether linked derivatives (24–26). Thus, the selective reacylation of the acyl analog after phospholipase A_2-catalyzed formation of the lyso intermediates could leave a mixture of 1-radyl-2-lyso-GPC that is enriched in 1-alkyl-2-lyso-GPC for subsequent reacetylation. Although this mechanism could contribute to the selective accumulation of 1-alkyl-2-acetyl-GPC in non-PMSF-treated cells, it is more difficult to reconcile with the loss of this selectivity in PMSF-treated cells, unless PMSF inhibited completely the acyl-CoA:1-acyl-2-lyso-GPC acyltransferase. This possibility is less likely, based on experimental evidence indicating that this biosynthetic enzyme from the liver of various species is not inhibited by diisopropylfluorophosphate (27).

By contrast, it is demonstrated in this paper (Fig. 4) that the enzyme that deacylates 1-acyl-2-acetyl-GPC is completely blocked in PMSF-treated cells. In attempts to further identify the responsible enzyme,

TABLE 1

Identification of Water-soluble Product Formed from 1-Acyl-2-Acetyl-GP[^{14}C]C

Compound	Rf values[a]	
	System I	System II
Choline	0.57	0.79
Glycerophosphocholine	0.66	0.19
Phosphocholine	0.38	0.44
PMN product	0.64	0.20; 0.32[b]
Platelet product	0.65	0.21; 0.34[b]

[a]The water-soluble product formed in incubations with sonicates from non-PMSF-treated cells was identified by cellulose thin-layer chromatography along with reference compounds as indicated. Identical results were obtained in two separate experiments. Solvent system I: ethanol/ammonia/water (6:3:1, v/v/v). Solvent system II: t-butanol/picric acid/water (20:1:5, v/w/v).

[b]Tentatively identified as acetyl-GPC. Mild alkaline hydrolysis of this compound quantitatively converted it into glycerophosphocholine.

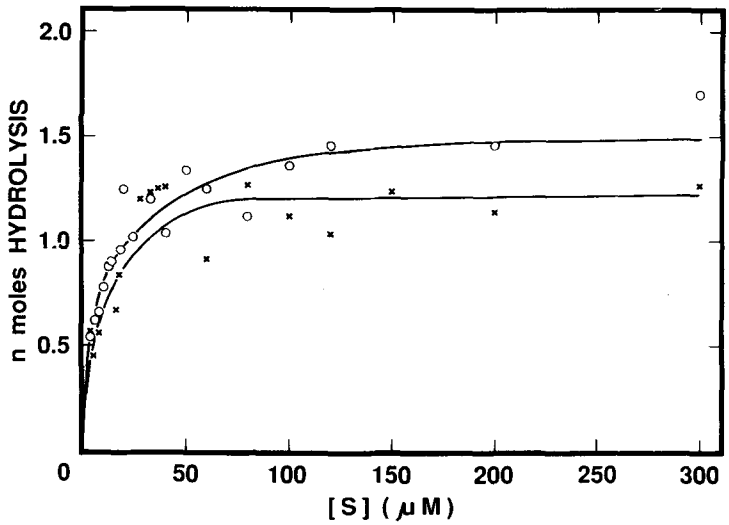


FIG. 6. Deacylation of 1-acyl-2-acetyl-GPC by lysophospholipase. The indicated concentrations of 1-palmitoyl-2-lyso-GP[^{14}C]C (o) and 1-palmitoyl-2-acetyl-GP[^{14}C]C (x) (spec. radioact. 5200 dpm/nmol) were incubated for 10 min at 37°C with 0.3 μg bovine liver lysophospholipase II in 0.5 mL of 20 mM potassium phosphate buffer (pH 7.5). Lysophospholipase II was purified as described (32). Hydrolysis of the 1-ester bond was calculated from the appearance of water-soluble radioactivity after lipid extraction and was corrected for blanks without enzyme.

we considered the possibility that it might be a lysophospholipase. These enzymes are not very specific (28) and do not require a free hydroxyl-function at the *sn*-2-position as evidenced by the fact that 1-palmitoylpropane-3-phosphocholine was hydrolyzed at a rate comparable to that of 1-palmitoyl-2-hydroxy-GPC (29). In addition, these enzymes are known to be inhibited by the serine-blocker diisopropylfluorophosphate (30,31). Therefore, we investigated whether 1-acyl-2-acetyl-GPC could be deacylated by a purified, homogeneous lysophospholipase from bovine liver (32). The results (Fig. 6) showed this to be the case. Indeed, 1-acyl-2-acetyl-GPC was deacylated at a rate comparable to that of 1-acyl-2-lyso-GPC. The water-soluble compound obtained from 1-acyl-2-acetyl-GP[^{14}C]C migrated on cellulose thin-layer plates developed with solvent system II (compare Table 1) with an Rf value of 0.34. Mild alkaline hydrolysis of this product yielded GPC (Rf 0.20), indicating that acetyl-GPC had been formed in the incubation with lysophospholipase (A.J. Aarsman, unpublished observations). The fact that the initially formed 1-lyso-2-acetyl-GPC has partially migrated into 1-acetyl-2-lyso-GPC cannot be excluded from the thin-layer chromatographic data.

These results and those of Table 1 imply that 1-acyl-2-acetyl-GPC was metabolized by sonicated non-PMSF-treated PMN and platelets both by direct deacylation and by deacylation of 1-acyl-2-lyso-GPC produced after removal of the 2-acetyl group by acetylhydrolase. As shown in Figure 6, both deacylations can be catalyzed by a lysophospholipase. Inhibition of this deacylation in PMSF-treated cells caused production of 1-ether linked and 1-ester linked analogs of PAF in a ratio nearly identical to that present in the cellular diradylGPC. This phenomenon disclosed the lack of selectivity for ether linked species of the phospholipase A_2 and acetyltransferase involved in PAF production under cellular conditions. This interpretation could only be developed by using a combination of PMSF-treatment and differentiating acetate incorporation into ether linked and ester linked analogs of PAF by phospholipase A_1 treatment. Previous reports have either studied the effect of PMSF without differentiating acetate incorporation in ether linked and ester linked PAF analogs (14,15), or have used this differentiation solely in non-PMSF treated cells (7,10-12). The results of these and our data are summarized in Table 2, and all indicate a four- to ten-fold selective utilization of ether linked species. However, based on evidence with PMSF-treated cells presented in this paper, we propose that

TABLE 2

Apparent Selectivity of Phospholipase A_2 and Acetyltransferase for Ether Linked Species in PAF Production in Non-PMSF-Treated Cells

Cell type	Ratio of 1-ether/1-ester linked species[a]		Ref.
	DiradylGPC	PAF analogs	
Rabbit platelet	0.15	0.7-1.3	10
Human platelet	0.22	2.3	7
	0.22	1.3	this paper
Rabbit neutrophil	0.35	3.0	11
	0.35	1.3	12
Human neutrophil	1.13	6.7	11
	1.13	13.8	this paper

[a]Comparison of 1-ether linked vs 1-ester linked species in cellular diradylGPC and accumulated PAF analogs upon stimulation of the indicated cell types. Ratios in cellular diradylGPC were calculated from published data: rabbit platelet (33), human platelet (23), rabbit neutrophil (12) and human neutrophil (22).

this selective accumulation of 1-ether linked PAF analogs is caused by a selective deacylation of the concomitantly produced 1-acyl analogs and intermediates, rather than by selective utilization of the 1-ether linked species by phospholipase A_2 and acetyltransferase. This interpretation would be in line with the observation that thrombin treatment of human platelets prelabelled with [^{14}C]stearic or [^{14}C]palmitic acid induced the formation of both radioactive lysophosphatidylcholine and free fatty acids (34). As suggested (34), the hydrolysis of the lysophosphatidylcholine by lysophospholipase also would explain why the amounts of lysophosphatidylcholine that accumulate in thrombin-treated platelets (35) are considerably lower than the amounts of arachidonate which are released. Likewise, A23187-stimulation of human neutrophils not only caused release of [^{3}H]arachidonate, but also of [^{14}C]palmitate from the diradylGPC pool of prelabelled cells (36).

ACKNOWLEDGMENT

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Regulation of the Biosynthesis of Platelet-Activating Factor in Alveolar Macrophages[1]

Takayuki Sugiura*, Ayako Ojima-Uchiyama, Yasuo Masuzawa, Masamichi Fujita, Yasuhito Nakagawa and Keizo Waku

Faculty of Pharmaceutical Sciences, Teikyo University, Kanagawa 199-01, Japan

Activities of enzymes which metabolize lysoplatelet-activating factor (lysoPAF) and platelet-activating factor (PAF) were studied in rabbit alveolar macrophage lysates. Substantial acetyltransferase activity was noted in the presence of 100 μM acetyl-coenzyme A (CoA), and this activity was increased in A23187-stimulated cell lysate. On the other hand, in the absence of exogenous acetyl-CoA, lysoPAF was mainly acylated through a transacylation pathway rather than by acetyltransferase in both control and A23187-stimulated cell lysates. We confirmed that the intracellular concentration of acetyl-CoA is relatively low. The observations suggest that the transacylation system may play an equally important role in the regulation of the availability of lysoPAF in intact cells. Intracellular lysoPAF was also maintained at relatively low levels. Interestingly, large amounts of PAF were produced even in unstimulated cells upon addition of an excess of exogenous lysoPAF, suggesting that generation of an adequate amount of lysoPAF within cells may be sufficient to trigger PAF synthesis in this type of cells.

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Platelet-activating factor (PAF) (1), chemically identified as 1-*O*-alkyl-2-acetyl-*sn*-glycero-3-phosphocholine (2-4), is a potent lipid mediator with a variety of biological activities. For instance, PAF was shown to induce aggregation, degranulation and infiltration of blood cells, increased vascular permeability, hypotension, bronchoconstriction and bronchial hyperreactivity *in vitro* or *in vivo* (5-7), and is now assumed to be an important mediator in various inflammatory and allergic reactions such as endotoxin shock and asthma.

Various types of mammalian cells, including polymorphonuclear leukocytes and macrophages, are known to generate considerable amounts of PAF upon stimulation (5-7). The mechanism of the biosynthesis of PAF in mammalian tissues and cells has been studied by several groups in the 1980s (5-7). The remodeling of the preexisting 1-alkyl-2-acyl-*sn*-glycero-3-phosphocholine (GPC) with a long acyl chain in position 2 to alkylacetyl-GPC (PAF) by action of phospholipase A_2 and acetyl-coenzyme A (CoA):1-alkyl-GPC acetyltransferase is considered to be the most important biosynthetic pathway for PAF in inflammatory cells upon stimulation.

The properties of the acetyltransferase have been studied by several investigators (8-13). Acetyltransferase activity was shown to increase in various stimulated cells and tissues (14-24) and is considered important in controlling the biosynthesis of PAF. A unique feature of the acetyltransferase is its ability to utilize only very short-chain acyl-CoA, such as acetyl-CoA, in the acylation of lysoPAF (1-alkyl-GPC) (8,9,12). Recent studies have also provided evidence for the presence of another enzyme which acylates lysoPAF with long-chain fatty acids derived from diacylglycerophospholipids (25-28). This enzyme, a cofactor-independent transacylase, transfers C_{20} and C_{22} polyunsaturated fatty acids from donor phospholipids to lysoPAF (25-29). Both the acetylation and the transacylation pathways may be operative and may metabolize lysoPAF in certain cell types, such as inflammatory cells.

However, precise comparative data on the activities of acetyltransferase and transacylase either in unstimulated cells or in stimulated cells are lacking. In fact, little information is available concerning the effect of cellular activation on the activity of the transacylase. It therefore seemed important to investigate these points in some detail in order to gain a better understanding of the regulation of the biosynthesis of PAF, as lysoPAF is a common substrate for both acetyltransferase and transacylase and because substrate availability could affect the amount of PAF formed.

In the present study, we first examined the activities of the enzymes which metabolize lysoPAF in rabbit alveolar macrophages. Secondly, we examined the effect of substrate availability on the biosynthesis of PAF. We found that lysoPAF was mainly acylated by transacylase rather than by acetyltransferase and that a considerable amount of PAF was formed even in unstimulated cells when a sufficient amount of lysoPAF was supplemented.

MATERIALS AND METHODS

Chemicals. All chemicals were of reagent grade. Acetyl-CoA, 1-hexadecyl-GPC (lysoPAF), alkaline phosphatase *(E. coli),* acetoacetate (lithium salt) and DL-β-hydroxybutyrate (sodium salt) were obtained from Sigma (St. Louis, MO). A23187 was from Calbiochem (La Jolla, CA). Eagle's minimal essential medium was from Nissui (Tokyo, Japan). PAF (16:0) was purchased from Bachem (Bubendorf, Switzerland). Pre-coated silica gel thin-layer chromatography (TLC) plates were from Merck (Darmstadt, Germany). 1-[^{3}H]Hexadecyl-2-acetyl-GPC ([^{3}H]-PAF) (40Ci/mmol) was purchased from New England Nuclear (Boston, MA). In order to obtain [^{3}H]lysoPAF, [^{3}H]PAF was deacetylated by brief treatment with sodium methoxide. After neutralization, the resultant [^{3}H]-lysoPAF was extracted and purified by TLC before use.

Cells. Alveolar macrophages were prepared from the pulmonary lavage of normal healthy rabbits (Japan white) as described earlier (30). For measurement of endogenous

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*To whom correspondence should be addressed at Faculty of Pharmaceutical Sciences, Teikyo University, Sagamiko, Kanagawa 199-01, Japan.

Abbreviations: BSA, bovine serum albumin; CoA, coenzyme A; GPC, *sn*-glycero-3-phosphocholine; GPE, *sn*-glycero-3-phosphoethanolamine; Hepes, *N*-2-hydroxyethyl piperazine-*N*'-2-ethane sulfonic acid; MEM, minimum essential medium; PAF, platelet-activating factor; PMN, polymorphonuclear leukocytes; TLC, thin-layer chromatography.

substrates, rabbits injected with 1 mL of Freund's complete adjuvant were used (27). Cells were suspended at 2×10^6 cells/mL in 20 mM *N*-2-hydroxyethyl piperazine-*N'*-2-ethane sulfonic acid-minimum essential medium (Hepes-MEM) (pH 7.4) and preincubated at 37°C for 10 min.

Enzyme assay. Macrophages were stimulated with A23187 (10 μM) for 2 min and subsequently cooled by adding ice-cold medium (4°C). After centrifugation at 1,200 rpm for 6 min, the supernatant was discarded and the sedimented cells were resuspended at 2×10^6 cells/mL in 20 mM Hepes-MEM. Then, the cell suspension was sonicated with a Branson Sonifier (setting 3, 20 sec, 50% pulse). The cell lysate (0.5 mL) was incubated with 20 μM 1-[^{3}H]alkyl-GPC (25,000 dpm) for 5 min in the presence (for acetyltransferase assay) or absence (for transacylase assay) of 100 μM acetyl-CoA. In an experiment where endogenous acetyl-CoA was used as the substrate, a more concentrated cell lysate (2×10^7 cells/mL) was incubated with [^{3}H]lysoPAF (70,000 dpm) for 2 min. The amount of acetyl-CoA was estimated by the method of Wieland and Weiss (31). In order to study the degradation of [^{3}H]lysoPAF and [^{3}H]PAF, the incubation was carried out for 5 min in the presence of 20 μM [^{3}H]lysoPAF (25,000 dpm) and [^{3}H]PAF (25,000 dpm), respectively, the latter of which was sonicated prior to the addition to the incubation mixture. The reactions were terminated by adding chloroform/methanol (1:2, v/v), and small amounts (10 nmol) of nonradiolabeled lysoPAF and PAF were added as carriers. After the extraction of total lipids by the method of Bligh and Dyer (32), neutral lipids, phosphatidylcholine, PAF and lysoPAF were separated from each other by TLC using chloroform/methanol/water (50:25:4, v/v/v) as developing solvent. In some experiments, lipids were separated by TLC with petroleum ether/diethyl ether/acetic acid (30:70:1, v/v/v) as solvent. Lipid spots were made visible with primuline, and the silica gel was scraped off into vials. Radioactivity was measured as described earlier (33).

PAF production. Macrophages (10^6 cells) were suspended in 0.5 mL of 20 mM Hepes-MEM and preincubated at 37°C for 10 min. Then cells were incubated in the presence or absence of A23187 (final 0.5 μM or 10 μM) or lysoPAF (final 20 μM) for a further 7.5–30 min. The incubation was terminated by adding chloroform/methanol (1:2, v/v), and total lipids were extracted by the method of Bligh and Dyer (32). Individual phospholipids were separated by TLC with chloroform/methanol/water (40:24:4, v/v/v). The area corresponding to authentic PAF was scraped off and the adsorbent extracted with chloroform/methanol/water (1:2:0.8, v/v/v). After Bligh and Dyer extraction (32), the chloroform layer was evaporated to dryness. The residue was resuspended in Hepes-buffered Tyrode's solution (pH 7.4) containing 0.25% bovine serum albumin (BSA). Amounts of PAF were estimated by measuring the aggregating activity toward washed rabbit platelets (34), using 16:0-PAF as standard. LysoPAF content was estimated by measuring lipid phosphorus of the lysolecithin fraction after treatment with HCl and NaOH.

RESULTS AND DISCUSSION

The enzymatic basis for PAF metabolism has been studied by many investigators (35). However, there apparently has not been a precise study in which individual enzyme activities in the same cells were compared. In the present study, we compared in detail the activities of the enzymes involved in PAF metabolism in alveolar macrophages. The cells are regarded as an important source of PAF in pulmonary tissue (36).

Table 1 shows the activities of various enzymes which metabolize 1-alkyl-GPC (lysoPAF) and PAF in macrophage lysate. Acetyltransferase activity was shown to be the highest, with the incubation mixture being supplemental with 100 μM acetyl-CoA. Acetyltransferase activity was increased 3.6-fold in A23187-stimulated cell lysate. This increase in enzyme activity may be due to protein phosphorylation as treatment of the microsomes from stimulated cells with alkaline phosphatase *(E. coli)* abolished the increase in enzyme activity (data not shown) as had previously been demonstrated for other cell types (19,24,37,38). On the other hand, the activities of the enzymes of lysoPAF degradation and acylation (with long-chain fatty acids), as well as of PAF degradation by acetylhydrolase were considerably lower than was acetyltransferase activity and did not increase in A23187-stimulated cell lysate, which would tend to favor PAF accumulation in stimulated cells. The product of the degradation of lysoPAF was mainly 1-alkylglycerol (data not shown), suggesting that the combined actions of lysophospholipase D and phosphatase are involved as they are in other tissues (39,40). The transacylation pathway could be responsible for the rapid acylation of lysoPAF with long-chain fatty acids, as has already been suggested by us (26,27,29) and by other groups (25,28).

The metabolic fate of lysoPAF in the absence of exogenous acetyl-CoA was shown to be, however, considerably different from the results obtained above. Table 2 shows the metabolism of lysoPAF in concentrated cell lysate in the absence of exogenous acetyl-CoA. LysoPAF was mainly metabolized through the transacylation pathway to form alkylacyl-GPC, and only a small amount of PAF was produced, though the formation of PAF increased in A23187-stimulated cell lysate.

The effects of acetyl-CoA concentration on the activities of acetyltransferase and transacylase are shown in Figure 1. The activity of acetyltransferase depends quite markedly on the concentration of acetyl-CoA, while this is not the case for transacylase. The Km values of acetyltransferase for acetyl-CoA were calculated to be

TABLE 1

Activities of Enzymes Involved in the Biosynthesis and Degradation of PAF in Alveolar Macrophage Lysate[a]

	pmol/min/10^6 cell lysate	
	Control	A23187
PAF formation (acetyltransferase)	280 ± 17	1021 ± 21
PAF degradation (acetylhydrolase)	87 ± 10	78 ± 14
LysoPAF degradation (lysophospholipase D + phosphatase)	62 ± 7	47 ± 14
LysoPAF acylation (transacylase)	86 ± 18	66 ± 10

[a]Cell lysate (10^6 cells/0.5 mL) obtained from unstimulated or A23187-stimulated macrophages was incubated in the presence of 20 μM [^{3}H]lysoPAF or [^{3}H]PAF (25,000 dpm) with (for the acetyltransferase assay) or without (for other assays) 100 μM acetyl-CoA. The data represent the means ± SD from three determinations.

TABLE 2

Metabolic Fate of [^{3}H]LysoPAF in Concentrated Macrophage Lysate[a]

Product	pmol/min/10^7 cell lysate	
	Control	A23187
PAF	5 ± 1	34 ± 5
Alkylglycerol	100 ± 6	115 ± 3
Alkylacyl-GPC	975 ± 70	805 ± 115

[a]Cell lysate (10^7 cells/0.5 mL) obtained from unstimulated or A23187-stimulated macrophages was incubated in the presence of 20 μM [^{3}H]lysoPAF (70,000 dpm) for 2 min. The data represent the means ± SD from three determinations.

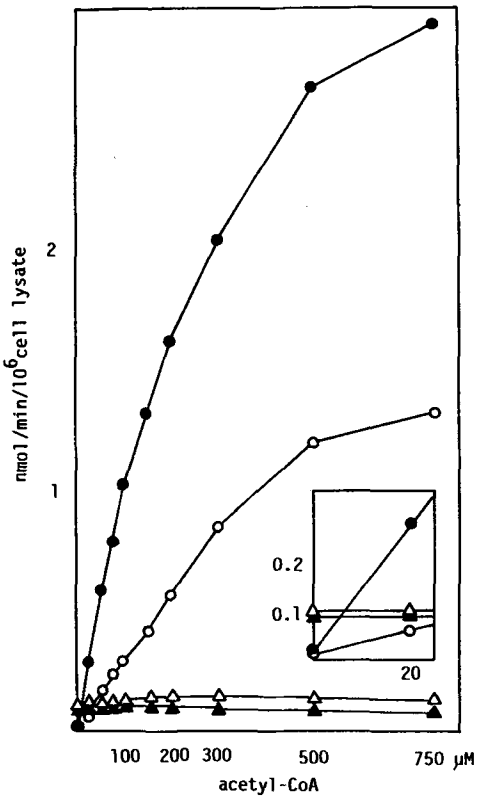


FIG. 1. Effects of the concentration of acetyl-CoA on the activities of acetyltransferase and transacylase. Macrophage lysate (10^6 cells/0.5 mL) was incubated with various concentrations of acetyl-CoA in the presence of 20 μM [^{3}H]lyso-PAF (25,000 dpm) for 5 min. After the extraction of total lipids, individual phospholipids were separated by TLC and radioactivities were estimated as described in the text. The data represent the means of three determinations. Acetyltransferase activities in control cell lysate (○—○) and in A23187-stimulated cell lysate (●—●), transacylase activities in control cell lystate (△—△) and in A23187-stimulated cell lysate (▲—▲).

around 320 μM and 250 μM in control and A23187-stimulated cell lysate, respectively. This observation is in general agreement with previous reports on other tissues and cells (9,13). However, the intracellular levels of acetyl-CoA in mammalian tissues are known to be usually low (41). A very low level of acetyl-CoA in macrophage lysate is also suggested by the results in Table 2. In fact, we found that the amount of acetyl-CoA in

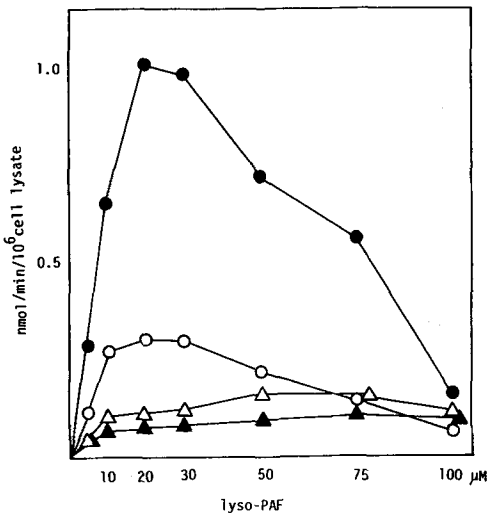


FIG. 2. Effect of lysoPAF concentration on the activities of acetyltransferase and transacylase. Macrophage lysate (10^6 cell lysate/0.5 mL) was incubated with various concentrations of [^{3}H]lysoPAF (25,000 dpm) in the presence of 100 μM acetyl-CoA. The data represent the means of three determinations. Acetyltransferase activities in control cell lysate (○—○) and in A23187-stimulated cell lysate (●—●), transacylase activities in control cell lysate (△—△) and in A23187-stimulated cell lysate (▲—▲).

rabbit alveolar macrophages is less than 4 nmol/10^8 cells (the packed volume of 10^8 cells corresponds to *ca.* 0.5 mL), using the method of Wieland and Weiss (31). It can be possible, therefore, that lysoPAF is preferentially metabolized by transacylase rather than by acetyltransferase also in living cells.

Figure 2 shows the effects of lysoPAF concentration on the activities of acetyltransferase and transacylase in the presence of 100 μM exogenous acetyl-CoA. The activities of both acetyltransferase and transacylase increased with the concentration of lysoPAF. However, the activity of acetyltransferase became maximal at 20 μM, and its activity was rather reduced at a higher concentration of lysoPAF, such as 50 μM or 100 μM, both in control and A23187-stimulated cell lysates. Such an inhibition was not observed in the case of transacylase, suggesting that acetyltransferase is much more sensitive to the detergent effect of lysoPAF. The inhibition of the activity of acetyltransferase by a high concentration of lysoPAF has also been observed in other types of cells (8,9,12,13). We measured the intracellular pool of lysoPAF by estimating the lipid phosphorus of unsaponifiable material of the lysophosphatidylcholine fraction and obtained values of 4.2 ± 0.8 nmol/10^8 cells and 6.2 ± 0.8 nmol/10^8 cells for control and A23187-stimulated cell lysate, respectively (the means ± SD from three determinations), both of which may be suboptimal concentrations for acetyltransferase, as shown in Figure 2.

Then we examined the effects of substrate availability on the PAF production. First we studied the effects, on the production of PAF, of ketone bodies which are known to be transporters of acetyl units in blood and are metabolized by various peripheral tissues to yield acetyl-CoA. As shown in Figure 3, the addition of 5 mM acetoacetate, β-hydroxybutyrate or acetate augmented PAF production. On the other hand, a relatively inactive

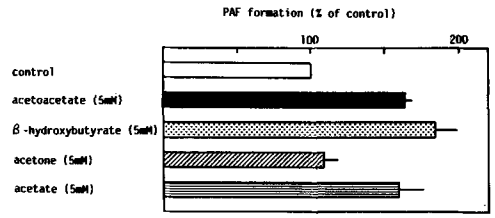


FIG. 3. Effects of ketone bodies and acetate on the production of PAF in A23187-stimulated macrophages. Macrophages (10^6 cells/0.5 mL) were stimulated with A23187 (10 μM) for 10 min in the presence of 5 mM ketone bodies or acetate. After the extraction of total lipids, PAF fraction was separated by TLC. The amounts of PAF were estimated by bioassay with washed rabbit platelets as described in the text. The data represent the means ± SD from three determinations.

ketone body, acetone, failed to enhance the formation of PAF. The addition of 5 mM acetoacetate to unstimulated cells did not affect the basal level of PAF (data not shown). The increased production of PAF induced by acetoacetate, β-hydroxybutyrate and acetate in A23187-stimulated cells may be due to the changes in acetyl-CoA level in cells induced by these materials. Several investigators have already shown that the treatment of sheep kidney with 5 mM acetoacetate (42) or rat heart with 5.5 mM β-hydroxybutyrate (43) resulted in a dramatic increase of acetyl-CoA in these tissues. The intracellular level of acetyl-CoA in rabbit alveolar macrophages is considerably lower than the optimal concentration for acetyltransferase as demonstrated in Figure 1 and Table 2. Thus, it is very likely that the increased level of acetyl-CoA in cells leads to the enhanced formation of PAF when these cells are stimulated. We also found that ionophore or opsonized zymosan-stimulated human peripheral polymorphonuclear leukocytes (PMN) produce much more PAF in the presence of acetoacetate, β-hydroxybutyrate or acetate (44). The effect of acetate or acetyl-CoA on the production of PAF has already been reported by Benveniste and his colleagues (17,45–47). In any case, the fact that the amount of PAF produced is affected by exogenous ketone bodies strongly suggests that the biosynthesis of PAF in peripheral tissues and cells is closely related to the status of lipid metabolism in the whole body, especially the liver.

The effects of lysoPAF on the biosynthesis of PAF are illustrated in Figure 4. Formation of PAF in A23187 (0.5 μM)-stimulated cells was considerably increased in the presence of 2 μM lysoPAF, suggesting that the availability of lysoPAF is not yet optimal for PAF synthesis, at least at this concentration of ionophore. On the other hand, the stimulative effect of lysoPAF was not as prominent in the case of a higher concentration of A23187 (10 μM). This is probably due to the fact that a sufficient amount of lysoPAF was available to the PAF-synthesizing enzyme, acetyltransferase. The enhanced synthesis of PAF in the presence of lysoPAF has already been reported for human polymorphonuclear leukocytes (46) and endothelial cells (48) exposed to various stimuli. In contrast, Mencia-Huerta *et al.* (45) found that the addition of lysoPAF did not accelerate PAF formation in murine peritoneal macrophages. The most striking observation in this study was, however, that considerable

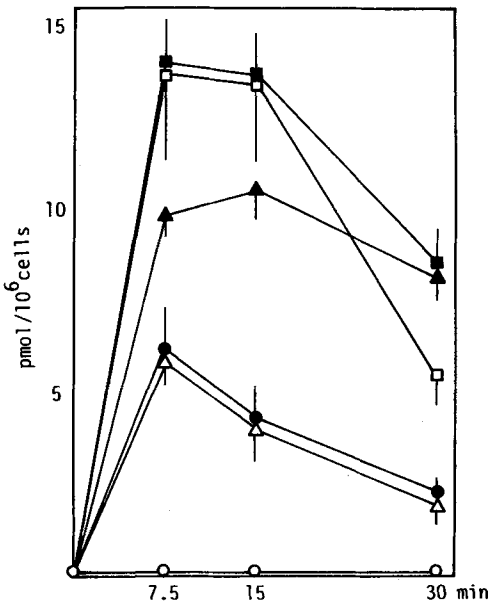


FIG. 4. Effects of exogenous lysoPAF on PAF production in unstimulated macrophages and in A23187-stimulated macrophages. Macrophages (10^6 cells/0.5 mL) were incubated with or without A23187 (0.5 μM or 10 μM) for 7.5–30 min in the presence or absence of lysoPAF (2 μM or 20 μM). The amounts of PAF were estimated by bioassay with washed rabbit platelets as described in the text. The data represent the means ± SD from three determinations. Unstimulated cells + no lysoPAF (○—○) and + 20 μM lysoPAF (●—●), A23187 (0.5 μM) + no lysoPAF (△—△) and + 2 μM lysoPAF (▲—▲), A23187 (10 μM) + no lysoPAF (□—□) and + 2 μM lysoPAF (■—■).

amounts of PAF were formed even in unstimulated cells, if these cells are incubated with a relatively high concentration of lysoPAF (Fig. 4). In fact, the amount of PAF produced in the presence of 20 μM lysoPAF alone was almost equal to that produced by 0.5 μM A23187 stimulation. This is quite different from the case for ketone bodies. We also confirmed that unstimulated human polymorphonuclear leukocytes can produce large amounts of PAF without increase in acetyltransferase activity, if these cells are incubated in the presence of lysoPAF, especially in the absence of albumin (Sugiura, T., manuscript in preparation).

These observations are of particular importance in considering the mechanism of the regulation of PAF synthesis. The level or localization of lysoPAF in unstimulated cells seems to be under strict regulation and only a small amount of PAF can be produced. Such a small amount of PAF may be rapidly degraded by acetylhydrolase, since rabbit alveolar macrophages contain relatively high acetylhydrolase activity (Table 1). In fact, the PAF level in stimulated macrophages also decreased with time (Fig. 4), suggesting that the degradation of PAF could be very important in the regulation of PAF levels, as suggested by other investigators (17,49). On the other hand, if excess amounts of lysoPAF were present within cells or near cells, significant amounts of PAF could be formed without additional stimulation, since a high activity of acetyltransferase was noted even in control cells. The elevation of acetyltransferase activity in stimulated cells shown in this study (Figs. 1 and 2 and Table 1) and in

others (14-24) could be favorable for the formation of additional PAF. However, this might not be necessary in some situations as described above. We also found that the treatment of human polymorphonuclear leukocytes with exogenous phospholipase A_2 and l-alkenyl-*sn*-glycero-3-phosphoethanolamine (GPE), the latter of which was shown to cause the degradation of alkylacyl-GPC to provide l-alkyl-GPC, produces small but significant amounts of PAF (Sugiura, T., manuscript in preparation). Taking all these results together, it would appear that the cellular availability of lysoPAF, which may be controlled by phospholipase A_2, the transacylation system, lysoPAF degrading activity, and intracellular localization, is one of the important factors which modulate the magnitude of PAF production.

In conclusion, we found that i) the concentrations of both acetyl-CoA and lysoPAF in living cells are suboptimal for acetyltransfer and that ii) increase of these substrates within cells augments PAF synthesis in ionophore-stimulated alveolar macrophages. We furthermore found that iii) the addition of an excess amount of lysoPAF *per se* triggers PAF production in unstimulated cells, which suggests that the generation of lysoPAF within cells can be enough to elicit for PAF synthesis in this type of cell, and that iv) the transacylation system could play a possible important role in regulating intracellular levels of lysoPAF.

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Platelet-Activating Factor Acetylhydrolase Activity in Human Tissues and Blood Cells[1]

Diana M. Stafforini*, Stephen M. Prescott, Guy A. Zimmerman and Thomas M. McIntyre
The Nora Eccles Harrison Cardiovascular Research and Training Institute and the Departments of Internal Medicine and Biochemistry, University of Utah School of Medicine, Salt Lake City, Utah 84112

Human tissues, blood cells, and plasma have enzymes that catalyze the hydrolysis of PAF (1-*O*-alkyl-2-acetyl-*sn*-glycero-3-phosphocholine). The activities are not due to phospholipases A_2 that hydrolyze long chain acyl groups at the *sn*-2 position of glycerophospholipids, since they are calcium-independent and are specific for hydrolysis of short chain acyl groups. We examined the biochemical properties of these PAF acetylhydrolase activities (EC 3.1.1.47) in homogenates of human liver and spleen, in white blood cells (neutrophils and monocytes), and in erythrocytes. The data suggest that the plasma and intracellular PAF acetylhydrolase activities are likely due to different proteins. Second, the intracellular PAF acetylhydrolase activities in liver and spleen share several biochemical features that differentiate them from the activities in blood cells. Third, the activities in monocytes and neutrophils have properties that differentiate them from the activity present in human erythrocytes. Finally, the erythrocyte activity has unique properties that place it in a separate category of short chain acylhydrolases. In conclusion, there is a family of distinct enzymes that can be identified as PAF acetylhydrolases based on their calcium-independence and specificity for a short residue at the *sn*-2 position of phospholipids.
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Platelet-activating factor (PAF, 1-*O*-alkyl-2-acetyl-*sn*-glycero-3-phosphocholine) is a bioactive phospholipid that is synthesized by a variety of mammalian cells (1-6). The biological effects mediated by PAF have been reviewed by Snyder (7) and Hanahan (8) and they include, among many other effects, aggregation of platelets, monocytes, and neutrophils; induction of hypotension; vasopermeability; and smooth muscle contraction. It has been suggested that PAF synthesized by endothelial cells mediates the adherence of neutrophils to the endothelium (9). PAF biosynthesis is under tight regulatory control (10), but there is also evidence that the accumulation of PAF is regulated by the rate of degradation in certain cells (11). Thus, the metabolism of PAF is an area of current interest. It has been recognized since 1980 that the degradation of PAF occurs by removal of the acetyl group at the *sn*-2 position of the glycerol backbone to produce the biologically inactive lysoPAF [1-*O*-alkyl-*sn*-glycero-3-phosphocholine, (12)]. This reaction is catalyzed by a specific PAF acetylhydrolase (1-alkyl-2-acetyl-glycerophosphocholine esterase, 1-alkyl-2-acetyl-*sn*-glycero-3-phosphocholine acetohydrolase, EC 3.1.1.47) present in plasma (13,14), in the cytosolic fraction of various mammalian tissues (12), and human blood cells (15).

We previously purified and characterized the human plasma PAF acetylhydrolase (16) and determined that PAF degradation in human blood and in plasma is entirely accounted for by this activity (17). However, the relationship of the plasma PAF acetylhydrolase and the intracellular PAF acetylhydrolase activities is currently undefined. The activities may be the expression of a single protein present in cells and fluids such as plasma, or there can be two or more proteins with the ability to specifically degrade PAF. The latter possibility is more likely, since inhibitor studies have shown a different response of the activities present in cells as compared to the plasma activity (18-20).

Blank *et al.* (12) described PAF acetylhydrolase activities in the cytosolic fractions of rat tissues. The activity present in kidney cortex was specific for hydrolysis of phospholipids with short chain acyl groups at the *sn*-2 position and the liver activity was calcium-independent. Thus, the plasma and the tissue PAF acetylhydrolases had properties that differentiated them from phospholipases A_2 that hydrolyzed phospholipids with long chain acyl groups at the *sn*-2 position. The substrate specificity and the Km of the activities present in rat tissues and in plasma were similar. However, the plasma activity had a higher apparent molecular weight than the intracellular activity (18). In addition, the rat plasma PAF acetylhydrolase was resistant to protease treatment, while the intracellular activity was sensitive (18). Nijssen *et al.* (19) described the presence of a calcium-independent PAF acetylhydrolase activity in rat lung cytosol. The activity was sensitive to treatment with serine esterase inhibitors but was not affected by the alkylating agent *p*-bromophenacylbromide. Recently, Yanoshita *et al.* examined the substrate specificity of PAF acetylhydrolases and found that the tissue-originated activities could be differentiated from the activities in blood cells and in plasma based on the rates with which the enzymes degraded PAF vs 1*S*-methyl-1-*O*-hexadecyl-2-acetyl-*sn*-glycero-3-phosphocholine (20). These observations suggest that there are at least two PAF acetylhydrolase activities—plasma and intracellular—but do not define their relationship to one another, nor do they suggest if there is more than one intracellular PAF acetylhydrolase.

In this paper we examine the biochemical properties of PAF acetylhydrolase activities in homogenates of human liver and spleen, and in blood cells. A comparison of the properties of plasma vs intracellular PAF acetylhydrolase activities suggests that they are likely due to different proteins. In addition, the PAF acetylhydrolase activities in liver and spleen share several biochemical features that differentiate them from the PAF acetylhydrolase activities in monocytes and neutrophils. Finally, the

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*To whom correspondence should be addressed.

Abbreviations: *p*-BPB, *p*-bromophenacylbromide; DEPC, diethylpyrocarbonate; DTNB, 5,5′-dithio-*bis*(2-nitrobenzoic acid); EDTA, ethylenediaminetetraacetic acid; EGTA, ethylene glycol-*bis*(β-aminoethyl ether)*N,N,N′,N′*-tetraacetic acid; LDL, low density lipoprotein; lysoPAF, 1-*O*-alkyl-*sn*-glycero-3-phosphocholine; PAF, 1-*O*-alkyl-2-acetyl-*sn*-glycero-3-phosphocholine.

erythrocyte activity has unique properties that place it in a separate category of short chain acylhydrolases. We conclude that there is a family of distinct enzymes that can be identified as PAF acetylhydrolases based on their calcium-independence and specificity for a short residue at the *sn*-2 position of phospholipids.

MATERIALS AND METHODS

Materials. 1-*O*-Alkyl-2-[^{3}H]acetyl-*sn*-glycero-3-phosphocholine ([^{3}H-*acetyl*]PAF) and 1-palmitoyl-2-[^{14}C]arachidonoyl-*sn*-glycero-3-phosphocholine were purchased from New England Nuclear (Boston, MA). 1-*O*-Alkyl-2-acetyl-*sn*-glycero-3-phosphocholine (PAF), and 1-palmitoyl-2-arachidonoyl phosphatidylcholine were from Avanti Polar Lipids (Birmingham, AL). Bovine trypsin was from Calbiochem-Behring (La Jolla, CA).

Assays. PAF acetylhydrolase assays were performed as described previously (16), except that the incubation time, unless otherwise stated, was 15 min rather than 30 min. The assays were performed at saturating concentrations of PAF, which ensured zero order kinetics with respect to substrate. A unit of enzymatic activity was defined as 1 μmol of acetate produced per hour at 37°C. Phospholipase A_2 assays were carried out as described (21), using 1-palmitoyl-2-arachidonoyl phosphatidylcholine as substrate. Protein content was determined by the method of Lowry *et al.* (22).

Preparation of tissue homogenates. Human polymorphonuclear neutrophils were isolated as described (23), and human monocytes were obtained by elutriation (11). Tissue samples of human liver and spleen were obtained from unused specimens removed by the Organ Transplantation Service, University of Utah Medical Center, and the tissues were frozen immediately after extraction. Homogenates were prepared by first slicing the tissues (1 g) into small pieces, and then homogenizing them with the aid of a Potter-Elvehjem homogenizer, in 10 mL of 50 mM Tris-HCl buffer (pH 7.5) containing 1% aprotinin and 0.1 mg/mL soybean trypsin inhibitor. When the homogenates were used to test the effect of proteases, aprotinin and trypsin inhibitor were omitted from the buffer. The preparations were centrifuged at 130,000 × *g* for 60 min at 4°C and the supernatants were used as the source of PAF acetylhydrolase.

For preparation of erythrocyte PAF acetylhydrolase, human blood was drawn from healthy volunteers using EDTA as anticoagulant. The cellular fraction was isolated by centrifugation at 2,000 × *g* for 20 min. After removing the plasma fraction, the buffy coat was aspirated and discarded. The red cells were washed three times with 0.9% NaCl and then lysed by osmotic shock in 0.83% NH_4Cl, sonication or freezing-thawing. For some experiments, the PAF acetylhydrolase activities were partially purified by ion exchange chromatography on DEAE-Sepharose CL-6B.

Chromatography and electrophoresis. Gel permeation chromatography was carried out on Sephacryl S-200 columns (1.5 × 60 cm) equilibrated with 50 mM Tris-HCl buffer (pH 7.5), at a flow rate of 10 mL/hr. Packed erythrocytes (500 μL of a preparation containing 278 mg/mL of protein) were diluted ten-fold in equilibrating buffer, lysed by sonication, and then centrifuged at 130,000 × *g* for 60 min. The supernatant, containing 0.5 U of PAF acetylhydrolase activity, was loaded on the column. Similarly, a liver homogenate prepared as described above was centrifuged at 130,000 × *g* for 60 min and 5 mL of the supernatant fraction containing 2.9 mg of protein and 0.2 U of PAF acetylhydrolase activity was loaded on the column. Human plasma low density lipoprotein fraction was prepared as described (17). A preparation containing 0.24 mg of protein and 0.28 U of PAF acetylhydrolase activity, in a total volume of 5 mL, was loaded on the column. Elution took place at 4°C. Fractions (1.8 mL) were collected and assayed for PAF acetylhydrolase activity.

Native polyacrylamide gel electrophoresis was performed under reducing conditions by the method of Davis (24). Samples of high speed supernatants from erythroctes and liver and a neutrophil lysate were separately loaded on slab gels (1.5 mm × 18 cm × 16 cm) of 7% acrylamide and electrophoresed at a constant current of 20 mA. After electrophoresis, the individual lanes were excised and sliced into 20–25 fragments of equal size. The gel slices then were assayed for PAF acetylhydrolase activity for 60 min at 37°C, in 0.1 M Hepes buffer (pH 7.2) using 50 μL of 0.1 mM [^{3}H-*acetyl*]PAF, in a total volume of 100 μL.

RESULTS

Human tissue homogenates and blood cells have PAF acetylhydrolase activity. We first determined that homogenates from human tissues and blood cells catalyze the hydrolysis of PAF. The assay developed for the determination of PAF acetylhydrolase activity in plasma was adequate for the assay of intracellular PAF acetylhydrolase activities. For example, using this assay we found that the time course of PAF hydrolysis catalyzed by a human erythrocyte lysate was linear up to 20 min and leveled off with longer incubation times (Fig. 1A). PAF hydrolysis increased linearly with the amount of protein added, up to 0.5 μL of erthrocyte lystate (Fig. 1B). The enzymatic activity increased with substrate concentration and then reached a plateau level (Fig. 1C). Optimal hydrolysis of PAF was observed at pH 7.6. Using this assay, we determined that human spleen and liver homogenates, as well as human monocytes and neutrophils, had detectable levels of PAF acetylhydrolase activity (not shown).

Since the activity we were measuring could have been the expression of phospholipase A_2-like activities that hydrolyze long as well as short fatty acyl chains at the *sn*-2 position of glycerophospholipids, we tested the effect of calcium ions added to the incubations. Calcium is an obligatory cofactor for activity of many phospholipases A_2 (25); however, addition of supplemental calcium did not stimulate PAF hydrolysis by intracellular PAF acetylhydrolases (Table 1). Furthermore, the chelating agents EDTA and EGTA did not significantly affect PAF hydrolysis. In addition, phosphatidylcholine (which had a long chain fatty acid at the *sn*-2 position) did not inhibit PAF hydrolysis by human neutrophils, monocytes, or erythrocytes. The liver and spleen PAF acetylhydrolase activities were only slightly inhibited by phosphatidylcholine.

Biochemical comparison of tissue PAF acetylhydrolase activities with the plasma activity. Agents known to

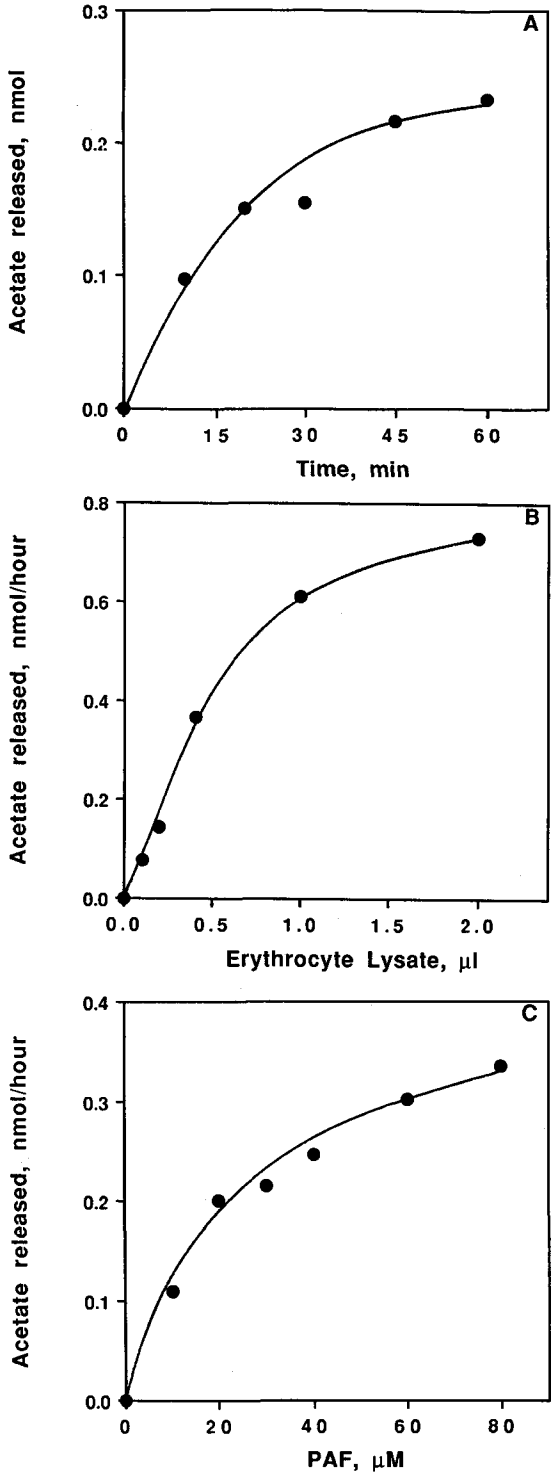


FIG. 1. Dependence of PAF hydrolysis on time, protein, and substrate concentration. (A) Time course: A fresh erythrocyte lysate (0.4 µL of a total lysate containing a protein concentration of 277 mg/mL) was incubated with 80 µM PAF at 37°C for the indicated periods of time (0–60 min). The assays were then continued as described (16). (B) Protein concentration: Varying amounts of a fresh erythrocyte lysate (containing 277 mg/mL of protein, 0–2 µL) were incubated for 30 min at 37°C in the presence of 80 µM [^{3}H-*acetyl*]-PAF. The assays were then continued as described (16). (C) Substrate concentration: Varying amounts of [^{3}H-*acetyl*]PAF (0–80 µM) were incubated for 30 min at 37°C using 0.4 µL of a fresh erythrocyte lystate containing a protein concentration of 277 mg/mL, as the enzyme source. The assays were then continued as described (16). Results are representative of at least two determinations.

TABLE 1

Effect of Calcium, EDTA, EGTA, and Phosphatidylcholine on Various Cellular PAF Acetylhydrolase Activities

	PAF acetylhydrolase activity[a] % of Control			
Tissue tested	$CaCl_2$ 2 mM	EDTA 10 mM	EGTA 10 mM	PC[b] 0.4 mM
Erythrocyte lysate	97	70	94	99
Neutrophil lysate	95	83	105	105
Monocyte lystate	81	96	101	91
Liver homogenate	96	108	102	79
Spleen homogenate	90	98	100	67

[a]Control values were obtained by adding buffer instead of the reagent indicated in each case. Results are representative of at least two determinations.

[b]PC: phosphatidylcholine.

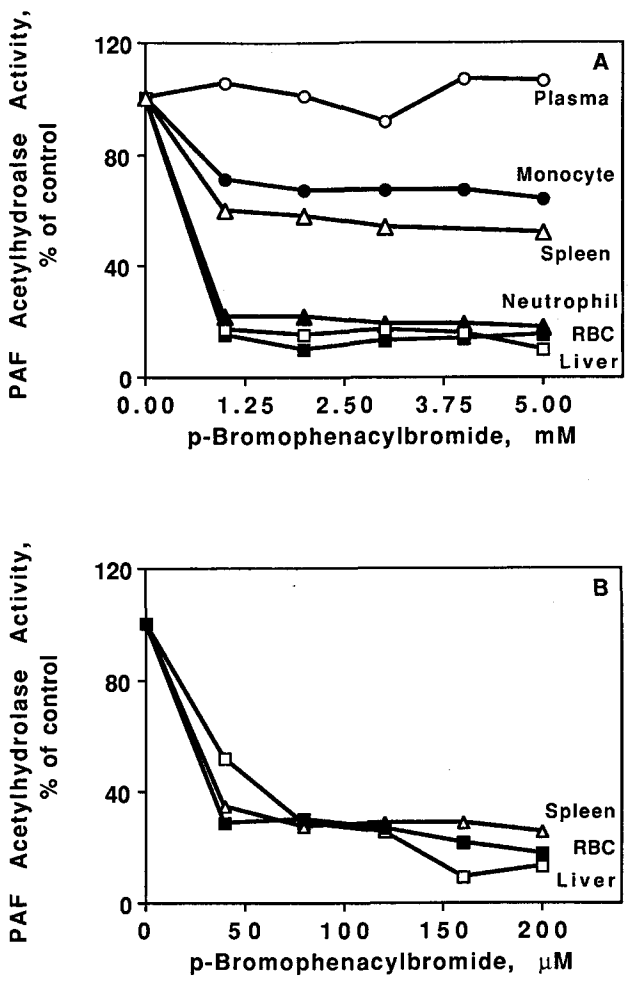


FIG. 2. Effect of *p*-bromophenacylbromide on PAF acetylhydrolase activity. Samples from various cellular fractions, prepared as described in Materials and Methods, were preincubated for 15 min at 37°C with *p*-bromophenacylbromide (*p*-BPB), at the concentrations shown. The assays then were continued as described (16). (A): crude homogenates or plasma; (B) partially purified preparations of PAF acetylhydrolase were used. Symbols: ○, plasma; ●, monocytes; △, spleen; ▲, neutrophils; ■, erythrocytes; and □, liver. Results are representative of at least two determinations.

inhibit phospholipase A_2 activity were tested for their effects on the various intracellular PAF acetylhydrolase activities. We first tested the effect of *p*-bromophenacylbromide (*p*-BPB), a nucleophilic reagent that has been shown to block phospholipase A_2 activity by derivatizing a histidine at the active site (Fig. 2A). The PAF acetylhydrolase activities from neutrophils, erythrocytes, and liver resembled one another in that they were almost completely inhibited by *p*-BPB. The monocyte and spleen activities were partially sensitive and the plasma activity was completely resistant to *p*-BPB. The partial inhibition obtained with the monocyte and spleen extracts could have been due to the presence of other proteins that bound the reagent, thus preventing it from fully inhibiting enzymatic activity. To address this issue, we subjected erythrocyte lysates and tissue homogenates to gel permeation chromatography on Sephacryl S-200 or ion exchange chromatography on DEAE Sepharose. Partial purification of the PAF acetylhydrolase activity in spleen resulted in higher sensitivity to *p*-BPB; the partially purified erythrocyte and liver activities remained sensitive to treatment with the inhibitor (Fig. 2B). In contrast, the purified plasma PAF acetylhydrolase (specific activity, 500 μmol/mg/hr) was resistant; concentrations of *p*-BPB up to 3 mM had no effect (not shown). Thus, the intracellular and plasma PAF acetylhydrolase activities had opposite sensitivities to *p*-BPB, suggesting that the activities were due to different proteins.

The presence of essential histidine residues at the active site of intracellular PAF acetylhydrolase activities was further examined by testing the effect of diethylpyrocarbonate (DEPC) on the enzymatic activities (Table 2). DEPC inhibited the partially purified intracellular PAF acetylhydrolase activities from human erythrocytes, neutrophils, and spleen, but it had no effect on the purified plasma activity (Table 2). Thus, the inhibitory effects of DEPC and *p*-BPB were in agreement and suggested the presence of essential histidine residues at the active sites of intracellular PAF acetylhydrolases, but apparently not the plasma activity.

We next examined the effect of 5,5′-dithio-*bis*(2-nitrobenzoic acid) (DTNB), an agent that inactivates enzymes containing essential sulfhydryl groups at the active site. We found that the activities in blood cells were sensitive to treatment with DTNB, in contrast to the tissue activities, which were only partially sensitive. The plasma PAF acetylhydrolase activity was resistant (Table 2). This suggested the occurrence of at least two kinds of intracellular PAF acetylhydrolase activities—the activity in blood cells such as neutrophils, erythrocytes and monocytes, which seemed to contain sensitive sulfhydryl groups, and the activity in tissues such as spleen and liver, with sulfhydryl groups partially protected from attack by DTNB or removed from the active site. The plasma activity did not seem to contain essential sulfhydryl groups.

Sodium fluoride has been reported to inhibit bee venom phospholipase A_2 (25) and Nijssen *et al.* (19) have reported that sodium fluoride partially inhibits the PAF acetylhydrolase activity in rat lung cytosol. We tested the effect of sodium fluoride on the PAF acetylhydrolase activities from plasma, tissues and blood cells (Table 2). The human erythrocyte activity was inhibited by sodium fluoride. In contrast, the intracellular PAF acetylhydrolase activities of every other source were quite resistant over a wide range of concentrations. Thus, the PAF acetylhydrolase in red blood cells has properties that differentiate it from the activity in white blood cells and from the plasma and tissue activities.

TABLE 2

Effect of Diethylpyrocarbonate, 5,5′-Dithio*bis*(2-Nitrobenzoic Acid), and Sodium Fluoride on PAF Acetylhydrolase Activities[a]

Enzyme source	Inhibitor	Activity % of Control
Plasma[b]	DEPC	80
Spleen[c]	DEPC	24
Neutrophils[d]	DEPC	28
Erythrocyte[e]	DEPC	24
Plasma	DTNB	91
Spleen	DTNB	69
Liver[f]	DTNB	61
Neutrophils	DTNB	31
Monocytes[g]	DTNB	35
Erythrocyte	DTNB	5
Plasma	NaF	100
Spleen	NaF	87
Liver	NaF	81
Neutrophils	NaF	64
Monocytes	NaF	77
Erythrocyte	NaF	7

[a]The enzyme source indicated in each case was preincubated with diethylpyrocarbonate (DEPC), 0.2–0.8 mM, 5,5′-Dithio*bis*(2-nitrobenzoic acid), DTNB, 1–2 mM, or NaF, 9–20 mM. The mixtures were preincubated for 15–30 min at 37°C. Then, [^{3}H-*acetyl*]PAF was added and the incubations were continued for 15–30 min at 37°C, as described (16). Results are representative of at least two determinations.

[b]The plasma PAF acetylhydrolase was purified from LDL to a final specific activity of 300 U/mg, as described (15). Each assay contained 0.4 ng of protein. Control activity, 12 nmol/mL/hr.

[c]The PAF acetylhydrolase activity present in high speed supernatants of spleen homogenates was purified two-fold by gel filtration on Sephacryl S-200, as described in Materials and Methods. The amount of protein per assay was 4.8 μg. Control activity, 30 nmol/mL/hr.

[d]Each assay contained 3×10^6 lysed neutrophils. Control activity, 2.3 nmol/mL/hr.

[e]The PAF acetylhydrolase activity in high speed supernatants of erythrocyte lysates was purified two-fold by gel filtration on Sephacryl S-200, as described in Materials and Methods. The amount of protein per assay was 28 μg. Control activity, 20 nmol/mL/hr.

[f]The PAF acetylhydrolase activity in high speed supernatants of liver homogenates was purified two-fold by gel filtration on DEAE Sepharose CL-6B, as described in Materials and Methods. The amount of protein per assay was 3.6 μg. Control activity, 12.2 nmol/mL/hr.

[g]Each assay contained 6×10^5 lysed peripheral blood monocytes. Control activity, 3.8 nmol/mL/hr.

Susceptibility of PAF acetylhydrolases to trypsin. Trypsin was tested for its effect on various PAF acetylhydrolase activities (Table 3). The plasma PAF acetylhydrolase activity was resistant to trypsin treatment, in contrast to the erythrocyte, liver, and spleen activities, which were sensitive. The activities in neutrophils and monocytes were only partially inhibited by trypsin

treatment. Thus, the tissue activities resembled one another and the activity present in red blood cells and were differentiated from the monocyte, neutrophil, and plasma activities.

Physical separation of various PAF acetylhydrolase activities. The cellular PAF acetylhydrolase activities were further compared by gel filtration and electrophoresis on non-denaturing polyacrylamide gels. The elution profiles of cellular PAF acetylhydrolase activities upon gel filtration chromatography on Sephacryl S-200 showed that the

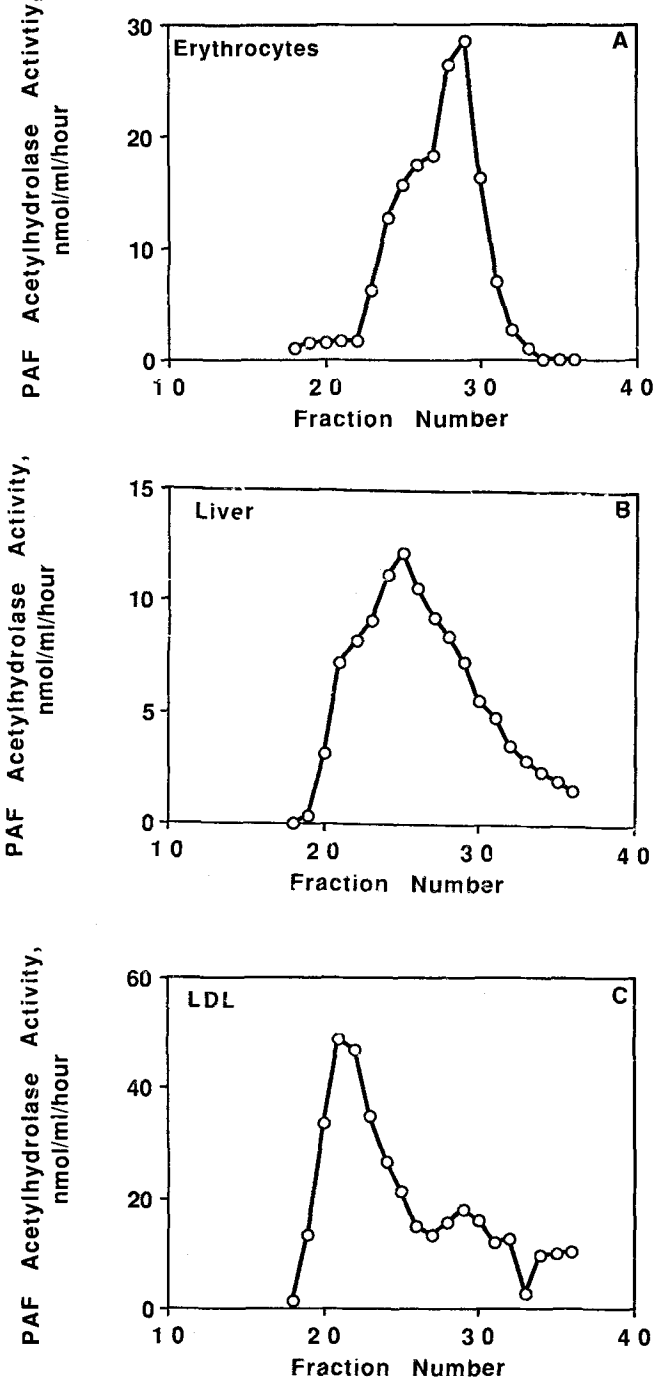


FIG. 3. Sephacryl S-200 column chromatography of human erythrocyte, liver, and LDL-associated PAF acetylhydrolase activities. Samples from the cellular fractions indicated were loaded on a Sephacryl S-200 column as described in Materials and Methods. (A) erythrocytes; (B) liver; and (C) LDL.

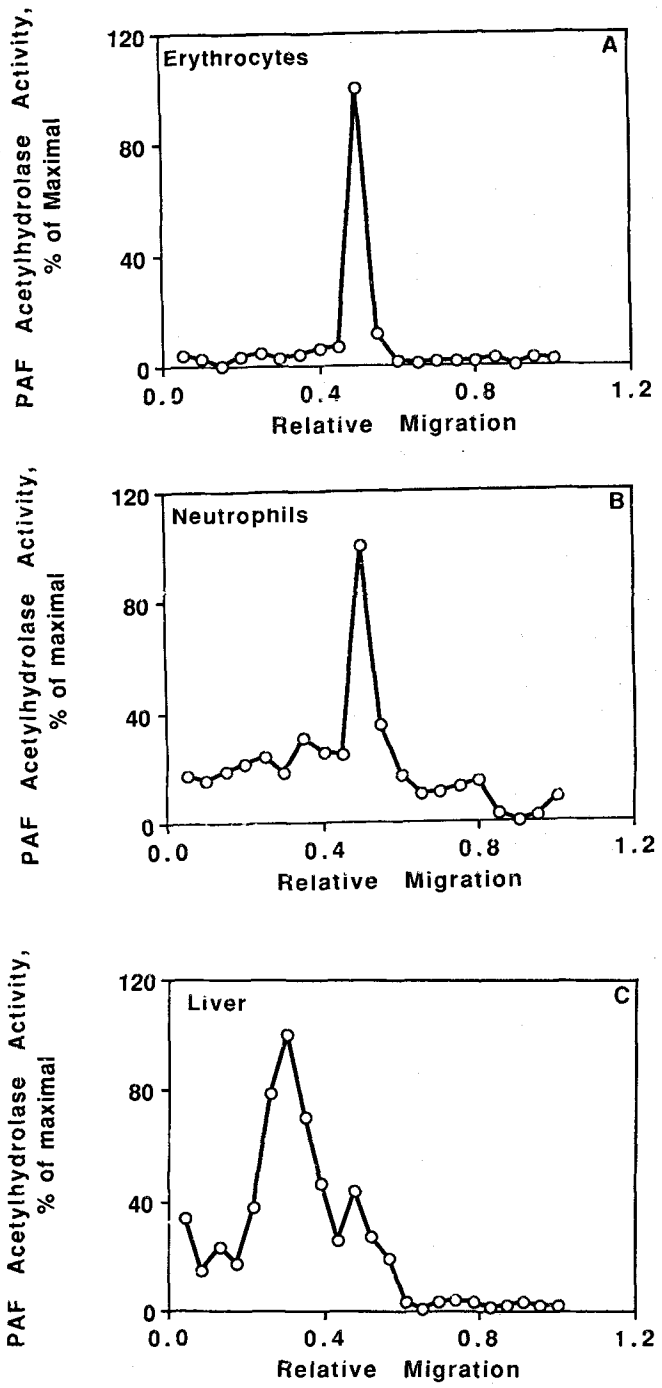


FIG. 4. Non-denaturing polyacrylamide gel electrophoresis of human erythrocyte, neutrophil, and liver PAF acetylhydrolase activities. Samples (5–100 μL) were loaded on native slab gels and electrophoresed as described in Materials and Methods. (A) Erythrocytes. The erythrocyte lysate sample (130,000 × *g* supernatant) contained 692 μg of protein; maximal activity—0.15 nmol/h. (B) Neutrophils. A total of 2×10^7 neutrophils were lysed by sonication and then subjected to electrophoresis; maximal activity—0.04 nmol/hr. (C): Liver. A crude homogenate containing 29 μg of protein was centrifuged at 130,000 × *g* for 60 min. The supernatant fraction then was subjected to electrophoresis. Maximal activity, 0.11 nmol/hr.

TABLE 3

Effect of Trypsin on PAF Acetylhydrolase Activity

Enzyme source	PAF acetylhydrolase activity[a] % of Control
Plasma	88
Spleen[b]	0
Liver[b]	7
Neutrophils	57
Monocytes	59
Erythrocytes	4

[a]Equal amounts of activity from each enzyme source were preincubated with buffer or various amounts of trypsin (0–0.025%), for 60 min at 37°C, in a total volume of 50 μL. Then, 20 μL-aliquots were incubated with 30 μL of 0.1 mM [^{3}H-*acetyl*]PAF for 60 min at 37°C, as described (16). Results reported represent the residual amount of PAF acetylhydrolase activity determined after trypsin action had reached a plateau. Results are representative of at least two determinations.

[b]The activities were partially purified as described in the legend to Table 2.

TABLE 4

Comparison of Intracellular and Plasma PAF Acetylhydrolase Activities

Location	Characteristics			
	Histidine reagents	Sulfhydryl reagents	Trypsin	NaF
Extracellular (e.g., plasma)	Resistant	Resistant	Resistant	Resistant
Intracellular				
Tissues (e.g., liver and spleen)	Sensitive	Partially sensitive	Sensitive	Resistant
Blood cells				
White blood cells (e.g., neutrophils and monocytes)	Sensitive	Sensitive	Partially sensitive	Resistant
Red blood cells (e.g., erythrocytes)	Sensitive	Sensitive	Sensitive	Sensitive

erythrocyte activity was eluted with a lower apparent molecular weight than the liver acetylhydrolase (Fig. 3). The plasma PAF acetylhydrolase was eluted at the void volume, since the activity associates with much larger lipoprotein particles (16). The elution positions of the liver and erythrocyte PAF acetylhydrolases indicated that the two activities could be differentiated from each other, in agreement with the inhibitor results. To compare the mass/charge ratio of the erythrocyte and neutrophil activities, we subjected cell lysates to electrophoresis in polyacrylamide gels that did not contain detergent (Fig. 4). The erythrocyte and neutrophil activities migrated with the same relative mobilities. The liver activity had a higher apparent mass/charge ratio, in agreement with the gel filtration results. Thus, in addition to the inhibitory profiles, the blood cell PAF acetylhydrolases can be differentiated from the tissue and plasma activities on the basis of size and mass/charge ratios.

CONCLUSION

We have shown that human tissues, white blood cells, and red blood cells have enzymes that catalyze the hydrolysis of PAF. The activities share two features with the plasma PAF acetylhydrolase activity—they are calcium-independent and only hydrolyze phospholipids with a short chain acyl group at the *sn*-2 position of the glycerol backbone. Thus, the activities are not an expression of phospholipase A_2 activities that can hydrolyze phospholipids with long chain acyl residues at the *sn*-2 position of the glycerol backbone.

The functional similarities between the plasma and intracellular PAF acetylhydrolase activities contrasted with our finding of marked differences in the physical and biochemical behavior of the enzymes (Table 4). First, the intracellular activities were differentiated from the plasma activity since the former are sensitive to histidine reagents and trypsin treatment, in contrast to the plasma activity, which is resistant. These differences were emphasized when comparisons where made with the partially purified preparations, where contamination with other proteins was less likely to contribute to the observed effects. Second, the tissue activities were differentiated from the blood cell activities, based on their size or mass/charge ratios and resistance to sulfhydryl reagents. Third, the activity in red blood cells is extremely sensitive to proteolysis and treatment with sodium fluoride, in contrast to the activities in white blood cells, which are only partially sensitive to these agents (Table 4).

It remains to be established whether the intracellular PAF acetylhydrolase activities are the expression of more than one protein, or whether their different behaviors are due to various stages in the processing of a single protein. However, it is apparent that a variety of cells and tissues express PAF acetylhydrolase activity, and that these various activities can be grouped into at least four species, according to their kinetic and physical behavior. It appears that the extracellular plasma activity, intracellular erythrocyte, white blood cell, and tissue activities comprise a family of PAF acetylhydrolases.

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Properties of PAF-Synthesizing Phosphocholinetransferase and Evidence for LysoPAF Acetyltransferase Activity in Rat Brain[1]

Gianfrancesco Goracci* and **Ermelinda Francescangeli**
Dipartimento di Medicina Sperimentale e Scienze Biochimiche, Università di Perugia, 06100 Perugia, Italy

Several reports have indicated that platelet-activating factor (PAF) may play a role in the physiopathology of nervous tissue. We previously have demonstrated the presence, in the microsomal fractions of rat brain, of a phosphocholinetransferase which is able to synthesize PAF by the *de novo* pathway. The presence of dithiothreitol in the medium increases the rate of PAF biosynthesis, whereas it inhibits the synthesis of long-chain alkylacyl- and diacyl-glycerophosphocholines (GPC), including dioctanoyl-GPC. This and other properties, such as pH dependence and thermal stability, indicate that rat brain may have two distinct enzymes for the synthesis of PAF and other choline phospholipids. The affinity of these enzymes for CDPcholine is similar to that reported for other tissues, the K_m being 42 μm and 55 μm with alkylacetylglycerol and dioctanoylglycerol as lipid substrates, respectively. The V_{max} values were 3.0 and 2.2 nmol/mg prot/min for PAF and dioctanoyl-GPC, respectively. In addition, it was shown that the microsomal fraction of rat brain contains an acetyltransferase which can convert lysoPAF to PAF. Since it has been reported previously that brain tissue possesses phospholipase A_2 activity that can hydrolyze alkylacyl-GPC to lysoPAF, we conclude that brain tissue has all enzymic activities for the synthesis of PAF by the "remodeling pathway". The role of the two routes of PAF biosynthesis in nervous tissue remains to be established.
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Different molecular species of PAF (1-*O*-alkyl-2-acetyl-*sn*-glycero-3-phosphocholine) have been detected in lipid extracts of bovine brain together with ten 1-acyl analogues (1,2). In brain of rats the basal level of PAF, as determined by bioassay, is about 0.25 pmol/g wet weight, but the concentration greatly increases upon treatment with chemoconvulsant drugs or upon electroconvulsion (3).

The role of PAF in the nervous tissue is not known, but several reports have indicated that this lipid mediator may serve some function under physiological and pathological conditions. In fact, it has been shown by Bussolino and colleagues (4) that neurotransmitters induce PAF biosynthesis in chick retina. Furthermore, Kornecki and Ehrlich (5) have shown that, at nanomolar concentrations, PAF can stimulate morphological differentiation of neural cells, but becomes cytotoxic at higher concentrations. On the basis of these findings, it was postulated that PAF might play a physiological role in neuronal development, but might also be involved in some pathological events. The latter possibility is consistent with the observations on the effect of the PAF antagonist BN52021 on cerebral ischemia-reperfusion damages in the gerbil (6). In fact, the injection of the antagonist at the onset of reperfusion clearly reduced brain free fatty acid levels (6), an increase of which has been considered as one of the biochemical indicators for ischemic brain damage (7,8).

Two pathways have been described for PAF biosynthesis in various cell types and tissues (9)—the *de novo* pathway, which produces PAF from CDPcholine and 1-*O*-alkyl-2-acetyl-*sn*-glycerol (alkylacetylG) *via* a diothiothreitol (DTT)-insensitive phosphocholinetransferase (10), and the "remodeling pathway" which leads to the formation of PAF from acetylCoA and 1-*O*-alkyl-*sn*-glycero-3-phosphocholine (lysoPAF) by a lysoPAF acetyltransferase (11).

We have recently demonstrated that a rat brain microsomal fraction contained a phosphocholinetransferase which could convert alkylacetylG and CDPcholine into PAF (12). Since Lee *et al.* (13,14) had previously reported that rat brain tissue can produce alkylacetylG, one can expect that brain should be able to synthesize PAF by the *de novo* route.

In the present study, we have further characterized the rat brain PAF-synthesizing phosphocholinetransferase which was compared with the enzyme responsible for the synthesis of other choline glycerophospholipids. Knowledge of the enzyme properties is essential for the correct assay of enzyme activities.

It is not clear whether nervous tissue can synthesize PAF by the "remodeling pathway". In fact, lysoPAF acetyltransferase activity has not been detected in the microsomal fraction from brain (11) or in synaptosomal preparations (3). On the other hand, PAF biosynthesis by lysoPAF acetyltransferase has been reported in chick retina (4).

In the present study, we provide some evidence for the presence of lysoPAF acetyltransferase in a rat brain microsomal fraction. This finding would indicate that brain tissue is able to synthesize PAF by the "remodeling pathway" as well.

MATERIALS AND METHODS

Brain microsomes were prepared from Sprague-Dawley rats (28–30-days-old) after intracardiac perfusion under light anesthesia (12). Protein concentrations were determined using bovine serum albumin as standard (15). Radioactivity of the products was measured by liquid scintillation counting.

Assay of phosphocholinetransferase. 1-*O*-Hexadecyl-2-acetyl-*sn*-glycerol and 1,2-dioctanoyl-*sn*-glycerol were from Novabiochem (Läufelfingen, Switzerland). Long-chain 1,2-diacyl-*sn*-glycerols were prepared from soybean lecithin and long-chain 1-*O*-alkyl-2-acyl-*sn*-glycerols from ratfish liver oil (16). Diradylglycerols were dispersed by sonication in a convenient volume of 0.03–0.12% Tween-20

[1]Based on a paper presented at the Third International Conference on Platelet-Activating Factor and Structurally Related Alkyl Ether Lipids, Tokyo, Japan, May 1989.

*To whom correspondence should be addressed at Dipartimento di Medicina Sperimentale e Scienze Biochimiche, Via del Giochetto, Università di Perugia, 06100 Perugia, Italy.

Abbreviations: DO, dioctanoyl; DTT, dithiothreitol; G, glycerol; GPC, glycero-3-phosphocholine; PAF, platelet-activating factor, 1-*O*-alkyl-2-acetyl-*sn*-glycero-3-phosphocholine.

depending on the experiments (12). Unless specified otherwise, phosphocholinetransferase activity was determined by incubating rat brain microsomes (0.05–0.3 mg protein) with 0.1 mM CDP[*methyl*-^{14}C]choline (spec. act. 1–2 nCi/nmol, Amersham, Little Chalfont, England) and 0.2-0.5 mM 1,2-diradylglycerol in a medium containing 60 mM Tris/HCl buffer (pH 8) and 20 mM $MgCl_2$ at 37°C using a final volume of 0.3 mL. Twenty mM DTT (Sigma, St. Louis, MO) was included when activity for PAF synthesis was tested; otherwise DTT was omitted. The concentration of Tween-20 in the incubation medium was 0.005%. Extraction, isolation of labeled choline glycerophospholipids, and their identification were as previously described (12). Lipids were separated by TLC on Silica gel G using chloroform/methanol/acetic acid/water (50:25:8:4, v/v/v/v) as developing solvent (10).

Assay of lysoPAF acetyltransferase. The enzymic activity was assayed as reported by Wykle *et al.* (11) by incubating the homogenate or microsomes from rat brain (0.3–0.5 mg protein) with 0.2 mM [1-^{14}C]acetyl-CoA (Amersham, Little Chalfont, England) and 0.03 mM 1-*O*-alkyl-*sn*-glycero-3-phosphocholine (lysoPAF, Novabiochem) in 100 mM Tris/HCl buffer (pH 6.9). The incubation was carried out at 37°C in a final volume of 0.5 mL. In some experiments acetyltransferase activity was measured in rat spleen microsomes which were prepared according to Wykle *et al.* (11) after having rinsed the isolated organ three times in 0.25 M sucrose containing 1 mM DTT.

The extraction of lipids was performed by the procedure of Bligh and Dyer (17), and labeled PAF was isolated as mentioned above. The reaction product, having the same R_F as authentic 1-*O*-hexadecyl-2-acetyl-*sn*-glycero-3-phosphocholine (PAF, Novabiochem), was further purified by TLC and/or HPLC (12) and then hydrolyzed by phospholipase C (*B. cereus*, Boehringer, Mannheim, Germany). The diethyl ether extract was then fractionated by TLC (silica gel G; chloroform/methanol, 98:2, v/v) and radioactivity was recovered together with authentic 1-*O*-hexadecyl-2-acetyl-*sn*-glycerol. The reaction product was able to induce aggregation of washed rabbit platelets, which, in turn, could be completely prevented in the presence of the specific antagonist of PAF receptor binding, BN 52021 (18).

RESULTS

We have previously reported some properties of rat brain phosphocholinetransferase activity which catalyzes the conversion of alkylacetylG to PAF (12). The enzyme is located in the microsomal fraction, requires Mg^{2+} to exhibit activity, and is inhibited by Ca^{2+}. These characteristics are identical to those of the enzymic activity responsible for the synthesis of long-chain diradyl-GPC in this tissue (19,20). Maximal activity of PAF-synthesizing phosphocholinetransferase activity was attained at pH 8 (12) and with 20 mM $MgCl_2$ (data not shown). We have also shown the effect of DTT on the synthesis of PAF, long-chain alkylacyl-GPC and long-chain diacyl-GPC (12). The data are summarized in Figure 1. The effect of DTT on the synthesis of dioctanoyl-GPC is also reported. Our results demonstrate that the presence of DTT in the incubation mixture increases the rate of PAF biosynthesis, whereas it inhibits the synthesis of the ether analogue

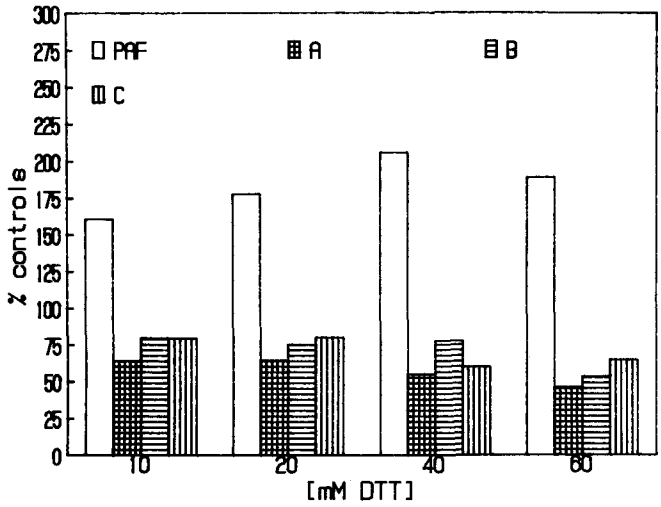


FIG. 1. The effect of DTT on the synthesis of PAF and other choline glycerophospholipids. A, long-chain alkylacyl-GPC; B, long-chain diacyl-GPC; C, dioctanoyl-GPC. Incubation conditions: for PAF, A and B, see reference 12; for dioctanoyl-GPC, 60 mM Tris-HCl buffer (pH 8), 20 mM $MgCl_2$, 0.5 mM dioctanoylG, 0.005% Tween-20, 0.1 mM labeled CDPcholine (spec. act. 1.4 nCi/nmol), 44 μg microsomal protein, 15 min at 37°C, final volume 0.3 mL. Data are representative of four experiments giving similar results.

with a long-chain fatty acid in C-2 position of the glycerol moiety. DTT also inhibits the synthesis of long-chain diacyl choline glycerophospholipids and dioctanoyl-GPC. However, this inhibition was not complete, even at relatively high DTT concentrations.

Because the physical properties of lipid substrates can greatly affect the rate of lipid incorporation into phospholipids by the phosphotransferase reaction, we have in some experiments assayed phosphocholinetransferase activity using 1,2-dioctanoylG as the lipid substrate and have compared the rate of its conversion to dioctanoyl-GPC with the rate of PAF biosynthesis under similar conditions.

Data on the effect of microsomal protein concentration on phosphocholinetransferase activity are presented in Figure 2. Under the conditions indicated, the rate of PAF biosynthesis is essentially linear up to 0.3 mg protein/mL, whereas the rate of 1,2-dioctanoyl-GPC synthesis can be considered proportional to protein concentration at least up to 1 mg protein/mL.

The dependence on CDPcholine concentration of PAF and dioctanoyl-GPC biosynthesis by brain phosphocholinetransferase is shown in Figure 3. Typical Michaelis-Menten kinetics have been obtained with either alkylacetylG or dioctanoylG as substrate. The K_m for CDPcholine was 42 μM with alkylacetylG. This value is similar to that reported for DTT-insensitive phosphocholinetransferase from rat renal inner medulla (21).

We have obtained a slightly higher K_m for CDPcholine when dioctanoylG was used as the lipid substrate (55 μM). Reddy and Horrocks (22) have measured the apparent K_m values for CDPcholine with dioctanoylG using microsomes from rat brain gray and white matter as function of age by assaying the enzyme activity at pH 7.6. With both preparations from 30-day-old rats the K_m was 32 μM.

Under the conditions indicated, the V_{max} values for PAF and dioctanoyl-GPC were 3.0 and 2.2 nmol/mg

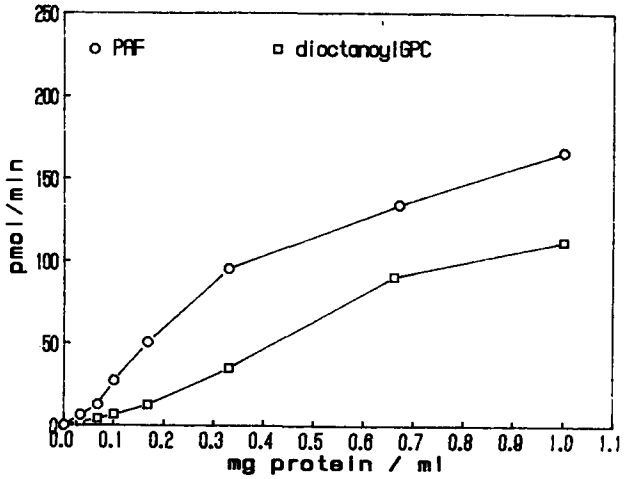


FIG. 2. Effect of microsomal protein concentration on the synthesis of PAF and dioctanoyl-GPC. Incubation conditions: 60 mM Tris-HCl buffer (pH 8), 1 mM alkylacetylG or dioctanoylG, 0.005% Tween 20, 10 mM $MgCl_2$, 0.1 mM labeled CDPcholine (spec. act. 2 nCi/nmol), microsomal protein at the indicated concentrations, at 37°C for 15 min in a final volume of 0.3 mL. PAF-synthesizing phosphocholine-transferase was assayed in the presence of 20 mM DTT. Data are from one experiment in duplicate.

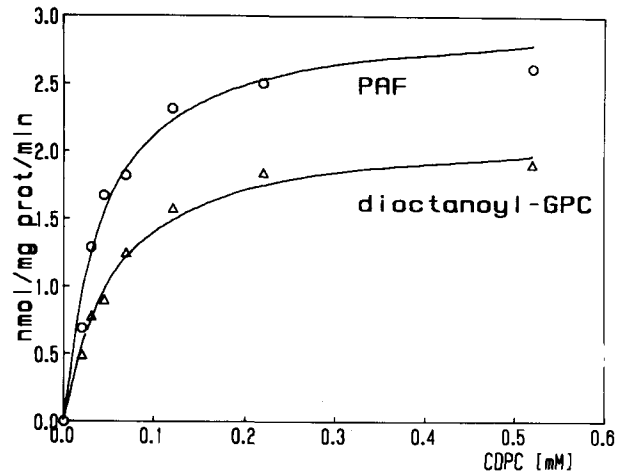


FIG. 3. Dependence of brain phosphocholinetransferase activity on CDPcholine concentration. Incubation conditions: 60 mM Tris-HCl buffer (pH 8), 0.5 mM diradylG, 0.005% Tween 20, 20 mM $MgCl_2$, labeled CDPcholine (spec. act. 50–1.9 nCi/nmol), 90 μg microsomal protein, 37°C for 5 min, in a final volume of 0.3 mL. PAF-synthesizing phosphocholinetransferase was assayed in the presence of 20 mM DTT. Data are the mean of duplicate measurements from one experiment which is representative of two others.

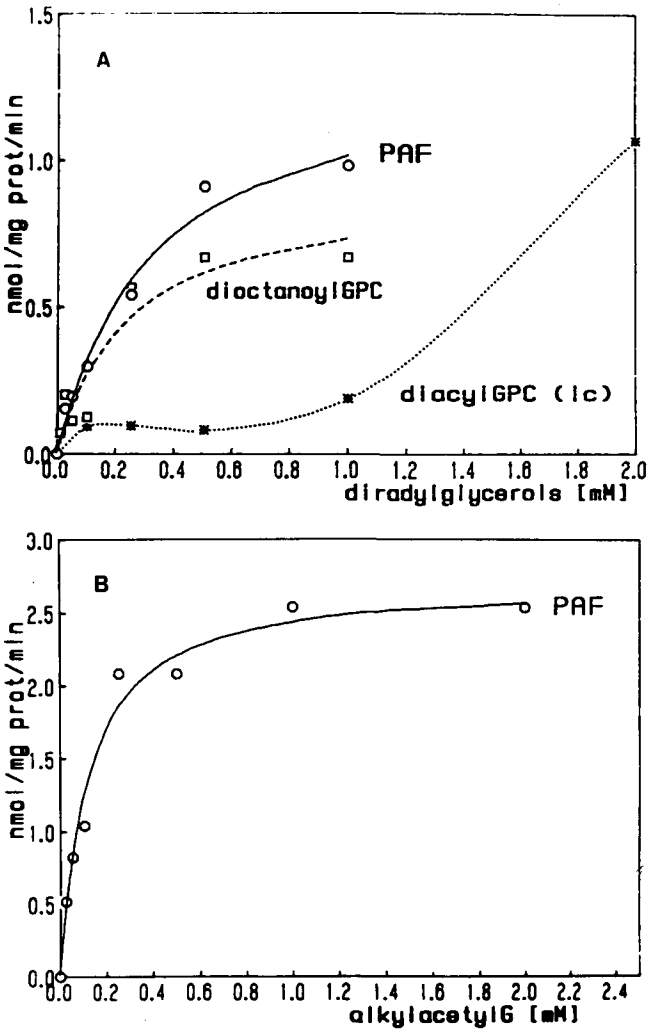


FIG. 4. Dependence of brain phosphocholinetransferase activity on diradylglycerols concentration. A, 60 mM Tris-HCl buffer (pH 8), alkylacetylG and dioctanoylG at the indicated concentrations, 0.005% Tween 20, 10 mM $MgCl_2$, 0.5 mM labeled CDPcholine (spec. act. 0.9 nCi/nmol), 0.12 mg microsomal protein, 37°C for 5 min, in a final volume of 0.3 mL. Data for long-chain diacylglycerol are from reference 12. PAF-synthesizing phosphocholinetransferase was assayed in the presence of 20 mM DTT. B, as above, but 20 mM $MgCl_2$ was used.

prot/min, respectively. Woodard *et al.* (21) found a V_{max} of 9.6 nmol/mg prot/min for PAF biosynthesis by the renal enzyme. Higher values of V_{max} than those given in the present paper have been reported using dioctanoylG as substrate and microsomal preparations from gray (5.1 nmol/mg protein/min) and white matter (4.7 nmol/mg protein/min) of brain from 30-day-old rats (22).

The dependence of brain phosphocholinetransferase activity on diradylG concentration is reported in Figure 4. Under the conditions indicated, saturation of the enzyme was achieved with 0.5 mM alkylacetylG or dioctanoylG. Using long-chain diacylG as the lipid substrate, kinetics did not satisfy the Michaelis-Menten equation (Fig. 4A). The apparent K_m for dioctanoylG (230 μM) was lower than that for alkylacetylG (312 μM). However, both values are much higher than those reported by Woodard *et al.* (21) for 1,2-dioleoylG and alkylacetylG with microsomes from rat inner renal medulla. The V_{max} values for alkylacetylG were higher than those for dioctanoylG, averaging 1.3 and 0.9 nmol/mg prot/min, respectively. However, at higher Mg^{2+} concentration (20 mM), V_{max} for alkylacetylG was 2.7 nmol/mg prot/min and the apparent K_m was 115 μM (Fig. 4B).

We have carried out experiments for studying thermal stability of phosphocholinetransferase using alkylacetylG or dioctanoylG as substrates. Preliminary experiments had indicated that preincubation of the microsomes for

15 min at 60°C caused an essentially complete inactivation of PAF-synthesizing enzyme and of DTT-sensitive phosphocholinetransferase. Further experiments were carried out by preincubating microsomes for various time-intervals at 60°C and then measuring phosphocholine-transferase activity with alkylacetylG or dioctanoylG as the lipid substrates (Table 1). In the absence of DTT, preincubation of microsomes for 5 min at 60°C did not cause inhibition of PAF biosynthesis, whereas synthesis of dioctanoyl-GPC was reduced to 80% of the control sample, which had not been preincubated. Preincubation of microsomes for 10 min or 15 min without DTT inhibited the synthesis of dioctanoyl-GPC more than that of PAF.

When DTT was present in the medium during the preincubation period at 60°C, incorporation of both dioctanoylG and alkylacetylG into the corresponding cholinephospholipids was much reduced and this became evident after 5 min. Similar results have been obtained by preincubating microsomes at 55°C. In this case, complete inactivation of the enzymic activities was achieved after 8 min when DTT was included in the incubation system. In its absence, the synthesis of dioctanoyl-GPC was reduced by about 35% after 15 min and no effect was observed on PAF biosynthesis.

Another series of experiments was performed to estimate the capability of rat brain microsomes to synthesize PAF by acetylCoA:lysoPAF acetyltransferase, the enzyme catalyzing the last step of the "remodeling pathway". The enzyme activity was determined according to the procedure described by Wykle *et al.* (11). The rate of the incorporation of the labeled acetyl group into PAF was three-fold higher in the microsomes than in the homogenate. As shown in Figure 5, the incorporation of the labeled precursor into PAF was essentially linear with time up to 10 min. No radioactivity co-migrated with authentic PAF after TLC analysis of samples that had not been incubated or had been incubated for 30 min with microsomes which had been boiled for 5 min. The acetyltransferase activity measured was proportional to microsomal protein concentration up to 0.6 mg prot/mL (Fig. 6).

The specific activity of rat brain microsomal acetyltransferase was 0.12 ± 0.013 (n=5) nmol/mg prot/min.

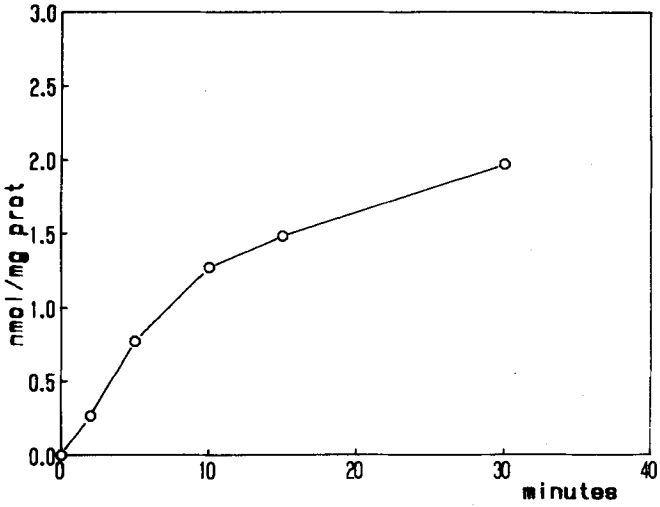


FIG. 5. Time course for the incorporation of labeled acetyl into PAF. Incubation conditions: 0.1 M Tris/HCl (pH 6.9), 0.03 mM 1-*O*-hexadecyl-*sn*-glycero-3-phosphocholine (lysoPAF), 0.2 mM [1-^{14}C]acetyl-CoA (spec. act. 2.04 nCi/nmol), 0.25 mg microsomal proteins, in a final volume of 0.5 mL. The incubation was carried out at 37°C. Data represent the mean of duplicate determinations from one experiment which is representative of another which gave similar results.

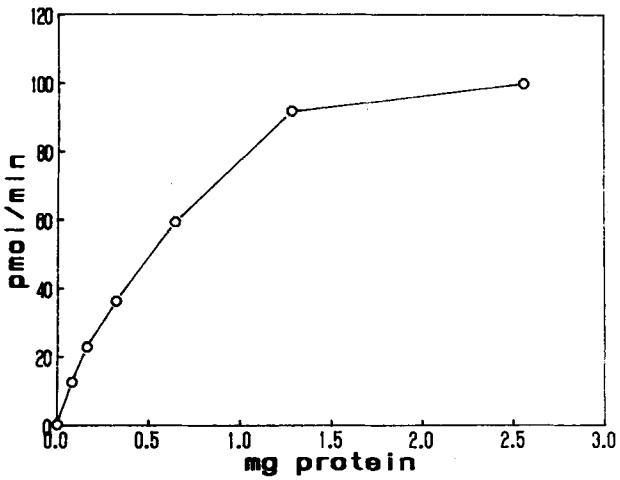


FIG. 6. Dependence of brain acetyltransferase activity on protein concentration. Incubation conditions: 0.1M Tris/HCl (pH 6.9), 0.03 mM lysoPAF, 0.2 mM [1-^{14}C]acetyl-CoA (spec. act. 2.04 nCi/nmol), microsomal protein at the indicated concentrations, 15 min, 37°C, 0.5 mL final volume. Data are the mean of duplicate determinations.

TABLE 1

Effect of Preincubation at 60°C of Brain Microsomes on Phosphocholinetransferase Activity[a]

Product	Addition	Preincubation time (min) 2	5	10	15
PAF	None	—	113.0	54.4	24.8
	DTT	95.5	5.5	1.6	1.5
DO-GPC	None	—	78.0	17.2	18.0
	DTT	87.7	3.9	1.4	1.2

[a]Microsomes were resuspended in 90 mM Tris/HCl (pH 8), or in the same buffer containing 20 mM DTT, and preincubated at 60°C for the indicated periods of time. The incubation was carried out at 37°C for 15 min in a medium containing: 60 mM Tris/HCl buffer (pH 8), 20 mM $MgCl_2$, 0.5 mM alkylacetylG or dioctanoylG, 0.005% Tween-20, 0.1 mM labeled CDPcholine (spec. act. 1.66 nCi/nmol) and 70 μg microsomal protein. Data (mean of duplicate samples) are expressed as % of the enzyme specific activity of non preincubated samples. DO-GPC, dioctanoyl-GPC.

This value is much lower than that reported for the enzyme in microsomes of rat spleen (11), but is very similar to that reported by Bussolino *et al.* (4) for the retina. We have done two experiments to assay acetyltransferase activity in rat spleen microsomes and, under our conditions, we measured a specific activity of 0.23 nmol/mg protein/min, which is very low with respect to the value reported by Wykle *et al.* (11), but closer to that reported by others (23).

DISCUSSION

There is some evidence (24,25) that in nervous tissue long-chain diacyl-GPC and long-chain alkylacyl-GPC are

synthesized by the same phosphocholinetransferase, but it is not known whether or not this enzyme catalyzes the conversion of alkylacetylG to PAF. We earlier reported the presence in rat brain of a phosphocholinetransferase activity that can convert CDPcholine and alkylacetylG to PAF (12). We also described some of its characteristics (*i.e.*, subcellular localization, Mg^{2+} requirement, inhibition by Ca^{2+}) that are similar to those identified for the synthesis of long-chain choline glycerophospholipids (19,20). However, other properties, such as the dependence of enzyme activity on pH and, more evidently, the effect of DTT, have indicated the possibility that a distinct enzyme catalyzes the biosynthesis of PAF in brain tissue (12). In fact, the presence of DTT in the incubation medium inhibits the incorporation of long-chain alkylacylG, long-chain diacylG and dioctanoylG into the corresponding choline glycerophospholipids, whereas it has a stimulatory effect on the incorporation of alkylacetylG into PAF. These findings confirm previous data obtained with microsomal preparations from other tissues (10,21) which suggested that the DTT-insensitive phosphocholinetransferase possesses a strong specificity for alkylglycerol having a very short carbon chain at the 2-position of the glycerol moiety. In addition, it should be pointed out that, even at relatively high concentrations, DTT is not able to completely inhibit the activity of the brain phosphocholinetransferase which synthesizes long-chain choline glycerophospholipids and dioctanoyl-GPC. Due to this, we cannot exclude a certain contribution of DTT-sensitive phosphocholinetransferase activity to the synthesis of PAF in our assay system where we have included 20 mM DTT, which inhibits this enzyme activity by 25–30% only. In the future, other sulfhydryl reagents should be tested to find a more discriminatory tool to distinguish the two phosphocholinetransferase activities.

The possibility that two distinct enzymes are present in rat brain with different specificity for alkylacetylG and other diradylG is substantiated by the studies on the thermal stability of the enzymes. In fact, the data reported in Table 1 indicate that the PAF-synthesizing enzyme possesses a higher stability since the preincubation of microsomes at 60°C causes an earlier inhibition of the synthesis of dioctanoyl-GPC with respect to that of PAF. This finding is in agreement with data by Woodard *et al.* (21) who also reported a higher thermal stability of PAF-synthesizing enzyme of rat kidney medulla by pretreating microsomes for 15 min at various temperatures. It is also of interest to re-emphasize the rapid inhibition of the synthesis of PAF and of dioctanoyl-GPC at 60°C when DTT was present in the incubation system. At present, we have no explanation for this phenomenon.

The studies of the kinetic properties of brain phosphocholinetransferase have shown that the K_m values for CDPcholine are very similar when alkylacetylG or dioctanoylG are used as the lipid substrates and that the values are also very close to those reported for microsomes from inner renal medulla with alkylacetylG or long-chain diacylglycerol (21). The V_{max} for the synthesis of PAF in brain is three times lower than that measured for the renal enzyme (21).

Brain tissue contains 1-*O*-alkyl-2-acyl-*sn*-glycero-3-phosphocholine which represents about 5% of the total choline glycerophospholipids (26). This ether lipid is considered to be the metabolic precursor for the synthesis of PAF by the "remodeling pathway" (27). In brain, it is synthesized by a phosphocholinetransferase which utilizes long-chain alkylacylG as the lipid precursor (25).

It has also been demonstrated that brain tissue possesses a phospholipase A_2 activity which hydrolyzes 1-*O*-alkyl-2-acyl-GPC to produce the corresponding 2-lyso derivative (28,29).

The present paper reports preliminary results showing the presence in the rat brain of an acetyltransferase activity which is able to convert lysoPAF into PAF. Its specific activity is very low if compared to the activities reported for other tissues, but it is possible that it has been underestimated since conditions have not been optimized. Furthermore, it is known that acetyltransferase of rat spleen microsomes is activated by phosphorylation (23), and it cannot be excluded that the brain enzyme is regulated by the same mechanism. In this case, a loss of activity might have occurred during homogenization by dephosphorylation of the enzyme.

On the basis of our findings, we can conclude that rat brain possesses the capability of synthesizing PAF by the "remodeling pathway" in addition to the *de novo* pathway. It will be necessary to establish the role of the two metabolic routes in the brain. This task is made more difficult because different brain areas and different cell populations are known to have very different functions and it is quite possible that the enzymic activities responsible for PAF biosynthesis by the two pathways may be of different importance in glial and neuronal cells and in different brain regions. For instance, we have demonstrated that the specific activity of phosphocholinetransferase for the synthesis of long-chain diacylGPC is higher in neuronal cells than in glial cells (20). The same is true for phospholipase A_2, which is about five-fold higher in neurons than in glia (30).

The regulation of brain choline glycerophospholipid synthesis takes place mainly at the level of the cytidylyltransferase reaction (20,31) which produces CDPcholine from phosphocholine and CTP. CDPcholine is a common substrate for the synthesis of long-chain choline glycerophospholipids and PAF by the *de novo* pathway. Since the affinity for CDPcholine of PAF-synthesizing phosphocholinetransferase is very similar to that of the enzyme responsible for the synthesis of diradyl-GPC, having a longer chain at C-2 of the glycerol moiety, the relative rate of PAF biosynthesis might depend on the availability of the lipid substrate alkylacetylG. This hypothesis is supported by the data reported by Bussolino *et al.* (32) who found an increase of the concentration of alkylacetylG after stimulation of the retina with acetylcholine or dopamine in parallel with an increased rate of PAF biosynthesis *via* phosphocholinetransferase.

The mechanism for the regulation of brain phospholipase A_2 is not known and it remains to be established whether the same enzyme is responsible for the degradation of diacyl-, alkenylacyl- and alkylacyl-GPC. However, it is known that under certain pathological conditions (*e.g.*, ischemia and epilepsy), brain phospholipase A_2 becames more active and releases fatty acids from membrane phospholipids (7,8,33). As in other cell types and tissues, the activation of phospholipase A_2 would lead to the production of lysoPAF for the synthesis of PAF by the activated form of acetyltransferase (27,34,35). It also should be considered that PAF levels in brain are

regulated by the activity of acetylhydrolase which inactivates specifically PAF (3,36).

In conclusion, it appears that brain tissue is capable of synthesizing PAF by the DTT-insensitive phosphocholine-transferase and by the "remodeling pathway". As proposed by Snyder (9), the first pathway might be responsible for the production of PAF to maintain physiological levels, whereas the second pathway might be responsible for the abnormal PAF levels produced under pathological conditions or/and in situations of metabolic stress.

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The Metabolism of 1-Acyl-PAF in Rabbit Platelets And Its Possible Interaction With PAF[1]

Lhousseine Touqui*, Clystenes Soares Silva[2] and Bernardo Boris Vargaftig
Unite de Pharmacologie Cellulaire, Unite Associee INSERM/Pasteur U.285, Institut Pasteur, 75015 Paris, France

The metabolism of 1-acyl-2-acetyl-*sn*-glycero-3-phosphocholine (1-acyl-PAF), a naturally occurring analogue of platelet activating factor (PAF), was investigated in rabbit platelets. Our studies showed that 1-acyl-[^{3}H]PAF (1-palmitoyl-2-acetyl-*sn*-glycero-3-phospho[*N-methyl*-^{3}H]-choline) was converted by platelets into phosphatidyl-[^{3}H]choline ([^{3}H]PC) in a time-dependent fashion. The formation of [^{3}H]PC occurred at a rate similar to that observed when lyso-[^{3}H]PC (palmitoyl-*sn*-glycero-3-phospho[*N-methyl*-^{3}H]choline) was used as substrate. In addition, a time-dependent increase in the level of water-soluble radioactivity was observed during the incubation of platelets with either 1-acyl-[^{3}H]PAF or lyso-[^{3}H]PC. This increase was parallel to the formation of [^{3}H]PC and was not observed in the presence of [^{14}C]PAF (1-octadecyl-2-acetyl-*sn*-glycero-3-phospho[*N methyl*-^{14}C]-choline). Analysis by thin-layer chromatography showed that the soluble radioactivity was mainly associated with glycerophosphocholine (GPC). On the other hand, the preincubation of platelets with phenylmethylsulfonyl fluoride, an inhibitor of the acetylhydrolase, reduced the hydrolysis of 1-acyl-[^{3}H]PAF to [^{3}H]GPC with a concomitant accumulation of radioactivity in 1-acyl-PAF. These findings suggest that 1-acyl-PAF is converted into PC through deacetylation-reacylation with lysoPC as an obligatory intermediate. The findings also indicate that the lysoPC resulting from 1-acyl-PAF is either reacylated to phosphatidylcholine (PC) or hydrolyzed to GPC by lysophospholipase. Finally, we showed that the stimulation of platelets with PAF led to a time- and concentration-dependent increase in the conversion of 1-acyl-[^{3}H]PAF to [^{3}H]PC. The stimulatory effect of PAF was not observed when platelets were lysed before incubation, suggesting that PAF enhances the metabolism of 1-acyl-PAF, probably by accelerating its translocation through the plasma membrane.
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PAF (platelet-activating factor, 1-*O*-alkyl-2-acetyl-*sn*-glycero-3-phosphocholine) is a potent mediator of platelet aggregation involved in inflammation and allergy. It is actively produced by a variety of cells, including platelets, through a calcium-dependent mechanism. The biosynthesis of PAF involves the deacylation-reacetylation of its inactive precursor 1-*O*-alkyl-2-acyl-*sn*-glycero-3-phosphocholine (alkylacyl-GPC) by a phospholipase A_2 and an acetyltransferase. Conversely, PAF is inactivated by a deacetylation reaction catalyzed by a calcium-independent acetylhydrolase found both in plasma and in the cytosolic fraction of the cell. This reaction leads to the generation of the inactive metabolite lysoPAF which, in turn, is reacylated by transacylases into alkylacyl-GPC (1,2). Polymorphonuclear leukocytes (PMN) (3,4) and platelets (5,6) produce a 1-acyl analogue of PAF (containing an ester linkage at the *sn*-1 position) upon stimulation with the calcium ionophore A23187. 1-Acyl-PAF (1-acyl-acetyl-*sn*-glycero-3-phosphocholine) represents approximately 40% and 30% of the total amount of PAF-like materials produced by stimulated PMN and platelets, respectively. Rat spleen microsomes contain an acetyltransferase activity which converts lysophosphatidylcholine (lysoPC) into 1-acyl-PAF (7,8). This enzyme seems to be identical to the acetyltransferase involved in the acetylation of lysoPAF. Unlike PAF, 1-acyl-PAF can be hydrolyzed by a phospholipase A_1 (PLA_1) leading to the formation of water-soluble acetylglycerophosphocholine (6). It has been shown that 1-acyl-PAF is 200- to 500-fold less active than PAF in inducing platelet aggregation (9,10), suggesting that this analogue might not have physiological functions. Although the biological role of this unique lipid molecule is not yet clear, it represents an important part of the total amount of PAF-like material produced in stimulated PMN and platelets (3–6) and probably in other cells. So, it will be of importance to compare the biochemical properties of PAF and that of 1-acyl-PAF in various types of cells.

MATERIALS AND METHODS

Materials. [^{3}H]PC (1,2 dipalmitoyl-*sn*-glycero-3-phospho-[*N-methyl*-^{3}H]choline, 40–80 Ci/mmol) and [^{14}C]PAF (1-*O*-octadecyl-2-acetyl-*sn*-glycero-3-phospho[*N-methyl*-^{14}C]choline, 50–60 mCi/mmol) were from Amersham Corp. (Arlington Heights, IL). 1-Acyl-[^{3}H]PAF (1-palmitoyl-2-acetyl-*sn*-glycero-3-phospho[*N-methyl*-^{3}H]-choline) and lyso[^{3}H]PC (1-palmitoyl-*sn*-glycero-3-phospho[*N-methyl*-^{3}H]choline) were prepared as indicated below. Unlabelled phospholipids (PC, lysoPC, PAF), bee venom phospholipase A_2 (PLA_2), and bovine serum albumin (BSA) were from Sigma Chemical Co. (St. Louis, MO). Synthetic 1-acyl-PAF was the generous gift of Dr. F. Heymans (Laboratoire de Pharmacochimie Moléculaire, Université Paris VII, France). Plastic silica gel plates for thin-layer chromatography (TLC) and organic solvents were from Merck, Ltd. (Darmstadt, West Germany).

Preparation of radioactive 1-acyl-PAF. [^{3}H]PC (50 μCi) was hydrolyzed by 30-min incubation with 10 μg of bee venom PLA_2 in Tyrode's buffer containing 2 mM Ca^{2+}. The lyso-[^{3}H]PC produced by this hydrolysis was then converted into 1-acyl-[^{3}H]PAF (1-palmitoyl-2-acetyl-*sn*-

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*To whom correspondence should be addressed at INSERM/Pasteur U.285, Institut Pasteur, 28 rue Dr. Roux, 75015 Paris, France.

[2]Present address: Disciplina de Pneumologia, Escola Paulista de Medicina, Rua do Barão de Taquari, 35. CEP 04125. São Paulo, Brazil.

Abbreviations: 1-acyl-PAF, 1-acyl-2-acetyl-*sn*-glycero-3-phosphocholine; DMSO, dimethylsulfoxide; EDTA, ethylenediaminetetraacetic acid; EGTA, ethylene glycol *bis*(aminoethyl ether)*N,N,N',N'*-tetraacetic acid; GPC, glycerophosphocholine; lysoPC, lysophosphatidylcholine; PAF, platelet activating factor; PC, phosphatidylcholine; PLA_1, phospholipase A_1; PLA_2, phospholipase A_2; PMN, polymorphonuclear leukocytes; PMSF, phenylmethylsulfonyl fluoride; TLC, thin-layer chromatography.

glycero-3-phospho[*N-methyl*-^{3}H]choline) by acetylation according to the method of Wientzek *et al.* (11). Briefly, 120 μL of pyridine, 80 μL of acetic anhydride and 2 μL of perchloric acid were added sequentially to a glass tube containing 30 μCi of radioactive lysoPC. After 60 min incubation at 70°C, the solvents were evaporated to dryness with nitrogen, and lipids were separated by phase partition according to Bligh and Dyer (12). The solvent of the organic (lower) phase was evaporated to dryness and the residue redissolved in 50 μL of chloroform/methanol (2:1, v/v) and subjected to TLC with chloroform/methanol/ammonia (70:35:7, v/v/v) as mobile phase. Unlabelled phospholipids (PAF, lysoPAF, 1-acyl-PAF and lysoPC) were applied onto the same plate as standards and visualized with iodine vapors. The plates were divided into 34 bands (0.5 cm width) which were scraped into scintillation vials containing 5 mL of ACS II and radioactivity was determined by scintillation counting. Radioactive 1-acyl-PAF migrated as a single band closely corresponding to PAF (Rf = 0.41). The fractions containing radioactive 1-acyl-PAF were scraped off and extracted as indicated above. The solvent of the organic phase was evaporated to dryness and the residue resuspended in 100 μL of ethanol and stored at −20°C until use.

Preparation and lysis of platelets. Blood was collected from the central ear artery of adult New Zealand rabbits directly into a 5 mM solution of ethylenediaminetetraacetic acid (EDTA) and the platelets were washed according to the method of Ardlie *et al.* (13). The final suspension (5 × 10^8 platelets/mL) was in calcium-free Tyrode's buffer containing 1 mM ethylene glycol *bis*(aminoethyl ether)*N,N,N',N'*-tetraacetic acid (EGTA) and 2.5 mg/mL gelatin. Aliquots of platelet suspensions were kept in an ice bath, lysed by sonication (30 seconds, twice), using a MSE sonifier at its maximum output, and then kept at −80°C until use.

Metabolic studies. Suspensions (2.5 mL) from intact or lysed platelets were incubated with 5 × 10^4 cpm/mL of 1-acyl-[^{3}H]PAF, lyso-[^{3}H]PC or [^{14}C]PAF added in ethanol solution (0.1% final). The final concentration of each lipid was adjusted to 10 nM by the addition of unlabelled compound. In some experiments, unlabelled PAF was added to platelets at the concentrations indicated in Figure 5 immediately before incubation with 1-acyl-[^{3}H]PAF (in the absence of unlabelled 1-acyl-PAF). At the times indicated (see Figures), aliquots (0.5 mL) were transfered into Eppendorf tubes containing 1 mL of methanol/chloroform (1:1, v/v) and maintained at 4°C. Phase partition was promoted by 2-min centrifugation at 12,000 rpm. Samples (100 μL) from the aqueous phases were carefully aspirated and their radioactivity measured by liquid scintillation counting. Finally, the lipids were extracted from the organic phases according to Bligh and Dyer (12). The radioactivity associated with the organic and aqueous phases were analyzed by TLC, as indicated below.

In other experiments, suspensions from lysed platelets were treated for 10 min at 37°C with increasing concentrations of phenylmethylsulfonyl fluoride (PMSF) or with dimethylsulfoxide (DMSO, 0.25% v/v) before addition of 1-acyl-[^{3}H]PAF or lyso[^{3}H]PC. After 30 min incubation, the reactions were stopped and the extracts were analyzed as indicated above.

TLC analysis. The organic phases of platelet extracts were evaporated to dryness under nitrogen and resuspended in 50 μL of chloroform:methanol (2:1, v/v). The radioactivity contained in 5 μL of these suspensions was measured by liquid scintillation spectrophotometry to determine the yield of extraction. The remaining suspensions (45 μL) were submitted to TLC on plastic silica gel plates using chloroform/methanol/ammonia (70:35:7, v/v/v; System A) as mobile phase. Unlabelled PAF, lysoPAF, lysoPC, 1-acyl-PAF and PC were also applied onto the same plates as standards. Hundred-μL aliquots from aqueous phases were boiled for 10 min (100°C) and then applied to plastic silica gel plates. The plates were developed in a solvent system consisting of methanol/2.4% NaCl/water/ammonia (50:12.5:17.5:5, v/v/v/v) (System B). This system was adapted from that described by Vance *et al.* (14) and allowed good separation of phosphocholine and glycerophocholine (Rf = 0.35 and 0.47, respectively). The plates were then divided into 17 bands and radioactivity of the fractions was determined by liquid scintillation counting.

RESULTS AND DISCUSSION

Rabbit platelets were incubated with radioactive 1-acyl-PAF for various time periods and then extracted by addition of chloroform/methanol (see Methods). The radioactivity associated with the organic phase was analyzed by TLC. It was shown that the content of 1-acyl-PAF decreased progressively with a concomitant increase in PC (Fig. 1). The radioactivity associated with lysoPC

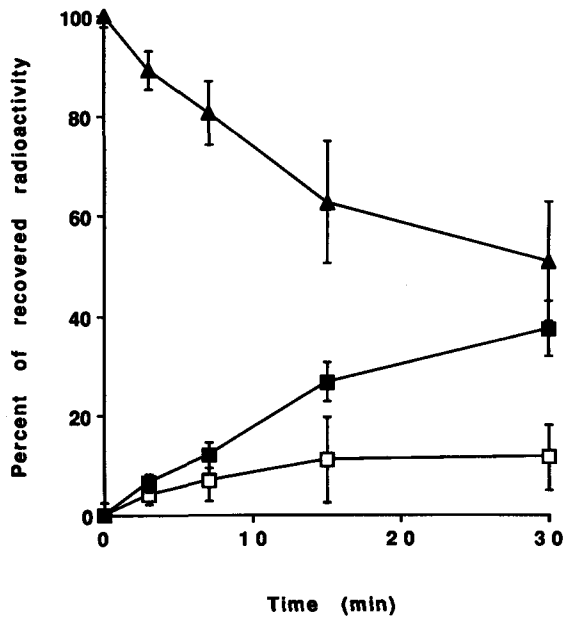


FIG. 1. Conversion of 1-acyl-PAF by intact platelets. The platelet suspension was incubated at 37°C with 1-acyl-[^{3}H]PAF (10 nM, 5 × 10^4 cpm/mL, final conc.). Aliquots (0.5 mL) were collected at the times indicated. The lipids were extracted and analyzed by TLC as described in the Methods section. The Figure shows the variation in the radioactivity associated to 1-acyl-PAF (▲), PC (■) and lysoPC (□) expressed as percentage of the radioactivity recovered from the organic phase. The results are representative for four separate experiments.

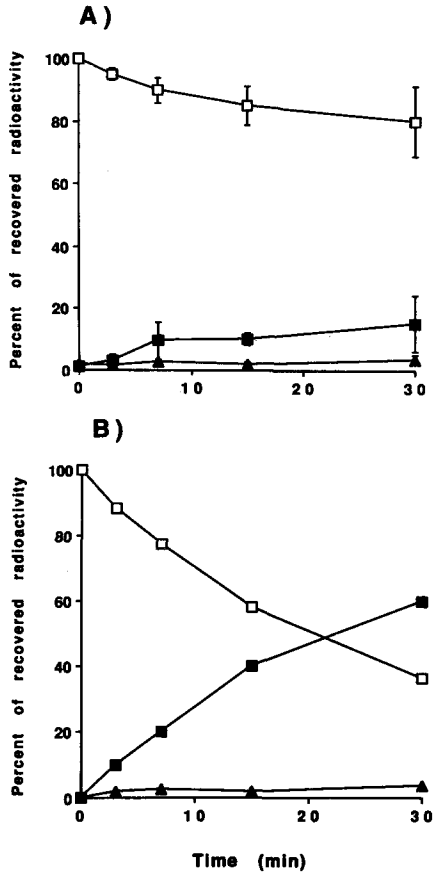


FIG. 2. Comparative metabolism of lysoPC in intact and lysed platelets. Suspensions from intact (A) or lysed (B) platelets were incubated at 37°C with lyso-[^{3}H]PC (10 nM, 5×10^4 cpm/mL). Lipid extraction and analysis were carried out as detailed in the Methods section. The Figure shows the radioactivity associated to PC (■) and lysoPC (□) expressed as percent of the radioactivity recovered from the organic phase. No significant radioactivity was associated with 1-acyl-PAF (▲).

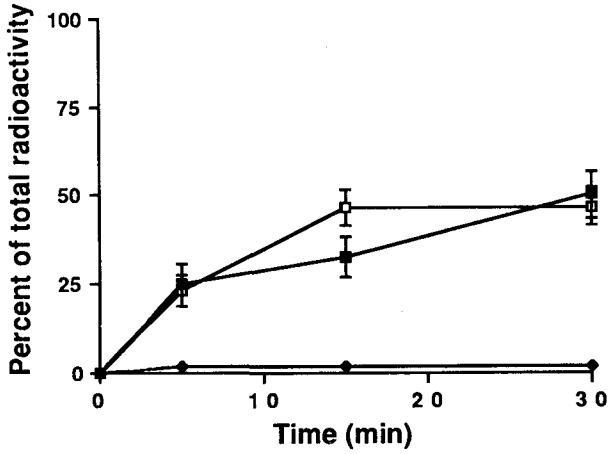


FIG. 3. Release of water-soluble radioactivity during the incubation of platelet lysates with 1-acyl-PAF, lysoPC or PAF. Platelet lysates were incubated separately with 1-acyl-[^{3}H]PAF, lyso-[^{3}H]PC or [^{14}C]PAF as indicated in the Methods section. After 30 min incubation, aliquots (0.5 mL) were transferred into Eppendorf tubes containing 1 mL of chloroform/methanol (1:1) and maintained at 4°C. Phase partition was promoted by centrifugation at 12,000 rpm for 2 min. Samples (100 μL) from the aqueous phases were carefully aspirated and their radioactivity measured by liquid scintillation counting. The Figure shows the recovery of radioactivity from the aqueous phase during incubation of platelet lysates with 1-acyl-[^{3}H]PAF (■), lyso-[^{3}H]PC (□) or [^{14}C]PAF (◆). All lipids were radiolabelled at the *sn*-3 position in the choline group.

remained constant over the time period examined. This suggests that platelets convert 1-acyl-PAF into PC through a deacetylation-reacylation reaction and that lysoPC is a transient intermediate in this conversion. However, intact platelets convert exogenous lysoPC into PC to a much lesser extent that is observed with 1-acyl-PAF (Fig. 2A); this conversion was markedly increased when platelets were lysed by ultrasonication before the addition of lysoPC (Fig. 2B). This result is in accordance with our previous findings (15) and supports the hypothesis that the platelet plasma membrane is poorly permeable to exogenous lysophospholipids. On the other hand, the recovery of radioactivity associated with the organic phase decreased following the incubation of platelets with 1-acyl-[^{3}H]PAF (data not shown), suggesting that 1-acyl-PAF was hydrolyzed to yield water-soluble products. Indeed, in contrast to PAF, 1-acyl-PAF can be hydrolyzed at the *sn*-1 position by *in vitro* incubation with PLA_1, which leads to the generation of water-soluble products (6). This procedure was used in the present study to investigate the ability of platelets to hydrolyze 1-acyl-PAF at the *sn*-1 position (i.e., PLA_1 or lysophospholipase). We showed that the incubation of platelet lysates with 1-acyl-[^{3}H]PAF led to a time-dependent increase of the water-soluble radioactivity which reached 35–45% of the total radioactivity added after 30 min (Fig. 3). This increase was parallel to that observed when platelets were incubated with lyso[^{3}H]PC instead of 1-acyl-[^{3}H]PAF (Fig. 3). However, no significant increase of water-soluble radioactivity was observed when platelets were incubated with [^{14}C]PAF (also labelled at the *sn*-3 position). TLC analysis (System B) showed that the water-soluble radioactivity derived either from 1-acyl-[^{3}H]PAF or lyso[^{3}H]PC comigrated with glycerophosphocholine (GPC) (Fig. 4). This suggests that a lysophospholipase-type activity was involved in the hydrolysis of 1-acyl-PAF. One would expect that 1-acyl-PAF is first deacetylated by the acetylhydrolase and that the resulting lysoPC is subsequently hydrolyzed by a lysophospholipase to produce GPC. However, it could be argued that 1-acyl-PAF could be first hydrolyzed at the *sn*-1 position by a PLA_1-like enzyme before its deacetylation. The results of Figure 5 show that preincubation of platelets with phenylmethylsulfonyl fluoride (PMSF), a potent inhibitor of acetylhydrolase (15), led to the inhibition of the hydrolysis of 1-acyl-[^{3}H]PAF to [^{3}H]GPC (Fig. 5) with a concomitant accumulation of radioactivity in 1-acyl-PAF (data not shown). This would support the hypothesis that 1-acyl-PAF is first deacetylated before its hydrolysis to GPC. However, this interepretation must be considered with caution because PMSF also inhibited lysophospholipase activity (i.e., hydrolysis of lysoPC to GPC) (Fig. 5). The sensitivity of this enzyme to serine protease inhibitors has been previously reported by van den Bosch *et al.* (16).

Finally, we examined whether the metabolism of 1-acyl-PAF could be regulated by the stimulation of platelets

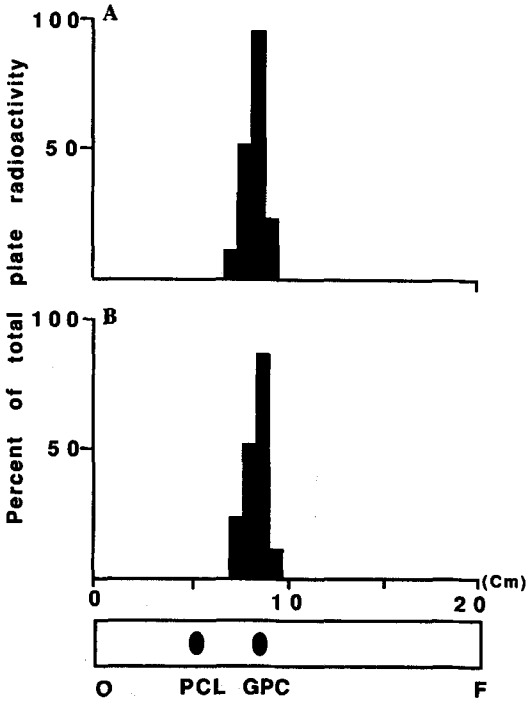


FIG. 4. Thin-layer chromatographic analysis of the water-soluble radioactivity released from 1-acyl-PAF (A) and lysoPC (B). Aliquots (0.5 mL) from platelet lysates were incubated with 1-acyl-[^{3}H]PAF or lyso-[^{3}H]PC for 30 min at 37°C. After phase partition (see above), samples (200 μL) were collected from the aqueous phase, boiled for 10 min and then applied onto plastic silica gel plates. The solvent system (System B) consisted of methanol/NaCl 2.5%/water/ammonia (50:12.5:17.5:5). The Figure shows thin-layer chromatographic distribution of the water-soluble radioactivity derived from 1-acyl-PAF (A) or from lysoPC (B). Similar results were obtained in three separate experiments. GPC and PCL refer to glycerophosphocholine and phosphocholine standards, respectively. O, origin; F, front.

with PAF. Figure 6 shows that addition of unlabelled PAF to intact platelets led to a marked increase of the conversion of 1-acyl-[^{3}H]PAF to [^{3}H]PC. This effect was dependent on the concentration of PAF added and reached maximal levels within 30 min. It occurred in the absence of extracellular calcium and was not

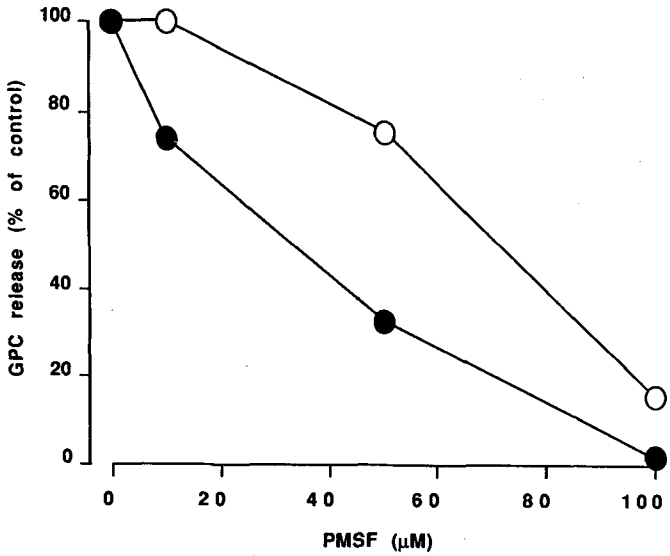


FIG. 5. Effect of PMSF on the hydrolysis of 1-acyl-PAF and lysoPC to GPC by lysed platelets. Suspensions from lysed platelets were pretreated for 10 min with increasing concentrations of PMSF or DMSO (0.25% final conc.) as control and then incubated for 30 min at 37°C with 1-acyl-[^{3}H]PAF or lyso-[^{3}H]PC. The recovery of [^{3}H]GPC in the aqueous phase was measured as indicated in the Methods section. The results show the effect of PMSF on the hydrolysis of 1-acyl-[^{3}H]PAF (○) or lyso-[^{3}H]PC (●) to [^{3}H]GPC and are expressed as percent of control (DMSO-treated platelets).

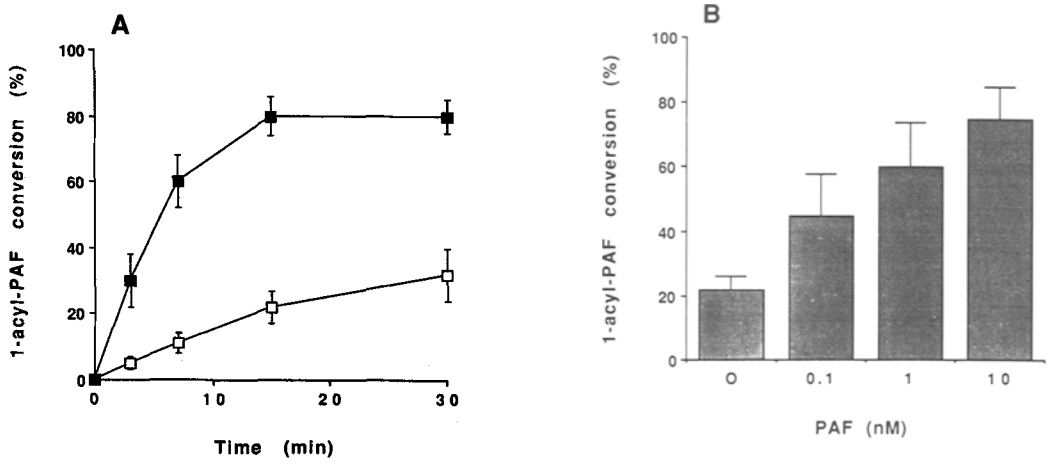


FIG. 6. Effect of PAF on the conversion of 1-acyl-PAF into PC by intact platelets. The time-course (A) and the concentration-dependent effect (B) are shown. (A) Platelets were incubated with 10 nM of unlabelled PAF or with BSA (0.25% final conc., as control) before addition of 1-acyl-[^{3}H]PAF. At the indicated time points, the reactions were stopped and the formation of [^{3}H]PC was measured as indicated above; [control (□), PAF-stimulated platelets (■)]. (B) 1-Acyl-[^{3}H]PAF was incubated together with platelets for 20 min in the presence of PAF at increasing concentrations. The results are expressed as percentage of the radioactivity recovered from the organic phase.

accompanyied by an aggregation or release reaction (data not shown). Thus, the effect of PAF on the conversion of 1-acyl-PAF seems to be independent of the platelet activation. On the other hand, the addition of PAF to lysed platelets had no significant effect on the conversion of 1-acyl-PAF (data not shown), suggesting that PAF stimulates the metabolism of 1-acyl-PAF, probably by accelerating its transmembrane movements. Such a process has been reported to be involved in the regulation by PAF of its own metabolism (17,18).

We conclude that rabbit platelets metabolize 1-acyl-PAF to PC through a deacetylation/reacylation reaction with lysoPC as intermediate. The deacetylation of 1-acyl-PAF is probably catalyzed by an acetylhydrolase similar to that involved in the hydrolysis of PAF. The ability of this enzyme to deacetylate 1-acyl-PAF has been suggested earlier by the studies of Blank *et al.* (19). Other studies (7) have shown that 1-acyl-PAF is formed by the acetylation of lysoPC, which, in turn, results from the hydrolysis of PC by PLA_2. Taken together, these findings suggest that 1-acyl-PAF is metabolized through a metabolic cycle similar to that previously described for PAF (1,2,14) and that 1-acyl-PAF may actively participate in the turnover of PC (Lands pathway). In addition, the lysoPC formed by hydrolysis of 1-acyl-PAF is also cleaved at the *sn*-1 position by a lysophospholipase-dependent reaction leading to the generation of GPC. However, the possibility that 1-acyl-PAF could be hydrolyzed at the *sn*-1 position before its deacetylation cannot be excluded. The presence of the ester linkage at the *sn*-1 position confer to 1-acyl-PAF a metabolic behavior different from that of PAF. The physiological significance of the catabolism of 1-acyl-PAF is not clear. Because intact platelets are more permeable to 1-acyl-PAF than to lysoPC, one could speculate that 1-acyl-PAF may play a role as a transport vehicle for lysoPC through the plasma membrane of platelets. Although the biological role of this unique lipid molecule is not yet clear, 1-acyl-PAF represents 40% of the total amount of PAF-like material produced in stimulated PMN and platelets. So, it will be of importance to compare the biochemical properties of PAF and 1-acyl-PAF in various types of cells.

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Platelet-Activating Factor (PAF) Stimulates the LysoPAF Acetyltransferase in Leukocyte-Rich Plasma: Use in PAF Antagonist Studies[1]

Thomas W. Doebber*, Margaret S. Wu, Anthony Mauriello and Alfred Alberts
Department of Biochemical Regulation, Merck Sharp & Dohme Research Laboratories, Rahway, New Jersey 07065-0900

Addition of platelet-activating factor (PAF; 1-*O*-alkyl-2-acetyl-*sn*-glycero-3-phosphocholine) to leukocyte-rich plasma from several species resulted in the rapid and pronounced activation of the PAF biosynthetic enzyme acetyl-CoA:1-*O*-alkyl-*sn*-glycero-3-phosphocholine acetyltransferase (EC 2.3.1.67). Activation of acetyltransferase by PAF occurred in leukocyte-rich plasma from human, chimpanzee, rhesus monkey, and dog. The neutrophil was indicated to be the major cellular source of the activatable acetyltransferase in leukocyte-rich plasma. The induction of acetyltransferase was substantial with 10 nM PAF, and maximal at 10–30 seconds. Measurable acetyltransferase activation was significantly greater when the PAF-activated cells were separated from the plasma by centrifugation before the acetyltransferase assay. This may be due in part to the removal of the PAF-specific acetylhydrolase present in plasma which can cleave the acetyl group from PAF. Measuring PAF activation of acetyltransferase in leukocyte-rich plasma can be useful to determine the potency of PAF antagonists with neutrophils in plasma compared to isolated neutrophils in aqueous buffer, and as an *ex vivo* assay to determine the efficacy and plasma concentration equivalents of antagonists administered to whole animals. The PAF antagonist L-659,989 was shown to be 3–5 times more potent in inhibiting PAF induction of acetyltransferase in isolated human neutrophils than in human leukocyte-rich plasma, with IC_{50} values of 10 nM and 40 nM, respectively. In the *ex vivo* assay, oral administration of the PAF antagonist L-667,131 to dogs resulted in very substantial inhibition of PAF induction of acetyltransferase in the leukocyte-rich plasma. Utilizing the *ex vivo* assay, oral administration of 1 mg/kg L-659,989 to rats was found to result in plasma concentration equivalents of approximately 200–300 nM L-659,989. Our findings offer a new approach for characterizing the *in vitro* and *in vivo* efficacy of PAF receptor antagonists and demonstrate that PAF may be able to activate neutrophils in the blood *in vivo*, further enhancing PAF synthesis.
***Lipids* 26, 997–1003 (1991).**

Platelet-activating factor (PAF) synthesis is induced by specific agonist-receptor interactions in several cell types. Such interactions include those of antigen-stimulated mast cells (1), immune complex-activated and opsonized zymosan-activated macrophages (2), complement component C5a-stimulated eosinophils (3), and opsonized zymosan-stimulated and complement component C5a-stimulated neutrophils (4,5). Cellular PAF synthesis results from a two-step pathway involving: (i) phospholipase A_2 cleavage of the fatty acid from 1-*O*-alkyl-2-acyl-glycero-3-phosphocholine to form 1-*O*-alkyl-glycero-3-phosphocholine (lysoPAF); and (ii) acetylation of lysoPAF to PAF by a specific acetyl-CoA:1-*O*-alkyl-*sn*-glycero-3-phosphocholine acetyltransferase (EC 2.3.1.67) which utilizes acetyl-CoA as the acetyl donor (6). This acetyltransferase is a highly regulated enzyme and its activation in intact cells by specific agonists closely parallels large increases observed in the synthesis of PAF. Specifically, opsonized zymosan stimulation of human neutrophils resulted in activation of the acetyltransferase that correlated closely with the induction of PAF synthesis with regard to agonist concentration and time of stimulation (7). Similar studies with human eosinophils demonstrated a close correlation between acetyltransferase activation and increased PAF synthesis upon cell stimulation with formyl-methionyl-leucyl-phenylalamine (fMet-Leu-Phe), complement component C5a, eosinophil chemotactic factor of anaphylaxis, and the calcium ionophore A23187 (3). The combined evidence, at least for leukocytes, indicates that activation of the acetyltransferase is a prerequisite for increased PAF synthesis.

More recently, we demonstrated that PAF itself is a potent stimulator of this specific acetyltransferase in isolated rat and human neutrophils, resulting in the *de novo* synthesis of very substantial amounts of PAF in rat neutrophils (8). The induction of PAF synthesis by PAF in human neutrophils has since been documented (9). We also discovered that PAF activation of acetyltransferase occurs in leukocyte-rich plasma from several different species. In the present study, we characterized this leukoctye-rich plasma response to PAF. We also describe the utility of measuring this PAF response for characterizing PAF receptor antagonists.

MATERIALS AND METHODS

Blood was collected from experimental animals and normal human volunteers by venipuncture using heparinized syringes or heparinized vacutainer tubes. Leukocyte-rich plasma was prepared by mixing whole blood with 1/3 volume of 3% dextran (average molecular weight of 510,000) in 0.15 M NaCl and then letting this mixture stand at room temperature for 1 hr. The resulting leukocyte-rich plasma was then pipetted from above the sedimented erythrocytes. Rat elicited polymorphonuclear PMN neutrophils (utilized in experiments described in Fig. 9 and Table 1) were prepared by the method of

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*To whom correspondence should be addressed at R80-B5, Merck Sharp & Dohme Research Laboratories, P.O. Box 2000, Rahway, NJ 07065-0900.

Abbreviations: BSA, bovine serum albumin; fMet-Leu-Phe, formyl-methionyl-leucyl-phenylalamine; lysoPAF, 1-*O*-alkyl-*sn*-glycero-3-phosphocholine; PAF, platelet-activating factor, 1-*O*-alkyl-2-acetyl-*sn*-glycero-3-phosphocholine; PMN, polymorphonuclear.

Cunningham *et al.* (10) using intraperitoneal injections of 9 mL of 12% sodium caseinate per rat. Human neutrophils were isolated from blood by methods previously described (8,11). Leukocyte concentrations were determined as described previously (12). Leukocyte DNA was measured by use of the Hoechst Dye H33258.

For determination of PAF stimulation of acetyltransferase, 0.4 mL of a leukocyte suspension (leukocyte-rich plasma or isolated neutrophils suspended in Hanks balanced salt solution) was transferred to 1.5 mL polypropylene tubes. To the leukocyte suspension was added 100 μL of Hanks containing 0.25% bovine serum albumin (BSA), 3 mM $CaCl_2$, 5 mM $MgCl_2$, and the indicated concentration of PAF. For measurement of the basal (unstimulated) level of acetyltransferase, 100 μL of the same solution without PAF was used. The resulting cell suspension was incubated at room temperature for the time period indicated. The cells were then rapidly pelleted in a microcentrifuge for 7 seconds and the plasma supernatant was completely removed. The packed cells were resuspended in 1 mL of a mixture of Hanks containing 0.25% BSA/0.1M Tris-HCl, pH 7.2 (1:1, v/v), and then lysed by sonication for 10 seconds (Branson Sonifier Cell Disruptor, W-350, Branson, Danbury, CT). If acetyltransferase activity was to be assayed soon thereafter, lysed cell suspensions were stored in ice. Otherwise, the suspensions were quick-frozen in dry-ice/ethanol and then stored at −70°C for later assay. The loss in acetyltransferase catalytic activity using the latter freeze treatment was 5% or less when compared to the assay on the fresh samples immediately after sonication. Acetyltransferase activity was determined as described previously utilizing 5 μM 1-*O*-alkyl-*sn*-glycero-3-phosphocholine and 100 μM [^{3}H]-acetyl-CoA (0.3 μCi, New England Nuclear, Boston, MA) (8,13). Acetyltransferase activity is expressed as the amount of [^{3}H]PAF synthesized per min by the equivalent of 10^7 lysed cells. Any deviation from this protocol and other details of the assay are given in the Figure legends.

For the oral administration of the PAF antagonist L-667,131 to dogs, an ethanol solution of L-667,131 was mixed with starch. After evaporation of the ethanol, the L-667,131 starch mixture was packed into gelatin capsules. A pre-dose blood sample was obtained followed by oral administration of the capsules to the dogs.

For the study of the L-659,989 concentration equivalents in plasma from rats dosed orally with L-659,989, this compound was suspended in a solution containing 0.9% NaCl, 0.5% carboxymethylcellulose, 0.4% Tween 80, and 0.9% benzyl alcohol to a final concentration of 0.1 mg/mL. This suspension was administered orally *via* stomach tube to female CD rats (Charles River Breeding Labs, St. Constant, Quebec, Canada). Blood samples were obtained by cardiac puncture, 30 min later, and plasma was prepared by centrifugation.

1-*O*-Alkyl-2-[*acetyl*-^{3}H(N)]-acetyl-*sn*-glycero-3-phosphocholine, for measuring acetylhydrolase activity was prepared enzymatically by incubation of rat spleen microsomal acetyltransferase with 1-*O*-alkyl-*sn*-glycero-3-phosphocholine and [^{3}H]acetyl coenzyme A. The resulting [^{3}H]PAF was extracted according to the procedure of Bligh and Dyer (16). L-659,989 was prepared and supplied by Dr. M. Ponipom (14). L-667,131 was prepared and supplied by Dr. D. Graham and Dr. Y.-C. Chiang (15).

RESULTS

Addition of increasing concentrations of PAF to leukocyte-rich plasma from different species resulted in concentration-dependent increases in acetyltransferase activity as demonstrated for human, rhesus monkey, dog, and chimpanzee (Fig. 1). While there were modest increases with 1 nM PAF, 10 nM PAF induced substantial increases in acetyltransferase activity, and the activity plateaued between 10^{-7} and 10^{-6} PAF. The exception was the human acetyltransferase, which continued to increase in activity with PAF concentrations up to 10^{-5}M. The increase in maximal acetyltransferase activity compared to basal activity ranged from 3.3-fold for the rhesus monkey to 10.6-fold for the human. The chimpanzee basal activity was zero.

For the human leukocyte-rich plasma, evidence indicates that the PMN neutrophil is the cell type predominantly responsible for the PAF-activatable acetyltransferase. Firstly, PMN neutrophils isolated from human blood displayed an acetyltransferase that is substantially activated by PAF (8). Secondly, the selective removal of PMN neutrophils from human leukocyte-rich plasma greatly reduced the amount of PAF-activatable acetyltransferase on a per cell basis. Specifically, in one experiment, the leukocyte-rich plasma, in which 76% of all leukocytes were PMN neutrophils, displayed a basal and a PAF-stimulated acetyltransferase activity of 1405 and 5272 pmole/min/10^7 cells, respectively. The net PAF-activatable acetyltransferase activity was thus 3867 pmole/min/10^7 cells. When PMN neutrophils were specifically pelleted from the leukocyte-rich plasma by layering the leukocyte-rich plasma over lymphocyte separation medium followed by centrifugation, 100% of the pelleted cells were PMN neutrophils (200 out of 200 cells counted) and 0% of the leukocytes in the remaining supernatant were PMN neutrophils (0 out of 205 cells counted). The latter supernatant exhibited a basal and a PAF-stimulated acetyl-

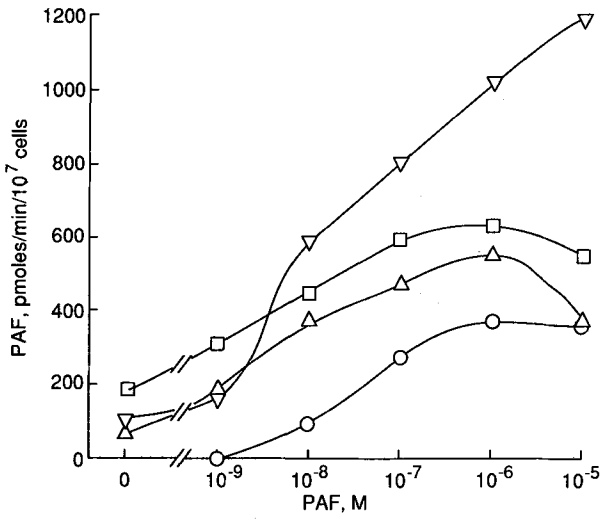


FIG. 1. PAF concentration-dependent induction of acetyltransferase activity in leukocyte-rich plasma samples from human (▽), rhesus monkey (□), dog (△), and chimpanzee (○). Leukocyte-rich plasma samples were activated for 30 seconds after addition of PAF. The reaction was stopped by the addition of 0.5 mL of 0.1M Tris-HCl, pH 7.2, followed by sonication to lyse the cells. The individual values represent the mean of triplicate determinations.

transferase activity of 1152 and 2088 pmoles/min/10^7 cells, respectively, for a net PAF-activatable acetyltransferase activity of 936 pmole/min/10^7 cells. Thus, the PMN-containing leukocyte-rich plasma on a cell basis had four times more PAF-activatable acetyltransferase than the PMN-free leukocyte-rich plasma. While this type of analysis was not performed on the three other species presented in Figure 1, the PMN differential counts in the leukocyte-rich plasma were quite similar to that for human—77% for chimpanzee, 68% for rhesus, and 78% for dog.

The PAF concentration-dependent increase in acetyltransferase differed considerably for leukocyte-rich plasmas from different human donors, as shown for three donors in Figure 2. Both the threshold PAF concentration that activated the acetyltransferase and the PAF concentration at which the activity plateaued differed for the three leukocyte-rich plasma samples. While there is variability among different human donors, the pattern for leukocyte-rich plasma samples obtained at different times from the same donor was much more consistent.

The effect of plasma on the acetyltransferase assay was followed by measuring the PAF concentration-dependent increase in acetyltransferase in cells that had been separated from plasma by centrifugation after PAF activation and in cells that remained in the plasma. The results depicted in Figure 3 demonstrate that the measurable acetyltransferase activation was considerably greater when the plasma was removed from the cells. The lower activity with plasma present may be due to the steady deacetylation of [^{3}H]PAF formed by the PAF-specific acetylhydrolase. This interpretation is consistent with the data shown in Figure 4 which demonstrate that human plasma, human leukocyte-rich plasma, and sonicated human leukocyte-rich plasma each degrade [^{3}H]PAF in a time-dependent manner. Plasma and leukocyte-rich plasma were about equally active in their ability to degrade [^{3}H]PAF, while the sonicated leukocyte-rich plasma was slightly less active. Because the undiluted sonicated leukocyte-rich plasma deacetylated only 28% of the [^{3}H]PAF by 10 min and because the leukocyte-rich plasma assayed (Fig. 3) was diluted five-fold for the

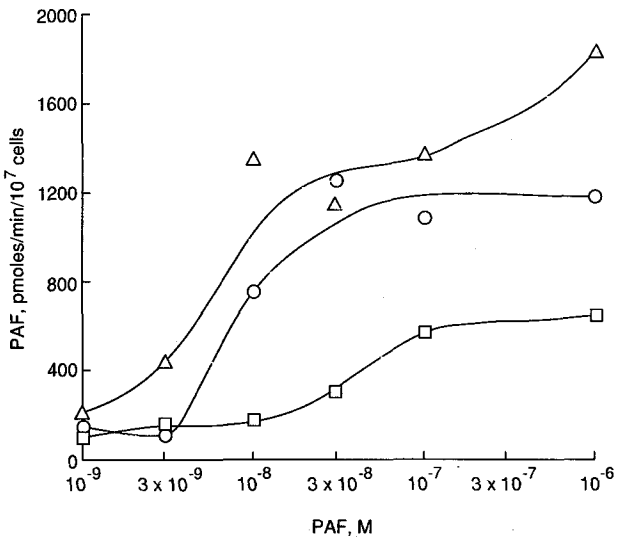


FIG. 2. PAF concentration-dependent stimulation of acetyltransferase activity in leukocyte-rich plasmas from three human donors. The PAF activation of the leukocyte-rich plasma was for 30 seconds. The individual values represent the mean of duplicate determinations.

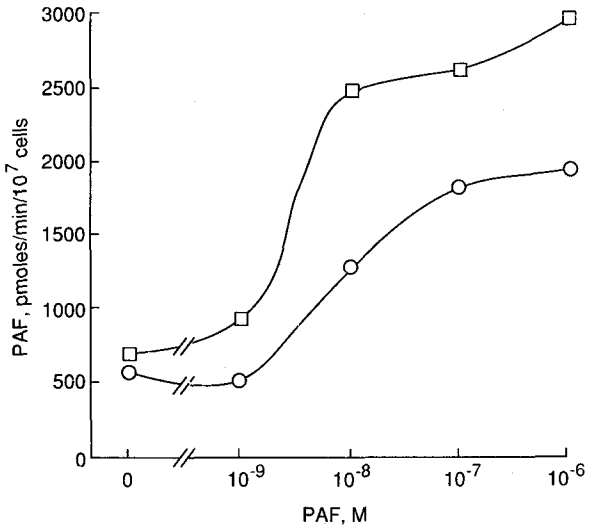


FIG. 3. Effect of plasma on the determination of human leukocyte-rich plasma acetyltransferase that was activated by different concentrations of PAF. Following a 1-min incubation with PAF, the cell suspensions were either diluted with 0.5 mL of 0.1 M Tris-HCl (pH 7.2) and then sonicated (○), or the cells were rapidly pelleted in a microcentrifuge, the plasma supernatant was removed, and the packed cells were then sonicated in 1 mL of a mixture of Hanks containing 0.25% BSA/0.1 M Tris-HCl, pH 7.2 (1:1, v/v) (□). The individual values represent the mean of triplicate determinations.

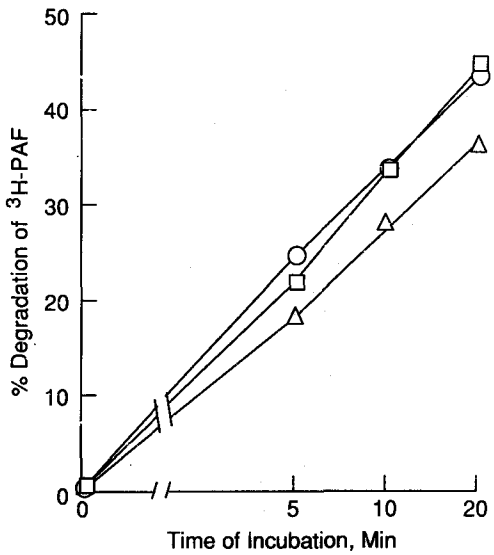


FIG. 4. Time-dependent deacetylation of [^{3}H]PAF by human plasma (○), human leukocyte-rich plasma (□), and human leukocyte-rich plasma in which the cells had been lysed by sonication (△). [^{3}H]PAF with the ^{3}H label in the acetate moiety (80,000 dpm) was added to 1.1 mL of human plasma or leukocyte-rich plasma and the mixture was incubated at 37°C. At 0, 5, 10, and 20 min of incubation, duplicate 0.1-mL aliquots were withdrawn and subjected to Bligh-Dyer extraction. The ^{3}H activity in the aqueous layer was counted as a measure of the [^{3}H]acetate released from [^{3}H]PAF. A control incubation of [^{3}H]PAF in Hanks containing 0.25% BSA resulted in negligible release of [^{3}H]acetate into the aqueous layer.

acetyltransferase assay, there appear to be other factors responsible for the much lower acetyltransferase activity that resulted when the plasma was not removed from the leukocytes before sonication and enzyme assay.

The time dependence for the 10 nM PAF induction of acetyltransferase in human leukocyte-rich plasma at room temperature and at 37°C is presented in Figure 5. At room temperature, the activation was discernable but not maximal at 10 seconds, and reached maximal values at 0.5 to 1 min. The state of activation steadily decreased at 3, 10, and 20 min. A different pattern occurred at 37°C with maximal activity at 10 seconds and substantial decreases at 0.5 and 1 min. The decrease in activity at 3, 10, and 20 min was quite parallel to the pattern that was seen at room temperature.

One of the applications of measuring PAF activation of acetyltransferase is the ability to determine the inhibition by PAF antagonists of this response to PAF in neutrophils in plasma compared to isolated neutrophils in buffer. Figure 6 shows the concentration-dependent inhibition of PAF activation of acetyltransferase in human leukocyte-rich plasma and isolated human neutrophils by the PAF antagonist L-659,989 (Scheme 1). Approximately three- to five-fold higher concentrations of L-659,989 were required to inhibit this response to PAF in human leukocyte-rich plasma as compared with isolated human neutrophils prepared from the same leukocyte-rich plasma. The L-659,989 concentration required for 50% inhibition of the PAF induction of acetyltransferase was 10 nM for isolated neutrophils and 40 nM for leukocyte-rich plasma. The decreased potency of L-659,989 in the leukocyte-rich plasma compared to isolated neutrophils is probably due to the fact that 97% of L-659,989 added to plasma is bound to plasma proteins. The inhibitory action of L-659,989 on PAF activation of acetyltransferase in human leukocyte-rich plasma was not due to inhibition of the acetyltransferase catalytic activity and was specific

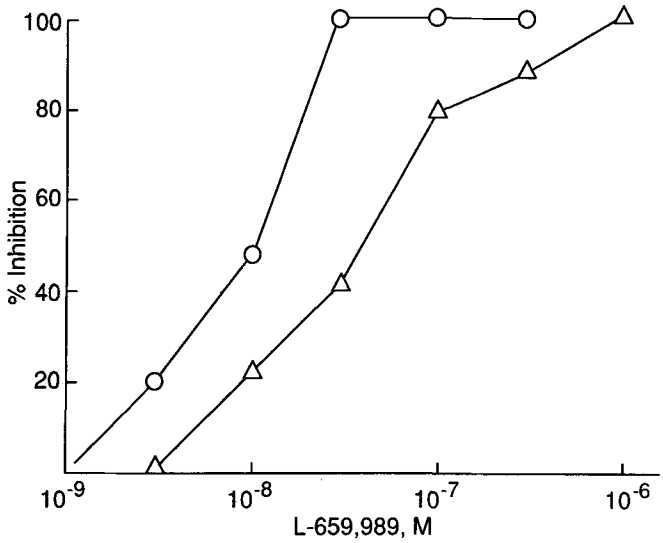
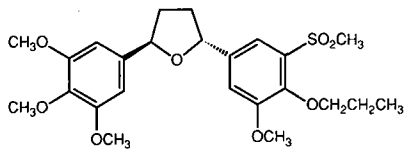


FIG. 6. Concentration-dependent inhibition by PAF antagonist L-659,989 of 10 nM PAF induction of acetyltransferase in isolated human neutrophils (○) and human leukocyte-rich plasma (△). The L-659,989 was preincubated with the cells 15 min before addition of PAF. The PAF activation of the cells was for 30 seconds. Each value depicts the mean of duplicate determinations for the isolated neutrophils and of triplicate determinations for the leukocyte-rich plasma.



SCHEME 1. Structure of L-659,989.

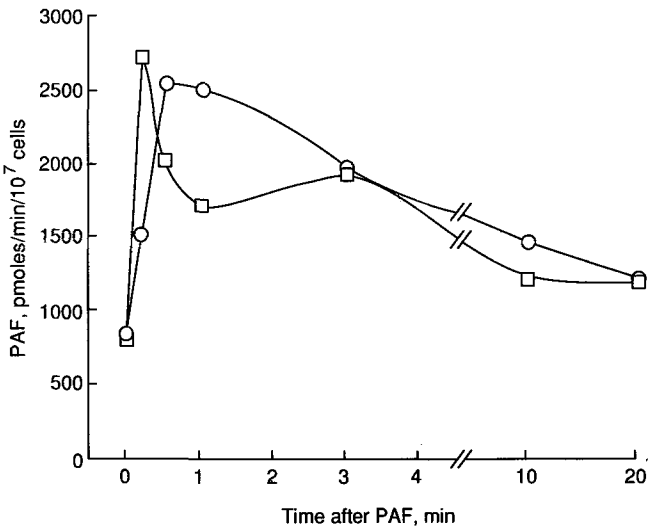


FIG. 5. Time-dependent activation of acetyltransferase in human leukocyte-rich plasma by 10 nM PAF at room temperature (○) and 37°C (□). For the 37°C study, the leukocyte-rich plasmas were preincubated at 37°C for 15 min before addition of PAF. All values represent the mean of triplicate determinations.

to PAF: i) The addition of L-659,989 (1 μM final concentration) to sonicated leukocytes from either unactivated or PAF-activated human leukocyte-rich plasma had essentially no inhibitory action on the acetyltransferase catalytic activity; and ii) the activation of acetyltransferase in human leukocyte-rich plasma by 10^{-8}M fMet-Leu-Phe was unaffected by the addition of 1 μM L-659,989 to the leukocyte-rich plasma before the fMet-Leu-Phe challenge.

Another application of the PAF activation of acetyltransferase in leukocyte-rich plasma is the *ex vivo* determination of the efficacy of PAF antagonists administered to experimental subjects. In this procedure, a control blood sample is taken followed by dosing with the antagonist and then subsequent blood samples are obtained at desired times. Leukocyte-rich plasma samples which are prepared from each blood sample are challenged with buffer and 10 nM PAF and the level of activation of acetyltransferase is determined. If a PAF antagonist is efficacious in this assay, the level of PAF activation of acetyltransferase in the leukocyte-rich plasma obtained after dosing of antagonist would be substantially lower than that in the pre-dose leukocyte-rich plasma. A prerequisite for results from such an assay to be meaningful is that the level of PAF activation of acetyltransferase be quite reproducible for leukocyte-rich plasma obtained from the same donor at different times. This requirement was

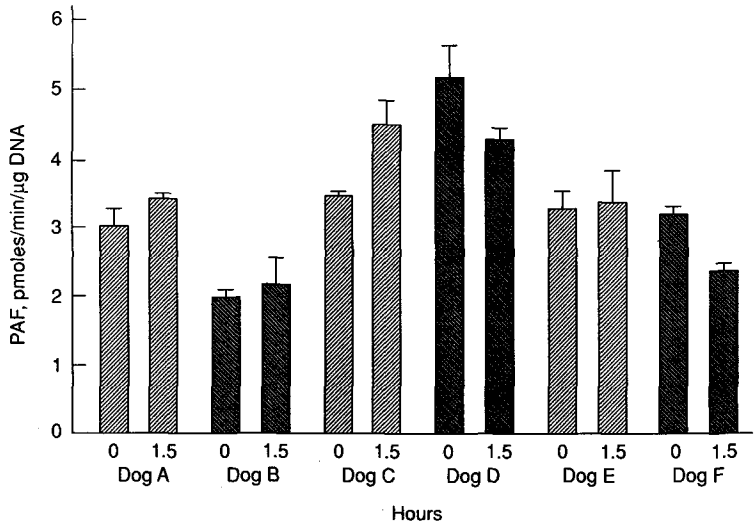
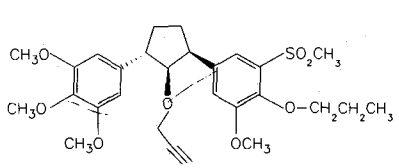


FIG. 7. Reproducibility of PAF activation of acetyltransferase in leukocyte-rich plasmas from six dogs. Two blood samples were obtained from each dog with 1.5 hr between blood drawings. The resulting leukocyte-rich plasma samples were stimulated with 10 nM PAF and buffer (for determination of basal activity) for 30 seconds. The acetyltransferase activity was calculated on the basis of μg of cellular DNA. The results are expressed as the net PAF activated acetyltransferase having subtracted the basal activity. The values represent the mean of triplicate determinations and the error bars depict the standard deviations. The basal activities ranged from 0.54 pmole/min/μg DNA for the 0 hr blood sample of dog B to 1.44 pmole/min/μg DNA for the 1.5 hr blood sample of dog D.

tested in dogs followed by the full *ex vivo* assay of the efficacy of a PAF antagonist administered orally to dogs. The reproducibility of the PAF activation of acetyltransferase in leukocyte-rich plasma obtained at two different time points was determined in six pure-bred beagles. The results in Figure 7 show the net PAF activation of acetyltransferase (after subtracting basal activity) for two blood samples obtained with a 1.5-hr interval. The leukocyte-rich plasma from dogs A, B, and E showed very good reproducibility. In comparing the 1.5-hr sample to the 0-hr sample, dog C exhibited a 28% increase and dogs D and F exhibited a 17% and 25% decrease, respectively. Next, the efficacy of the PAF antagonist L-667,131 upon oral administration at 1 mg/kg in two pure-bred beagles was determined (Scheme 2). The results in Figure 8, again expressed as the net PAF activation, demonstrate a very substantial reduction in the PAF activation of acetyltransferase in leukocyte-rich plasmas from blood samples obtained 0.5, 2 and 4 hr after the antagonist dosing as compared to the predose level of PAF activatable acetyltransferase. Dog A showed decreases in PAF activatable acetyltransferase of 85%, 93%, and 99% for the 0.5, 2, and 4 hr blood samples, respectively, compared to the 0 hr predose level, while the corresponding decreases for dog B were 28%, 56%, and 56%. Except for the 0.5 hr value of dog B, these decreases are clearly much larger than the variability displayed in the activatable acetyltransferase



SCHEME 2. Structure of L-667,131.

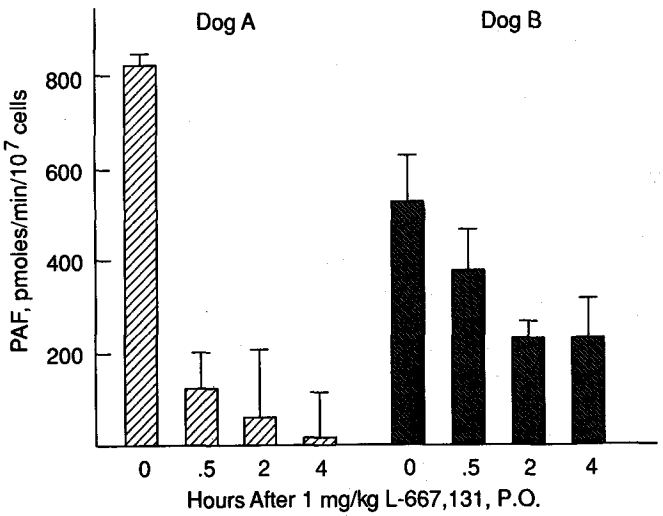


FIG. 8. PAF activation of acetyltransferase in leukocyte-rich plasma from dogs that were orally dosed with L-667,131. Immediately after the 0 hr blood sample was drawn, each dog was dosed with the capsule that contained L-667,131. Subsequent blood samples were obtained at 0.5, 2, and 4 hr after dosing. The leukocyte-rich plasma derived from each blood sample was stimulated with 10 nM PAF for 30 seconds. The results are expressed as the net PAF activated acetyltransferase after subtraction of the basal activity. The values indicate the mean of triplicate determinations and the error bars represent the standard deviations. The basal activities ranged from 144 pmole/min/10^7 cells for the 2 hr blood sample of dog B to 576 pmole/min/10^7 cells for the 0 hr blood sample of dog A.

TABLE 1

Inhibition of PAF Activation of Rat Neutrophil Acetyltransferase by Plasma from Rats Dosed Orally with L-659,989[a]

Plasma sample	Net PAF activatable acetyltransferase	% Inhibition
Control rat	318 ± 51 pmole PAF/min/10^7 cells	—
L-659,989-dosed Rat 1	215 ± 17 pmole PAF/min/10^7 cells	32%
L-659-989-dosed Rat 2	236 ± 6 pmole PAF/min/10^7 cells	26%

[a]Rat neutrophils were preincubated at room temperature with diluted plasma from the control rat or L-659-989-dosed rats for 15 min before addition of PAF to a final concentration of 10 nM. The time of PAF stimulation was 1 min. The results are expressed as the net PAF activated acetyltransferase after subtraction of the basal activity and represent the mean of triplicate determinations along with the standard deviation. The basal acetyltransferase values were 64, 56, and 53 pmole PAF/min/10^7 cells for the incubations with plasma from the control rat, rat 1, and rat 2, respectively.

in the two blood samples from each of the six control animals in Figure 7.

A modification of this latter assay permits the determination of the concentration equivalents of a PAF antagonist in plasma of animals that were dosed with the antagonist. For this assay, two rats were dosed orally with 1 mg/kg of L-659,989 and then bled 1/2 hr later. A control rat received an equal volume of vehicle. The resulting plasma samples from L-659,989-dosed rats and control rats were diluted 16.7-fold with Hanks containing 0.25% BSA, and these dilutions were added to isolated neutrophils prepared from other rats. The neutrophil suspensions were challenged with 10 nM PAF and the extent of activation of the acetyltransferase was determined. The results in Table 1 demonstrate that the plasma samples from the L-659,989-dosed rats inhibited the PAF activation of neutrophil acetyltransferase by 32% and 26%. The concentration-dependent inhibition by exogenously added L-659,989 of PAF activation of acetyltransferase in neutrophils suspended in the same dilution (16.7-fold) of control plasma was performed and the results are presented in Figure 9. By knowing the amount of inhibition given by the plasma from the L-659,989-treated rats (Table 1), the factor of the dilution of plasma added to the neutrophils, and the amount of inhibition by known amounts of L-659,989 (Fig. 9), the L-659,989 concentration equivalents in the plasma from the L-659,989-dosed rats can be determined. The 32% inhibition exhibited by the diluted plasma from rat 1 that received the oral dose of L-659,989 (Table 1) is the interpolated level of inhibition given by 16 nM L-659,989 added exogenously to neutrophils (Fig. 9). Multiplication of the 16 nM value by the 16.7-fold dilution of the plasma added to the neutrophils results in a value of 267 nM plasma equivalents of L-659,989 for rat 1. The same exercise performed for rat 2 indicates a plasma equivalents level of 225 nM L-659,989. The term equivalents is used since it is not known if intact L-659,989 or a metabolite that is an active PAF antagonist is the inhibitory component in the plasma from the orally-dosed rats.

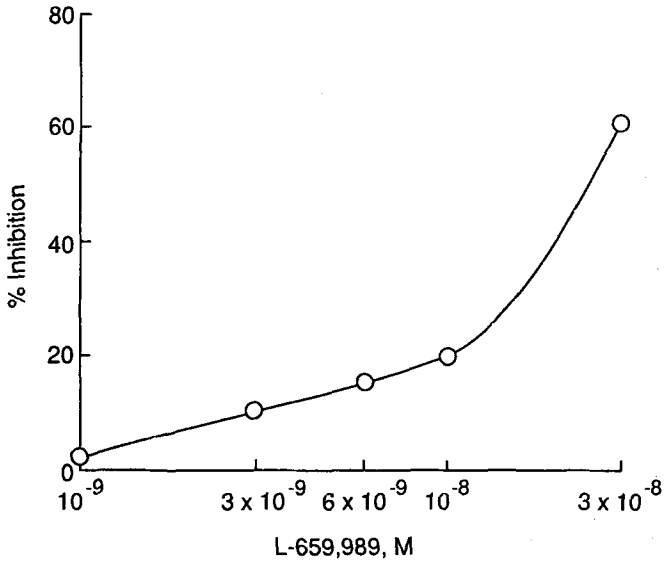


FIG. 9. Concentration-dependent inhibition by L-659,989 of 10 nM PAF induction of acetyltransferase in isolated rat neutrophils. The L-659,989 dilutions and control plasma were preincubated with the neutrophils for 15 min. The PAF activation was for 1 min. All samples contained 6% control rat plasma, the same plasma concentration present in the acetyltransferase assays of Table 1. Each point is the mean of triplicate determinations.

DISCUSSION

As we demonstrated previously with isolated human and rat neutrophils (8), PAF was now shown to activate the PAF synthesizing acetyltransferase in leukocyte-rich plasma from several species, including human, rhesus monkey, dog, and chimpanzee. This activation occurred at rather low PAF concentrations (10 nM) and reached maximal levels of activation after very brief exposure to PAF (10 seconds at 37°C and 30 seconds at room temperature), as was true for the activation of acetyltransferase in isolated neutrophils (8). For human leukocyte-rich plasma, the neutrophil was the primary cell responsible for the increased acetyltransferase levels following PAF stimulation.

The detectability of the level of activation of the PAF-stimulated acetyltransferase in human leukocyte-rich plasma was substantially greater when the plasma was removed from the cells before cell lysis by sonication (Fig. 3). The continued deacetylation by the PAF-specific

acetylhydrolase in plasma of the [^{3}H]PAF formed during the acetyltransferase assay was considered a partial factor for the dampening effect of plasma. Other explanations could include the deactivation of the stimulated acetyltransferase and alternate uses of the substrates used for the acetyltransferase assay by enzymes present in the plasma.

While the extent of acetyltransferase activation is almost the same for PAF stimulation of human leukocyte-rich plasma at room temperature and 37°C, the time-dependency is quite different. The activation and subsequent deactivation occur more rapidly at 37°C than at room temperature. Other laboratories have indicated that the acetyltransferase in spleen microsomes and human neutrophils is activated by kinases and deactivated by phosphatases (17-19). Possibly, the more rapid activation and deactivation of acetyltransferase at 37°C compared to room temperature may be due to the higher catalytic activity at 37°C of neutrophil kinases and phosphatases that may be responsible for activation and deactivation, respectively, of the acetyltransferase.

While PAF does activate the acetyltransferase in human leukocyte-rich plasma up to 10-fold over the basal level, we were unable to detect any PAF stimulation of PAF synthesis in human leukocyte-rich plasma as measured by incorporation of [^{3}H]acetate into PAF. A major reason for not detecting any PAF synthesis is that the [^{3}H]acetate method requires a neutrophil concentration of at least 10^7 cells/mL for significant synthesis by isolated neutrophils of [^{3}H]PAF to occur. Typical neutrophil concentrations in human leukocyte-rich plasmas are only 1-3 × 10^6 cells/mL.

A main benefit of this response of the leukocyte-rich plasma acetyltransferase to PAF is the ability to characterize PAF antagonists. The potencies of antagonists in inhibiting the PAF induction of acetyltransferase in leukocyte-rich plasma can be compared with that in isolated neutrophils. This is quite important since an antagonist that is quite potent with isolated cells in plasma-free buffers may be much less potent in the presence of plasma because of extensive binding of antagonist to plasma proteins and/or metabolism by enzymes present in plasma. Indeed, this was the case for the studies with L-659,989 (Fig. 6). These studies can be performed by examining antagonist inhibition of PAF induction of aggregation of washed platelets and platelet-rich plasma. However, it is important to examine the neutrophil system as well as the platelet system since the studies of Hwang (20) indicate that PAF receptors on platelets may be different from those on neutrophils.

The PAF induction of acetyltransferase in leukocyte-rich plasma also provides a useful *ex vivo* assay for determining the efficacy and plasma concentration equivalents of antagonists administered to whole animals. This capability was demonstrated here for the PAF antagonists L-667,131 and L-659,989. The very good efficacy of the PAF antagonist WEB 2086 was demonstrated in humans utilizing the *ex vivo* efficacy of PAF induction of aggregation of platelet-rich plasma (21). Similar studies can be performed in humans or experimental animals using the PAF induction of leukocyte-rich plasma acetyltransferase. The advantages of the latter assay are: i) Only 4 mL of blood are needed for triplicate determinations of basal and PAF activatable acetyltransferase; ii) the PAF challenge of the leukocyte-rich plasma can be performed very quickly and the sonicated neutrophils can be quick-frozen and then assayed for acetyltransferase activity at a later time; and iii) determination of the neutrophil DNA concentration provides a convenient basis for expression of the acetyltransferase activity.

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Kinetic Studies of Human and Rat Neutrophil LysoPAF Acetyltransferase Using LysoPAF and DansyllysoPAF as Substrates[1]

Peter W. Schindler[a,*] and **Ewa Ninio**[b]

[a]Pharma Forschung, Hoechst AG, 6230 Frankfurt 80, Germany, and [b]INSERM U200, Université Paris-Sud, 92140 Clamart, France

Enzyme kinetic studies of lysoPAF acetyltransferase from microsomal preparations of human and rat neutrophils were carried out using lysoPAF or dansyllysoPAF as substrate. With the human enzyme, incomplete conversion of the substrate into the product was observed at 37°C with both substrates. The acetyltransferase was inactivated at 37°C in the absence of substrate with a half-life of 7.5 min. However, the initial rate of product formation under the assay conditions was linear up to 10 min. Both enzymes were optimally active at 40 μM concentration with either substrate, but enzyme activity was inhibited at higher substrate levels. At a constant substrate concentration (40 μM), the K_m (μM) and V_{max} (nmol product/min/mg protein) values for the human acetyltransferase, with respect to acetyl-CoA were 132 and 23.1, respectively, with lysoPAF as substrate, and 105 and 26.7, respectively, when dansyllysoPAF was used. The K_m and V_{max} values for the rat enzyme were 105 and 6.5, respectively, with lysoPAF as substrate, and 120 and 5.4, respectively, when dansyllysoPAF was used. Under our standard conditions, lysoPAF required 1 mg of BSA per mL in the assay, whereas full activity of both enzymes was seen with dansyllysoPAF even in the absence of BSA. The results show that dansyllysoPAF can replace lysoPAF in the assay without any significant changes in kinetic parameters.

Lipids 26, 1004–1010 (1991).

Paf-acether (platelet-activating factor, PAF) has been implicated in the development of a variety of pathological conditions, such as endotoxic shock (1), anaphylaxis (2), asthma (3), and thrombi formation (4). Thus, interest has grown in recent years in the development of PAF antagonists with pharmacological potential (2). However, little attention has been paid to inhibitors of PAF biosynthesis. Acetyl-CoA:1-*O*-alkyl-*sn*-glycero-3-phosphocholine acetyltransferase (EC 2.3.1.67) is an enzyme which plays a key role in Paf-acether formation (5,6) Inhibition of this enzyme could decrease Paf-acether production, and thus lessen certain disease symptoms. Only one preliminary report has been published on inhibitors of microsomal acetyltransferase (7), and only few compounds have been shown to inhibit PAF biosynthesis in neutrophils (8–10). The mode of action of these inhibitors is not yet fully understood.

Large-scale screening for enzyme-specific inhibitors requires that a high number of assays can be performed within a short period of time. A newly developed assay for measuring acetyltransferase activity based on a fluorophore-labeled lysoPAF derivative, *i.e.*, dansyllysoPAF, was recently described (11). This acetyltransferase assay appears useful for the screening of potential enzyme inhibitors. In the present study, the dansyllysoPAF assay was compared with a standard assay based on the incorporation of [^{3}H]acetyl from [^{3}H]acetyl-CoA into lysoPAF (12). It was our objective to compare the kinetics of the acetyltransferase reaction for both substrates, and for the enzymes from both human and rat neutrophils.

EXPERIMENTAL PROCEDURES

1-(*N*-Dansyl-11-amino-1-undecyl)-*sn*-glycero-3-phosphocholine (dansyllysoPAF) and its acetylated derivative (dansylPAF) were prepared as previously described (11). LysoPAF (1-*O*-octadecyl-*sn*-glycero-3-phosphocholine) and [^{3}H]-acetyl-CoA were purchased from Bachem (Bubendorf, Switzerland) and New England Nuclear (Boston, MA), respectively. [^{14}C]PAF was the product of Amersham International (Amersham, U.K.), and Ficoll-Hypaque was obtained from Pharmacia (Uppsala, Sweden). Plasmagel® was the product of Laboratoire R. Bellon (Neilly s/Seine, France). Acetyl-CoA, essentially fatty acid free bovine serum albumin (BSA), leupeptin and soybean trypsin inhibitor were purchased from Sigma (Deisenhofen, Germany). Hank's balanced salt solution (HBSS), sodium caseinate and ionophore A23187 were purchased from Gibco (Karlsruhe, Germany), Roth (Karlsruhe, Germany) and Calbiochem (La Jolla, CA), respectively. Thin-layer plates (type 5641) were purchased from Merck (Darmstadt, Germany). All other chemicals were reagent grade. Stock solutions of lysoPAF (1 mM) and dansyllysoPAF (1.6 mM) were prepared in 0.9% NaCl containing 0.25% BSA and water, respectively, and further diluted as indicated.

Protein was quantified by the method of Lowry *et al.* (13). Bovine serum albumin was used as the protein standard.

Preparation of acetyltransferase from human polymorphonuclear leukocytes (PMN). Venous blood from healthy donors was collected in tubes containing ethylenediaminetetraacetic acid (EDTA) (5 mM, final concentration) as anticoagulant. After sedimentation for 30 to 45 min in the presence of 3% gelatin (Plasmagel®), the supernatant (20 mL) was layered onto 10 mL Ficoll-Hypaque (density 1.077) and centrifuged at 400 × *g* for 20 min at 20°C. The cell pellet was washed in washing buffer, pH 7.4, containing (in mM) KCl (2.6), NaCl (137), glucose (5.6), and HEPES (4.2). Erythrocytes were lysed by adding 3 vol of distilled water per 1 vol of cell suspension for 40

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*To whom correspondence should be addressed at Hoechst AG, Postfach 800320, D-6230 Frankfurt/M 80, Germany.

Abbreviations: BSA, bovine serum albumin; dansyllysoPAF, 1-(*N*-dansyl-11-amino-1-undecyl)-GPC; dansylPAF, 1-(*N*-dansyl-11-amino-1-undecyl)-2-acetyl-GPC; EDTA, ethylene diamine tetraacetic acid; GPC, *sn*-glycero-3-phosphocholine; HBSS, Hank's balanced salt solution; lysoPAF, 1-*O*-alkyl-GPC; PAF, platelet-activating factor, Paf-acether, 1-*O*-alkyl-2-acetyl-*sn*-glycero-3-phosphocholine; PMN, polymorphonuclear leukocytes; PMSF, phenylmethylsulfonyl fluoride; TLC, thin-layer chromatography.

sec, and lysis was stopped by the addition of 1 vol of NaCl (3.5%). The cells were then washed twice with washing buffer (400 × *g* × 10 min at 20°C) and resuspended in washing buffer supplemented with $CaCl_2$ (1.3 mM) and $MgCl_2$ (1 mM) at a cell concentration of 1×10^6/mL. All preparations contained more than 98% PMN as determined by the examination of May Grunwald-Giemsa-stained smears. Cells (8×10^6 in 8 mL) were preincubated for 5 min at 37°C and then challenged with ionophore A23187 (3 μg/mL) for 5 min. Stimulation was stopped by washing with 0.15 NaCl at 4°C. Cell lysates were obtained from the ionophore-stimulated PMN (8×10^7 cells), and suspended in 0.5 mL of 0.25 M sucrose containing phenylmethylsulfonyl fluoride (PMSF) (2 mM) and leupeptin (20 μg/mL) by sonication in an ice bath (3 pulses of 15 sec). The postnuclear supernatant was fractionated by centrifuging twice at 500 × *g* for 10 min at 4°C in 0.25 M sucrose. Granules and mitochondria were first removed by centrifugation (15,000 × *g* for 10 min) at 4°C. The 15,000 × *g* supernatant was collected and centrifuged at 200,000 × *g* for 30 min at 4°C to separate the microsomal fraction (pellet) from the cytosol (supernatant). The microsomal fraction was resuspended in 0.15 N NaCl to yield a final concentration of approximately 1 mg protein/mL.

Preparation of acetyltransferase from rat peritoneal PMN. Rat peritoneal PMN were obtained 16 hr after intraperitoneal injection of 2% sodium caseinate (14). The cells were lavaged from the peritoneum with HBSS buffer, centrifuged, and exposed to hypotonic medium to remove erythrocytes as described above. The cells were washed twice with HBSS buffer and resuspended in the same buffer at 10^8 cells/mL. The purity of PMN was better than 95% based on May Grunwald-Giemsa-stained smears. PMN were stimulated with ionophore A23187 (3 μg/mL) for 5 min at 37°C (12). All subsequent steps were performed at 4°C. The stimulated cells were washed with ice-cold buffer A [HBSS buffer pH 7.0 which contained 0.5 mg/mL of soybean trypsin inhibitor (15) and 25 mM NaF (16)]. The cell-free preparations were obtained by sonication of the cells suspended in buffer A in an ice bath (3 pulses of 10 sec). Cell debris was removed from crude lysate by pelleting at 1,000 × *g* for 10 min. The resulting supernatant was further fractionated by centrifugation at 15,000 × *g* for 30 min. The microsomal fraction was obtained from the 15,000 × *g* supernatant by centrifugation at 105,000 × *g* for 1 hr. The microsomal fraction was resuspended in buffer A to yield a final concentration of approximately 1 mg protein/mL.

Acetyltransferase assay. The acetyltransferase activity of microsomal fractions obtained from ionophore-stimulated PMN was measured using two different substrates. The radiolabeled assay (assay 1) was performed as previously described (12). Briefly, 50 μL of microsomal fraction containing approximately 50 μg protein was added to the reaction mixture (0.5 mL final volume, pH 7.0) containing (in mM): HEPES (4.2), NaCl (137), KCl (2.6), $CaCl_2$ (0.65), $MgCl_2$ (0.5), 0.5 μCi [^{3}H]acetyl-CoA, and acetyl-CoA and lysoPAF as indicated in the legends to the Figures. When not stated otherwise, the assay contained BSA at a concentration of 1 mg per mL of assay. Samples without lysoPAF served as blanks. The reaction was carried out at 37°C for 10 min, and was stopped by the addition of 2 mL of chloroform/methanol (1:1, v/v) containing [^{14}C]PAF (3,000 dpm) as internal standard. The extraction procedures used were those described in ref. 12. After substraction of blanks, the amount of [^{3}H]PAF assayed also was corrected for isolation losses. Acetyltransferase activity was calculated as the average of duplicate determinations with an intraexperimental variation of less than 10%. The fluorophore assay (assay 2) for acetyltransferase was performed at 37°C in 60 μL of buffer A, which contained dansyllysoPAF (40 μM), acetyl-CoA (200 μM) and enzyme as previously described (11). The standard assay 2 was run in the absence of BSA. Incubations were carried out up to the indicated time points and terminated by directly spotting a 5-μL aliquot of the reaction mixture onto thin-layer plates. Substrate and product were separated using the solvent system chloroform/methanol/acetic acid/water (50:25:4.5:4.5, by vol) and were quantified by scanning plates in a fluorescence densitometer (CD 50, Desaga, Heidelberg, Germany) with the excitation wave length set at 256 nm and an emission cut-off filter at 400 nm. Both microsomal preparations of human and rat enzyme were devoid of transacylase activity when tested as previously described (11).

When not stated otherwise, results are expressed as nmol product formed per min per mg of protein. Kinetic data were analyzed by weighted fits to the appropriate function with a non-linear regression analysis program adapted from KINFIT (17).

RESULTS AND DISCUSSION

Time course experiments. Typical results of the time course experiments for human acetyltransferase using assay 1 and assay 2 are shown in Figure 1. Both assays gave similar results with respect to product formation over time. A linear relationship between initial velocity and human leukocyte microsomal protein concentration has already been established previously for assay 1 (18). A similar linear relationship was found in a plot of initial velocities of dansylPAF formation (calculated from the progress curves in Fig. 1b) as a function of protein up to 56 μg protein (data not shown). However, in both assays, the total amount of PAF or dansylPAF formed at the end of the incubation time decreased as protein concentrations decreased. Incomplete conversion of lysoPAF to PAF has previously been observed (18,19). Similar results were obtained for rat PMN acetyltransferase (P.W. Schindler, unpublished results).

Effects of assay temperature on the activity of the human enzyme. Since the microsomal preparation of acetyltransferase was devoid of any significant PAF acetylhydrolase (data not shown), the possibility could be excluded that PAF or dansylPAF were hydrolyzed again after being formed in the assay. Therefore, we addressed the question whether the observed incomplete production formation may be due to thermal instability of the enzyme at the incubation temperature. As shown in Figure 2, preincubating the stock microsomal fraction (0.676 mg/mL) at 37°C for up to 50 min resulted in a rapid loss of enzyme activity. A plot of log [fractional enzyme activity] *vs.* time gave a straight line, regardless of the type of substrate used. In both cases, the half life of the human enzyme was 7.5 min. Thus, the rapid inactivation of human acetyltransferase at 37°C may account for the incomplete product formation which is apparent from Figure 1. We presently do not know whether the loss of

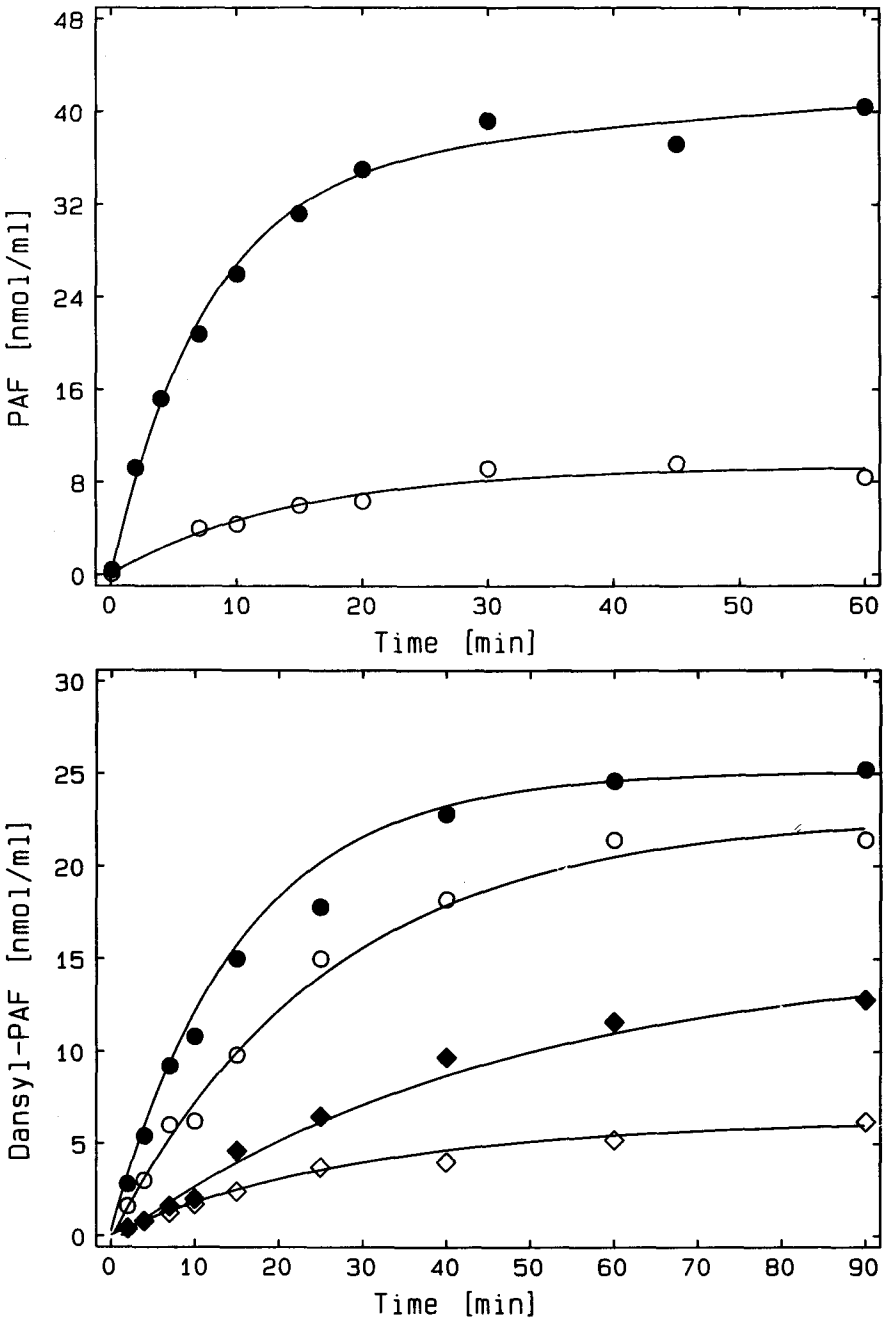


FIG. 1. Time course of paf-acether formation as a function of human acetyltransferase concentration. a) Standard assay 1 using lysoPAF (40 μM); the assay mixture (500 μL) contained 120 μg of protein (closed circles) and 24 μg of protein (open circle), respectively. b) Standard assay 2 with dansyllysoPAF as the substrate; the assay mixture (60 μL) contained the following amount of protein (μg): 56.4 (closed circles), 28.2 (open circles), 11.3 (closed squares), and 5.7 (open squares).

enzyme activity is due to intrinsic thermal instability of the protein at 37°C, to facilitated deactivation [for example, by dephosphorylation of the enzyme (15,16,20)], or to other factors. Recently Ninio *et al.* (18) showed that complete conversion could be achieved when microsomal proteins of high specific activity from ionophore-stimulated human neutrophils are used.

Effect of BSA. Assays of acetyltransferase with lysoPAF as substrate are routinely performed in the presence of BSA, which binds PAF as it is formed (18). By comparison, BSA was found dispensable in assay 2 when rat PMN acetyltransferase with dansyllysoPAF as substrate was measured (11). When we compared the effect of various BSA concentrations on the activity of human acetyltransferase measured by the two assays (Fig. 3), we found that human acetyltransferase was virtually inactive in the absence of BSA with lysoPAF as substrate. The activity sharply rose when BSA was added at concentra-

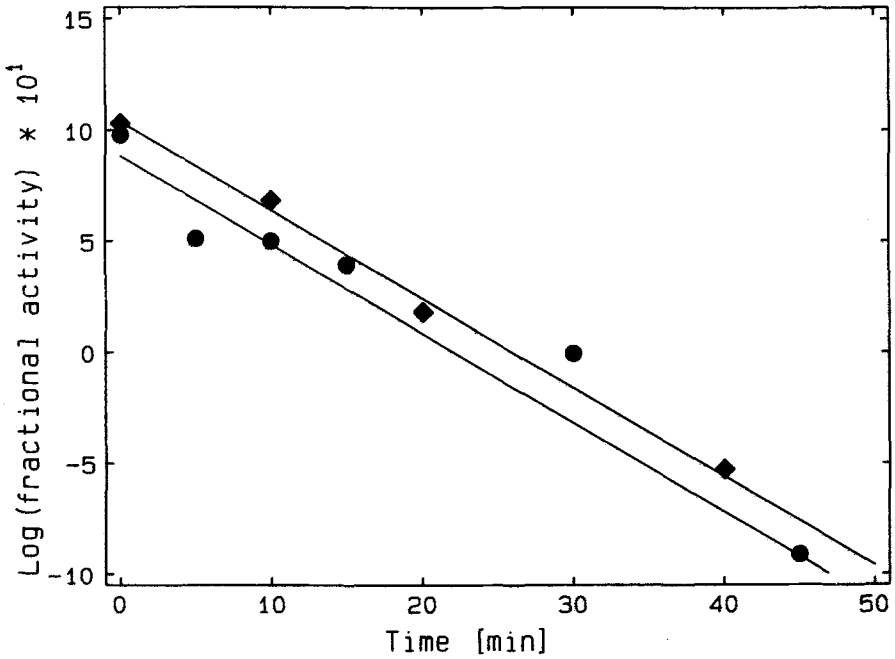


FIG. 2. Time course of the inactivation of human acetyltransferase at 37°C. A stock microsomal fraction (0.676 mg/mL) was incubated at 37°C. At the intervals indicated, samples were withdrawn and assayed using lysoPAF (closed circles) or dansyllysoPAF (closed squares).

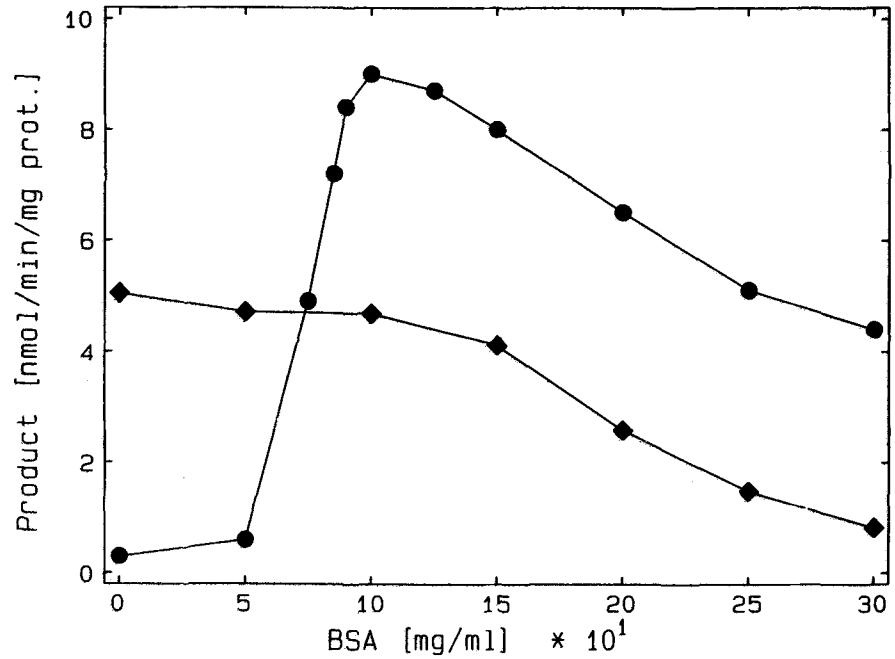


FIG. 3. Activity of human acetyltransferase as a function of BSA assayed with lysoPAF (closed circles) or dansyllysoPAF (closed squares).

tions above 0.5 mg/mL. Maximum enzyme activity occurred at 1 mg BSA per mL assay. At BSA concentrations ranging from 1–3 mg/mL, enzyme activity declined rapidly. Since lysoPAF is quite insoluble in aqueous medium below its critical micellar concentration (18), the results suggest that a critical BSA/lysoPAF ratio exists in assay 1 which provides for an optimal dispersion of the lysoPAF substrate. Contrary to the results from assay 1, full acetyltransferase activity was observed in assay 2 in the absence of BSA. However, BSA concentrations exceeding 1 mg/mL led to diminution of dansylPAF formation. Contrary to lysoPAF, dansyllysoPAF is readily soluble in water at least up to 1.6 mM. Therefore, the data using assay 2 suggest that BSA-mediated dispersion of substrate is not required if the substrate is readily soluble. The decline in activity of the enzyme observed with both substrates at BSA concentrations exceeding 1 mg/mL was beyond the scope of our present study. However,

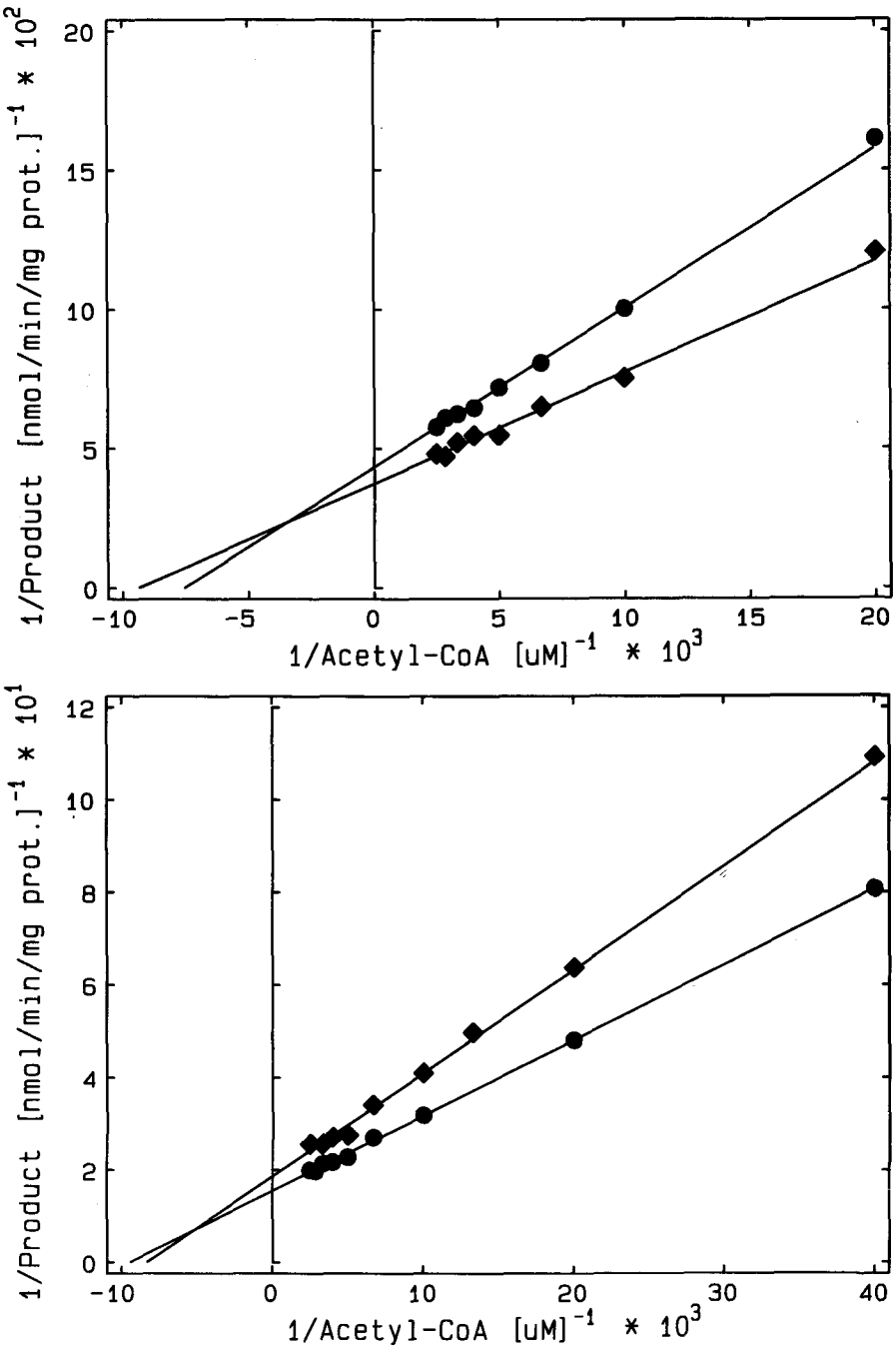


FIG. 4. a) Activity of human acetyltransferase as a function of acetyl-CoA at fixed concentrations (40 μM) of lysoPAF (closed circles, assay 1) or dansyllysoPAF (closed squares, assay 2). b) Activity of rat acetyltransferase as a function of acetyl-CoA at fixed concentrations (40 μM) of lysoPAF (closed circles, assay 1) or dansyllysoPAF (closed squares, assay 2).

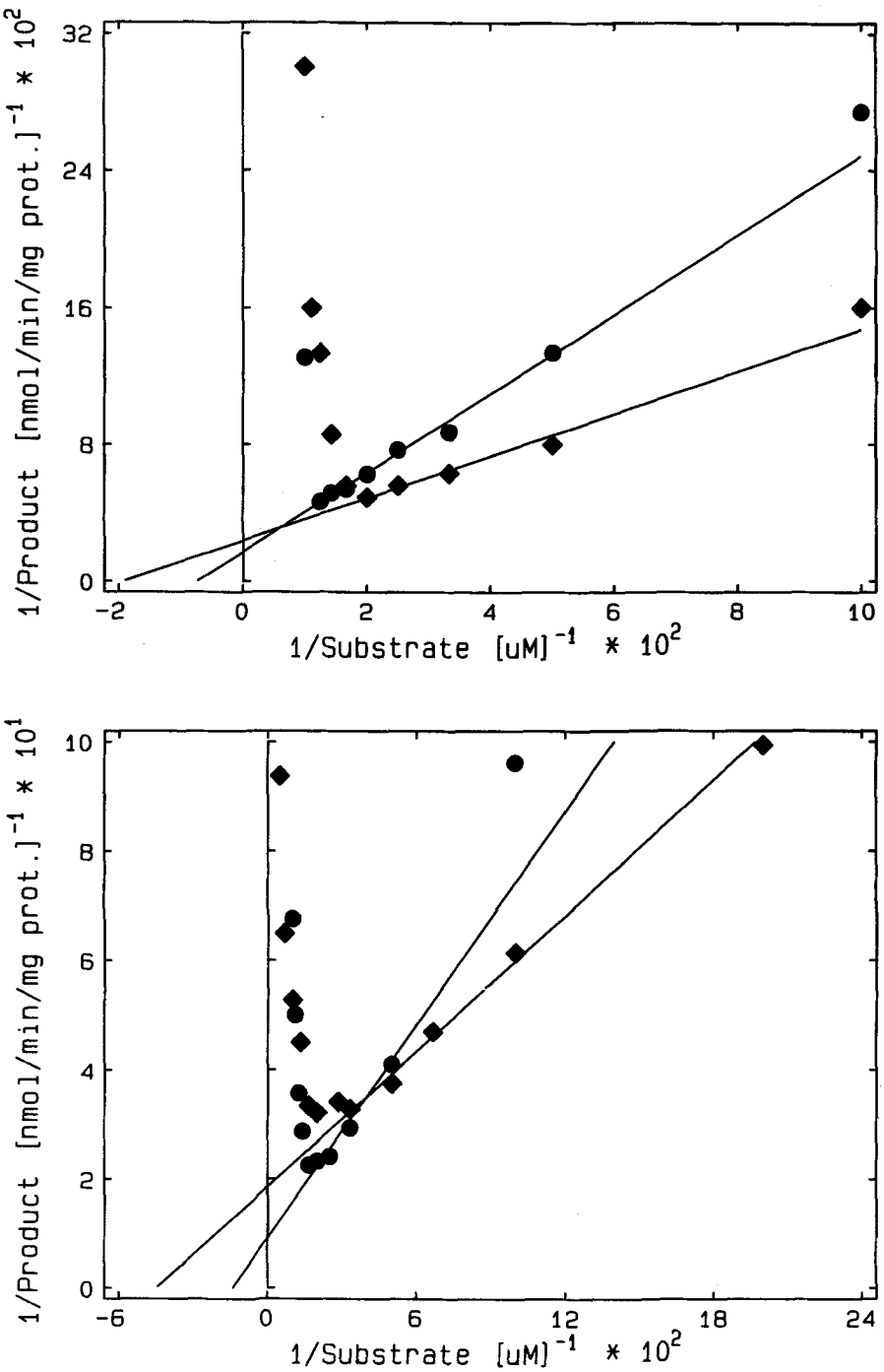


FIG. 5. a) Activity of human acetyltransferase as a function of lysoPAF (closed circles) or dansyllysoPAF (closed squares) at fixed concentrations (200 μM) of acetyl-CoA. b) Activity of rat acetyltransferase as a function of lysoPAF (closed circles) or dansyllysoPAF (closed squares) at fixed concentrations (200 μM) of acetyl-CoA.

TABLE 1

Comparison of Kinetic Parameters for Human and Rat PMN Acetyltransferase Activity as a Function of Acetyl-CoA[a]

Substrate		K_m for Acetyl-CoA (μM)		V_{max} nmol × mg^{-1} × mg^{-1}	
		Human	Rat	Human	Rat
LysoPAF	(+BSA)	132	105	23.1	6.5
DansyllysoPAF	(−BSA)	105	120	26.7	5.4

[a]Kinetic data were obtained from experiments shown in Figure 4. Initial velocity data were analyzed by weighted fits to the Michaelis-Menten equation using a non-linear regression analysis program adapted from KINFIT (17).

this effect may have been caused from the formation of tightly BSA-bound substrates unavailable to the substrate-binding site of the enzyme.

Activity of acetyltransferase as a function of acetyl-CoA. When the activity of acetyltransferase was determined at acetyl-CoA concentrations ranging from 25 to 400 μM at a fixed concentration of lysoPAF or dansyllysoPAF (40 μM), both human and rat acetyltransferase exhibited classical Michaelis-Menten kinetics with this cosubstrate (Fig. 4). The kinetic parameters derived from these experiments are summarized in Table 1. As can be seen from the data, both enzymes exhibited similar K_m values for acetyl-CoA regardless of which substrate was used. Also, the V_{max} values for the human or rat acetyltransferase were similar when assayed with either lysoPAF or dansyllysoPAF. However, the specific activity of rat PMN microsomal acetyltransferase is considerably less than the specific activity of the human enzyme.

Activity of acetyltransferase as a function of lyso ether lipid. Figure 5 shows data of experiments obtained when the concentration of lysoPAF or dansyllysoPAF was varied between 5 and 100 μM, and the concentration of acetyl-CoA (200 μM) was fixed. As can be seen from this Figure, the increase in the activity of lysoPAF acetyltransferase with increasing substrate concentrations apparently followed simple saturation kinetics only up to about 50 μM. At concentrations of lyso compound exceeding 50 μM a drop in enzyme activity was observed that was consistent with previously published data (19,21,22). However, the limited range of substrate concentrations that seemed to follow Michaelis-Menten kinetics was not suitable to calculate meaningful values for K_m or V_{max}. Since the data obtained could not be fitted to a function representing substrate inhibition, factors other than unproductive substrate binding may be responsible for the drop in activity at higher substrate concentrations.

The data presented in this paper demonstrate that no significant differences in the kinetic behavior of acetyltransferase are apparent when either lysoPAF or dansyllysoPAF serve as the substrate. Therefore, the introduction of the dansylamino group into the omega position of the alkyl side chain of lysoPAF does not affect the rate-limiting step of product formation with either human or rat acetyltransferase. Thus, dansyllysoPAF should be a suitable substrate for high throughput screening of potential inhibitors of acetyltransferase from both rat and human neutrophils.

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The Effect of Inhibitors of Platelet Aggregation on the Metabolism of Platelet-Activating Factor (PAF) in Washed Rabbit Platelets[1]

C. O'Neill[a,*], A.J. Ammit[a], R. Korth[b], S. Fleming[a], and X. Wells[a]

[a]Human Reproduction Unit, Royal North Shore Hospital of Sydney, St. Leonards, 2065, NSW Australia and [b]Inserm U200, Universite Paris, Sud, F-92140 Clamart, France

The rabbit platelet metabolizes platelet-activating factor (PAF) intracellularly. PAF is deacetylated to produce lysoPAF which, in turn, can be acylated to produce 1-*O*-alkyl-2-acyl-*sn*-glycero-3-phosphocholine (alkylacyl GPC). Some PAF receptor antagonists have been shown to inhibit this metabolic conversion. In the present study we examined whether the PAF receptor antagonists SRI 63-441 and WEB 2086 would inhibit the metabolism of PAF by intact rabbit platelets. In addition, we examined whether iloprost, a stable analogue of prostaglandin I_2 (PGI_2), and a potent inhibitor of platelet activation induced by a range of agonists, would also inhibit PAF metabolism. We found that SRI 63-441 and WEB 2086 caused an almost complete inhibition of the conversion of PAF to alkylacyl GPC. Iloprost caused up to a 50% inhibition of PAF metabolism compared to antagonist-free controls. Iloprost (and PGI_2) is thought to inhibit platelet response by elevation of cAMP, while receptor antagonists act by blocking PAF binding to its receptor. Since iloprost caused partial inhibition of PAF metabolism, the results of this study suggest that inhibition of PAF metabolism does not occur solely due to competitive inhibition of PAF binding to its receptor.
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Platelet-activating factor (PAF) is generally acknowledged to have a short half-life *in vivo*. This is due to the broad distribution of the enzyme PAF acetylhydrolase (1), both extracellularly and intracellularly. Acetyl hydrolysis results in the conversion of PAF to lysoPAF, the latter being cytotoxic (2). To avoid cell and tissue damage, rapid processing of lysoPAF is therefore required. LysoPAF in cells is usually reacylated *via* a transacylase to form 1-*O*-alkyl-2-acyl-*sn*-glycero-3-phosphocholine (alkylacyl GPC). The fatty acid utilized in this esterification in most cells is preferentially, but not exclusively, arachidonic acid (3,4). Membrane alkylacyl GPC may then serve as a precursor for PAF synthesis *via* phospholipase A_2 and acetyltransferase (E.C. 2.3.1.67), thus creating the PAF cycle (5). However, this pattern of metabolism may not be universal. Activation of washed human platelets by PAF does not result in this type of cellular PAF metabolism (6).

Some PAF receptor antagonists inhibit the metabolism of PAF by rabbit platelets (7,8) and a variety of other cell types, preventing the production of both lysoPAF and alkylacyl GPC. The dose of antagonist required is generally higher than that needed to inhibit platelet activation (8). It has been suggested that the PAF receptor antagonist prevents internalization of PAF by rabbit platelets, and thus exposure of PAF to intracellular metabolic enzymes (7,8). In the case of human platelets, however, PAF receptor antagonists, such as WEB 2086 or ginkgolides, did not inhibit PAF metabolism, nor did they inhibit serum acetylhydrolase (6,9).

The present study, examined (for the first time) the capacity of the PAF receptor antagonists SRI 63-441 and WEB 2086 to inhibit the metabolism of PAF by washed rabbit platelets. Since other agents which activate platelets, such as thrombin, can also promote metabolism of PAF by platelets (10), the ability of the prostaglandin I_2 (PGI_2) stable analogue, iloprost (ZK 36374), which is a very potent inhibitor of platelet activation, was also examined.

MATERIALS AND METHODS

Preparation of platelets. Blood (7 vol) from a male New Zealand white rabbit was collected into 1 vol of acidified citrate dextrose (ACD; pH 6.4, 4°C) (6). The platelets were centrifuged at 100 × *g* for 15 min to produce platelet-rich plasma (PRP). They were then washed three times in Tyrodes buffer containing ACD (9:1, v/v; pH 6.4) (6). After the last wash, the platelets were resuspended ($250 \times 10^3/\mu L$) in Tyrodes buffer. Tyrodes buffer contained 137 mM NaCl, 2.6 mM KCl, 11.9 mM $NaHCO_3$, 1.0 mM $MgCl_2$, 0.41 mM NaH_2PO_4, 0.5 mM dextrose, and 5.0 mM HEPES (Sigma Chemical Co., St. Louis, MO). The buffer was supplemented with 0.25% (w/v) bovine serum albumin (BSA, CSL, Melbourne, Australia) and adjusted to pH 7.4. This medium is referred to as Tyrodes-BSA (6).

[^{3}H]PAF and inhibitors. [^{3}H]PAF (1-*O*-[hexadecyl-1′, 2′-^{3}H(N)]-; 56.7 Ci/mmol) was obtained from NEN Research Products (Wilmington, DE). [^{3}H]PAF in chloroform was placed into siliconized glass test tubes, the solvent was evaporated under N_2, and PAF was resuspended in Tyrodes-BSA. Three inhibitors were used in the study: i) SRI 63-441 which is *cis*-(±)-1-[2-hydroxy [tetrahydro-5-[(octadecylaminocarbonyl)oxy]methyl]-furan-2-yl]methoxy-phosphinyloxy]ethyl]quinolinium hydroxide, inner salt (Sandoz Research Institute, East Hanover, NJ); ii) WEB 2086 which is 3-[4-(2-chlorophenyl)-9-methyl-6H-thieno[3,2-f][1,2,4]triazolo-[4,3-a][1,4]-diazepin-2-yl]-1-(4-morpholinyl)-1-propanone (Boehringer Ingelheim, Ingelheim, Germany); and iii) iloprost which is 5-(E)-(1S,5S,6R,7R)-7-hydroxy-6-(E)-(3S,4RS)-3-hydroxy-4-methyl-oct-1-en-6-yn-yl-bicyclo-3.3.0-octano-3-yliden-pentanoic acid (Schering AG, Berlin, Germany). Iloprost was provided as a 0.1 mg/mL saline solution, and SRI 63-441 and WEB 2086 were prepared as 1 mg/mL solutions

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*To whom correspondence should be addressed.

Abbreviations: ACD, acidified citrate dextrose; alkylacyl GPC, 1-*O*-alkyl-2-acyl-*sn*-glycero-3-phosphocholine; BSA, bovine serum albumin; HPLC, high-performance liquid chromatography; PAF, platelet-activating factor, 1-*O*-alkyl-2-acetyl-*sn*-glycero-3-phosphocholine; PGI_2, prostaglandin I_2; PRP, platelet-rich plasma.

in phosphate buffered saline. The inhibitor concentrations required were attained by serial dilution with saline. The potency of the three inhibitors was tested in a platelet aggregation assay as previously described (11).

PAF metabolism assay. The washed platelet suspension (250 μL) was placed into a 37°C teflon chamber and stirred using a magnetic stirrer (approximately 500 rpm). [^{3}H]PAF (5 μL) in Tyrodes-BSA was added to give a final concentration of 1.3 nM. Immediately thereafter, 45 μL of saline containing the appropriate concentration of inhibitor was added. For zero time assessment, 125 μL of the platelet suspension was removed and added immediately to 19 vol of methanol to stop metabolism and to extract the lipids. The remaining platelet suspension was incubated, and the reaction was stopped at various time points by addition to methanol.

Lipid extraction. The addition of methanol caused precipitation of proteins which were removed by centrifugation (2,500 × *g*, 4°C, 30 min). The supernatant (1 vol) was added to H_2O and chloroform (0.8:0.95, v/v) to effect phase separation. The chloroform phase was recovered and solvent was removed in a rotary evaporator; the lipids were resuspended in 100 μL of methanol and chromatographed by high-performance liquid chromatography (HPLC).

Chromatography. Ion exchange HPLC was used to separate PAF from its metabolites (adapted from ref. 12), utilizing an LKB system with a Whatman Partisil SCX (covalently bound benzene sulfonate residues) column (250 × 4.6 mm, 10 μM silica; Activon, Sydney, Australia). The mobile phase was $CH_3CN/CH_3OH/H_2O$ (300:150:35, v/v/v) at a flow rate of 1.5 mL/min. Fractions (1.5 mL) were collected for 40 min and the radioactivity in each fraction was quantitated by scintillation counting. Figure 1 shows a typical elution profile for a number of phospholipids, including [^{3}H]lysoPAF. The putative acylated derivative of lysoPAF (alkylacyl GPC) eluted at approximately 11–12 min, PAF at 18–21 min and lysoPAF at 28–31 min. Over 85% of all counts recovered were associated with these three regions. Counts eluting at the solvent front were generally less than 10%.

RESULTS

The first experiments were designed to determine the time dependence of PAF metabolism by washed rabbit platelets. These experiments were done in the absence of PAF antagonists (Fig. 2). A significant amount of PAF conversion had already occurred by 15 min when most of the PAF-derived label was found in the lysoPAF fraction. PAF degradation proceeded in an almost linear fashion between 15 and 65 min, and by 80 min most of the label migrated close to the phosphatidylcholine fraction. Based on the results of previous studies, it appeared likely that the latter fraction was alkylacyl GPC (13). Only a small amount of lysoPAF was present at this time, confirming that lysoPAF does not accumulate in platelets. Further experiments used 80 min as the end-point, and the conversion of [^{3}H]PAF to putative alkylacyl GPC was used as a measure of metabolic activity.

Figure 3 shows the relative potency of inhibition of PAF induced platelet aggregation by the three antagonists used in this study. Both iloprost and WEB 2086 were more potent than SRI 63-441, while iloprost was significantly more potent than WEB 2086 (iloprost IC_{50} 13.9 nM; WEB 2086 IC_{50} 55 nM).

Figure 4 shows that the PAF receptor antagonists SRI 63-441 and WEB 2086 almost completely inhibited conversion of [^{3}H]PAF to putative alkylacyl GPC at the highest concentrations tested. The amount of lysoPAF present was not different from the zero time control or inhibitor-free preparation (results not shown). There was

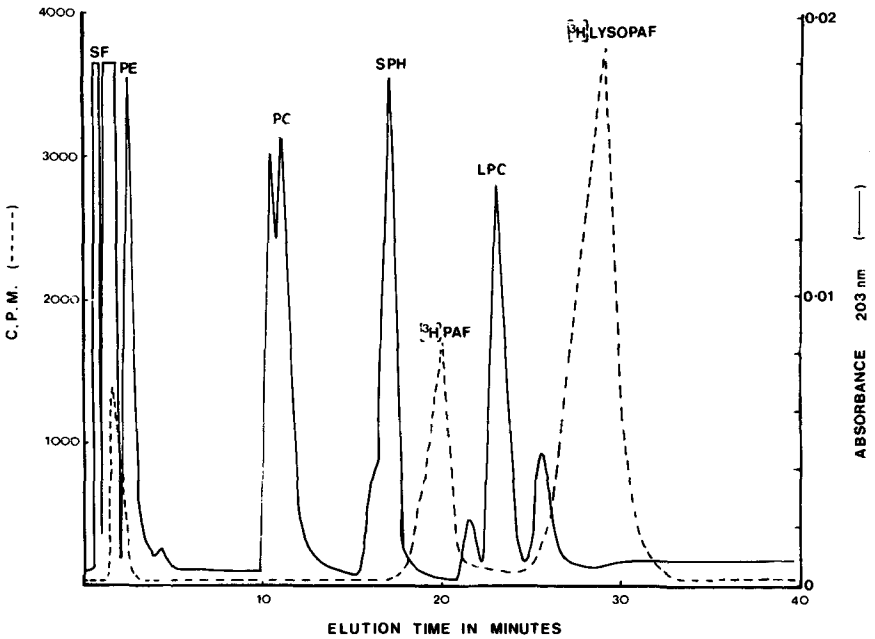


FIG. 1. Typical elution of [^{3}H]PAF and [^{3}H]lysoPAF (broken line) and phospholipid standards (solid line) from a Whatman Partisil strong cation-exchange column with acetonitrile/methanol/water (300:150:35, v/v/v) at a flow rate of 1.5 mL/min.

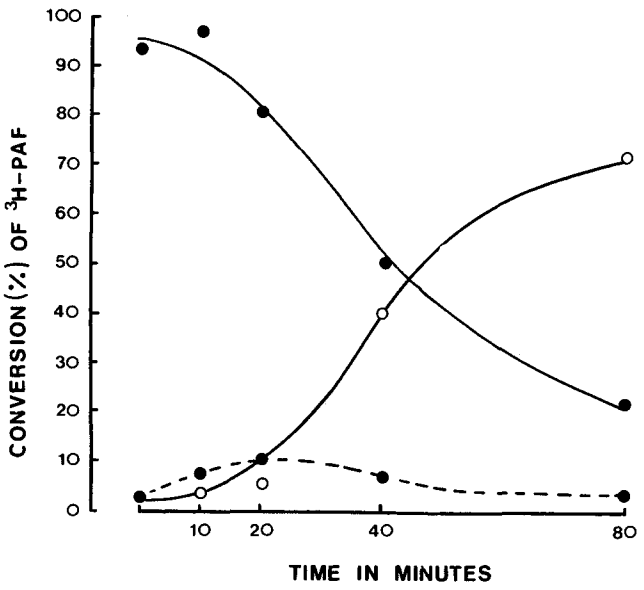


FIG. 2. Redistribution of label from [^{3}H]PAF by washed rabbit platelets as percentage of radioactivity added as a function of time. PAF (●-●), lysoPAF (●--●), or alkylacyl GPC fraction (O-O). Each point is the mean of three replicates.

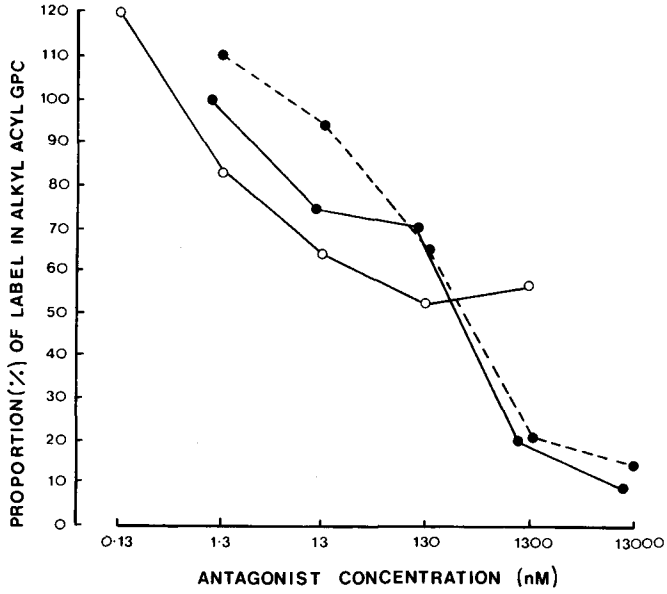


FIG. 4. The effect of PAF inhibitors (iloprost O-O; WEB 2086 ●-●; SRI 63-441 ●- -●) on the conversion of [^{3}H]PAF to putative alkylacyl GPC relative to its conversion in the absence of inhibitors, *i.e.*, % conversion with inhibitor/% conversion without inhibitor × 100. Each point is the mean of three replicates, except for WEB 2086 g, which was the mean of four replicates.

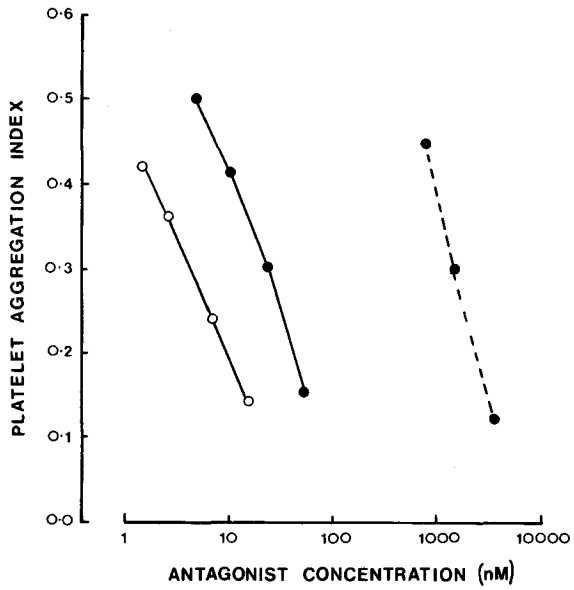


FIG. 3. The effect of increasing concentrations of the PAF inhibitors, iloprost (O-O), WEB 2086 (●-●) and SRI 63-441 (●- -●) on the platelet aggregation induced by the EC_{50} for PAF (0.023 μM) after 15 min incubation with citrated rabbit blood. Each point is the mean of at least three replicates. The platelet aggregation index is 1−(platelet count at 15 min/platelet count at 0 min). An index of 0 signifies no aggregation, and 1 is complete aggregation.

little difference in the relative potencies of response of the two receptor antagonists (which is in contrast to the relative potencies of inhibition of platelet aggregation by these two agents; Fig. 3). For both of these antagonists, the maximum inhibition of metabolism was at a 1,000-fold higher dose than its non-effective concentration. Iloprost caused significant inhibition of metabolism at lower doses than SRI 63-441 and WEB 2086. In contrast to that of the two receptor antagonists, the maximum inhibition caused by iloprost was only approximately 50% of that of antagonist-free controls. WEB 2086 and SRI 63-441 caused a 90% inhibition of metabolism as compared with controls. As with the receptor antagonists, iloprost displayed its maximum inhibitory effect at a 1,000-fold higher dose than its non-effective concentration.

DISCUSSION

The action of PAF on blood platelets is receptor mediated (6,9,14). This signal is transduced by a GTPase (G protein) (15,16) which appears to activate phospholipase C (17). The consequent generation of inositol polyphosphates and diacylglycerol is well documented, as is the resulting increase in cellular calcium concentration and activation of protein kinase C (for a review, see ref. 18).

PAF receptor antagonists compete with PAF for platelet receptors, but do not stimulate GTPase activity upon binding (19), thus inhibiting signal transduction and preventing cell activation. By comparison, iloprost is a potent stimulant of adenylate cyclase (20), resulting in increased cellular cAMP concentrations. Treatment of platelets with dibutyryl cAMP suppresses their response to PAF, and also suppresses PAF induced GTPase activity (19). cAMP also inhibits diacylglycerol dependent activation of protein kinase C (21). Thus, by different mechanisms, the PAF receptor antagonists and iloprost prevent PAF induced activation of cells by inhibition of its signal transduction and secondary messengers.

It was suggested that the binding of PAF to its receptor stimulates the internalization of PAF (and other phospholipids), resulting in its exposure to metabolic enzymes (8,22). The PAF receptor antagonist, BN 52021, inhibited conversion of PAF to putative alkylacyl GPC in intact platelets, but not with cell lysates (7).

A possible explanation for the inhibition of PAF metabolism by receptor antagonists is that the antagonists compete with PAF for the receptor, thus reducing the amount of PAF that can be internalized. It was shown that structural analogues of PAF, U66985 and lysoPAF had only marginal effects on the metabolism of PAF (10). However, these agents also compete for the receptor, but had either minimal or no inhibitory effect on platelet activation, respectively (10). Furthermore, desensitization of platelets to PAF, by prior exposure in the absence of calcium, actually enhanced the metabolism of PAF upon stimulation of platelets with thrombin, collagen or A23187 (9). From such results it appears that the effects of receptor antagonists on PAF metabolism are not simply due to competitive inhibition at the receptor level, but are also dependent upon inhibition of platelet activation.

If this is the case, it may be expected that other inhibitors of platelet activation would also inhibit PAF metabolism. To test this hypothesis we have examined the effect of iloprost on PAF metabolism. Iloprost was the most potent inhibitor of platelet aggregation, with an IC_{50} of 13.9 nM (Fig. 3), and was also the most potent inhibitor of PAF metabolism by platelets (Fig. 4).

All three inhibitors showed a similar pattern of response at low concentrations. For iloprost, however, a maximum response of only about 50% inhibition of metabolism was observed. Further increases in the concentration caused no additional effect. At low doses, iloprost stimulates intracellular platelet cAMP levels (23). At higher concentrations, however, while there was a rapid production of cAMP, the concentration subsequently returned to normal (14). Such a biphasic response is consistent with our inability to completely inhibit PAF metabolism with iloprost. It was not determined in this study whether iloprost affected PAF binding to platelets, although it previously has been shown that iloprost increases intracellular cAMP (24) and that dibutyryl cAMP may indirectly reduce PAF binding by platelets (19). Since iloprost caused partial inhibition of metabolism, the results of this study suggest that inhibition of PAF metabolism does not occur solely due to competitive inhibition of PAF binding to its receptor.

In situ, there is extensive extracellular metabolism of PAF to lysoPAF by serum acetylhydrolase (1). The resulting lysoPAF can cross membranes independently of the PAF receptor (8), albeit at a slower rate than PAF. Therefore, the inhibition of metabolism by PAF antagonists at the cellular level may be of limited significance. However, PAF binding molecules in serum (molecular weight 160–180 kilodalton) which protect PAF from metabolism by serum acetylhydrolase and, hence, limit extracellular metabolism, has been reported (25). If PAF were bound to a molecule protecting it from serum acetylhydrolase, the presence of a high concentration of an inhibitor of PAFs action (and hence cellular metabolism) would extend the half-life of PAF *in situ* considerably. This could facilitate PAFs local accumulation and potentially accentuate its action.

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The Hormonal Regulation of Platelet-Activating Factor Acetylhydrolase Activity in Plasma[1]

Shuichi Miyaura, Noriei Maki, William Byrd and **John M. Johnston***
The Departments of Biochemistry, Obstetrics-Gynecology and The Cecil H. & Ida Green Center for Reproductive Biology Sciences, The University of Texas Southwestern Medical Center, Dallas, Texas 75235-9038

We have previously reported that certain fetal tissues including the lung and kidney have an increased platelet-activating factor (PAF) content and enzymatic mechanism for its elevated biosynthesis during the latter stages of pregnancy. In contrast, in the maternal plasma compartment of both the rabbit and human, a decreased capacity to inactivate PAF has been demonstrated. The PAF acetylhydrolase in the fetal plasma is also suppressed. The present study was undertaken to determine the mechanism(s) involved in the regulation of PAF acetylhydrolase. The 17α-ethynylestradiol was administered (intraperitoneal [i.p.] 2.5 mg/kg body wt 5 days) to female and male rats. The plasma PAF acetylhydrolase activity decreased 5-fold. A decrease was observed when a concentration of the estrogen as low as 50 μg/kg was employed. The injection of dexamethasone (i.p., 1.3 mg/kg body wt, 5 days) to male and female rats resulted in a 3-fold increase in the plasma PAF acetylhydrolase activity. The activity returned to the values prior to hormone treatment 4 days after cessation of treatment. Testosterone and progesterone were without effect on plasma acetylhydrolase activity. The change in PAF acetylhydrolase activity caused by estrogen and the glucocorticoid was reflected by a change in the activity in the HDL fraction and not due to the presence of an inhibitor or activator in the plasma of the hormone-treated animals. Human serum obtained from a group of women, in which the 17β-estradiol concentration was elevated in preparation for an *in vitro* fertilization procedure, showed an inverse relationship between the plasma estrogen concentration and the PAF acetylhydrolase activity. It is suggested that estrogen is responsible for the regulation of PAF acetylhydrolase and the decrease in the plasma PAF acetylhydrolase during the latter stages of pregnancy in both the maternal and fetal plasma caused by the hyperestrogenic state that occurs during this period. The observed increase in PAF acetylhydrolase by dexamethasone may account for, in part, the known anti-inflammatory properties of this steroid by decreasing the concentration of this potent autacoid.
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The name "platelet-activating factor (PAF)" was first utilized to describe a mediator that was released from basophils following IgE sensitization which would cause platelet aggregation (1). The structure was subsequently shown to be 1-*O*-alkyl-2-acetyl-*sn*-glycero-3-phosphocholine by Demopoulos *et al.* (2) and Benveniste *et al.* (3). During this same period, Snyder and colleagues demonstrated that the polar renal-medullary antihypertensive lipid was PAF (4). Subsequently it was demonstrated that a variety of cells release PAF upon stimulation (for recent reviews, see ref. 5-7). It is now well established that this active autocoid is involved in a variety of biological phenomena including anaphylaxis and the inflammatory response including neutrophil and eosinophil stimulation (8-10), vascular permeability (11-13), and induction of hypotension (4). Its physiological role has also been suggested in reproductive biology in both animals and humans including implantation (14) and fetal lung maturation, and the initiation and maintenance of parturition (15). Other investigators have suggested a role for PAF in ovulation (16) and spermogenesis (17).

The enzymatic mechanisms involved in PAF biosynthesis in various tissues have been reported (for review, see ref. 18). PAF can be synthesized by two distinct mechanisms referred to as the remodeling and the *de novo* pathways. Until recently, the regulation of PAF concentration in most tissues has focused on the biosynthetic pathways. The key regulatory enzymes of the remodeling pathway and *de novo* pathway are thought to be acetylCoA:lysoPAF acetyltransferase and acetylCoA: 1-alkyl-2-lyso-*sn*-glycero-3-P acetyltransferase, respectively (7).

The inactivation of PAF is catalyzed by the enzyme PAF acetylhydrolase (18). Two forms of the enzyme exist: one is found in the cytosolic fraction of most cells, and an extracellular form of the enzyme is present in plasma. Several reports have appeared concerning the change in activity of PAF acetylhydrolase in plasma in association with certain biological events. A significant increase in activity has been reported during a "stress" reaction of the lizard (19). The activity also increased during platelet aggregation (20) and in patients with ischemic cerebrovascular disease (21). A decrease in PAF acetylhydrolase activity in maternal plasma was found during the latter half of pregnancy in the rabbit (22) and human (23). The activity of this enzyme is increased dramatically in the plasma of the rabbit neonate shortly after delivery (22). A decrease in plasma acetylhydrolase activity has also been reported in asthmatic children (24), and the deficiency in serum PAF acetylhydrolase is transmitted as an autosomal recessive trait. An increase in PAF acetylhydrolase has been reported in insulin-dependent diabetes mellitus (25) and also in spontaneously hypertensive rats (26) and hypertensive Caucasian males (27). The properties of PAF acetylhydrolase and its potential role in human disease have recently been reviewed in detail (28).

To investigate the regulation of the circulating or tissue levels of PAF, we have examined the activity of PAF acetylhydrolase as it may be regulated by various hormones during pregnancy. Increases in estrogens and progesterone are known to occur in both the fetal and maternal compartments during the latter stages of pregnancy. During this time period the activity of PAF acetylhydrolase

[1]Based on a paper presented at the Third International Conference on Platelet-Activating Factor and Structurally Related Alkyl Ether Lipids, Tokyo, Japan, May 1989.

*To whom correspondence should be addressed at The Departments of Biochemistry, Obstetrics-Gynecology and The Cecil H. & Ida Green Center for Reproductive Biology Sciences, The University of Texas Southwestern Medical Center, 5323 Harry Hines Boulevard, Dallas, TX 75235-9038.

Abbreviation: PAF, platelet-activating factor.

is low in fetal plasma and decreasing in the maternal plasma (22). We have examined the role of various steroid hormones on the activity of PAF acetylhydrolase in rat plasma and in the plasma of women undergoing hormonal manipulation in preparation for an *in vitro* fertilization procedure.

MATERIALS AND METHODS

Chemicals. [^{3}H]PAF (1-*O*-hexadecyl-2-[^{3}H]acetyl-*sn*-glycero-3-phosphocholine; 10 Ci/mmol; 1 Ci = 37 GBq) was purchased from New England Nuclear, Boston, MA. Nonradiolabeled PAF was obtained from Avanti Polar Lipids, Pelham, AL. Bovine serum albumin (fatty acid-poor), 17α-ethynylestradiol, progresterone, testosterone and dexamethasone were purchased from Sigma Chemical, St. Louis, MO.

Treatment of rats with hormones. Hormones were dissolved in ethanol and made up to desired concentrations with dimethylsulfoxide. The final concentration of ethanol was 18%. Hormones were administered to both male and female Wistar rats (270 g) for 5 days by daily intraperitoneal injection. Control rats received the vehicle only. Blood was collected from the tail vein into heparinized capillary tubes. Samples were collected 22 hr prior to the first, second and fourth injections and after the last injection. The plasma was immediately separated by centrifugation.

Patient selection. Twelve patients requesting treatment by *in vitro* fertilization and embryo transfer were given luprolide acetate (TAP Pharmaceuticals, North Chicago, IL) subcutaneously to inhibit gonadotrophin secretion, 1 mg/day for a minimum of 14 days followed by 0.5 mg a day until the administration of human chorionic gonadotrophin. Follicular development was stimulated following suppression of endogenous luteinizing hormone (LH) and follicle stimulating hormone (FSH) by injection of 3 ampoules of Pergonal (preparation of human gonadotrophins containing equal amounts of LH and FSH) per day (Serono, Randolf, MA) for the three days, followed by variable doses of Perganol depending on the patient's estradiol response. Ovulation was induced by 5,000 IU of hCG when two or more follicles reached 18 mm in diameter as determined by sonography.

Hormone assays. Estradiol and progesterone were measured using the Delfia (Pharmacia ENI, Columbia, MD) solid phase fluoroimmunoassay procedure. Blood samples were taken from patients at indicated times, and allowed to clot at room temperature. The serum separated by centrifugation. Intra-assay variation was 5.7% for progesterone and 8.7% for estradiol. Inter-assay variation was 7.1 and 9.9%, respectively.

Assay for PAF acetylhydrolase activity. The activity of PAF acetylhydrolase (EC 3.1.1.48) in plasma was assayed according to the method of Miwa *et al.* (24) with minor modifications. The assay mixture contained Tris-HCl (30 mM, pH 7.5), 1-*O*-hexadecyl-2-[^{3}H]acetyl-*sn*-glycero-3-phosphocholine (50 μM, 4.0 μCi/μmol) in bovine serum albumin (0.13% final concentration), and various amounts of plasma in a total vol of 0.5 mL. The assay mixtures and controls (without enzyme) were incubated for 20 min at 37°C and terminated by addition of 0.5 mL of 14% trichloroacetic acid. The supernatant was obtained by centrifugation for 5 min at 3,000 × *g* at 4°C. A 0.1 mL aliquot of the supernatant was removed and mixed with 5 mL scintillation cocktail (Budget-Solve; Research Products International Corp.). The water-soluble [^{3}H]acetate released from [^{3}H]PAF was assayed by liquid scintillation spectroscopy.

Preparation of cytosolic and lipoprotein fractions. Liver, kidney, lung, spleen and brain were removed following hormone treatments and stored at −70°C until used for assay. Tissues were homogenized in 6 vol of a cold 0.25 M sucrose using a PT 10/20 Polytron homogenizer at maximum speed. All procedures were performed at 4°C. The homogenate was centrifuged at 600 × *g* for 10 min. The resulting supernatant fraction was further centrifuged at 18,000 × *g* for 15 min and then at 105,000 × *g* for 60 min to obtain the cytosolic fraction. The lipoprotein fractions were prepared by ultracentrifugation on a KBr density gradient as described (29). PAF acetylhydrolase activity in the cytosolic fraction was assayed as described above.

RESULTS

Effect of hormones on PAF acetylhydrolase. Previously Pritchard (30) had injected 17α-ethynylestradiol to male rats in order to establish that the activity of PAF acetylhydrolase was closely associated with the lipoprotein fraction. In our initial studies, 2.5 mg/kg of 17α-ethynylestradiol, 50 mg/kg of progesterone, 5 mg/kg of testosterone and 1.3 mg/kg of dexamethasone were injected

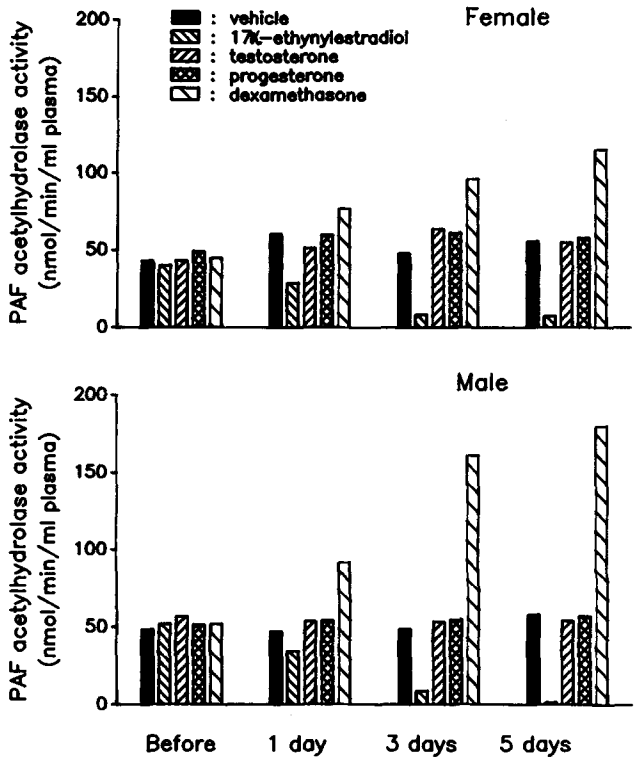


FIG. 1. Effect of various steroid hormones on the plasma PAF acetylhydrolase activity of the rat. Rats received vehicle, 17α-ethynylestradiol (2.5 mg/kg), testosterone (5 mg/kg), progesterone (40 mg/kg) or dexamethasone (1.3 mg/kg) as detailed in Experimental Procedures. PAF acetylhydrolase activities in plasma were assayed periodically, and the values are the averages of results from two rats.

i.p. in male and female rats for 5 days, and the plasma PAF acetylhydrolase was assayed (Fig. 1). The specific activities of PAF acetylhydrolase in male and female rats before treatment were 52 ± 1.5 (means ± SEM; n = 10) and 50 ± 2.1 nmol × min^{-1} × mL^{-1} of plasma (n = 10), respectively. The activity of the enzyme decreased during the 5 days of treatment with 17α-ethynylestradiol to 1.2 and 7.5 nmol × min^{-1} × mL^{-1} plasma in male and female rats, respectively. When dexamethasone was administered for similar time periods, a significant increase in PAF acetylhydrolase activity was observed: from 52 to 180 nmol × min^{-1} × mL^{-1} plasma in male rats and from 50 to 115 in female rats after a 5-day treatment. Progesterone (50 mg/kg) or testosterone (5 mg/kg) administration under similar conditions did not alter the activity of PAF acetylhydrolase in either male or female rats. This experiment was repeated several times and similar results were obtained. In addition, we observed no effect of progesterone when given in combination with 17α-ethynylestradiol over and above the predicted decrease in activity when 17α-ethynylestradiol was administered alone (data not shown). However, when 17α-ethynylestradiol and dexamethasone were administered simultaneously, no change in PAF acetylhydrolase activity was observed (Fig. 2). It is suggested that the combination of these two hormones, one of which inhibits and one of which increases PAF acetylhydrolase activity, resulted in no net change in the activity of this enzyme.

To clarify whether or not hormones have some effect on the intracellular PAF acetylhydrolase activity, we determined the activity of the cytosolic enzyme in various tissues obtained from rats treated with the various hormones. In control male rats, specific activities of PAF acetylhydrolase were: kidney 4.2, lung 3.0, brain 1.4, spleen 0.88 and liver 0.28 nmol × min^{-1} × mg^{-1} protein. Although these values were lower than that previously reported (31), the relative distribution pattern among tissues was very similar. No change of intracellular PAF acetylhydrolase activity in any tissues was observed after hormone treatment.

In order to determine whether the changes of the enzyme activity in plasma from rats treated with either 17α-ethynylestradiol or dexamethasone were due to an activator or inhibitor, a mixing experiment was performed (Table 1). The activity of PAF acetylhydrolase was assayed following the mixing of plasma samples obtained from the hormone-treated group with the control samples in various proportions. The specific activities in the plasmas obtained following treatment for 5 days with 17α-ethynylestradiol from the 17α-ethynylestradiol and control rats were 4.5 and 57 nmol × min^{-1} × mL^{-1} plasma, respectively. When equal volumes of each plasma were mixed and assayed, the specific activity was 31 nmol × min^{-1} × mL^{-1} plasma (theoretical value, 31). Similar theoretical values were obtained when samples from a variety of known mixtures were analyzed. When mixtures of control and dexamethasone plasma were assayed in a similar manner, the results again agreed with the theoretical values. It is, therefore, concluded that the plasma obtained from the 17α-ethynylestradiol- and dexamethasone-treated animals contained neither activators nor inhibitors of PAF acetylhydrolase that account for the change in activity following hormone treatment.

TABLE 1

PAF Acetylhydrolase Activity in Mixtures of Plasma from Rats Treated with Hormones[a]

Combinations	Mixing ratio by vol				
	1:0	3:1	1:1	1:3	0:1
Vehicle/estrogen	57	45	31	18	4.5
Vehicle/dexamethasone	57	93	120	154	177
Dexamethasone/estrogen	177	131	101	50	4.5

[a]Plasma obtained from rats injected with either vehicle, 17α-ethynylestradiol or dexamethasone (see Experimental Procedures) was mixed in the ratios indicated and their PAF acetylhydrolase activities were determined. Activities are expressed as nmol × min^{-1} × mL^{-1} plasma.

We next examined the dose response of 17α-ethynylestradiol and dexamethasone on PAF acetylhydrolase activity. As indicated in Figure 3, the administration of 17α-ethynylestradiol at doses as low as 0.05 mg/kg resulted in a significant decrease of enzyme activity. Maximum inhibition was observed at doses of 0.25 mg/kg. Dexamethasone increased PAF acetylhydrolase when this hormone was administered at doses as low as 0.65 mg/kg. Within three days following the suppression or induction of PAF acetylhydrolase activity by either hormone, the values returned to the levels observed prior to hormone treatment.

Distribution of PAF acetylhydrolase activity in lipoprotein fractions. It was previously reported that in the human plasma PAF acetylhydrolase is a Ca^{2+}-independent phospholipase A_2 associated with the lipoprotein fraction with specificity for substrates with a short-chain acyl group (7,32,33). In the human, PAF acetylhydrolase activity is primarily associated with the LDL fractions (28,32,33), while in the rabbit (22) and rat (30) the activity is present in the HDL fraction. The distribution of PAF acetylhydrolase in the various plasma fractions of the rat is illustrated in Figure 4. The PAF acetylhydrolase activity in the control samples following hormone treatment is present in the HDL fraction in rat plasma (85%) and less than 5% in the LDL fraction. PAF acetylhydrolase activities in plasma obtained from the 17α-ethynylestradiol- and dexamethasone-treated animals were altered to 6 and 216 % of the control animals, respectively.

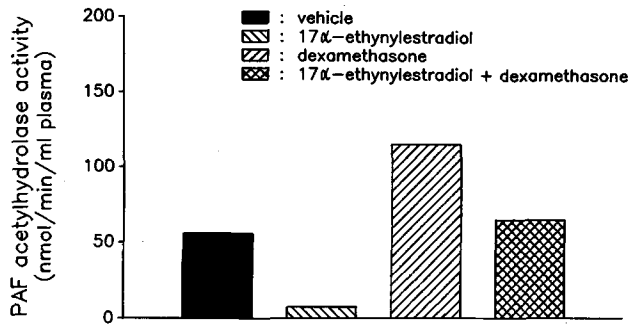


FIG. 2. Effect of combination hormone treatment on the plasma PAF acetylhydrolase activity of the rat. Rats received vehicle, 17α-ethynylestradiol (2.5 mg/kg), dexamethasone (1.3 mg/kg) or a mixture of 17α-ethynylestradiol and dexamethasone for 5 days. PAF acetylhydrolase activity in plasma was assayed 22 hr after the last injection (experiment repeated 3 times with similar results).

However, the activity was still primarily associated with the HDL fraction. Based on these findings, it is suggested that the suppression and induction of the enzyme by these hormones resulted in a change in the activity without changing the plasma distribution of the enzyme from that present in the untreated animal.

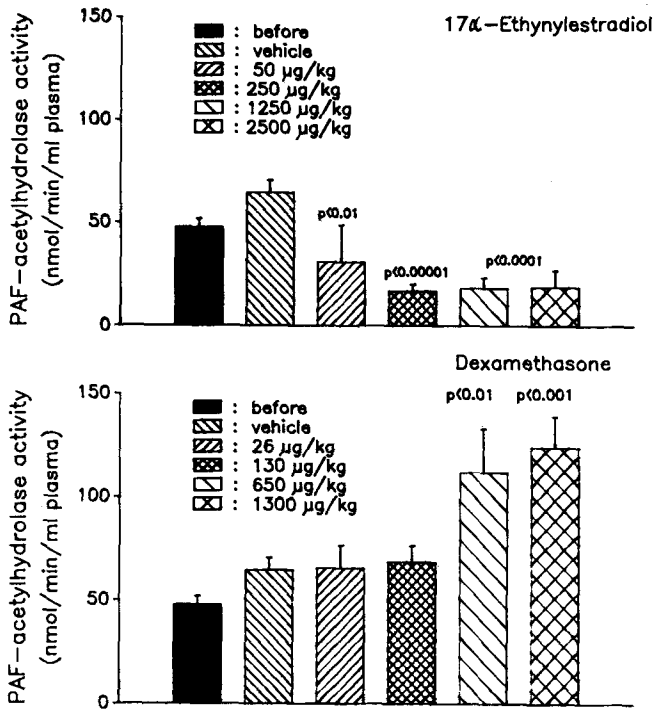


FIG. 3. Dose response of 17α-ethynylestradiol and dexamethasone on the plasma PAF acetylhydrolase in male rat plasma. Male rats received vehicle, 17α-ethynylestradiol or dexamethasone at various concentrations for 3 days by daily intraperitoneal injection. Blood was collected from the tail vein 22 hr before the 1st injection and after the last injection. PAF acetylhydrolase activity in plasma was assayed, and statistical analysis was carried out by Student's t-test for unpaired samples. Values are the means ± SD of results from 4 rats.

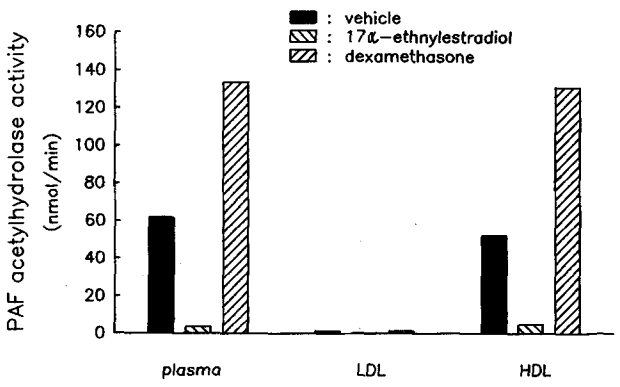


FIG. 4. Distribution of PAF acetylhydrolase activity in rat plasma. Rats received 17α-ethynylestradiol at 0.25 mg/kg or dexamethasone at 0.65 mg/kg for 5 days. Blood was collected from heart of unanesthetized rats, mixed with 1 mg EDTA per mL and placed on ice. Lipoproteins were separated by ultracentrifugation on a KBr density gradient as described (29). Values in plasma and lipoprotein fractions are expressed as total activities obtained from 1 mL of plasma (experiment repeated 2 times with similar results).

Plasma PAF acetylhydrolase activity and estrogen concentration in the human. In an attempt to determine whether or not the activity of PAF acetylhydrolase could be correlated to circulating hormone concentrations in the human, we examined the serum PAF acetylhydrolase activity in a group of women whose serum 17β-estradiol concentrations were known. These women were undergoing follicular stimulation in preparation for *in vitro* fertilization. Serum 17β-estradiol levels were suppressed to less than 0.2 nmol/L following the subcutaneous administration of Lupron prior to follicular stimulation with Pergonal. Following Pergonal stimulation, samples were taken throughout the cycle, and the estradiol and progesterone were measured. The PAF acetylhydrolase activity expressed as a function of the serum 17β-estradiol concentration in these women is illustrated in Figure 5. As can be seen, there is an inverse relationship between the serum estradiol concentration and the activity of PAF acetylhydrolase. In Table 2 the relationship of progesterone and estradiol was examined in these same women. The activity of the PAF acetylhydrolase in the first three groups was significantly different from each other ($p < 0.001$). We found no relationship between the serum progesterone levels and the activity of plasma PAF acetylhydrolase activity.

We have also determined the level of estradiol and PAF acetylhydrolase activity in aspirates from preovulatory follicles in women undergoing *in vitro* fertilization. We found that the estradiol levels in the follicular aspirates had a mean value of 140.9 ± 25.3 nmol/L with a corresponding value of 1.3 ± 0.8 nmol × min^{-1} × mL^{-1} of PAF acetylhydrolase activity. When the estradiol levels from serum and follicular fluid were compared to PAF acetylhydrolase activities, there was a correlation of $r = 0.856$ ($p < 0.0001$).

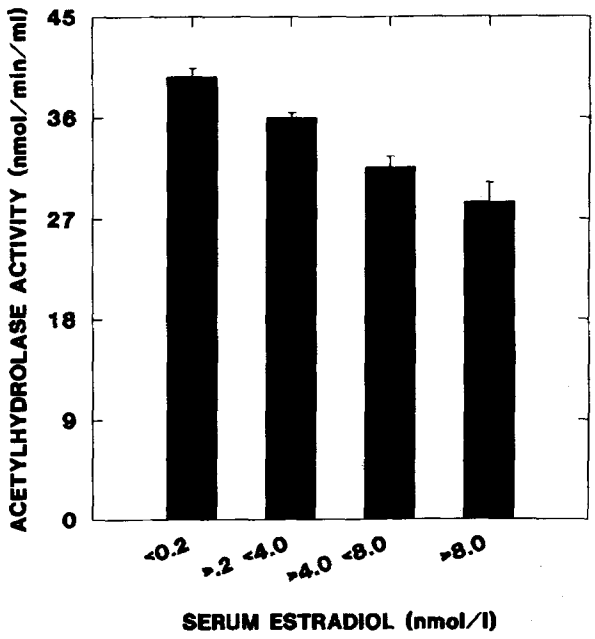


FIG. 5. Acetylhydrolase activity and estradiol levels in women undergoing follicular stimulation wtih Pergonal. Serum samples were obtained daily from 12 women undergoing follicular stimulation.

TABLE 2

Plasma PAF Acetylhydrolase Activity During the Menstrual Cycle in Women

	Estradiol nmol/L	Progesterone nmol/L	PAF Acetylhydrolase nmole × min^{-1} × mL^{-1}
Day 1 (start Pergonal)	0.01 ± 0.01[b]	0.8 ± 0.49[d]	40.05 ± 31[f]
Day 11 (hCG)	7.35 ± 4.45[a]	2.14 ± 0.93[d]	30.24 ± 6.41[e]
Day 21 (Mid-Luteal)	0.055 ± 0.35[b]	45.37 ± 12.55[c]	38.56 ± 7.56[f]

Mean ± SD.

[a]Significantly different from b ($p < 0.001$) ANOVA (analysis of variance).

[c]Significantly different from d ($p < 0.001$) ANOVA.

[e]Significantly different from f ($p < 0.02$) ANOVA.

DISCUSSION

The importance of PAF in reproductive and developmental biology has recently been reviewed (14,15,34). We have previously observed that PAF is present in the amniotic fluid of women at term and in labor but not present in the amniotic fluid of women at term and not in labor (35). These findings were confirmed by Nishihara *et al.* (36). We also reported that PAF is present in the amniotic fluid of women with preterm labor and premature rupture of membranes (37). The source of the PAF in amniotic fluid is thought to be at least, in part, the fetal lung (38). It has been observed that PAF will stimulate PGE_2 formation in amnion tissue (39) and amnion cells in culture (40). PAF is also one of the most potent stimuli of myometrial contraction in rat (36), guinea pig (41) and human (42). In support of its role in stimulating myometrial contraction, we have recently demonstrated the presence of PAF receptors in myometrium, and shown that PAF is a potent stimulator of Ca^{2+} uptake and myosin light-chain phosphorylation in isolated human myometrial cells (43). It has also been demonstrated that the length of labor in rats was increased 3- to 5-fold by the administration of the PAF receptor antagonist L-659,989 (44).

We have suggested that the increase in PAF production occurs in several fetal tissues, including the lung, during the latter stages of gestation (15,38). The increase in PAF is most likely due to an elevation in its biosynthesis due to an increase in the activities of the enzymes of both the remodeling and *de novo* pathways in fetal tissue (38,45). No change in the intracellular activity of PAF acetylhydrolase in fetal lung tissues was observed during this period. Thus, the capacity for PAF biosynthesis is increased in certain fetal tissues during the latter stages of pregnancy (23).

In contrast, it is suggested that there is a decreased capacity to inactivate PAF in the maternal plasma during the latter stages of pregnancy in the rabbit (22) as well as the human (23) since the activity of PAF acetylhydrolase is significantly reduced. It is suggested that the combination of an increase in the biosynthesis of PAF in fetal tissues and a decrease in the capacity of the maternal plasma to inactivate PAF is of critical importance in the initiation and maintenance of parturition (23). The decidua may be the site in which the PAF is inactivated in early and mid-pregnancy because of its "lush" blood supply. During the latter stages of pregnancy, a decrease in the capacity to inactivate PAF occurs due to the reduced activity of PAF acetylhydrolase in maternal plasma (22). The mechanism involved in the regulation of this potent autacoid during development is of importance. It is well documented that during the latter stages of pregnancy dramatic hormonal changes occur in both the fetal and maternal compartments. Estrogen would be a prime candidate for hormonal regulation of plasma PAF acetylhydrolase since its concentration increases at a similar time as the changes in PAF acetylhydrolase. Support for a possible role for estrogen in regulating PAF acetylhydrolase was provided by the observation by Pritchard (30) who injected 17 α-ethynylestradiol to male rats, and demonstrated a decrease in the lipoprotein fraction and PAF acetylhydrolase activity in plasma.

In the present study, it has been demonstrated that the intraperitoneal administration of 17α-ethynylestradiol to both male and female rats results in a significant decrease in the plasma PAF acetylhydrolase activity. The decrease was noted within the first 24 hr and was maximal following 3 days of estrogen administration. The activity returns to pretreatment levels (within 3 days) when the hormone treatment was discontinued. The administration of progesterone by itself or in combination with the estrogen did not alter the activity over that expected by 17α-ethynylestradiol injection. Thus, the combined treatment was not necessary for the decreased expression of the enzyme activity. Testosterone administration was also without effect.

In contrast, the administration of dexamethasone to both male and female rats resulted in an increase in the specific activity of plasma PAF acetylhydrolase. The increase was seen within 24 hr, and was maximal after 3 days of treatment. Like estrogen administration, the activity returned to the preinjection levels within 3 days following the suspension of dexamethasone treatment. The importance of PAF as an autacoid in the inflammatory response is well established (6). As discussed, the activity of PAF acetylhydrolase increases during a "stress" reaction in the lizard which is related to an increase in glucocorticoids (19). Based on the reported observations that dexamethasone increases PAF acetylhydrolase activity, it is suggested that this synthetic glucocorticoid may function, in part, as an anti-inflammatory agent by increasing the activity of PAF acetylhydrolase, and thus decrease the concentration of this potent mediator of the inflammatory response in plasma and certain tissues.

During pregnancy the estrogen levels in the maternal compartment are increasing. In this study we have demonstrated a decrease in PAF acetylhydrolase activity by estrogen administration which would be consistent with a central role for estrogens in the regulation of this enzyme during the latter stages of pregnancy. An additional role for estrogen in the regulation of the activity of this enzyme in fetal plasma might also be invoked. The PAF acetylhydrolase activity in rabbit fetal plasma is low during most of gestation (22) and then increases to values which are two to three times that of the adult nonpregnant rabbit within 10 days after birth (23). The activity remains elevated in the newborn until the time of sexual maturity. This would be the expected pattern of PAF acetylhydrolase activity of the neonate and maturing rabbit if the plasma enzyme were regulated by estrogen, since the estrogen concentration in the fetus is high during the latter stages of gestation. Following delivery, the estrogen concentration decreses in the newborn since the placenta is the primary site of its biosynthesis. It is suggested that the high circulating levels of PAF acetylhydrolase present in neonatal rabbits may be the explanation of the increased resistance of neonate and young animals to PAF administration when compared to adult animals (45). We have also noted an inverse relationship between the serum 17β-estradiol levels in women in which the estrogen level has been significantly elevated in preparation for *in vitro* fertilization (Table 2, Fig. 5). The role of estrogens in the regulation of PAF acetylhydrolase in relation to the initiation and maintenance of parturition and to certain complications of pregnancy is presently under investigation.

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REVIEW

Recent Advances in Our Understanding of the Biochemical Interactions Between Platelet-Activating Factor and Arachidonic Acid[1]

Floyd H. Chilton*, Marc Cluzel and Massimo Triggiani
The Johns Hopkins University School of Medicine, The Johns Hopkins Asthma and Allergy Center, Baltimore, Maryland 21224

In the last few years, it has become increasingly apparent that the biochemistry of PAF (platelet-activating factor) and that of arachidonic acid are interrelated in a number of inflammatory cells. Experiments presented here further point out that arachidonic acid plays a crucial role in the catabolism and biosynthesis of PAF. In addition, they suggest that the same phospholipid molecular species may serve as a source for both arachidonic acid and 1-alkyl-2-lyso-*sn*-glycero-3-phosphocholine during cell activation. Finally, they reveal that there may be common regulatory mechanisms for the biosynthesis of PAF and arachidonic acid metabolites. Taken together, studies examining the relationship between PAF and arachidonic acid suggest it may be difficult to consider the biochemistry of PAF without considering arachidonic acid metabolism and vice versa.
Lipids 26, 1021–1027 (1991).

Five years ago, we and others proposed that the biochemistry of PAF (platelet-activating factor) and arachidonic acid may overlap at several points (Fig. 1). Two observations strongly supported this hypothesis: (i) PAF is catabolized by several inflammatory cells by removing the 2-acetyl group and specifically replacing it with arachidonate; and (ii) cells mobilize 1-alkyl-2-lyso-GPC (glycerophosphocholine) and free arachidonic acid from a common precursor molecule, 1-*O*-alkyl-2-arachidonoyl-GPC by a phospholipase A_2 reaction upon Ca^{++} stimulation. Both 1-alkyl-2-lyso-GPC and free arachidonic acid may then serve as precursors for bioactive lipids, PAF and eicosanoids, respectively. Since these initial findings, several studies have been performed in a number of cells to test and to better characterize these pathways. Progress has been made in several areas which support the hypothesis; these areas include our understanding of: (i) the role of arachidonate in the catabolism of PAF; (ii) the role of arachidonate in the biosynthesis of PAF; (iii) phospholipid sources of arachidonic acid used for eicosanoid biosynthesis and their relationship to PAF sources; and (iv) common regulatory mechanisms for PAF and arachidonic acid metabolites. This review briefly examines some recent developments in each of these areas.

ROLE FOR ARACHIDONATE IN THE CATABOLISM OF PAF

Several cells and tissues including human neutrophils (1), rabbit macrophages (2–4), human (5) and rabbit (6) platelets, human (7) and mouse (8) mast cells and the isolated perfused lung (9) have been shown to metabolize PAF by removing the acetate moiety at the *sn*-2 position and replacing it with arachidonate. Studies utilizing microsomal preparations from a variety of cells now indicate that the key enzyme responsible for the selective acylation of 1-alkyl-2-lyso-GPC with arachidonate is a CoA-independent transacylase (2–4). This enzyme selectively transfers arachidonate from 1-*O*-acyl-2-arachidonoyl-GPC to 1-alkyl-2-lyso-GPC to form 1-alkyl-2-arachidonoyl-GPC. In addition to arachidonate, other C_{20}–C_{22} fatty acids such as 20:5, 22:4, and 22:6 are transferred as well from 1,2-diacyl-GPC to 1-alkyl-2-lyso-GPC in a CoA-independent manner (10). In support of these data, whole cell studies indicate that certain C_{20}–C_{22} carbon fatty acids can replace arachidonate in the acylation reaction of 1-alkyl-2-lyso-GPC if they are present at comparable levels with arachidonate within the cellular phosphoglycerides (8,11). In these studies, the membrane content of ω-3 fatty acids was increased in cells *in vitro* by supplementing them in culture or *in vivo* with diets enriched in ω-3 fatty acids such as 22:6 or 20:5. Subsequently, the cells were examined for their ability to catabolize PAF. Shown in Figure 2 are the HPLC (high performance liquid chromatography) separations of molecular species of newly synthesized 1-alkyl-2-acyl-GPC produced during the catabolism of PAF by mast cells supplemented in culture with arachidonic acid (A), docosahexaenoic acid (B) or linoleic acid (C). Data from this study demonstrate that docosahexaenoate can replace arachidonate in the transacylation reaction if it is present at comparable levels within the cellular phospholipids. In the case of linoleic acid (C), HPLC profiles were identical to cells that had not been supplemented in culture. All of these findings suggest that the nature of the acylation in the whole cell depends on both the fatty acid specificity of the transacylase (C_{20}–C_{22} fatty acids) and also the availability of C_{20}–C_{22} fatty acids within cellular phospholipids.

The CoA-independent transacylation reaction may

*To whom correspondence should be addressed at The Johns Hopkins University School of Medicine, The Johns Hopkins Asthma and Allergy Center, 301 Bayview Boulevard, Baltimore, MD 21224.

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Abbreviations: AMP, adenosine 5′-monophosphate; ATP, adenosine 5′-triphosphate; GPC, glycerophosphocholine; GPE, glycerophosphoethanolamine; HPLC, high performance liquid chromatography; PAF, platelet-activating factor; PC, phosphatidylcholine; PMA, phorbol myristate acetate.

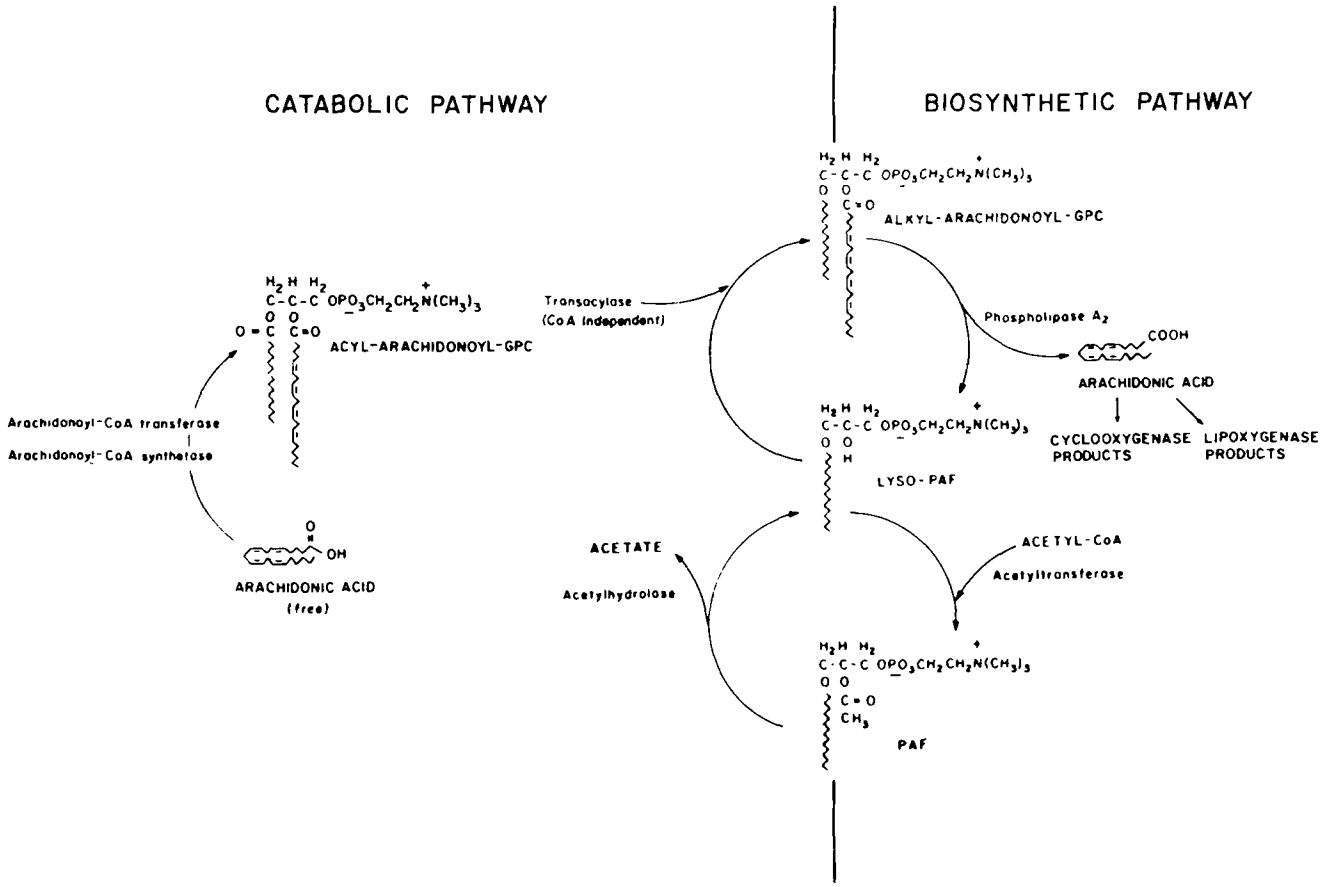


FIG. 1. Proposed biochemical interactions between PAF and arachidonate. (Chilton, F.H., and Murphy, R.C., *J. Biol. Chem. 261*, 7771, 1986).

explain how C_{20}–C_{22} fatty acids such as arachidonic acid accumulate in the large 1-alkyl pools of certain inflammatory cells. Until recently, little was known about this process. There is now evidence that arachidonic acid is incorporated into choline-linked subclasses by three different enzymatic steps (12). Initially, arachidonic acid is converted to arachidonoyl-CoA by a specific arachidonoyl-CoA synthetase step (13–15). The arachidonoyl-CoA is then incorporated into 1-acyl-2-lyso-GPC in a step that involves an arachidonoyl-CoA:lysophosphatidylcholine acyltransferase reaction that is selective for 1-acyl-linked glycerophosphocholine (12,16). These pathways help explain why free arachidonic acid or arachidonic acid from arachidonoyl-CoA is initially incorporated into 1,2-diacyl-linked species (12,17–19). Furthermore, there is now evidence that the initial acylation of 1-acyl-2-lyso-GPC may be regulated during cell activation (20,21). As discussed above, arachidonate is subsequently transferred from 1-acyl-2-arachidonoyl-GPC into the 1-alkyl-linked fraction by a CoA-independent transacylation step. Figure 3 shows the distribution of exogenously-provided arachidonic acid in choline-linked phospholipid molecular species of the neutrophil after addition for 5 min or 120 min. It is clear from these data that 1-acyl-linked pools are initially labelled; however, this arachidonate shifts into the larger 1-alkyl-linked pools during an additional 120 min incubation period. Further experiments will be necessary to determine which steps in the turnover of arachidonate are altered during cell activation.

THE ROLE OF ARACHIDONIC ACID IN THE BIOSYNTHESIS OF PAF

The discovery of the selectivity for arachidonate in acylation of 1-alkyl-2-lyso-GPC during PAF catabolism led to the question of whether the reversal of this reaction might take place during cell activation. Specifically, would 1-*O*-alkyl-2-arachidonoyl-*sn*-glycero-3-phosphocholine serve as a common source to 1-alkyl-2-lyso-GPC and arachidonic acid? The fact that approximately 65% of the total arachidonate in choline containing lipids is associated with 1-alkyl-2-arachidonoyl-GPC suggests that the neutrophil contains ample substrate to support such a pathway (22,23). Moreover, composition studies in other cell types indicate that arachidonate is preferentially associated with 1-ether-linked phospholipids when 1-ether-linked phospholipids are present within the cell (24–29). In order to test this

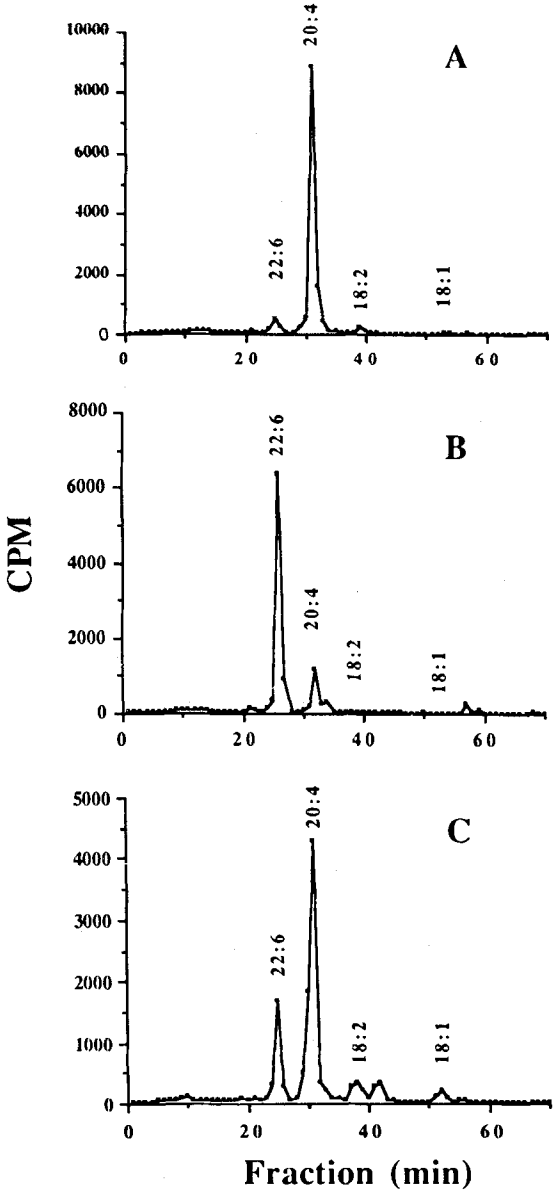


FIG. 2. HPLC separation of molecular species of 1-alkyl-2-acyl-glycerobenzoate derived from newly-synthesized 1-alkyl-2-acyl-GPC formed during the catabolism of PAF by mast cells supplemented in culture with arachidonic acid (A), docosahexaenoic acid (B) or linoleic acid (C). Murine mast cells (PT-18) were supplemented in culture with arachidonic acid, docosahexaenoic acid or linoleic acid (10 μM for 5 days) in addition to fetal calf serum. These cells were removed from culture, washed and incubated with 1-[^{3}H]hexadecyl-2-acetyl-GPC for 30 min. Newly synthesized 1-*O*-hexadecyl-2-acyl-GPC was isolated and converted to benzoate derivatives. The various glycero-benzoate molecular species containing different fatty acyl chains at the *sn*-2 position were isolated by HPLC. The retention times of the 1-alkyl-2-acyl-glycerobenzoate derivatives containing 22:6, 20:4, 18:2 and 18:1 at the *sn*-2 position are indicated on the chromatogram. (Triggiani, M., and Chilton, F.H., *Biochim. Biophys. Acta. 1006*, 41, 1989).

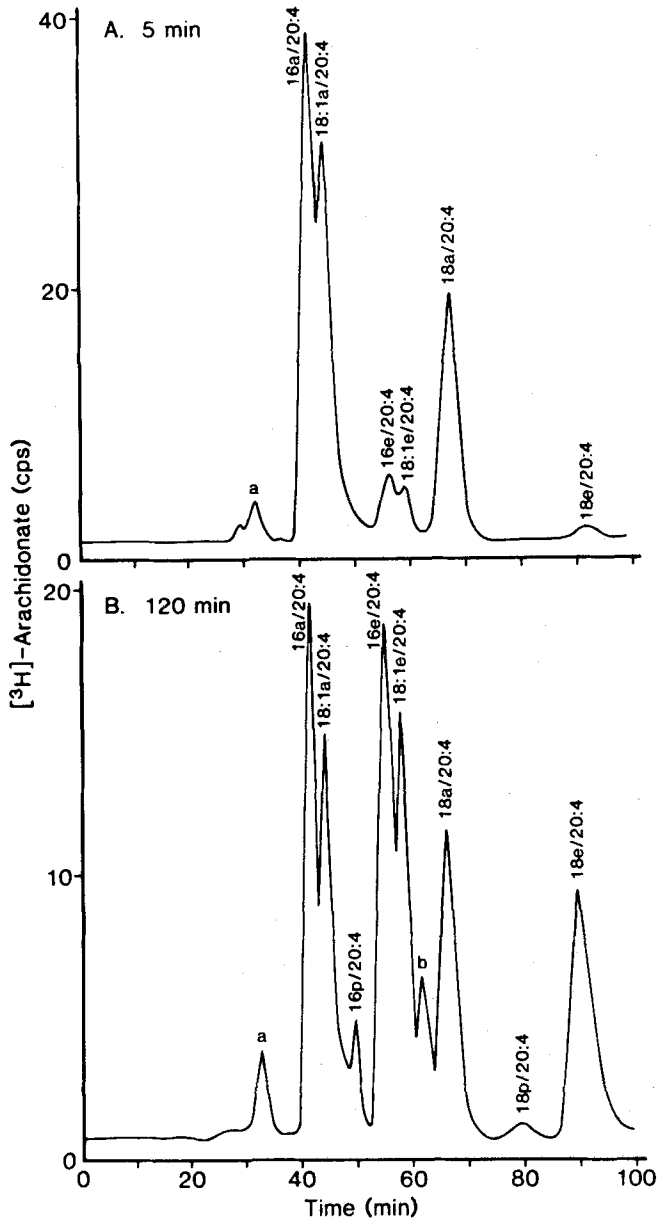


FIG. 3. Incorporation of exogenous arachidonic acid into choline-linked phosphoglyceride molecular species. Neutrophils were labeled for 5 min and 120 min as described (Chilton, F.H., and Murphy, R.C., *J. Biol. Chem. 261*, 7771, 1986). Choline-linked phosphoglycerides were purified by normal phase HPLC. The various 1-radyl-2-[^{3}H]arachidonoyl-GPC species were separated by reverse phase HPLC. The *top panel* shows the distribution of label in the various molecular species from cells which were pulse-labeled for 5 min. The *bottom panel* shows the distribution of label in the various molecular species from cells which were pulse-labeled followed by an additional 120-min incubation. The newly synthesized 1-radyl-[^{3}H]arachidonoyl-GPC contained 16:0, 18:0, and 18:1 fatty acid chains at the *sn*-1 position with *a* representing an acyl linkage at the *sn*-1 position, *e* representing an alkyl ether linkage at the *sn*-1 position, and *p* representing an alk-1-enyl ether linkage at the *sn*-1 position. Arachidonic acid (20:4) esterified at *sn*-2 is indicated following the terminal number. These data are representative of five separate experiments. (Chilton, F.H., and Murphy, R.C., *J. Biol. Chem. 261*, 7771, 1986).

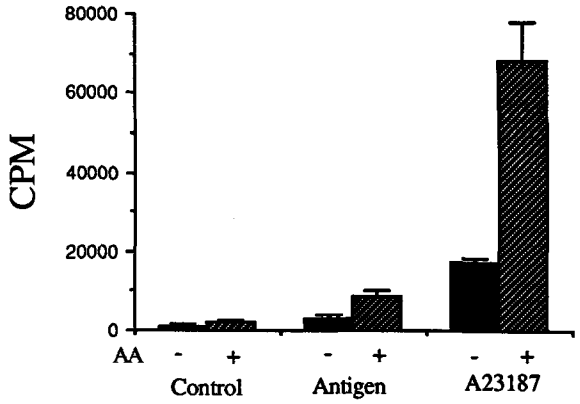


FIG. 4. PAF biosynthesis by mast cells supplemented in culture with arachidonic acid. Murine mast cells (PT-18) were supplemented in culture with (+) and without (−) arachidonic acid (10 μM for 5 days) in addition to fetal calf serum. These cells were removed from culture, washed and then stimulated in the presence of 7 μCi of [^{3}H]acetate with antigen (DNP7-HSA, 2μg/mL) or ionophore A23187 (5 μM). Labeled phospholipids were separated by TLC on silica gel G plates developed in chloroform/methanol/acetic acid/water (50:25:8:3, by vol). The radioactivity in the PAF region was determined by liquid scintillation counting. The data are the mean ± SEM of four experiments.

common precursor hypothesis, we labeled neutrophils with 1-alkyl-2-lyso-GPC. This rapidly converted to 1-alkyl-2-arachidonoyl-GPC which was incorporated into cellular phospholipids. Subsequently, these cells were stimulated, and a large portion of 1-alkyl-2-arachidonoyl-GPC was deacylated at the *sn*-2 position to form arachidonic acid and 1-alkyl-2-lyso-GPC. The 1-alkyl-2-lyso-GPC was then either converted to products such as PAF, or rapidly reacylated with unsaturated fatty acids (primarily 18:1 and 18:2) (30). This provided evidence that 1-alkyl-2-arachidonoyl-GPC could be a primary source of precursors for PAF and arachidonic acid. Studies in using similar prelabeling strategies suggest this pathway is functional in other cells (31).

While the aforementioned experiments suggest that 1-alkyl-2-arachidonoyl-GPC could be a source of 1-alkyl-2-lyso GPC which forms PAF, these experiments provide little evidence that arachidonate at the *sn*-2 position is an absolute requirement for PAF biosynthesis. However, circumstantial evidence suggests that arachidonate at the *sn*-2 position of a phospholipid may be an important factor in whether PAF is formed from that phospholipid. The requirement of arachidonate for PAF biosynthesis has been tested in an indirect manner in dietary and culture supplementation experiments where PAF production is measured in cells in which the composition of fatty acids within phospholipids is altered. Ramesha and Pickett (32) have demonstrated that rat neutrophils depleted of arachidonic acid produce substantially less PAF and leukotriene B_4 than normal neutrophils. In addition, PAF and leukotriene B_4 biosynthesis could be partially restored by incubating the cells with arachidonic acid. We have performed similar experiments in mast cells supplemented in culture with arachidonic acid, and found that the amount of PAF produced correlates with the levels of arachidonate in choline-containing phospholipids of the mast cell (Fig. 4). Similar findings have been reported by Suga and colleagues (33) in the cultured HL-60 cell.

We have recently found that the human lung mast cell produces primarily 1-acyl-2-acetyl-GPC and not 1-alkyl-2-acetyl-GPC upon immunologic activation (34). This finding raises new questions about the relationship of arachidonate to other PAF-like molecules such as 1-acyl-2-acetyl-GPC. Preliminary data indicate that most of the arachidonate found in choline-linked phospholipids of the human lung mast cell is associated with 1-acyl-2-arachidonoyl-GPC subclasses. In contrast to the human lung mast cell, the murine mast cell (PT-18) synthesizes primarily 1-alkyl-2-acetyl-GPC during antigen stimulation. As with the neutrophils, the bulk of arachidonate found in choline-linked phospholipids of the murine mast cell is found with 1-alkyl-2-acyl-GPC. These data support the hypothesis that the relative distribution of arachidonic acid within phospholipid subclasses might influence the subclass of PAF produced.

In addition to 1-alkyl-2-lyso-GPC, 1-alkyl-2-acetyl-GPC and arachidonic acid, 1-alkyl-2-acyl-glycerol was also formed from 1-alkyl-2-arachidonoyl-GPC during cell activation (30). A great deal of research activity has recently gone into understanding the biosynthesis and the biological function of 1-alkyl-2-acyl-glycerol. These studies suggest that this molecule may be formed either by a novel PC (phosphatidylcholine)-specific phospholipase C reaction (35) or a phospholipase D reaction followed by phosphohydrolase activity (36,37). However, it is not yet known if the phospholipase(s) C or D shows any specificity for 1-alkyl-2-acyl-GPC with arachidonate at the *sn*-2 position. Recent studies have compared the mole quantities of alkyl-acyl-glycerol and diacyl-glycerol produced upon cell stimulation (38–40). These studies indicate that a large proportion of radylglycerols formed during cell activation is 1-alkyl-linked. The 1-alkyl-2-acyl glycerols have been proposed to inhibit diacylglycerol-stimulated protein kinase C activity (41–43), to modulate the respiratory burst in neutrophils (44,45), to cause differentiation of human myeloid leukemia cells in culture (46) and to regulate arachidonic acid release in human neutrophils (47). Taken together, these studies suggest that the stimulated degradation of 1-alkyl-2-arachidonoyl-GPC eventually results in the production of at least three—PAF, arachidonic acid, and 1-alkyl-2-acyl glycerol—potentially bioactive molecules.

PHOSPHOLIPID SOURCES OF ARACHIDONIC ACID USED FOR EICOSANOID BIOSYNTHESIS AND THEIR RELATIONSHIP TO PAF SOURCES

There is little concrete information regarding the source of endogenous arachidonate which enters into eicosanoid pathways during cell activation. A major question which has been asked in relation to 1-alkyl-2-arachidonoyl-GPC is whether arachidonic acid which is lost from this molecule during cell activation is important in eicosanoid biosynthesis. We have found that there are at least 16 major arachidonate-containing

phospholipid molecular species in the human neutrophil (17). This large diversity of arachidonate-containing phospholipids makes it difficult to determine which of these lose arachidonate during cell activation. As previously discussed, 1-ether-linked phospholipids contain the major pools of arachidonate in a number of inflammatory cells including the neutrophil. Studies in several cell types indicate that labeled arachidonic acid is lost from both 1-acyl-2-arachidonoyl-GPC and 1-alkyl-2-arachidonoyl-GPC during cell activation (23, 48–54). By mass analysis, we have recently found that 1-alk-1′-enyl-2-arachidonoyl-GPE (glycerophosphoethanolamine) and 1-alkyl-2-arachidonoyl-GPC (23) are the major phospholipid subclasses which lose arachidonate during neutrophil activation (Fig. 5). In experiments where we have measured the mass loss of arachidonate, released LTB_4 and 20-OH LTB_4 account for only 10–35% of the arachidonate lost from phospholipids of the neutrophil during cell activation (23). Because released leukotrienes represent, by mass, such a small proportion of the total arachidonate lost from all phospholipids with ionophore stimulation, data measuring arachidonate loss from phospholipids does not allow one to determine which, if any, of the potential phospholipid subclasses is playing a dominant role in providing arachidonate for leukotriene biosynthesis. In order to get a better idea of which subclasses contribute arachidonate to the leukotrienes, we labeled the neutrophil so that the 6 major arachidonate-containing subclasses were at different radiospecific activities (55). The cells were then activated and the radiospecific activities of the phospholipid subclasses, as well as newly synthesized leukotrienes, were measured. Data from these studies indicated that of the 6 major arachidonate-containing phospholipid subclasses in the neutrophil, only the radiospecific activity of 1-alkyl-2-arachidonoyl-GPC matches that of LTB_4 and 20-OH LTB_4 (Fig. 6). However, it is still possible in these experiments that there are multiple phospholipids subclasses that contribute to the leukotrienes, and the average specific activity is reflected in the specific activity of the leukotrienes. In any event, 1-ether-linked phospholipids must contribute a large proportion of the arachidonate which forms the leukotrienes in the neutrophil since the radiospecific activities of the leukotrienes are well below that of 1-acyl-linked phospholipids. In a similar set of experiments in the platelet, Colard and colleagues (52) have found that the radiospecific activity of released arachidonic acid correlated closely with the specific activity of two phospholipids, 1-alkyl-2-acyl-GPC and 1,2-diacyl-GPE. Both experiments in the neutrophil and platelet provide evidence that 1-ether-linked phospholipids and, in particular, 1-alkyl-2-arachidonoyl-GPC may be an important source of arachidonate for eicosanoid biosynthesis.

COMMON REGULATORY MECHANISM FOR PAF AND ARACHIDONIC ACID

Further evidence for the coupling of PAF and leukotriene B_4 biosynthesis in the neutrophil has come from a study by McIntyre and co-workers (56) which demonstrates that activators of protein kinase C such as PMA (phorbol myristate acetate) enhance A23187-induced PAF and leukotriene B_4 biosynthesis. Furthermore, inhibitors of protein kinase C inhibit ionophore-induced synthesis of PAF and leukotriene B_4. This

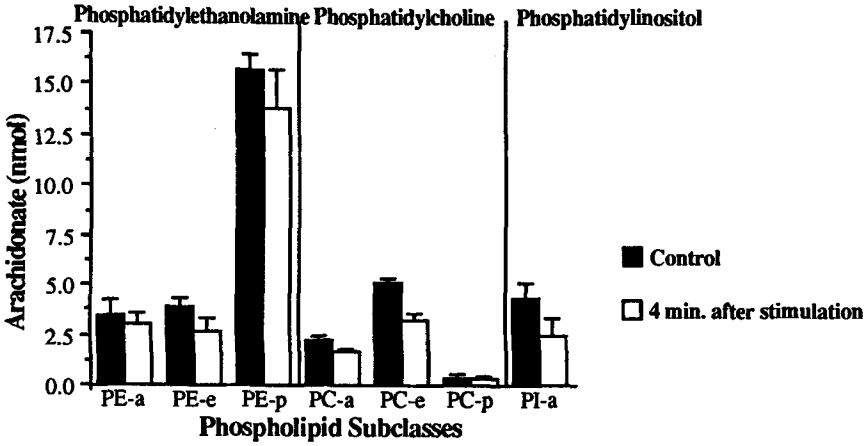


FIG. 5. Loss of endogenous arachidonate from phospholipid subclasses upon challenge with ionophore A23187. Neutrophils were incubated for 4 min with ionophore A23187 (2 μM) or no stimulus. Lipids were extracted from the cell pellet, and glycerolipid classes were separated by normal-phase HPLC. PE and PC were further separated by TLC as diradylglycerols into 1-acyl, 1-alkyl, and 1-alk-1′-enyl subclasses. The nanomole quantities of arachidonate in 1-acyl (a), 1-alkyl (e), and 1-alk-1′-enyl (p) subclasses were determined by gas chromatography/mass spectrometry. Quantity of arachidonate in an individual subclass after a 4 min incubation with no stimulus ■; quantity of arachidonate in an individual subclass after incubation with ionophore A23187 for 4 min □. The data are the mean ± SEM of four experiments (Chilton, F.H., *Biochem. J. 258*, 327, 1989).

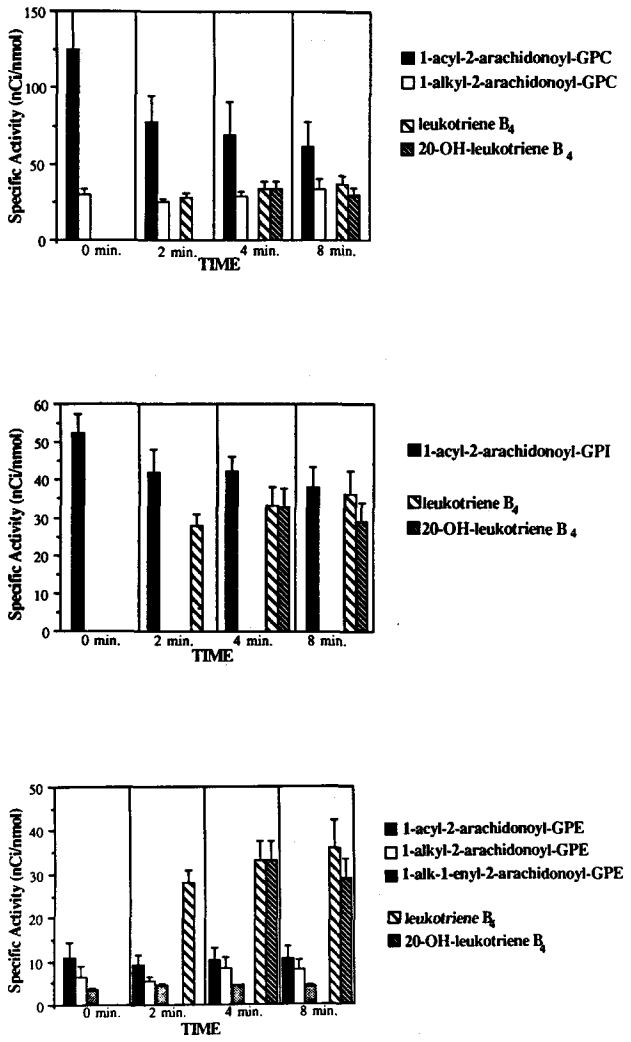


FIG. 6. Specific radioactivities of [^{3}H]arachidonic acid in the three major subclasses of cell-associated PC, PI and PE during cell activation. Neutrophils were labelled with [^{3}H]arachidonic acid as described (Chilton, F.H., *Biochem. J. 258*, 327, 1989) so that all the major arachidonate-containing subclasses had different specific radioactivities. The specific radioactivities of arachidonate in 1-acyl, 1-alkyl and 1-alk-1′-enyl-2-arachidonoyl-GPC, GPI, GPE as well as LTB_4 and 20-OH-LTB_4 were determined at various periods of time after ionophore stimulation. ■, □ and ▨ represent the specific radioactivity of arachidonate in 1-acyl-, 1-alkyl- and 1-alk-1′-enyl-2-arachidonoyl-GPC, GPI, and GPE respectively. ▧ and ▩ represent the specific radioactivity of LTB_4 and 20-OH-LTB_4 respectively. There is no value for the specific radioactivity of 20-OH-LTB_4 at 2 min owing to the fact that only small quantities of this leukotriene were produced at this time point. (Chilton, F.H., *Biochem. J. 258*, 327, 1989).

inhibition could be reversed for both PAF and leukotriene B_4 with the addition of the protein kinase C activator PMA. This study suggests that protein kinase C may regulate a step common to both mediators such as phospholipase A_2. Alternatively, activators of protein kinase C have recently been shown to inhibit the acylation of arachidonic acid into phospholipids (57). This mechanism could enhance both the production of PAF and eicosanoids by allowing for greater availability of lyso PAF and arachidonic acid during cell activation.

We have recently found that raising intracellular cyclic AMP in the mast cell will inhibit both PAF and leukotriene biosynthesis (58). In addition, this inhibition appears to occur at a mechanism independent of that which underlies cyclic AMP (adenosine 5′-monophosphate)-mediated inhibition of the rise in cytosolic calcium or histamine release. Similarly, we have found that agents which increase cyclic AMP in the neutrophil inhibit PAF biosynthesis in response to ionophore A23187 stimulation (unpublished observations). It was also found that the two nonenzymatic hydrolysis products of LTA_4 as well as LTB_4 were inhibited. All of these effects occurred in the absence of measurable effects on the ionophore-induced rises in cytosolic calcium. Given that ionophore-induced synthesis of these diverse lipid mediators—PAF, 6-*trans* LTB_4 and LTB_4—shares one common enzymatic step, namely phospholipase A_2, both results in the mast cell and neutrophil suggest that cyclic AMP regulates lipid biosynthesis at this step. Conversely, Nieto and colleagues (59) have demonstrated that acetyl transferase can be enhanced by the catalytic subunit of cyclic AMP-dependent protein kinase in the presence of magnesium ions and ATP (adenosine 5′-triphosphate). Studies with the neutrophil and mast cell implicate that regulation of C kinase and A kinase activities may influence a common step in the synthesis of both PAF and leukotrienes. However, further experiments will be needed to determine whether this step is a phospholipase A_2 reaction with specificity for 1-alkyl-2-arachidonoyl-GPC.

SUMMARY AND FUTURE DIRECTIONS

Studies in the last 5 yr from several laboratories suggest that PAF and arachidonic acid share common pathways in a number of inflammatory cells. For example, PAF is catabolized in cells by first removing the acetate moiety of the molecule by a cytosolic acetyl hydrolase, and replacing it with arachidonate. The acylation of 1-alkyl-2-lyso-GPC appears to take place via a CoA-independent transacylation reaction. In this reaction, it is thought that arachidonate is transferred from 1-acyl-2-arachidonoyl-GPC to 1-alkyl-2-lyso-GPC. In the biosynthesis portion of the pathway, 1-alkyl-2-lyso-GPC and free arachidonic acid are mobilized from 1-alkyl-2-arachidonoyl-GPC upon Ca^{++} activation. Both 1-alkyl-2-lyso-GPC and arachidonic acid may serve as precursors for PAF and eicosanoids, respectively. While a number of studies have now provided a plethora of direct and indirect evidence to support these pathways, many important questions still remain. For example, it has not been unequivocally established that 1-alkyl-2-arachidonoyl-GPC is the major precursor of PAF. The 1-alkyl-2-acyl-GPC with other fatty acid at the *sn*-2 position may work equally well. Although a few studies now suggest that the arachidonic acid released from 1-alkyl-2-arachidonoyl-GPC plays a role in eicosanoid formation, it is still not clear how tightly this arachidonic acid is coupled to eicosanoid production. In addition, recent studies suggest that some

cells make other 2-acetylated phospholipids such as 1-acyl-2-acetyl-GPC and 1-alk-1′-enyl-2-acetyl-GPE (60). It is not clear what role arachidonate plays in the biosynthesis of these molecules. Finally, several studies now indicate that 1-alkyl-2-arachidonoyl-GPC may undergo other reactions (phospholipase C and phospholipase D) during cell activation. These findings suggest that new avenues may be added to this pathway. Further studies in these directions should continue to provide valuable insight into the biochemistry of PAF and arachidonic acid.

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REVIEW

Inositol Phospholipid Turnover in PAF Transmembrane Signalling[1]

Shivendra D. Shukla
Department of Pharmacology, School of Medicine, University of Missouri, Columbia, MO 65212

In a variety of cells and tissues, platelet activating factor (PAF) stimulates phospholipase C catalyzed breakdown of phosphoinositides. This results in the generation of the second messengers, inositol trisphosphate and diglyceride. This process occurs independently of extracellular Ca^{2+}. A number of PAF structural analogues, receptor antagonists and drugs have been utilized to pharmacologically probe the activation of phospholipase C. PAF stimulation of the phosphoinositide turnover was shown to be sensitive to pertussis toxin in some systems, but not in others. The involvement of guanine nucleotide binding protein(s) and tyrosine kinase(s) in this process have also been postulated. These developments give new insights into PAF-receptor function at the molecular level, and also provide leads towards a better understanding of the cellular responses to PAF.
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A number of developments led to the discovery and the biochemical characterization of platelet-activating factor (PAF) as a unique biological mediator (1–7). After the elucidation of the structure of PAF as 1-*O*-alkyl-2-acetyl-*sn*-glycero-3-phosphocholine (AGEPC) in 1979 (8–10), one of the important facets of PAF research has been the identification of the molecular mechanism(s) of PAF action in cells. Ligand binding studies and various antagonists led to the pharmacological recognition of PAF receptors (7). Next, PAF receptor-associated transmembrane signalling mechanism, mediated *via* phosphoinositide (PPI) turnover, has been shown to be active in many systems. Since its discovery by Hokin (11), followed by the historical proposals by Michell (12) and Berridge and Irvine (13), it is now established that in response to many agonists inositol phospholipids [the primary one being phosphatidylinositol-4,5-*bis*phosphate (PIP_2)] are broken down by phosphoinositide specific phospholipase C (PLC). The generated products, inositol trisphosphate (IP_3) and diglyceride (DG), serve as second messengers and play a role in intracellular Ca^{2+} mobilization and in the activation of protein kinase C, respectively (13,14). It appears that this mechanism, mediated by PLC, is operative in several systems which respond to PAF (Table 1). It has further been documented that PAF action can also be mediated by phospholipase A_2 in some cells. However, a discussion of the PLA_2 pathway is not within the scope of this article. The present review summarizes recent developments which specifically relate to PAF stimulated PLC mediated phosphoinositide turnover and emphasizes the importance of this mechanism in PAF action.

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Abbreviations: AGEPC, 1-*O*-alkyl-2-acetyl-*sn*-glycero-3-phosphocholine; DG, diglyceride; IP_2, inositol diphosphate; IP_3, inositol trisphosphate; IP_4, inositol tetraphosphate; IP_5, inositol pentaphosphate; IP_6, inositol hexaphosphate; PA, phosphatidic acid; PAF, platelet activating factor; PMA, phorbol myristate acetate; PI, phosphatidylinositol; PIP, phosphatidylinositol-4-phosphate; PIP_2, phosphatidylinositol-4,5-bisphosphate; PKC, protein kinase C; PLA_2, phospholipase A_2; PLC, phospholipase C; and PPI, phosphoinositides.

PAF STIMULATED PHOSPHOINOSITIDE TURNOVER

Three biochemical indicators, namely production of inositol polyphosphates (primarily IP_3), diglyceride (DG) and phosphatidic acid (PA) have been followed as criteria in PPI turnover studies (14). In the case of PA, this metabolite may also be generated by a phospholipase D action (45) in some systems. However, this issue has not been addressed in studies involving PAF, and therefore remains open at this stage.

In the initial studies with rabbit platelets, it was demonstrated that PAF enhanced the incorporation of ^{32}P into platelet phosphoinositides (PI, PIP and PIP_2), indicating an increased turnover of these lipids (15). The level of PA also increased as monitored by both its mass and ^{32}P radioactivity. Subsequently, utilizing [^{3}H]inositol labeled (16) and ^{32}P-labeled platelets (17), it was observed that PAF caused a rapid (within 5 seconds) and transient decrease in radioactive PIP_2. Thus the earliest response to PAF was a decrease in PIP_2. This decrease was apparently mediated through the PLC pathway because the level of IP_3 increased within the same time frame (18).

Investigations with human and horse platelets have further focussed on this mechanism. Addition of PAF to ^{32}P-labeled horse platelets induced rapid formation of [^{32}P]PA. When platelets were labeled with [^{14}C]arachidonic acid, PAF caused production of [^{14}C]PA (26). It was further shown that PAF caused a transient decrease in [^{32}P]PIP_2 in horse platelets (27). When ^{32}P-labeled human platelets were exposed to PAF, the level of [^{32}P]PA increased. A decrease in [^{3}H]PI was observed when [^{3}H]arachidonate labeled human platelets were treated with PAF (19). Several other investigators have subsequently confirmed PAF stimulated PPI turnover in human platelets (21–25). LysoPAF (1-*O*-alkyl-glycero-3-phosphocholine) was without effect on phosphoinositide metabolism in platelets.

In a number of other cells and tissues, PAF has been shown to stimulate inositol lipid turnover (Table 1). In guinea pig pancreatic lobule (an exocrine secretory gland), PAF exhibited effects similar to those of carbamylcholine. It enhanced the incorporation of [^{32}P]orthophosphate into PA, PI and polyphosphoinositides and stimulated the formation of diglyceride (40). Interestingly, addition of PAF caused a transient increase (within 10–15 seconds) in [^{3}H]PI, [^{3}H]PIP, and to a lesser extent in [^{3}H]PIP_2. This was followed by a decrease within 60–120 seconds

TABLE 1

PAF Stimulation of Phosphoinositide Turnover in Various Cells and Tissue

Cells/Tissues	Reference
Platelets	
Rabbit	Shukla and Hanahan (15); Shukla and Hanahan (16); Mauco *et al.* (17); Shukla (18)
Human	MacIntyre and Pollock (19); Simon *et al.* (20); Kloprogge and Akkerman (21); Brass *et al.* (22); Shukla *et al.* (23); Tysnes *et al.* (24); see also Rao *et al.* (25)
Horse	Lapetina (26); Billah and Lapetina (27)
Neutrophils	
Rabbit	Naccache *et al.* (28)
Human	Rossi *et al.* (29); Verghese *et al.* (30); see also Lad *et al.* (31)
Macrophages	
Normal bone marrow derived	Huang *et al.* (32)
Peritoneal	Prpic *et al.* (33)
Human monocytic leukemic U-937 cells	Barzaghi *et al.* (34)
Bovine pulmonary artery endothelial cells	Grigorian and Ryan (35)
Aortic smooth muscle cells	Kawaguchi and Yasuda (36)
Renal epithelial cells (LLC-RK1)	Kawaguchi and Yasuda (36)
Cultured rat renal juxtaglomerular cells	Pfeilschifter *et al.* (37)
Renal mesangial cells	Bonventre *et al.* (38)
Ileal plasmalemmal vesicles	Kester *et al.* (39)
Guinea pig pancreatic lobule	Soling *et al.* (40)
Porcine thyroid cells	Haye *et al.* (41)
Rat liver cells, Kupffer cells	Shukla *et al.* (42); Gandhi *et al.* (43); Fisher *et al.* (44)

in all three lipids. In these experiments, the level of $[^3H]IP_3$ and $[^3H]IP_2$ increased within 10–20 seconds and then returned to basal levels. The level of [^{3}H]IP continued to increase over the 2 min period studied (40). Experiments with rat hepatocytes also showed that PAF caused a transient decrease in $[^{32}P]PIP_2$ (42), which recently was reported to be due to a PLA_2 mediated lipid breakdown (46).

Exposure of cultured renal mesangial cells to PAF also rapidly increased free arachidonate, diglyceride and IP_3 within 2 min (38). In normal bone marrow derived macrophages, addition of PAF to cells prelabeled with [^{3}H]inositol caused a marked increase in $[^3H]IP_3$ levels within 15 seconds (32). Human monocytic leukemic U-937 cells express PAF receptors when differentiated with dimethylsulfoxide to a macrophage-like state. In these cells, PAF causes rapid, but transient, increases in intracellular Ca^{2+} (47) which can be blocked by PAF receptor antagonists (48). In the differentiated U-937 cells, PAF induced hydrolysis of phosphoinositides and production of inositol phosphates within 5–15 seconds (34). Multiple forms of IP_3, including inositol 1,3,4-trisphosphate and inositol 1,4,5-trisphosphate were generated by PAF in U-937 cells (34) and in peritoneal macrophages (33). It is of interest to note that in rabbit platelets (49), U-937 cells (34) and peritoneal macrophages (33), a small increase in IP_4 and in bone marrow derived macrophages an increase in $IP_4 + IP_5$ (32) by PAF were also observed. Although the physiological significance of IP_4, IP_5 and IP_6 remains to be fully established, it is worth noting that a role for IP_4 in Ca^{2+} influx (50,52), and for IP_5 and IP_6 as neurotransmitters in blood pressure regulation (51), have been proposed (50,52). The role of IP_4 and IP_5 in PAF action remains unknown.

Thus, in the last few years, PAF has been reported to stimulate the metabolism of phosphoinositides in a wide spectrum of cells originating from blood, kidney, lung, liver, epithelium, intestine, etc. (Table 1). PLC catalyzed PIP_2 breakdown was noted to be one of the earliest events in the PAF response, and points toward a common mechanism underlying the PAF receptor signal transduction pathway in physiologically different cell systems.

PHARMACOLOGICAL MANIPULATIONS

Several agents, including PAF receptor antagonists, activators of adenylate cyclase and PKC, Ca^{2+} channel blockers and various inhibitors have been utilized to manipulate PAF stimulated phosphoinositide turnover (Table 2). PAF receptor antagonists CV-3988 (18,34), CV-6209, SRI 63-441, SRI 63-675 (59) and BN-52021 (20) have been shown to inhibit the PAF stimulated increases in IP_3 (18,34,59) and PA (20). It was also established

TABLE 2

Pharmacological Manipulation of PAF Stimulated Phosphoinositide Turnover

Drug/Chemical	Effect	Reference
Antagonists		
CV3988	Inhibition	Shukla (18); Morrison and Shukla (49); Barzaghi *et al.* (34); Tokumura *et al.* (53)
CV6209	Inhibition	Morrison and Shukla (49)
SRI 63-441	Inhibition	Morrison and Shukla (49)
SRI 63-675	Inhibition	Morrison and Shukla (49)
BN 52021	Inhibition	Simon *et al.* (20)
U 66985	Inhibition	Tokumura *et al.* (53)
U 66982	Inhibition	Tokumura *et al.* (53)
Forskolin	Inhibition	Shukla (18); Haye *et al.* (41)
Prostaglandin I_2	Inhibition	Lapetina (26)
Phorbol myristate acetate (PMA)	Inhibition	Huang *et al.* (32); Morrison *et al.* (54)
Staurosporine	Potentiation	Morrison *et al.* (54)
Genistein	Inhibition	Shukla *et al.* (55); Dhar *et al.* (56)
Indomethacin	No effect	Shukla (18); Lapetina (26)
Neomycin	Inhibition	Tysnes *et al.* (24)
EDTA or EGTA	No effect	Shukla (18); Barzaghi *et al.* (34)
Trifluoperazine	No effect	Lapetina (26)
Manoalide	Inhibition	Barzaghi *et al.* (34)
Pertussis toxin:		
	Inhibition	Brass *et al.* (22); Huang *et al.* (32); Lad *et al.* (31)
	No inhibition	Barzaghi *et al.* (34); Dhar *et al.* (56); Houslay *et al.* (57)
Serine protease inhibitors:		
Diethyl *p*-nitrophenyl phosphate (DNPP)	Partial inhibition	Sugatani *et al.* (58)
N-acetyl-l-tryptohpan ethyl ester (NATrpEE)	Partial inhibition	Sugatani *et al.* (58)

that both [^{3}H]PAF binding and [^{3}H]IP_3 production induced by PAF in rabbit platelets were inhibited by the antagonists and that PAF receptor occupancy correlated with PLC stimulation (59). It was proposed that low levels of receptor occupancy triggered a substantial activation of PLC, and it was speculated that a high affinity PAF receptor binding component is involved in the process (59). It has also been reported that full receptor occupancy may not be required for a maximal platelet aggregation response (60).

Activators of adenylate cyclase, which elevate cellular cyclicAMP, *e.g.*, forskolin or PGI_2, caused inhibition of PAF stimulated PPI turnover (18,41) and Ca^{2+} rise in the cells (61). Interestingly, PAF also has an inhibitory effect on adenylate cyclase activity (62). Activation of protein kinase C, *e.g.*, by phorbol myristate acetate (PMA), inhibits PPI turnover (32,54) and is negatively coupled to the stimulation of PLC. The specific mechanism for this remains to be explained. In neutrophils, activated PKC downregulates the high affinity PAF receptors which correlates with a decrease in Ca^{2+} transients (63). This issue has been further examined through the use of staurosporine, a putative inhibitor of PKC. This agent inhibited PAF→→PKC mediated protein phosphorylation and potentiated PAF stimulation of [^{3}H]IP_3 production (54). This led to the suggestion that the basal level of PKC activity in the cells imparts constraints on the activation of PLC (54). Further, the cyclooxygenase inhibitor indomethacin had little effect on PAF response, indicating that PLC activation is not secondary to production of a cyclooxygenase metabolite (18,26).

The turnover of phosphoinositides by PAF appears to be independent of extracellular Ca^{2+} because neither EGTA nor EDTA affected it (18,34). This is in accord with the PPI turnover observed in response to several other agonists (14). In the presence of 1 mM external Ca^{2+}, PAF caused an increase in platelet cytoplasmic Ca^{2+} ranging from 0.5–1.0 μM within 5 seconds (64). In the absence of external Ca^{2+}, this increase is about 25% of that seen in the presence of Ca^{2+} (34,64,65) and would represent Ca^{2+} released from internal stores by IP_3 (13, 14). Thus, increase in cytoplasmic Ca^{2+} from stored sites may initiate several biochemical reactions in the cell (14), but is inadequate to elicit the full PAF response. This points toward the importance of Ca^{2+} influx in PAF response (67). It should be noted that in one study Ca^{2+}

channel blockers, *e.g.*, verapamil, inhibited the PAF induced increase in Ca^{2+} (37), while in another study these blockers had no effect (34). An explanation for these differences remains to be sought. It is relevant to note that PAF is postulated to open, in some manner, Ca^{2+} channels in the plasma membrane (68,69). The relationship between such a Ca^{2+} channel and PPI turnover, if any, has yet to be explored. In platelets a correlation between PAF stimulated PA production (a metabolite of PPI turnover) and Ca^{2+} influx has been demonstrated (67,70).

Several other agents have also been investigated for their influence on lipid turnover (Table 2). Aminoglycosides, *e.g.*, neomycin, which bind to inositol lipids, inhibited PAF activated PPI turnover (24). In U-937 cells, manoalide, a purported inhibitor of PLC, also inhibited PAF stimulated PPI turnover (34). In the future, discovery of specific PLC inhibitors will be of great use to target this enzyme. In a new development, genistein, a putative tyrosine kinase inhibitor (71), inhibited PAF stimulated aggregation and PPI turnover (55,56). This may provide a new insight into the mechanism(s) of PAF action as outlined in the following section.

MECHANISM OF PHOSPHOLIPASE C ACTIVATION BY PAF

In a variety of systems in which hormonal activation of PPI-PLC has been observed, the involvement of guanine nucleotide binding proteins (G-proteins) has been proposed. Such a mechanism has also been considered for the PAF activation of PLC. It is of interest to note that GTPγS has been shown to activate PLC in several permeabilized systems, *e.g.*, platelets and neutrophils (72), in which PAF is known to stimulate PPI turnover. One of the arguments in this regard is that Gi or Ni (the inhibitory G protein of adenylate cyclase) itself is involved in control of PLC activity. In other studies, the possibility of a different G protein, termed Gp, in stimulation of PLC has also been raised (73). Both Gs and Gi have oligomeric structures consisting of α, β and γ subunits. Upon receptor activation, GTP binds to α subunit and dissociates it from β and γ subunits, and the dissociated α subunit becomes functional. Next, the intrinsic GTPase activity in the α subunit cleaves GTP favoring a reassociation of the three subunits. Pertussis toxin ADP-ribosylates the α subunit of Gi and renders it inactive, for example, in activating PLC. Several studies involving PAF have employed pertussis toxin to probe this question. The results are inconclusive. For example, in studies involving platelets, PAF stimulated PPI turnover was reported to be both sensitive (22) and insensitive (56,57) to pertussis toxin treatment. In macrophages (32) and neutrophils (28), the turnover was sensitive to the toxin while U-937 cells (34) were insensitive to this treatment. In neutrophils, pertussis toxin does block functional changes induced by PAF (31) but does not block Ca^{2+} elevation (28). Other agonists which are considered coupled to G protein and activate PLC also stimulate GTPase. PAF stimulates GTPase in human (74,75) and rabbit platelets (76) and in neutrophil membranes (74). The PAF stimulated GTPase activity in human platelet membranes was insensitive to pertussis toxin (74,22) while it was sensitive in neutrophil membranes. This has led to the suggestion that there may be multiple forms of PAF receptors in various cells and their interactions with G proteins could also vary. Existence of a family of G proteins is well recognized in this respect. The role and nature of G protein(s) in PAF stimulation of PLC therefore warrants further investigation.

Another approach to the mechanism of PAF stimulation of PLC has focused on the involvement of tyrosine kinases. Certain growth factors cause PPI turnover and the tyrosine kinase(s) have been implicated in the mechanism of PLC activation (77). In fact, certain forms of PPI-PLC are claimed as a substrate for the tyrosine kinase (78), although the exact nature of the PLC activation has yet to be defined. It has been reported that genistein is a putative inhibitor of tyrosine-specific protein kinases (71). Studies with genistein have indicated that this compound inhibited PAF stimulated IP_3 production in rabbit platelets (55,56). Interestingly, PAF was shown to phosphorylate two proteins of apparent molecular weight 50 K and 60 K which reacted with a monoclonal antibody (mAb) to phosphotyrosine. Genistein blocked the PAF stimulated reactivity of these two proteins to mAb. Further, phosphoamino acid analysis indicated that PAF increased the [^{32}P]phosphotyrosine residues in these two proteins (56). Of considerable interest is a recent report that the binding sites of PAF receptor in rabbit platelets resides in the polypeptide of Mr 52 K (79). Platelets have high levels of tyrosine kinase activity even though they are not a proliferating cell. It is therefore likely that tyrosine kinase is involved in signalling processes in this cell. The question whether PAF receptor is a tyrosine kinase or a substrate for tyrosine kinase will be worthy of investigation. Further, the role of tyrosine kinase in PLC activation and the consequential responses remain to be established.

SIGNIFICANCE OF PPI TURNOVER IN PAF RESPONSES

It is obvious from the preceding sections that changes in phosphoinositide turnover are among the earliest responses to PAF in many cells and tissues. IP_3 and DG generated by this pathway are involved in Ca^{2+} mobilization and in activation of PKC mediated protein phosphorylation, respectively (13,14,83). In addition to the above role, the significance of this phenomenon has also been explored towards the desensitization of cells to PAF. Prior exposure of platelets to PAF renders these cells desensitized (refractory) to any further PAF response, *e.g.*, aggregation (80). In a series of experiments it was observed that the PAF receptor coupled activation of PLC is desensitized under these conditions (49), and so is the protein phosphorylation (66). Thus, the PAF stimulation of PLC, the initial biochemical event after receptor occupancy, could well be the underlying mechanism for PAF desensitization. It is noteworthy that platelets desensitized to PAF still exhibited PPI turnover caused by thrombin (49). This observation has provided evidence that the activation of PLC and its regulation by PAF and thrombin may be distinct. The molecular mechanism of desensitization is poorly understood. In one study, the affinity of [^{3}H]PAF binding was altered in desensitized platelets (81), while in another study the characteristics of [^{3}H]PAF binding sites were reported to be unaltered in desensitized platelets (76). The possibility that PKC is involved in this mechanism appears

unlikely since inhibition of PKC by staurosporine failed to prevent the desensitization of PLC by PAF (54).

The relationship between a pathophysiological state and PAF stimulated PPI turnover has yet to be established. In a study involving platelets from asthmatics it was reported that these platelets had higher basal values of $[Ca^{2+}]i$ and IP_3 and showed a higher stimulation of both components by PAF. Thus, platelets of asthmatics showed increased PPI turnover in response to PAF (82). PAF stimulated PPI turnover has also been studied in diabetic human platelets. Compared to normal (control) human platelets, diabetic human platelets exhibited hypersensitivity (increased sensitivity) to PAF as monitored by the aggregation response and production of phosphatidic acid (Shukla, S.D., Paul, A., and Klachko, D.M., unpublished). Thus, the altered sensitivity of cells or tissues to PAF may result from alterations in the signal transduction pathway involving PPI turnover. The significance of PAF receptor coupled phosphoinositide turnover in pathophysiological conditions must await future investigations.

CONCLUDING REMARKS

In conclusion, studies with different cells and tissues have provided evidence that stimulation of PPI-PLC by PAF is a receptor dependent, extracellular Ca^{2+}-independent phenomenon. This pathway generates at least two second messengers, IP_3 and DG, which play roles in Ca^{2+}-homeostasis, protein phosphorylation and in other biochemical events of the cell. Elevation of cellular cAMP or activation of PKC negatively influences PLC activation. Although the mechanism for PLC activation by PAF is poorly defined, the involvement of G protein(s) and tyrosine kinase(s) have been suggested as depicted in Figure 1. In the future, it should be possible to probe this PAF transmembrane signalling pathway in depth and to relate its significance in PAF receptor mediated mechanisms and in the cellular response to PAF.

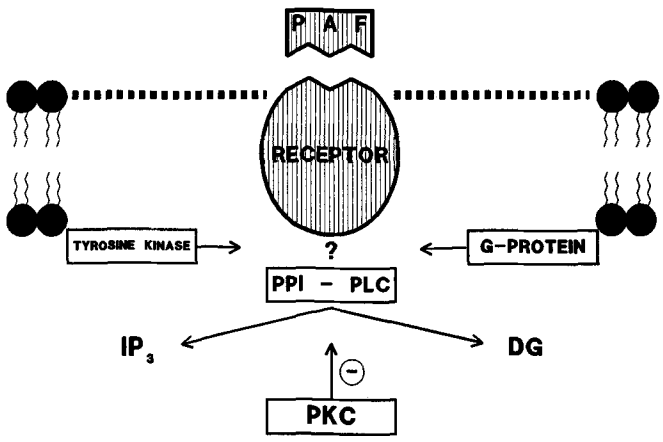


FIG. 1. A hypothetical model of PAF receptor coupled phosphoinositide specific phospholipase C. The model is simplified to depict the possible role of G-protein and/or tyrosine kinase in PAF receptor coupled stimulation of phosphoinositide specific phospholipase C (PPI-PLC). The question mark indicates that the nature of interaction(s) among these components is unknown. IP_3, inositol trisphosphate; PKC, protein kinase C; DG, diglyceride; and G-protein, guanine nucleotide binding protein.

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REVIEW

Transmembrane Signalling and Paf-Acether Biosynthesis[1]

Ewa Ninio* and **Francine Joly**
Institut National de la Santé et de la Recherche Médical U200, 92140 Clamart, France

Expression of lyso paf-acether (lyso paf):acetyl-CoA acetyltransferase and its activation above basal levels by specific agonists controls the rate of paf biosynthesis in proinflammatory cells. Acetyltransferase activation in these cells is due to the rapid postranslational modification of an inactive precursor by phosphorylation, most probably catalyzed by a cAMP-dependent kinase. However, the possibility exists that a calcium/calmodulin-dependent kinase can be implicated as well. Unlike murine cultured mast cells, human neutrophils form paf when stimulated with phorbol myristate acetate (PMA) or diacylglycerol. In both cell types, acetyltransferase is activated by PMA. Controversy exists as to whether PMA activates the remodeling pathway, *i.e.* the activation of phospholipase A_2 and acetyltransferase, or the *de novo* route through CDPcholine cholinephosphotransferase action on alkylacetylglycerol. There is some indication that PKC might regulate paf biosynthesis. The implication of a GTP-regulated protein has also been postulated in signal transduction leading to paf formation in endothelial cells, neutrophils, and mast cells. The topography of paf formation is discussed in light of the subcellular distribution of acetyltransferase in neutrophils and Krebs II cells. ***Lipids 26,*** **1034–1037 (1991).**

Studies on various cell types have revealed the importance of expression of lyso paf-acether (lyso paf):acetyl-CoA acetyltransferase (EC 2.3.1.67) and its activation above basal levels by specific agonists in controlling paf biosynthesis (1). When activation of acetyltransferase is suppressed by pharmacological means, paf is not formed. We have shown this using antigenically stimulated mouse bone marrow-derived mast cells treated with ketotifen (an anti-allergic drug from Sandoz, Basel, Switzerland) (2). A similar conclusion was drawn from studies in which dexamethasone was shown to reduce by 50% both acetyltransferase activation and paf formation in the same cell model (3). The action of ketotifen was mainly due to the inhibition of Ca^{2+} influx into the cells during antigen challenge. Dexamethasone treatment of mast cells decreased by 50% the number of IgE receptors on the cell surface available for sensitization, suggesting that the occupancy of all receptors is required to exhibit maximal response to antigen.

Acetyltransferase activation in proinflammatory cells is due to the rapid posttranslational modification of an inactive precursor (4), with the level of the enzyme being rate-limiting in paf biosynthesis (5). The acetyltransferase proenzyme is modified by phosphorylation in murine spleen microsomes (6,7), exocrine glands (8), and mast cells (9). The enzyme responsible for phosphorylation(s) is most probably the cAMP-dependent protein kinase (PKA). This is suggested by direct incorporation of phosphate into acetyltransferase purified from rat spleen (7); however, the possibility exists that calcium/calmodulin-dependent protein kinase could be involved as well (8).

TRANSMEMBRANE SIGNALLING AND ACETYLTRANSFERASE ACTIVATION

In contrast to earlier studies on murine macrophages (10) and human neutrophils (11), which were not performed under optimized conditions with respect to phorbol myristate acetate (PMA) concentration and the length of incubation, our more recent experiments have indicated that acetyltransferase is stimulated in intact mast cells (9) or in human neutrophils (12) by treatment with phorbol esters or synthetic diacylglycerol. In human neutrophils, this stimulation was accompanied by lyso paf and paf formation (Fig. 1). Phorbol didecanoate, an analog of PMA that cannot activate PKC, is also incapable of stimulating acetyltransferase in neutrophils and in mast cells. All reactions leading to paf formation in human neutrophils induced by PMA or opsonized zymosan were inhibited as much as 40 to 50% by a 15-min treatment with 25 μM sphingosine (12), a relatively specific protein kinase C (PKC) inhibitor (13). Thus PKC may be involved in both lyso paf and paf formation and acetyltransferase activation in neutrophils stimulated with phagocytable particles. This study also showed that the molecular species of paf formed during the opsonized zymosan- or PMA-induced stimulation were similar, with C_{16} alkyl species representing 84.3 ± 5% and 80.7 ± 3.5% (mean ± S.E., n = 3), respectively, and the balance being C_{18}paf (12). This suggests that similar precursors may be involved. The role for PKC in phospholipase A_2 (PLA_2) activation was also suggested by McIntyre *et al.* (14) who showed that, while activation of PKC was not sufficient to induce paf and leukotriene B_4 synthesis, its action was required to couple the rise in intracellular Ca^{2+} to their synthesis. Failure to detect paf secreted by PMA-stimulated neutrophils could be due to the use of labeled acetate to quantitate paf instead of the much more sensitive bioassay based on aggregation of washed rabbit platelets (15). Nieto *et al.* (16) suggested that paf formed by PMA-activated neutrophils could be sythesized *via* the *de novo* pathway implicating CDPcholine cholinephosphotransferase. This observation was based on labeling studies showing that in PMA-stimulated cells, while [^{3}H]acetate was not incorporated into paf, [^{3}H]choline was incorporated. In contrast, the opsonized zymosan- or the ionophore

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*To whom correspondence should be addressed.

Abbreviations: GPC, glycerophosphocholine; G protein, GTP-regulated protein; IP_3, inositol 1,4,5-triphoshate; IP_4, inositol 1,3,4,5-tetraphosphate; lyso paf, lyso paf-acether, 1-*O*-alkyl-*sn*-glycero-3-phosphocholine; paf, paf-acether, 1-*O*-alkyl-2-acetyl-*sn*-glycero-3-phosphocholine; PKA, cAMP-dependent protein kinase; PKC, protein kinase C; PLA_2, phospholipase A_2; PLC, phospholipase C; PMA, phorbol myristate acetate.

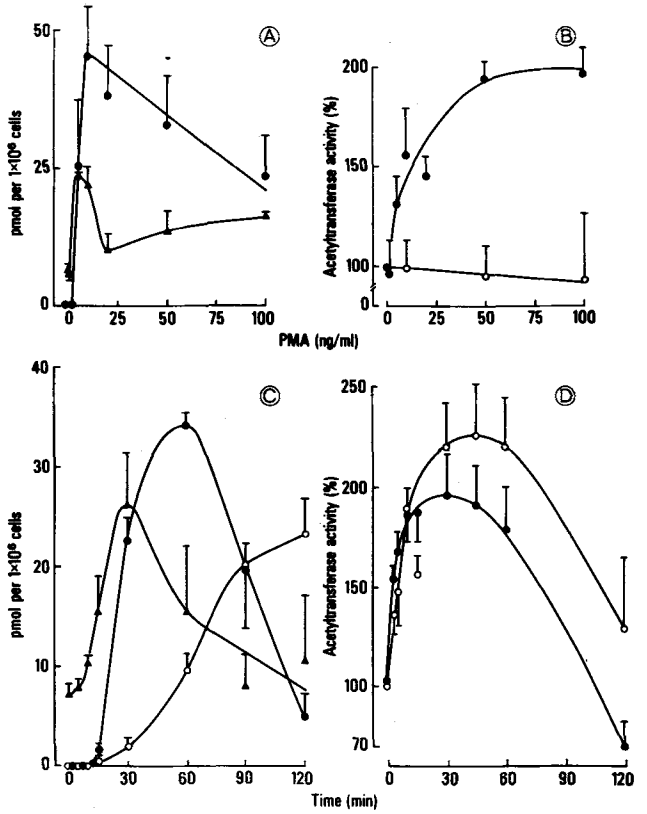


FIG. 1. Dose-dependency (A and B) and time-course (C and D) of paf, lyso paf biosynthesis, and acetyltransferase activation in human neutrophils stimulated with phorbol myristate acetate (PMA) or phorbol didecanoate (PDD). A: 1×10^6 cells/mL were stimulated for 60 min at 37°C. Amounts of total lyso paf (▲) and total paf (●). B: 1×10^6 cells/mL were simulated for 10 min at 37°C with PMA (●) or PDD (○). C: Cells were stimulated with 10 ng/mL of PMA. Amounts of released paf (○), cell-associated paf (●) and cell-associated lyso paf (▲). D: 2×10^6 cells/mL were stimulated with PMA 10 ng/mL (○) or 50 ng/mL (●). Results are the means ± SEM of four to five experiments. (Reproduced from Leyravaud *et al.* (1989) *J. Immunol. 143,* 245-249; with permission of The American Association of Immunologists)

A23187-stimulated neutrophils formed paf that was only labeled with [^{3}H]acetate. However, in the latter study (16) the major difficulty was to distinguish between the *de novo* incorporation of labeled choline into paf from the choline incorporated by the exchange reaction catalyzed by phospholipase D, which is activated by phorbol esters (17). The question also arose why paf formed in PMA-stimulated neutrophils was not labeled by [^{3}H]acetate. This finding is inconsistent with the involvement of an alkylglycerol:acetyl-CoA acetyltransferase that would be responsible for *de novo* formation of alkylacetylglycerol (18). From our more recent data using 2×10^7 neutrophils per incubation (19), it appears that both ionophore and PMA induce incorporation of labeled acetate into paf. In addition the specific activities (dpm [^{14}C]acetate incorporated into paf/paf mass quantified by bioassay) of paf formed were similar irregardless of the stimulus. When cells were prelabeled with [^{3}H]choline, the paf formed was weakly labeled for both stimuli, but only PMA stimulation triggered a several-fold increase in lyso paf and alkylacyl-GPC labeling.

In contrast to human neutrophils, mouse mast cells do not form paf when stimulated with phorbol esters in spite of substantial but delayed stimulation of acetyltransferase (20). This observation may be linked to the inability of PMA to induce lyso paf formation. However, short pretreatment of mast cells with PMA at a concentration that did not stimulate acetyltransferase, prior to the antigen challenge, increased by about 30% both acetyltransferase stimulation and paf formation, again without changing the level of lyso paf. Overnight treatment of mast cells with PMA (200 ng/mL) caused depletion of PKC in both cytosol and membranes. When such cells were stimulated further with antigen, acetyltransferase activation and paf formation were only partially decreased. In contrast, the PKC inhibitors chlorpromazine and staurosporine inhibited to a significant extent paf biosynthesis. We concluded that PKC-dependent mechanisms contribute only in part to paf biosynthesis in cultured mast cells. The PKC-dependent signal directly generated by PMA or diacylglycerol is not sufficient to trigger the full cell response, which is obtained only through receptor-mediated antigen challenge.

The receptor-mediated stimulation of PKC requires the coupling of receptors and phospholipase C (PLC) *via* the GTP-regulated proteins (G protein(s)). Diacylglycerol, inositol 1,4,5-triphosphate (IP_3) and inositol 1,3,4,5 tetraphosphate (IP_4) derived from phosphatidylinositol-4,5-*bis*-phosphate contribute directly or indirectly to the anchoring of PKC in the plasma membrane and the subsequent phosphorylation of various proteins (reviewed in ref. 21). In human neutrophils, the calmodulin-binding protein MARCKS is phosphorylated by PKC (22) and subsequently displaced from the plasma membrane into the cytosol (23). It thus participates in the propagation of transmembrane signalling within the cell and may cause displacement of bound calmodulin, possibly leading to the activation of Ca^{2+}-calmodulin-dependent processes (22). Adenylate cyclase was also shown to be among the proteins that are activated by phosphorylation due to PKC (24), suggesting that "crosstalk" takes place between the two corresponding transmembrane signalling pathways. The rise in cAMP level, in turn, promotes the increase of activity of PKA which might phosphorylate the acetyltransferase. Indeed, one of the earliest events being triggered by antigen bridging of adjacent IgE on murine peritoneal mast cell surfaces is a transient increase in intracellular cAMP level (25). However, controversies remain (reviewed in ref. 26) and, unlike cultured mast cells (9,20), the peritoneal counterparts (27) do not form paf.

A long-lasting stimulation of adenylate cyclase or inhibition of phosphodiesterase decreased paf formation in neutrophils (28) as well as in mast cells (29) and is a consequence of inhibition of PLA_2-like activity that generates lyso paf and arachidonate (29,30). In rat mast cells both type-I and type-II PKA are present in the cytoplasm and are activated after IgE-mediated cell triggering (reviewed in ref. 26). However, increased intracellular cAMP following addition of prostaglandin D_2 or theophylline does not activate the protein kinases (31). The latter finding might explain the apparently conflicting results concerning the role of cAMP in activating and inhibiting paf synthesis. Similarly, when rat basophilic leukemia cells were treated with cholera toxin, the level of inositol phosphate increased in concert with antigen-

stimulated $^{45}Ca^{2+}$ influx, cAMP level, and degranulation suggesting that cholera toxin-sensitive G protein might be an integral component of the mechanism responsible for the antigen-stimulated calcium influx (32). However, the effects of cholera toxin on calcium influx and secretion do not appear to be mediated *via* cAMP as a variety of agents capable of elevating cellular cAMP levels to a similar extent do not potentiate the antigen-stimulated calcium influx, and they partially inhibit stimulated secretion (32). Influx of Ca^{2+} is an obligatory step in stimulus-receptor coupling leading to paf biosynthesis (1,2,9,33,34). Paf biosynthesis can be activated solely by rise in Ca^{2+} level, as it was shown using the calcium ionophore A23187 (reviewed in ref. 1). When neutrophils are stimulated with PMA or diacylglycerol, paf is formed without a detectable rise in Ca^{2+} concentration (unpublished observation from our laboratory).

G proteins couple hormone-receptor binding to opening of membrane Ca^{2+} channels in endothelial cells, leading to paf synthesis (34). The formation of paf initiated by phagocytosis or formylmethionyl-leucylalanine in neutrophils was inhibited by pertussis toxin, suggesting that pertussis toxin-sensitive G protein might be involved (33). In murine cultured mast cells, acetyltransferase activation and paf biosynthesis are triggered by direct activators of G proteins (presented at the Third International Conference on Platelet-Activating Factor and Structurally Related Alkyl Ether Lipids, Tokyo, Japan, May 1989).

MECHANISM OF LYSO PAF FORMATION

The phospholipases A_2 are a family of enzymes which hydrolyze the ester bond at the glycerol *sn*-2 position of various phospholipids and thus participate in fatty acid release and lysophospholipid formation. The preferential release of arachidonic acid as compared to other fatty acids was observed in stimulated proinflammatory cells (35). Moreover, a close relationship between this release and paf synthesis was demonstrated (36,37). Consistent with this, in stimulated human HL-60 granulocytes only alkylarachidonoyl-GPC was hydrolyzed to an appreciable extent (38). In mammalian cells, two different types of PLA_2 have been purified and cloned: (i) a low molecular weight PLA_2 (13.5–14 kD), that was also found in inflammatory fluids, is active in the presence of Ca^{2+} at mM concentrations and is unspecific for fatty acids (39); (ii) a high molecular weight PLA_2 (105–110 kD) of cytosolic origin that is translocated into membranes and is active at Ca^{2+} μM concentrations, and exhibits high specificity for arachidonate containing phospholipids (40,41). The latter PLA_2 could be thus implicated in arachidonate release and lyso paf formation. However, the CoA-independent transacylases, which are specific for polyunsaturated fatty acids (42–45), might also be involved, as was suggested recently by Wykle's group (46,47), who demonstrated that lyso paf formation is induced by arachidonate transfer from alkyl-arachidonoyl-GPC to added lysophosphatidylethanolamine. In this context, PLA_2 might generate a lyso compound and thus indirectly participate in the deacylation of paf precursor.

G protein(s) can be implicated in the coupling of external receptors with intracellular responses, which leads to activation of certain types of PLA_2 (reviewed in ref. 48). The question arises as to whether the PLA_2 which participates in the generation of lyso paf is directly coupled to G protein(s), or whether its activation is not rather related to the increase in Ca^{2+} concentration due to IP_3 and IP_4 formation *via* the phospholipase C (PLC) route. The latter course appears more plausible given that acetylation of lyso paf through the specific acetyltransferase occurs in the non-plasma membranes, at least in human neutrophils (49), as was confirmed by Mollinedo *et al.* (50). The intracellular location of acetyltransferase would argue in favor of a similar topography for lyso paf formation as suggested by Record *et al.* (51). In addition, the endoplasmic reticulum is the biosynthetic site for alkylacyl-GPC which is formed from alkylacylglycerol *via* CDPcholine cholinephosphotransferase (52,53). If lyso paf formation depended on the plasma membrane PLA_2 directly coupled to G protein(s), it would be necessary to shuttle this newly formed lyso paf into the endoplasmic reticulum. In Krebs II cells, high levels of alkylacyl-GPC are found in the inner leaflet of the plasma membrane. By contrast, the acetyltransferase is located in the internal membranes, which served to explain impaired paf formation by Krebs II cells (51). Thus we believe that lyso paf is formed in the proximity of the acetyltransferase. The question still remains as to how PMA activates lyso paf formation. Late release of arachidonate (within 30 to 120 min) has been described in PMA-challenged Madin-Darby canine kidney cells (54) and neutrophils (19). This late release of arachidonate is compatible with delayed paf formation induced by PMA in human neutrophils (12) and might be linked to the PKC-dependent activation of PLA_2 (55). Another explanation for PMA-induced activation of PLA_2 might be derived from the results of Leslie and Channon (56) who showed that anionic phospholipids and diacylglycerol dramatically decreased the calcium concentration (from mM to nM range) required for full activity of partially purified PLA_2 acting on alkylarachidonoyl-GPC. Changes in the packing characteristics of the substrates induced by diacylglycerol correlate with increase in PLA_2 activity (57). A part of phorbol ester action on lyso paf accumulation (12) might also be attributed to the modulation of a CoA-independent transacylase which either competes with acetyltransferase for their common substrate or (19) generates lyso paf by itself (47). Indeed, in the ionophore A23187-stimulated neutrophils only a portion of generated lyso paf was acetylated to form paf, the balance being reacylated by various fatty acids to form phosphatidylcholine (58).

We believe that research on the regulation of paf biosynthesis will allow a better understanding of the role of paf in human health and desease. It should also stimulate the search for new drugs with anti-acetyltransferase properties. These drugs might become valuable tools for evaluating the roles of paf and other mediators such as leukotrienes or histamine, that are released from inflammatory cells during pathological processes.

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PAF Effects on Transmembrane Signaling Pathways in Rat Kupffer Cells[1]

Chandrashekhar R. Gandhi and **Merle S. Olson***
Department of Biochemistry, University of Texas Health Science Center at San Antonio, San Antonio, Texas 78284

Platelet activating factor (PAF) was found to stimulate the metabolism of inositol phospholipids via deacylation and phospholipase C in Kupffer cells, the resident macrophages in liver. PAF-induced phosphoinositide metabolism occurred in two phases. Within seconds after stimulation, in the absence of extracellular Ca^{++}, platelet activating factor caused the phosphodiester hydrolysis of phosphatidylinositol 4,5-bisphosphate and phosphatidylinositol 4-phosphate with the release of inositol 1,4,5-trisphosphate and inositol 1,4-bisphosphate. This was followed by an extracellular Ca^{++}-dependent release of glycerophosphoinositol, inositol monophosphates and inositol bisphosphates. Various Ca^{++}-mobilizing agonists failed to evoke hydrolysis of phosphoinositides. Platelet activating factor also stimulated the synthesis and release of prostaglandins from these cells. Platelet activating factor-stimulated phosphodiester metabolism of phosphoinositides and prostaglandin synthesis was inhibited by treatment with pertussis toxin and cholera toxin. Pertussis toxin also inhibited platelet activating factor-induced glycerophosphoinositol release. Cholera toxin, in contrast, stimulated platelet activating factor-induced glycerophosphoinositol release and prostaglandin synthesis and synergistically stimulated the effect of platelet activating factor on these processes. The results suggest that platelet activating factor-induced metabolism in the Kupffer cells occurs via specific receptors and may be mediated through the activation of different G-proteins.
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Hepatic responses of platelet activating factor (PAF; 1-*O*-alkyl-2-acetyl-*sn*-glycero-3-phosphocholine) present several intriguing questions concerning both intra- and intercellular signaling mechanisms. This agonist stimulates glucose production in the perfused rat liver *in situ* but fails to evoke such a response in isolated hepatocytes. PAF-induced glycogenolysis is accompanied by rapid, transient increases in portal pressure and alterations in oxygen consumption (1). Unlike various classical Ca^{++}-mobilizing agents such as phenylephrine, vasopressin and bradykinin, which stimulate glycogenolysis in perfused liver and isolated hepatocytes (2), PAF-induced hydrolysis of phosphoinositides occurs through the activation of phospholipase A_2 and not via the classical phosphodiester pathway in the isolated parenchymal cells (3).

Several hormones, neurotransmitters and secretagogues exert their effects on the target cell through the stimulation of a phosphatidylyinositol 4,5-bisphosphate (PIP_2)-specific phosphoplipase C, and many of these signal-transduction events are coupled through the activation of a G-protein. PIP_2 is hydrolyzed to two intracellular second messengers: inositol 1,4,5-trisphosphate (Ins 1,4,5-P_3), which is released into the cytosol and causes the mobilization of Ca^{++} from an intracellular non-mitochondrial pool and diacylglycerol (DAG), which remains in the plasma membrane and stimulates a Ca^{++}– and phospholipid-dependent protein kinase C (4–6). The increased concentration of intracellular Ca^{++} stimulates phosphodiester hydrolysis of phosphatidylinositol 4-phosphate (PIP) and phosphatidlyinositol (PI) to inositol 1,4-bisphosphate (Ins 1,4-P_2) and inositol 1-phosphate (Ins 1-P), respectively, and DAG. Ins 1,4,5-P_3 may be hydrolyzed sequentially to Ins 1,4-P_2, a conversion sensitive to 2,3-diphosphoglyceriate (7), Ins 1- and/or Ins 4-P and then to free inositol. Dephosphorylation of Ins 1,4-P_2 and Ins-P is known to be sensitive to Li^+ ions (8–13). Inositol is reutilized for the synthesis of PI. Alternatively, Ins 1,4,5-P_3 may be phosphorylated to inositol 1,3,4,5-tetrakisphosphate (Ins 1,3,4,5-P_4), which has been implicated as causal for the influx of Ca^{++} (14) and for Ca^{++} sequestration into intracellular organelles (15). Ins 1,3,4,5-P_4 may be hydrolyzed to Ins 1,3,4-P_3, the physiological function of which is not clear. DAG can be acted upon by specific lipases (16) resulting in the release of arachidonic acid or can be phosphorylated to phosphatidic acid in a kinase reaction. Phosphatidic acid may be converted to PI via CDP-DAG to complete the cycle.

Differential effects of PAF on hepatic and hepatocytic glycogenolysis in the perfused liver and in isolated hepatocytes suggest a somewhat indirect mechanism of action. On the basis of an extensive series of experiments, we have concluded that PAF exerts its hemodynamic and subsequent glycogenolytic effects on the liver independent of its ability to stimulate arachidonic acid release and metabolism to prostaglandins (17). There is little question that PAF stimulation of the intact liver or isolated hepatic Kupffer cells causes an increase in production of prostaglandins D_2 and E_2 and thromboxane B_2; this enhanced production of arachidonic acid metabolites seems to have little effect on the ability of PAF to evoke its hemodynamic and glycogenolytic responses in the isolated liver. Additionally, several recent reports illustrate the glycogenolytic action of eicosanoids, the lipoxygenase- and cycloxygenase-derived metabolites of arachidonic acid (18–23). Eicosanoids are synthesized by Kupffer and endothelial cells, but not hepatocytes, although the latter can metabolize these compounds (24). The precise role of the primary hepatic eicosanoids following their production during Kupffer cell stimulation is a subject for additional experimental

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*To whom correspondence should be addressed at: Department of Biochemistry, The University of Texas Health Science Center, 7703 Floyd Curl Drive, San Antonio, TX 78284.

Abbreviations: DAG, diacylglycerol; EGTA, ethylene glycol *bis*-(β aminoethyl ether)*N,N,N',N'*-tetraacetic acid; GBSS, Gey's balanced salt solution; GPI, glycerophosphoinositol; GPIP, glycerophosphoinositolphosphate; $GPIP_2$, glycerophosphoinositolbisphosphate; HPLC, high performance liquid chromatography; Ins 1-P, inositol 1-phosphate; Ins 4-P, inositol 4-phosphate; Ins 1,3-P_2, inositol 1,3-bisphosphate; Ins 1,4-P_2, inositol 1,4-bisphosphate; Ins 3,4-P_2, inositol 3,4-bisphosphate; Ins 1,3,4-P_3, inositol 1,3,5-trisphosphate; Ins 1,4,5-P_3, inositol 1,4,5-trisphosphate; Ins 1,3,4,5-P_4, inositol 1,3,4,5-tetrakisphosphate; PAF, platelet activating factor (1-*O*-alkyl-2-acetyl-*sn*-glycerophosphocholine); PG, prostaglandins; PI, phosphatidylinositol; PIP, phosphatidylinositol 4-phosphate; PIP_2, phosphatidylinositol 4,5-bisphosphate; TCA, trichloroacetic acid.

inquiry. Moreover, information regarding the second messenger signaling pathway in Kupffer cells is inadequate. Hence the present contribution concerns PAF-induced phosphoinositide metabolism and prostaglandin synthesis in the primary cultures of Kupffer cells.

MATERIALS AND METHODS

Materials. Collagenase (Type IV from Clostridium histolyticum), protease (Type XIV, from Streptomyces griseus), bovine serum albumin (fraction V), nifedipine, ethylene glycol *bis*-(β aminoethyl ether)*N,N,N',N'*-tetraacetic acid (EGTA), and heat-inactivated fetal bovine serum were purchased from Sigma Chemical Co. (St. Louis, MO). Metrizamide, which is 2-(3-acetamido-5-N-methylacetamido-2,4,6-triiodobenzamido)-2-deoxy-D-glucose, was from Nyegaard and Co. (Oslo, Norway), William's medium E from Gibco Labs (New York, NY). [2-^{3}H]-*myo*-Inositol (20 Ci/mmol) was purchased from Amersham (New York, NY) and [5,6,8,9,11,12,14,15-^{3}H(N)]-arachidonic acid (100 Ci/mmole) from DuPont (Boston, MA). Methyl formate was purchased from Eastman Kodak (Rochester, NY). All other chemicals and reagents purchased locally were of highest purity available.

Isolation and culture of Kupffer cells. Kupffer cells were isolated from livers of male Sprague-Dawley rats (200–250 g) using a modification of the centrifugal elutriation procedure of Knook and Sleyster (25). Briefly, livers were first perfused aseptically at 37°C through the portal vein and inferior vena cava with calcium- and magnesium-free Krebs-Henseleit buffer containing 0.2% glucose and 0.2% bovine serum albumin gassed with 95% O_2/5%CO_2 at a rate of 30 mL/min. This was followed by recirculating perfusion with the above buffer containing 0.03% collagenase and 0.02% protease in the presence of 2 mM Ca^{++}. The digestion was continued for 5 min, after which the perfusion was stopped, the liver capsule opened and the cells were dispersed in Krebs-Henseleit buffer. The cell suspension was filtered through two layers of nylon mesh and the filtrate was centrifuged at 525 × *g* for 45 seconds, followed by the centrifugation of the supernatant at 830 × *g* for 6 min. The pellet was suspended in Gey's balanced salt solution (GBSS) without NaCl containing 17.5% metrizamide. One mL of GBSS was layered over 6 mL of the above cell suspension and after centrifugation for 20 min at 1400 × *g*, nonparenchymal cells which collected at the interphase were aspirated and washed with Krebs-Henseleit buffer. Kupffer and endothelial cells were separated by centrifugal elutriation (25).

Isolation of hepatocytes. Hepatocytes were prepared by collagenase perfusion of the rat livers exactly as described by Berry and Fried (26).

Primary culture of Kupffer cells, endothelial cells and hepatocytes. Kupffer cells, endothelial cells and hepatocytes were suspended in William's medium E supplemented with 2 mM L-glutamine, 10% heat-inactivated fetal calf serum, 5000 U/mL penicillin and 5000 μg/mL streptomycin at 2 × 10^6, 2.5 × 10^6 and 1 × 10^6 cells/mL, respectively. Two-mL portions of this cell suspension were plated in 35 mm tissue culture dishes and placed in an incubator at 37°C in an atmosphere of 95%O_2/5%CO_2. Cells were allowed to attach for 2–3 hr and were washed with the incubation medium. After an overnight incubation, the attached cells were washed twice and placed in fresh medium containing 2% fetal calf serum and [^{3}H]-arachidonic acid [for prostaglandins (PG) determinations] or [^{3}H]inositol (for determination of inositol phosphates). Following incubation for 24 hr, cells were washed twice with nonradioactive medium and incubated. After 30 min in the incubator, cells were stimulated with agonists and the reaction was terminated with the addition of ethanol for the PG determinations (27), or with 5% ice-cold trichloroacetic acid (TCA) following rapid aspiration of the medium for the analysis of inositol phosphates.

Extraction of prostaglandins. Prostaglandins were extracted essentially as described by Powell (27). Cells were scraped from the culture plates and both the medium and cells were quantitatively transferred to tubes. The plates were washed with an additional 1 mL ethanol and water was added to the combined extracts to bring the final concentration of ethanol to 15%. The extract was centrifuged and the pellet was washed with 3–4 mL 15% ethanol. The combined supernatants were acidified to pH 3.5 with 1 M HCl, applied to SEP-PAK columns (C18 cartridges from Waters Associates, Milford, MA), washed with 15% ethanol and, finally, prostaglandins were eluted with methyl formate.

Analysis of prostaglandins. Methyl formate was evaporated under N_2, the residue was dissolved in 140 μL 36% acetic acid (0.1%, v/v) in acetonitrile and was filtered (0.45 μM Millipore Type HV filter). The filtrate (80–90 μL) was used for the analysis of prostaglandins by high performance liquid chromatography (HPLC). The analysis of prostaglandins was performed essentially as described by Peters *et al.* (28) using a Varian HPLC (Varian Associates, Palo Alto, CA) with an Ultrasphere ODS column (4.6 mm × 25 cm) and a pre-column. The solvent system used was 0.1% (v/v) aqueous acetic acid (pH 3.7) and acetonitrile with a flow rate of 1.5 mL/min. Half-minute fractions were collected and quantified by liquid scintillation spectroscopy. Scintillation counting was performed using a Beckman 5801 counter linked to a Beckman Data Transfer Module (Beckman Instruments, Fullerton, CA). The data was transferred directly to an IBM PC XT-type computer for analysis. Various prostaglandins were identified by spiking the cell extracts with a mixture of [^{3}H]PG standards.

Extraction and identification of [^{3}H]inositol phosphates. Cells were scraped from culture plates and the plates were washed with an additional 0.5 mL TCA. The combined extracts were centrifuged and the supernatant was washed with water-saturated diethyl ether, lyophilized and stored at −70°C. [^{3}H]Inositol phosphates were analyzed by HPLC essentially as described by Dean and Moyer (29). The identities of Ins 1-P, Ins 4-P, Ins 1,4-P_2 and Ins 1,4,5-P_3 were confirmed by spiking cell extracts with a mixture of [^{3}H]inositol phosphate standards. Other peaks observed were glycerophosphoinositol (GPI) which eluted between inositol and Ins 1,P, Ins 1,3,4-P_3 and Ins 1,3-P_2.

Analysis. Lyophilized extracts were dissolved in 250 μL of distilled water and filtered (0.45 μm Millipore Type HV filter). Samples of the extract (200 μL) were analyzed by HPLC essentially as described by Dean and Moyer (29). Buffers were made using HPLC grade ammonium phosphate (Fisher Scientific, Fair Lawn, NJ) and water purified with a Milli-Q* system (Millipore Corporation, Bedford, MA). Buffers were filtered (0.45 μM Millipore

TABLE 1

Effect of PAF on the Inositol Phospholipid Metabolism of Kupffer Cells, Endothelial Cells and Hepatocytes[a]

Cell type	GPI	IP	IP_2
Hepatocytes	118 ± 6 (235 ± 18)	103 ± 9 (199 ± 12)	100 ± 3 (45 ± 3)
Endothelial cells	135 ± 12 (141 ± 11)	237 ± 32 (212 ± 8)	284 ± 48 (58 ± 9)
Kupffer cells	204 ± 22 (241 ± 15)	527 ± 34 (278 ± 18)	418 ± 31 (79 ± 5)

[a]Hepatocytes, Kupffer cells and endothelial cells were prepared and cultured, as described in Materials and Methods at a cell density of 1×10^6 cells/mL (hepatocytes), 2×10^6 cells/mL (Kupffer cells) or 2.5×10^6 cells/mL (endothelial cells) (2 mL/dish). The cells were labeled with [^{3}H]inositol (2.5 μCi/mL), stimulated with 20 nM PAF, the reaction was terminated and the inositol phosphates were analyzed as described in Materials and Methods. The results are expressed as the percentage of the unstimulated control values ± S.E.M. of triplicate determinations from representative experiments. The values in parentheses represent radioactivity (DPM) in the inositol phosphates of the nonstimulated control samples.

Type HA filter) and degassed prior to use. A Waters HPLC system was used in conjunction with a 25 cm × 0.4 cm Partisil 10 SAX column (Phenomenex, Rancho Palos Verdes, CA). The A_{254} of the HPLC effluent was measured, and 30 second fractions were collected and quantified by scintillation spectroscopy.

RESULTS AND DISCUSSION

Table 1 shows the effect of PAF on the metabolism of [^{3}H]inositol-labeled lipids from hepatocytes, Kupffer cells and endothelial cells in primary culture. PAF treatment stimulated phosphoinositide hydrolysis in Kupffer and endothelial cells via deacylation and phospholipase C action, the Kupffer cells being more responsive to such stimulation. Consistent with the previous observations (3,30), hepatocytes did not respond to PAF, although a slight increase in their GPI content was observed. High affinity receptors for PAF have been reported on the liver plasma membranes (31) and these receptors may be coupled to phospholipase A_2. Interestingly, coupling of phospholipase A_2 to specific G-proteins has been shown (32–34).

Figure 1 illustrates the PAF-stimulated metabolism of phosphoinositides at 5 seconds and at 20 min in the cultured Kupffer cells. At 5 seconds, a sharp increase occurred in the Ins 1,4,5-P_3 and Ins 1,4-P_2 levels without a significant change in the inositol monophosphates or GPI, suggesting an early hydrolysis of PIP_2 and PIP. At 20 min, increases in the levels of Ins 1-P, Ins 4-P, Ins 1,4-P_2 and GPI were observed in the PAF-stimulated cells. At this time point the Ins 1,4,5-P_3 peak returned to the basal level with the appearance of Ins 1,3,4-P_3 and Ins 1,3-P_2, suggesting that Ins 1,4,5-P_3 was metabolized to these species via Ins 1,3,4,5-P_4. No significant change in the Ins 1,3,4,5-P_4 moiety was observed at any time during the stimulation of Kupffer cells with PAF (results not shown), indicating rapid metabolism of this species. EGTA inhibited phosphodiester metabolism as

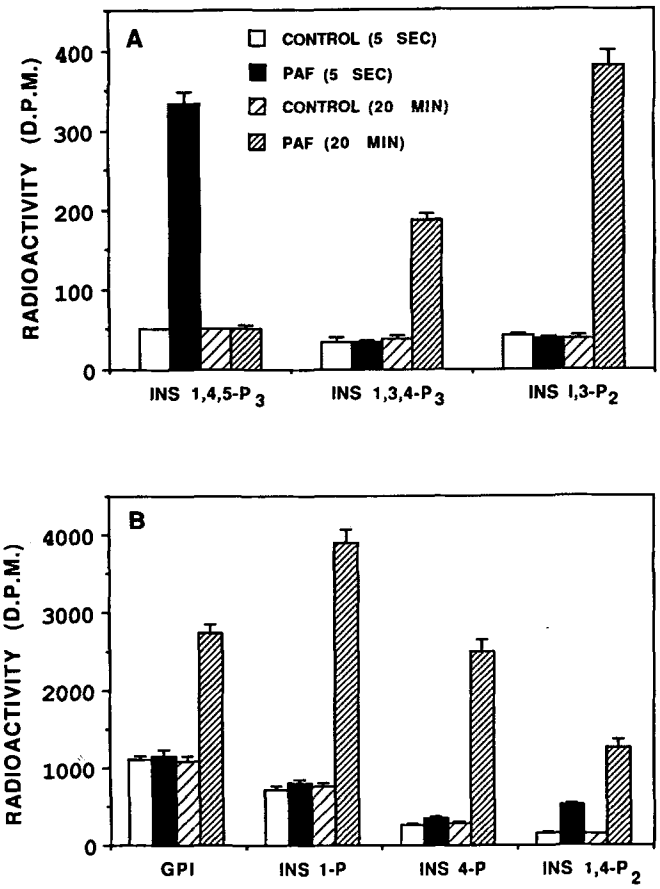


FIG. 1. Effect of PAF on the metabolism of phosphoinositides in Kupffer cells. Kupffer cells were isolated, cultured and labeled with 10 μCi/mL [^{3}H]inositol as described in the legend for Table 1. Cells were washed twice and stimulated with 20 nM PAF in the presence of 10 mM LiCl. Reaction was terminated at 5 seconds (A) and 20 min (B), and the inositol phosphates were extracted and analyzed essentially as described in Materials and Methods. The results are expressed as the means of triplicate determinations from a representative experiment ± S.E.M.

well as deacylation of phosphoinositides at 20 min, but not at 5 seconds (results not shown). The results indicate that PAF-induced metabolism of phosphoinositides in Kupffer cells involve distinct mechanisms. These assumptions were confirmed by thin-layer chromatography of [^{3}H]inositol-labeled lipids after PAF-stimulation of Kupffer cells for 5 seconds and 20 min as shown in Figure 2. Thus, at 5 seconds there was a decrease in PIP_2 and PIP but not in PI, indicating an extracellular Ca^{++}-independent hydrolysis of polyphosphoinositides. With continual stimulation, PIP hydrolysis progressed and PI hydrolysis also occurred to a large extent (Fig. 2). Since EGTA inhibited the release of inositol phosphates at the longer time interval, this metabolic activity was probably dependent on the extracellular Ca^{++}. Furthermore, these results suggest that two separate phospholipase C activities or two forms of the same enzyme (Ca^{++}-dependent and Ca^{++}-independent) must be present in the Kupffer cell membrane. A small but significant increase in lysophosphatidylinositol occurred at this time (results not shown). In another experiment, Kupffer cells were labeled with [^{3}H]glycerol and then stimulated with

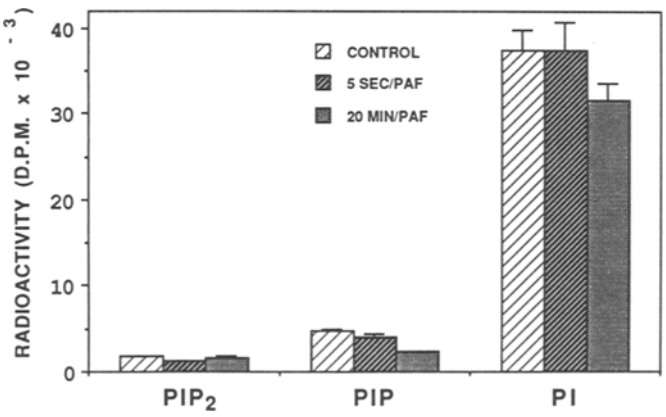


FIG. 2. Effect of PAF on [^{3}H]inositol-labeled phospholipids in Kupffer cells. Kupffer cells were isolated, cultured and labeled with [^{3}H]inositol (5 μCi/mL) for 24 hr. After washing, cells were stimulated with 20 nM PAF for indicated times. The reaction was terminated with the addition of TCA as described in the legend for Figure 1. Lipids from the TCA pellet were extracted with chloroform/methanol (2:1, v/v). Following addition of 2.4 M HCl and vortexing, phases were separated by low-speed centrifugation. Organic phase was dried under nitrogen and lipids were dissolved in chloroform/methanol/1M HCl (75:25:2, v/v/v). Phospholipids were separated by thin-layer chromatography on silica gel HL plates (Analtech) using the solvent system (chloroform/methanol/20% methylamine, 65:36:10, v/v/v). Authentic standards (Sigma Chemical Co., St. Louis, MO) were used to identify inositol phospholipid fractions which were scraped from the plates. Radioactivity was determined by liquid scintillation spectroscopy. The results are means of duplicate determinations ± S.E.M. from a representative experiment.

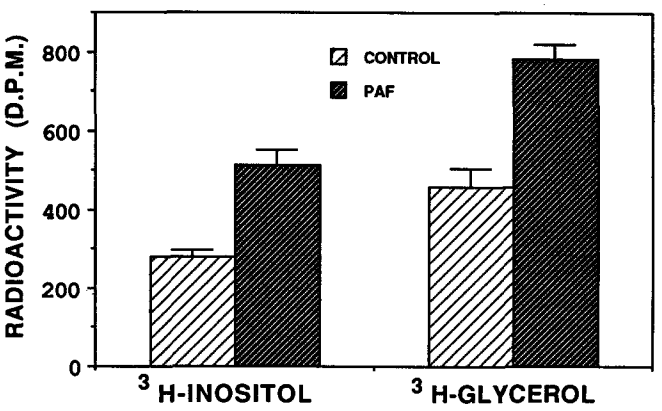


FIG. 3. Effect of PAF on the release of glycerophosphoinositol from [^{3}H]inositol- and [^{3}H]glycerol-labeled Kupffer cells. Kupffer cells were labeled with 2.5 μCi/mL [^{3}H]inositol or 5 μCi/mL [^{3}H]glycerol. Cells were stimulated with 20 nM PAF for 20 min. The reaction was terminated with TCA and GPI was analyzed in diethyl ether-washed extract by HPLC. For details, see legend for Figure 1 and Materials and Methods. The results are means of triplicate determination ± S.E.M. from one experiment.

PAF to evaluate the precursor of GPI. The results of this experiment are depicted in Figure 3. The extent of stimulation of GPI release was identical when the cells labeled with both radioactive inositol or glycerol were stimulated with PAF (Fig. 3). Glycerophosphoinositolphosphate (GPIP) or glycerophosphoinositolbisphosphate ($GPIP_2$) peaks (from cells labeled with radioactive inositol or glycerol) were not observed on the HPLC profiles,

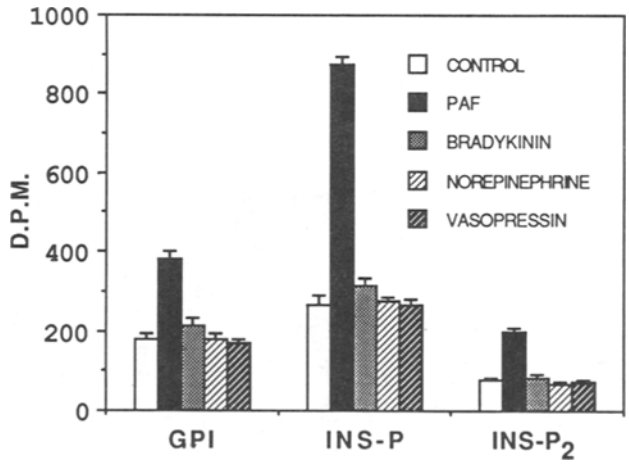


FIG. 4. Effect of Ca^{++}-mobilizing hormones on the metabolism of phosphoinositides in Kupffer cells. Kupffer cells were prepared and labeled with [^{3}H]inositol (2.5 μCi/mL) as described in Materials and Methods. Cells were then stimulated with PAF (20 nM), bradykinin (2 μM), norepinephrine (10 μM) and vasopressin (1 μM). Reaction was terminated at 20 min and the inositol phosphates were extracted and analyzed as described. The results are expressed as the means of triplicate determinations from a representative experiment ± S.E.M.

suggesting that deacylation of PIP or PIP_2 did not occur upon stimulation of Kupffer cells with PAF, or that GPIP and $GPIP_2$ were very rapidly converted to GPI.

Figure 4 illustrats a comparison of Ca^{++}-mobilizing hormones with PAF-stimulated phosphoinositide metabolism in Kupffer cells. These glycogenolytic hormones (vasopressin, norepinephrine and bradykinin) have been shown to induce phospholipase C-mediated metabolism of inositol lipids in hepatocytes (4,35). None of these hormones except bradykinin had an appreciable effect on Kupffer cell phosphoinositide turnover and the extent of the bradykinin effect was much less than observed for PAF.

Many receptor-mediated transmembrane signaling events occur through the activation of G-proteins. Pertussis toxin and cholera toxin alter such processes by causing ADP-ribosylation of the GTP-binding proteins (36). Table 2 shows the effect of pertussis toxin and cholera toxin on PAF-stimulated metabolism of phosphoinositides in the Kupffer cells. Pertussis toxin strongly inhibited both PAF-stimulated release of GPI and inositol phosphates suggesting the involvement of G-proteins in this transmembrane signaling process. In contrast, cholera toxin and PAF synergistically stimulated GPI release and cholera toxin inhibited the phospholipase C-mediated response induced by PAF. Consistent with these observations, cholera toxin and PAF had an additive effect on prostaglandin synthesis in Kupffer cells when the toxin was present in the cellular incubations throughout the duration of the experiment (C. R. Gandhi and M. S. Olson, unpublished observation).

On stimulation of [^{3}H]arachidonic acid-labeled Kupffer cells with PAF, a release of radioactivity (40 ± 8 over basal) in the medium occurred. However, only 1–3% of this released radioactivity was actually converted into prostaglandins (unpublished observation). Since arachidonic acid is attached at the *sn*-2 position in the

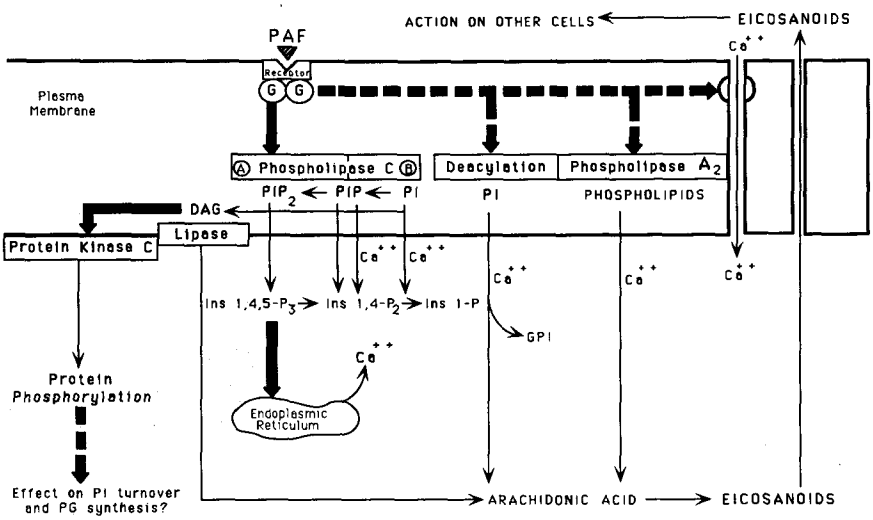


FIG. 5. A general scheme of the possible events occurring in Kupffer cells following stimulation with PAF.

TABLE 2

Effect of Pertussis Toxin and Cholera Toxin on PAF-induced Phosphoinositide Metabolism in the Kupffer Cells[a]

Treatment	GPI	IP	IP_2
Control	198 ± 12	221 ± 17	59 ± 3
PAF	360 ± 23	876 ± 34	178 ± 9
PTX	187 ± 8	241 ± 21	51 ± 5
PTX/PAF	204 ± 18	468 ± 29	89 ± 8
CTX	273 ± 15	199 ± 12	49 ± 2
CTX/PAF	423 ± 28	428 ± 36	45 ± 3

Kupffer cells were prepared and labeled with [^{3}H]inositol (2.5 μCi/mL) as described in the Materials and Methods. After washing, the cells were incubated in the medium containing 2 μg/mL cholera toxin or 100 ng/mL pertussis toxin for 3 hr. The cells were then stimulated with 20 nM PAF in the presence of LiCl. Other details of the procedure were described in the legend for Table 1. The results are expressed in DPM as means of duplicate determinations ± S.E.M. from a representative experiment of four experiments performed.

TABLE 3

Effect of EGTA and Nifedipine on PAF-Induced Prostaglandin Synthesis in Kupffer Cells[a]

Treatment	PGE_2	PGD_2
Control	277 ± 13	198 ± 15
PAF	798 ± 29	1162 ± 53
EGTA/PAF	246 ± 8	211 ± 17
Nifedipine/PAF	299 ± 19	257 ± 22

[a]Kupffer cells in primary culture were labeled with 0.5 μCi/mL [^{3}H]-arachidonic acid described in Materials and Methods. EGTA (5 mM) and nifedipine (100 μM) were added 5 min prior to the stimulation with PAF (20 nM). Reactions were terminated with the addition of ethanol and the prostaglandins were extracted and analyzed by HPLC as described. The results are expressed in DPM as mean values of triplicate determinations ± S.E.M. from a representative experiment of three experiments performed.

phospholipids, its release with PAF suggests stimulation of phospholipase A_2 activity. Phosphoinositides are rich in arachidonic acid and metabolically very active. Apart from the phospholipase A_2 pathway, arachidonic acid is released from DAG by a specific lipase. Alternatively, DAG is converted to phosphatidic acid and then may be hydrolyzed by phospholipase A_2 to release arachidonic acid. Thus, both deacylation and phospholipase C-mediated hydrolysis of phosphoinositides seem to be important for the PAF-stimulated prostaglandin synthesis in Kupffer cells. Table 3 illustrates the effect of the Ca^{++} ion chelator, EGTA, and the plasma membrane Ca^{++} channel antagonist, nifedipine, on the PAF-stimulated synthesis of prostaglandins in Kupffer cells. PAF-stimulated prostaglandin synthesis was completely inhibited by EGTA and nifedipine indicating the requirement of extracellular Ca^{++} for this process and suggesting that plasma membrane Ca^{++} channels may be regulated by PAF. The intracellular concentration of free arachidonic acid, which is the precursor of eicosanoids, is low (37). Therefore, in stimulated cells arachidonic acid is generated from the deacylation of membrane phospholipids by phospholipase A_2. This process has been reported to be receptor-mediated and modulated by G-proteins in certain cells (33,34). The intracellular concentration of Ca^{++} is increased through its mobilization from the cellular stores by Ins 1,4,5-P_3 and/or through an activation of plasma membrane Ca^{++} channels. Inhibition of PAF-stimulated synthesis of prostaglandins in Kupffer cells by EGTA and nifedipine (Table 3) implicates a requirement of extracellular Ca^{++} for this process and an activation of a membrane Ca^{++} channel by PAF. Involvement of Ca^{++} mobilized by Ins 1,4,5-P_3 in PAF-induced prostaglandin synthesis may be ruled out since EGTA was found not to inhibit early (5 second) PAF-stimulated hydrolysis of phosphoinositides, but strongly inhibited this process at later (20 min) times (results not shown). Although inositol lipids form a relatively minor pool of phospholipids, PAF-induced metabolism of these lipids may be important for prostaglandin synthesis and release since the phosphoinositides are rich

in arachidonic acid and are metabolically very active.

The results described in this study are outlined in the cartoon shown in Figure 5. PAF stimulates the early (5 seconds) hydrolysis of PIP_2 and PIP to Ins 1,4,5-P_3 and Ins 1,4-P_2, respectively, a process independent of extracellular Ca^{++} and mediated via the activation of a G-protein. PAF also induces Ca^{++} influx into the cells, which stimulates phosphodiester hydrolysis of PIP and PI, deacylation of PI (20 min), and the synthesis of prostaglandins. Inhibition of these processes by treatment with pertussis toxin suggests an involvement of G-protein(s). At this point it is not clear whether plasma membrane Ca^{++} influx, phospholipase A_2 activation, prostaglandin synthesis, or all of these processes involve G-protein(s). Also, it is not clear whether the same or different G-proteins are operating the above-mentioned processes. Stimulation of prostaglandin synthesis and GPI release by cholera toxin and the additive effect of cholera toxin and PAF on these processes suggest a possible involvement of cAMP. However, in separate experiments (data not shown) treatment of cells with dibutyryl cyclic AMP failed to stimulate GPI release or eicosanoid synthesis in Kupffer cells, suggesting an alternative mechanism of action of cholera toxin in these cells. Furthermore, in view of the recent reports concerning the involvement of protein kinase C in receptor-mediated events (38–44), such a mechanism of action in the PAF-induced Kupffer cell metabolism may not be ruled out.

ACKNOWLEDGMENT

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Platelet-Activating Factor May Participate in Signal Transduction Processes in Rabbit Leukocytes[1]

Alastair G. Stewart* and **Trudi Harris**
Department of Physiology, University of Melbourne, Victoria, Australia

The bacterial chemotactic peptide, formyl-methionyl-leucyl-phenylalanine (fMLP), induces the generation of platelet-activating factor (PAF), the mobilization of arachidonic acid and generation of superoxide anion (O_2^-) in rabbit polymorphonuclear leukocytes (PMNs). The PAF receptor antagonists, WEB 2086 (10–100 μM) and CV 6209 (1–10 μM), reduced the mobilization of arachidonic acid and the O_2^- generation in response to fMLP but not that in response to A23187. Pretreatment of PMNs with the phospholipase A_2 inhibitor, chloroquine, or the serine protease inhibitor, tosyl-phenylalanine chloromethyl ketone, reduced the fMLP-stimulated generation of PAF and also reduced the generation of O_2^-. The respiratory burst induced by a submaximal concentration of phorbol myristate acetate was not affected by these compounds. These data are consistent with the suggestion that endogenous PAF may contribute to the signal transduction cascade initiated by fMLP. ***Lipids 26,*** **1044–1049 (1991).**

Membrane-derived mediators such as cyclooxygenase and lipoxygenase arachidonic acid metabolites and platelet-activating factor (PAF) are considered to play a key role in a variety of inflammatory conditions involving macrophages and polymorphonuclear leukocytes (PMNs). In addition to their extracellular actions, it has been proposed that lipoxygenase products may contribute to cellular transduction processes (1–3).

Several features of the biology of PAF suggest that it may have a role within the cell in addition to its suggested role as an extracellular mediator of inflammation. Although it is now well-established that pathophysiological stimuli elicit the synthesis of PAF in a range of cell types including endothelium, macrophages and PMNs (4), the release of PAF into the extracellular medium varies considerably in magnitude. Furthermore, once released, PAF may be rapidly inactivated by plasma acetylhydrolase (5) or its actions may be prevented by the presence of endogenous PAF inhibitors (4). The potency of PAF in causing platelet aggregation is considerably higher in washed platelets than in platelet rich plasma (6) suggesting that physiological concentrations of serum albumin greatly reduce the potency of PAF by binding it in a less active form. These characteristics of PAF may diminish its importance as a mediator of inflammation.

We have recently reported that PAF receptor antagonists reduce prostacyclin (PGI_2) generation in adherent macrophages (7–9). These unstimulated adherent macrophages had no detectable PAF in the supernatants, but PAF was readily detectable in cellular extracts. Thus, we put forward the hypothesis that, under certain conditions, PAF may be acting as part of the intracellular transduction mechanism for eicosanoid and superoxide anion (O_2^-) generation rather than, or in addition to, its role as an extracellular mediator (7–10). In this study, we have examined further the possible intracellular role of PAF using the phospholipase A_2 inhibitor, chloroquine (11) and the serine protease inhibitor tosyl-phenylalanine chloromethyl ketone (TPCK), since these compounds have been reported to inhibit PAF synthesis (12,13). Furthermore, we have investigated the effects of the cyclooxygenase inhibitor indomethacin and the 5-lipoxygenase inhibitor CGS 8515 (14) on O_2^- generation by rabbit PMNs.

MATERIALS AND METHODS

Chemicals. All reagents and solvents were of analytical or higher grade. Chemicals were obtained from the following sources: A23187; BSA, grade 5, essentially fatty acid free; cytochrome C (horse heart type III); dextran, clinical grade C: formyl-methionyl-leucyl-phenylalanine (fMLP); indomethacin; superoxide dismutase (Sigma Chemical Co., St. Louis, MO); hexadecyl platelet-activating factor (PAF, Bachen, Bubendorf, Switzerland); [1-^{14}C]arachidonic acid, 58.3 mCi/mmol (Amersham International, Amersham, U.K.); 1-*O*-[1,2-^{3}H]hexadecyl-2-acetyl-*sn*-glycero-3-phosphocholine, 59.5 Ci/mmol; [14,15-^{3}H]leukotriene B_4,32 Ci/mmol (New England Nuclear, Boston, MA); leukotriene B_4 antiserum (Dr. J. Salmon, Wellcome Research Laboratories, Beckenham, U.K.); leukotriene B_4 (Dr. J. Rokach, Merck, Frosst Laboratories, Montreal, Canada); HEPES (*N*-2-hydroxyethylpiperazine-*N*-2-ethanesulfonic acid, BDH Chemicals, Melbourne, Australia); heparin (Fisons Pty. Ltd., Melbourne, Australia); CV 6209 (Takeda Chemical Industries Ltd., Osaka, Japan); WEB 2086 (Boehringer Ingelheim, Ingelheim, West Germany).

Isolation of platelets and polymorphonuclear leukocytes. Rabbits of either sex (2–4 kg) were anaesthetized using propanidid and exsanguinated *via* a sterile cannula placed in the carotid artery. The blood was anticoagulated with citrate (0.38% w/v, final concentration), platelet-rich plasma was obtained by centrifugation (100 × *g*, 20 min, 20°C), and washed platelets were prepared as described previously (7,15). Polymorphonuclear leukocytes (PMNs) were prepared by centrifugation through Ficoll-Paque or Lymphoprep (endotoxin-tested) as described (7,9) and finally resuspended in HEPES (5mM)-buffered Tyrode solution (0.25% bovine serum albumin); composition (mM): 137, NaCl; 11, D(+)-glucose; 11.9, $NaHCO_3$; 2.7, KCl; 0.26, $MgCl_2$; 0.4, NaH_2PO_4; 1.8, $CaCl_2$.

Superoxide anion generation. The generation of O_2^-

[1]Based on a paper presented at the Third International Conference on Platelet-Activating Factor and Structurally Related Alkyl Ether Lipids, Tokyo, Japan, May 1989.

*To whom correspondence should be addressed at Department of Physiology, University of Melbourne, Parkville 3052, Victoria, Australia.

Abbreviations: ANOVA, analysis of variance; fMLP, formyl-methionyl-leucyl-phenylalanine; HEPES, *N*-(2-hydroxyethyl) piperazine-*N*'-2-ethanesulfonic acid; HPLC, high-performance liquid chromatography; LTB_4, leukotriene B_4; NADPH, reduced nicotinamide adenine dinucleotide phosphate; PAF, platelet-activating factor; PGI_2, prostacyclin; PMA, phorbol myristate acetate; PMNs, polymorphonuclear leukocytes; SOD, superoxide dismutase; TPCK, tosyl-phenylalanine chloromethyl ketone.

was measured by the superoxide dismutase (SOD)-inhibitable reduction of cytochrome C (9,16). PMNs were suspended at 2–3 × 10^6/mL in Tyrode solution containing 80 μM cytochrome C and were pre-incubated with antagonists and inhibitors for 15 min at 37° before stimulation with fMLP (0.1–1000 nM) or phorbol myristate acetate (PMA, 0.1–1000 nM) for 15 min. The response to fMLP was complete within 5 min and the O_2^- generation by PMA-treated PMNs was stopped by the addition of 10 μg/mL SOD. The absorbance (550 nm) of the supernatants was determined in a Pye Unicam or Hitachi U2000 spectrophotometer. The data for O_2^- in response to fMLP (1 μM) and PMA (1 μM) in nmol/10^6 cells was 4.55 ± 0.75, n = 16 and 61.0 ± 9.4, n = 13, respectively and the basal value was 1.07 ± 0.40, n = 16.

Bioassay of PAF. Rabbit-washed platelets were suspended in Tyrode buffer containing indomethacin (2.8 μM) at 2 × 10^8/mL and a concentration-response curve for synthetic hexadecyl PAF (2–200 fmol)-induced aggregation (Chronolog 540 aggregometer) was constructed. PAF was assayed in supernatants without further purification and in cell extracts as previously described in detail (8–10,17). PAF was extracted into 80% ethanol and the identity of the extracted PAF was assessed by sensitivity of platelet aggregation induced by the extracted material to inhibition by the specific PAF receptor antagonist WEB 2086 (1 μM) and by the co-migration of this material with authentic PAF on a thin-layer chromatogram (chloroform/methanol/water/acetic acid, 65:35:6:0.1, by vol; Whatman LKD silica gel).

Radioimmunoassay of leukotriene B_4. The release of leukotriene B_4 into the supernatants of fMLP-stimulated PMNs was measured by radioimmunoassay of non-extracted PMN supernatants using previously described anti-serum and methods (9,18). None of the compounds used interfered with the radioimmunoassay. The limit of detection was 0.025 pmol (corresponding to 0.005–0.01 pmol/10^6 PMNs). The inter-assay co-efficient of variation was 11.5% and the intra-assay co-efficient of variation was 7% (n=7 assays).

Prelabelling of PMNs with [^{14}C]arachidonic acid. PMNs (10^7/mL) were incubated with 0.2 μCi/mL [^{14}C]arachidonic acid for 120 min during which time 20.4 ± 3.9% (n=5) of the label was taken up into phospholipid stores. At the end of this incubation period, the non-incorporated [^{14}C]arachidonic acid was removed by centrifuging the cells (1,000 × *g*, 5 min, ambient temperature) and decanting the supernatant. Residual non-incorporated [^{14}C]arachidonic acid was removed by resuspending the cells in Tyrode buffer and centrifuging to remove the supernatant as before. Finally the cells were resuspended in Tyrode buffer at a concentration of 2–3 × 10^6/mL and stimulated as described in the Results section. At the end of the stimulation period, the cells were pelleted by centrifugation and the ^{14}C-content of the supernatants was determined by liquid scintillation spectrometry (Packard). In some experiments the composition of the released radioactivity was examined by passing the supernatants through C^{18} Sep-paks to isolate the arachidonic acid and metabolites which were subsequently separated on reverse-phase high-performance liquid chromatography (HPLC) using a Nova-pak C^{18} column in a radial compression module and an initial mobile phase of methanol/water/acetic acid (65:35:0.1, v/v/v; pH 5.4) for the first 16 min followed by a 5-min linear gradient to 99% methanol containing 1% acetic acid for a further 20 min at a constant flow rate of 1 mL/min. Fractions were collected and counted for ^{14}C and products were identified by comparing their elution times with those of authentic ^{3}H standards. The released ^{14}C comprised [^{14}C]arachidonic acid (55%, elution time 29 min) and its metabolites (LTB_4; 6%, 9.5 min), an unidentified peak (12%) that eluted at 4 min but was distinguishable from leukotriene C_4 and 5-Hydroxy-eicosatetraenoic acid (10%, 26 min).

Statistical analyses. Concentration-response curves were analyzed by analysis of variance (ANOVA) with repeated measures. Single comparisons were made using Student's t-test. Differences were considered to be significant when $P<0.05$. There was considerable variability between rabbits in the amount of O_2^- generated and the release of [^{14}C]arachidonic acid. Therefore, these data have been normalized by expressing formation or release as a percentage of that observed in control incubations (no pretreatments) for individual experiments.

RESULTS

The bacterial chemotactic peptide fMLP concentration-dependently stimulated the generation of O_2^- (Fig. 1). PAF generation was also stimulated by fMLP (10 nM) (Table 1). To examine whether the PAF generated in fMLP-stimulated PMNs contributed to activation of O_2^- generation, PMNs were pretreated for 15 min before stimulation with the PAF receptor antagonists, WEB 2086 (10 μM) and CV 6209 (1 μM). The PAF receptor antagonists reduced fMLP (10 nM)-induced O_2^- generation, whereas only CV 6209 reduced the basal O_2^- (Table 2).

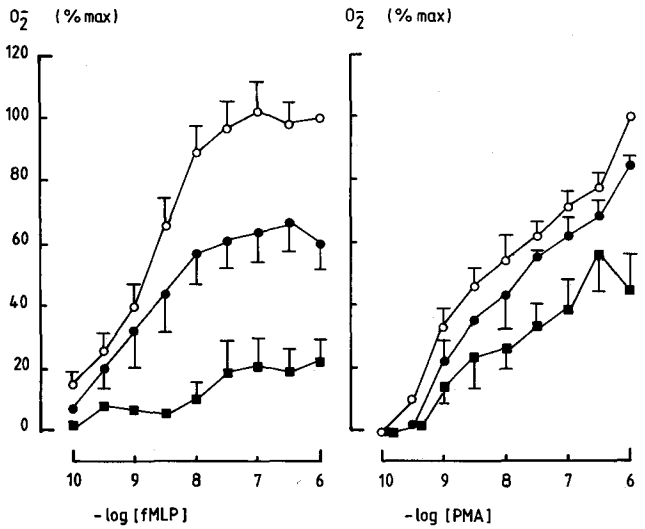


FIG. 1. fMLP and PMA (open circles)-induced O_2^- generation in a concentration-dependent manner. In cells which were pre-incubated in chloroquine (10 μM, filled circles; 100 μM, filled squares) the stimulation of O_2 generation by fMLP was significantly reduced. Responses to PMA were reduced only by the higher concentration of chloroquine. The data are expressed as percentages of the respective maximum control (no chloroquine) responses to 1 μM of the stimulus (fMLP or PMA) and are presented as the means and standard errors of the means (n = 4–11).

TABLE 1

Effects of PAF Antagonists on O_2^- Generation

Stimulus	Increase in O_2^- generation[a] (nmol/10^6 cells)	O_2^- generation (% control) WEB 2086 10 μM	CV 6209 1 μM
basal	[b]	101 ± 9	46 ± 12[c]
fMLP 10 nM	2.76 ± 0.46[d]	80 ± 6[c]	50 ± 7[c]
A23187 10 μM	5.72 ± 1.17[d]	98 ± 4	118 ± 13

[a]Data are presented as the means ± the standard errors of the means and represent the generation of O_2^- in the presence of antagonists expressed as a percentage of that in the absence of antagonists (*i.e.*, control); n = 7-12.

[b]Basal generation of O_2^- was 1.29 ± 0.26 nmol/10^6 cells.

[c]$P<0.05$, paired Student's t-test, compared with O_2^- generation in the presence of antagonists (*i.e.*, control).

[d]$P<0.05$, paired Student's t-test, compared with basal O_2^- generation.

TABLE 2

Effects of the Serine Protease Inhibitor on O_2^- Generation and Cell-Associated PAF Levels

Stimulus	O_2^- generation[a] (nmol/10^6 cells) Control	TPCK 10 μM	PAF (pmol/10^6 cells) Control	TPCK 10 μM
basal	2.09 ± 1.14	1.45 ± 0.56	0.005 ± 0.002	0.007 ± 0.004
fMLP 10 nM	5.78 ± 1.95	2.04 ± 0.94[b]	0.022 ± 0.013	0.014 ± 0.009[b]
PMA 0.3 nM	10.35 ± 3.03	11.04 ± 3.36	NT[c]	NT[c]

[a]Data are presented as the means ± standard errors of the means of 4-5 observations.

[b]$P<0.05$, paired Student's t-test, compared with increases observed in control PMNs.

[c]NT, not tested.

We examined the inhibitory effects of the PAF antagonists on ionophore A23187-induced O_2^- generation to check the specificity of these compounds. A23187 is considered to circumvent early steps in the signal transduction pathway by directly elevating intracellular Ca^{2+} and subsequently protein kinase C activity (19). In contrast to inhibition of fMLP responses, A23187-induced O_2^- generation was unaffected by the PAF receptor antagonists (Table 1).

Following 5 min of exposure to fMLP (10 nM), PAF was detected in cell extracts (0.024 ± 0.013 pmol/10^6 cells) and in supernatants (0.021 ± 0.012 pmol/10^6 cells). Thus, it was possible that PAF released during stimulation of PMNs by fMLP could augment the O_2^- response to fMLP. However, PAF (0.1 μM) alone elicited a barely detectable increase in O_2^- generation (0.26 ± 0.08 nmol/10^6 cells, n = 7) which was completely prevented by WEB 2086 (10 μM) or CV6209 (1 μM). Furthermore, we examined the possibility that PAF release early in the fMLP response could enhance the subsequent fMLP stimulation of O_2^- generation. However, PAF (0.1-1,000 nM) pretreatment of PMNs for 5 min before exposure to fMLP failed to augment the ensuing O_2^- response (n = 2, data not shown).

The activity of phospholipase A_2 appears to be rate-limiting for the formation of PAF. Thus, we used the inhibitor of phospholipase A_2, chloroquine, to reduce PAF formation induced by fMLP and then examined the generation of O_2^-. Chloroquine (10 and 100 μM) pretreatment produced a significant ($P<0.05$, ANOVA) inhibition of the O_2^- generation in response to fMLP, whereas only the higher concentration (100 μM) inhibited the response to PMA (Fig. 1). Parallel experiments confirmed that chloroquine inhibited the generation of PAF by fMLP (10 nM)-stimulated PMNs (% inhibition 10 μM, 58 ± 24; 100 μM, 95 ± 5). Recent studies indicate that serine proteases contribute to the regulation of enzymes involved in PAF synthesis. Pretreatment of PMNs with an inhibitor of serine proteases, TPCK (10 μM), caused the expected reduction in the generation of PAF in response to fMLP (Table 2). In addition, fMLP-induced O_2^- generation was inhibited by TPCK (Table 2), whereas the response to the activator of protein kinase C, phorbol myristate acetate (PMA, 0.3 nM), was unaffected (Table 2).

Chloroquine reduces PAF generation by inhibition of phospholipase A_2, probably by interacting with the substrate (11). Thus, this compound would be expected to reduce both the fMLP-induced mobilization of arachidonic acid from membrane phospholipids and its subsequent oxygenation to products implicated in PMN signal transduction processes (1-3). We confirmed that chloroquine reduced the release of [^{14}C]arachidonic acid (Table 3). It is interesting to note that chloroquine pretreatment gave only a marginal inhibition of A23187-induced [^{14}C]arachidonic acid release from PMNs (Table 3).

The possible importance of cyclooxygenase or 5-lipoxygenase products in the fMLP-stimulated O_2^- generation

TABLE 3

Mobilization of [^{14}C]Arachidonic Acid by Pre-labelled PMNs (n = 5)

Stimulus	dpm × 10^{-3} Control	[^{14}C]arachidonic acid release[a] (% control) WEB 2086 (μM) 10	WEB 2086 (μM) 100	CV 6209 (μM) 1	CV 6209 (μM) 10	Chloroquine (μM) 300
basal	[b]	103 ± 2	95 ± 4	101 ± 2	107 ± 2	99 ± 2
PAF 0.1 μM	0.44 ± 0.12[c]	7 ± 7[d]	25 ± 15[d]	30 ± 21[d]	8 ± 5[d]	13 ± 7[d]
fMLP 1 μM	1.09 ± 0.32[c]	83 ± 5[d]	69 ± 5[d]	73 ± 4[d]	37 ± 10[d]	13 ± 3[d]
A23187 10 μM	3.30 ± 0.70[c]	99 ± 2	100 ± 5	100 ± 1	88 ± 4[d]	74 ± 4[d]

[a]Data are presented as the means ± standard errors of the means and represent the release of [^{14}C]arachidonic acid in the presence of antagonists expressed as a percentage of that in the absence of antagonists (*i.e.*, control).

[b]Basal release of [^{14}C]arachidonic acid was 2.27 ± 1.02 (dpm × 10^{-3}).

[c]P<0.05 paired Student's t-test, compared with basal [^{14}C]arachidonic acid release.

[d]P<0.05 paired Student's t-test, compared with control (*i.e.*, 100%).

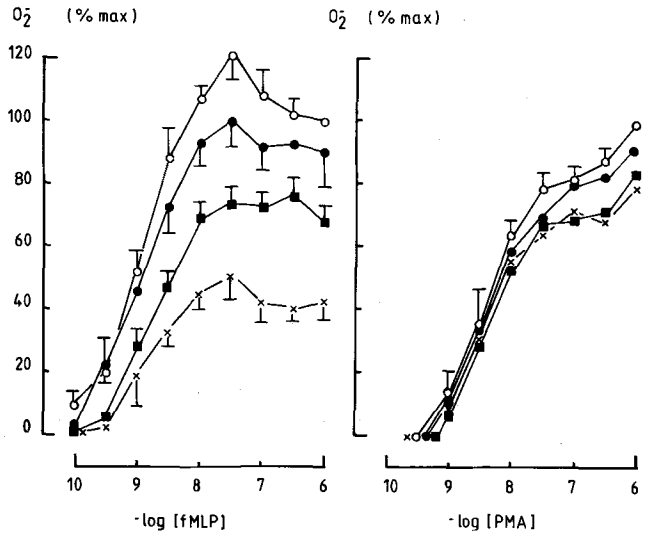


FIG. 2. The effects of pre-incubation of the cells with indomethacin (2.8 μM, filled squares), CGS 8515 (1 μM, filled circles) or their combination (crosses) on O_2^- generation induced by fMLP and PMA (control responses, open circles) have been assessed. Data are expressed as percentages of the respective maximum control (non-pretreated cells) responses to 1 μM of the stimulus (fMLP or PMA). Standard error bars for PMA experiments have been omitted for the sake of clarity (n = 5–7).

was investigated using pretreatment with either indomethacin (2.8 μM), CGS 8515 (1 μM) or their combination (Fig. 2). The 5-lipoxygenase inhibitor, CGS 8515, had no significant effects on O_2^- generation stimulated by either fMLP or PMA. On the other hand, the cyclooxygenase inhibitor, indomethacin, reduced fMLP and, to a lesser extent, PMA-induced O_2^- generation. Furthermore, the combination of these inhibitors produced greater inhibition of fMLP responses than indomethacin alone. Effective inhibition of 5-lipoxygenase by CGS 8515 was confirmed by examining its effect on immunoreactive LTB_4 generation by fMLP-stimulated PMNs (control 22.2 ± 5.2 pmol/10^6 cells, n = 6; CGS 8515 1 μM, 1.4 ± 0.9 pmol/10^6 cells, n = 6; P<0.05, paired Student's t-test).

Our previous studies in guinea-pig peritoneal macrophages suggested that endogenous PAF may have a role in the mobilization of arachidonic acid in response to receptor-operated stimuli (7–9). To examine whether PAF may play a similar role in PMNs, the PAF receptor antagonists, WEB 2086 and CV 6209, were incubated with PMNs for 15 min before stimulation with fMLP. The fMLP-induced mobilization of pre-incorporated [^{14}C]arachidonic acid was inhibited by WEB 2086 or CV 6209 as was [^{14}C]arachidonic acid release induced by PAF (Table 3). In contrast, A23187-induced mobilization of [^{14}C]arachidonic acid was not inhibited by these compounds indicating that phospholipase A_2 was not non-specifically inhibited.

DISCUSSION

Our findings suggest that PAF may contribute to the signal transduction cascade initiated by fMLP in rabbit PMNs. Several independent lines of evidence support this proposal. Firstly, the structurally distinct PAF receptor antagonists, WEB 2086 and CV 6209, reduced both arachidonic acid mobilization and O_2^- generation in response to fMLP. Secondly, we observed a reduction in O_2^- generation following pretreatment with the serine protease inhibitor, TPCK (12), and the phospholipase A_2 inhibitor, chloroquine (11), which concomitantly reduced the generation of PAF by fMLP-stimulated PMNs. Finally, in our previous studies, fMLP stimulated PMNs to generate PAF with a time course parallel to that of arachidonic acid mobilization and leukotriene B_4 (LTB_4) generation (9).

The specificity of the PAF antagonists has been established in detailed studies of platelet aggregation (20,21). In this study, evidence for a specific action is suggested by the differential inhibition of both PAF and fMLP-induced responses compared with those induced by the ionophore A23187. Inhibition of A23187-induced

O_2^- generation and arachidonic acid mobilization by PAF receptor antagonists could have been expected since A23187 is an effective stimulus for PAF generation (9,12,13,22). However, A23187 induces a large and persistent increase in cytosolic Ca^{2+} (23) and may activate protein kinase C (19). These combined actions appear to be sufficient stimuli for mobilization of arachidonic acid and generation of O_2^-, irrespective of the concomitant generation of PAF. The failure of the PAF receptor antagonists to reduce either arachidonic acid mobilization or O_2^- generation in response to A23187 indicates that these compounds do not inhibit non-specifically phospholipase A_2 or reduced nicotinamide adenine dinucleotide phosphate (NADPH) oxidase, or act as free radical scavengers (9).

Investigation of the role of PAF, particularly that of intracellular PAF, has been hampered by a lack of specific PAF synthesis inhibitors. Nevertheless, several compounds which reduce PAF generation have been described (11,24). PAF synthesis by PMNs is considered to involve activation of phopholipase A_2 and acetyl transferase (22) although non-physiological stimuli such as A23187 and the phorbol ester PMA may also activate the cholinephosphotransferase pathway (19,24). In our study, inhibition of phopholipase A_2 using chloroquine reduced both PAF synthesis and the mobilization of arachidonic acid. Furthermore, chloroquine concentration-dependently reduced O_2^- generation induced by fMLP, but inhibited responses to PMA to a lesser extent. The serine protease inhibitor, TPCK (12), reduced both PAF synthesis and O_2^- generation induced by fMLP, supporting our suggestion that PAF may be an important component of the signal transduction cascade.

The interpretation of the actions of chloroquine does not exclude a role of arachidonic acid itself, or its oxygenated products, both of which have received some consideration as second messengers (1–3). We examined a possible contribution of 5-lipoxygenase products in the activation of O_2^- generation using the selective inhibitor, CGS 8515 (14). An effective concentration of this compound had little effect on O_2^- generation stimulated by either fMLP or PMA, but it appeared to enhance the small inhibitory effect of indomethacin. These data do not support an important role for 5-lipoxygenase products in rabbit PMN signal transduction. It has been suggested that the actions of indomethacin on PMN function, including inhibition of aggregation and NADPH oxidase (25), may be unrelated to cyclooxygenase inhibition.

An involvement of arachidonic acid in the signal transduction cascade does not preclude a role for endogenous PAF, since PAF appears to be involved in arachidonic acid mobilization (7–10) and may therefore represent an earlier component of the transduction process.

We have previously proposed that PAF acts intracellularly to initiate mobilization of arachidonic acid in endothelial cells (10) and macrophages (8). These cells retain PAF intracellularly following activation by stimuli such as fMLP and bradykinin. However, in this and other studies (26), PMNs released a significant proportion of the PAF generated in response to fMLP. In contrast, Lynch and Henson (27) reported that rabbit PMNs release PAF only following pretreatment with cytochalasin B. This difference may be explained by the relatively insensitive assay ([^{3}H]serotonin release) used in the latter study. An effect of the released PAF in the generation of O_2^- seems unlikely, since even high concentrations of exogenous PAF (up to 0.1 μM) produced only 10% of the response to fMLP. Furthermore, although low concentrations of PAF are reported to prime human PMNs for enhanced O_2^- generation (28), we were unable to detect a similar enhancement of responses in rabbit PMNs. In parallel experiments, the possibility that the PMNs had already been primed by exposure to reagents containing endotoxin was excluded since low concentrations of added endotoxin (1–10 ng/mL) were able to prime PMNs (Stewart and Harris, unpublished observations). Thus, our findings support an intracellular rather than an extracellular site of action for PAF in rabbit PMNs.

Previous speculations on the role of intracelluar PAF include a possible receptor-independent regulation of membrane fluidity and expression of receptors (27). Our data with PAF receptor antagonists suggest that intracellular PAF may act by receptor stimulation. However, the possibility that PAF receptor antagonists inhibit PAF formation cannot be excluded. Indeed, recent evidence suggests that PAF stimulates its own synthesis in human PMNs, and PAF receptor antagonists inhibit this autogeneration (29). The possibility that PAF formed in response to unrelated stimuli such as fMLP could elicit autogeneration has yet to be explored.

In an early study, PAF release was shown to occur later than either degranulation or O_2^- generation in response to PMN activation, and the amount of PAF released did not correlate with either functional response (30) suggesting to these authors that PAF was not causally related to these responses. However, it is now clear that a significant proportion of the PAF synthesized by PMNs is retained and its formation precedes release (27). More recently, the synthesis of intracellular PAF has been temporally linked to the increase in PMN adhesion induced by A23187 and fMLP (26). Nevertheless, the lack of PAF synthesis in response to a number of stimuli known to increase adhesion (PMA, LTB_4, endotoxin, tumor necrosis factor α, platelet-derived growth factor) led these authors to suggest that PAF was not important for adhesion responses. This interpretation does not allow for the possibility that there are several signal transduction processes which may operate independently. Furthermore, we have confirmed the observation by Nieto *et al.* (24) that PMA is a potent stimulus for PAF formation in human PMNs (Stewart and Harris, unpublished observations). Both endotoxin (28) and tumor necrosis factor α (13) also elicit generation of measurable levels of PAF as detected by the platelet aggregation assay for PAF. Thus, the detection limit of the [^{3}H]acetate labelling assay may be too insensitive to detect PAF formed by these stimuli. Alternatively, the latter stimuli may use the cholinephosphotransferase pathway which may not be readily detected by [^{3}H]acetate labelling. Irrespective of the reason for these discrepancies, there is no unequivocal evidence to suggest that these stimuli for increased PMN adhesion do not produce PAF.

In conclusion, the data from these studies support a role for endogenous PAF in the mobilization of arachidonic acid and the generation of O_2^- by fMLP-stimulated rabbit neutrophils. The mechanism of action of endogenous

PAF is not clear, but appears to involve intra- rather than extracellular effects.

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Inhibition by the PAF Antagonist WEB 2086 of PAF Induced Inositol-1,4,5-trisphosphate Production in Human Platelets[1]

F.W. Birke* and **H.A. Ensinger**
Boehringer Ingelheim KG, Department of Biochemistry, D-6507 Ingelheim, Germany

Platelet-activating factor (PAF) activates human platelets by binding to a putative PAF receptor which evokes the rapid formation of inositol-1,4,5-trisphosphate (IP_3) by phospholipase C mediated phosphatidylinositol-4,5-bisphosphate (PIP_2) hydrolysis. Stimulation of [^{3}H]inositol-labeled human platelets by PAF (1 nM–1μM) resulted in a concentration-dependent increase of intracellular IP_3, IP_2 and inositolmonophosphate (IP_1). IP_1 levels increased up to three-fold upon maximum stimulation by 100 nM PAF. The EC_{50} concentration for PAF was 1.2 ± 0.3 nM. Addition of the hetrazepinoic PAF antagonist, WEB 2086, inhibited PAF stimulated hydrolysis of PIP_2 in a dose-dependent manner. WEB 2086 (100 μM) blocked inositol-1,4,5-trisphosphate formation down to baseline levels (IC_{50}=33 ± 12 μM WEB 2086). In thrombin and ADP stimulated platelets, inositol phosphate (IP) generation was not influenced by WEB 2086. It is concluded that WEB 2086 selectively antagonizes PAF-induced increases in IP and does not interfere directly with intracellular signal transduction. Instead, WEB 2086, which has been shown to bind specifically and with high affinity (K_i 15 nM) to human platelets, acts as a competitive antagonist at the PAF receptor level.
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Platelet activating factor (PAF, 1-*O*-alkyl-2-acetyl-*sn*-glycero-3-phosphocholine) is a potent phospholipid mediator which is synthesized by various cells and which is able to promote a number of biological effects including platelet activation, bronchoconstriction and hypotension (1). Induction of platelet activation by PAF is mediated by specific binding to a PAF receptor (2). PAF binding seems to exert its effects through guanine nucleotide binding (G) proteins. PAF induced effects on platelet adenylate cyclase are reported to be mediated by G_i (3). However, Houslay *et al.* (4) found that platelet GTPase activity associated with PAF stimulation was only slightly altered by treatment with pertussis toxin. They concluded that no G_i, but a different G protein named G_p, may be the predominant guanine nucleotide binding protein mediating the PAF signal. PAF binding was shown to be closely associated with the rapid cleavage of phosphodiester bonds in inositol phospholipids, especially phosphatidylinositol-4,5-bisphosphate (PIP_2) (5). The reaction, which involves phospholipase C cleavage, is thought to be mediated by G_p proteins and leads to the release of inositol-1,4,5-trisphosphate (IP_3). This second messenger IP_3 was shown to mobilize Ca^{2+} from intracellular stores of numerous cells, including platelets (6).

Two other platelet activators, thrombin and adenosine diphosphate (ADP), served as controls for PAF stimulation. ADP triggers platelet aggregation and is involved in amplifying the platelet response to other agonists such as thrombin or PAF (7).

Activation of platelets by thrombin and ADP has been shown to be mediated by activation of phospholipase C as is PAF stimulation, and unlike epinephrine activation, which involves the adenylate cyclase system. This process is independent of and dissociated from arachidonic acid metabolism (8). Furthermore, PAF and thrombin use the same pool of phosphatidylinositol-4,5-bisphosphate in rabbit platelets (9). Thrombin pretreatment evokes the phenomenon of desensitization both for thrombin and PAF stimulation (10). Therefore, thrombin and ADP stimulation seem to be relevant controls for PAF stimulation of human platelets.

WEB 2086 is a well characterized PAF antagonist with a hetrazepinoic structure. WEB 2086 was demonstrated to inhibit PAF induced effects *in vitro* and *in vivo* with high potency and specificity (11,12). WEB 2086 was shown to inhibit PAF binding to the PAF receptor in a competitive and specific manner in human platelets (13,14), neutrophils (15) and in eosinophils (16).

In the present study we examined whether WEB 2086, in addition to its PAF receptor antagonistic properties, has direct effects on the G protein/phospholipase C complex.

MATERIALS AND METHODS

Platelet activating factor (PAF, AGEPC, 1-*O*-alkyl-2-acetyl-*sn*-glycero-3-phosphocholine) was supplied as C_{16}PAF by Serva (Heidelberg, Germany). Human thrombin with 30 IU/mL activity was from Behring (Marburg, Germany). Myo-[2-^{3}H]inositol (spec. act. 3.3 TBq/mmol) was purchased from Amersham Buchler (Braunschweig, Germany). Dowex resins (1-X8, 200–400 mesh, pa.) and prostacyclin were obtained from Serva. All other chemicals and solvents used were supplied by Merck (Darmstadt, Germany) in the highest analytical grade available. WEB 2086, which is 3-[4-(-2-chlorophenyl)-9-methyl-6H-thieno[3,2-f][1,2,4]triazolo[4,3-a][1,4]diazepine-2-yl]-1-(4-morpholinyl)-1-propanone, was synthesized in the Department of Chemistry at Boehringer Ingelheim.

Isolation of human platelets. Blood was freshly drawn from healthy volunteers. Citrated blood (200 mL) was centrifuged at room temperature for 15 min at 160 × *g* for preparation of platelet rich plasma. Then the platelets were centrifuged at 800 × *g* for 10 min.

[^{3}H]Inositol labeling of platelets. The pellet of a 800 × *g*/10 min centrifugation, containing about 2 × 10^{10} platelets, was resuspended in 5 mL labeling buffer, pH 7.4 (20 mM Hepes, 137 mM NaCl, 2.68 mM KCl, 1 mM $MgCl_2$, 1 mM EGTA, 11.9 mM $NaHCO_3$, 0.42 mM NaH_2PO_4, and 5.55 mM glucose). [^{3}H]Inositol (18.5 MBq) was added and the mixture was incubated for 2 hr

[1]Based on a paper presented at the Third International Conference on Platelet-Activating Factor and Structurally Related Alkyl Ether Lipids, Tokyo, Japan, May 1989.

*To whom correspondence should be addressed.

Abbreviations: ADP, adenosine diphosphate; DAG, diacylglycerol; IP_3, inositol-1,4,5-trisphosphate; IP, inositol phosphates; PAF, platelet-activating factor, 1-*O*-alkyl-2-acetyl-*sn*-glycero-3-phosphocholine; PIP_2, phosphatidylinositol-4,5-bisphosphate; PKC, protein kinase C; PLC, phospholipase C.

at 37°C. The labeled platelets were washed twice by centrifugation (800 × *g*, 10 min) in labeling buffer supplemented with myoinositol (5 mmol/L), heparin (5000 U/L) and PGI_2 (0.25 nM).

Stimulation of labeled platelets. The resulting pellet was resuspended in assay buffer [labeling buffer supplemented with 0.1 mM $CaCl_2$ and 0.1% bovine serum albumin (BSA)] and used immediately for platelet stimulation. Platelets (10^9) were incubated in 1 mL final volume. The mixture contained PAF (concentrations as indicated), or thrombin (0.05 U/assay) or ADP (1 μM), and, additionally, the PAF antagonist WEB 2086 in inhibition experiments. Viability of labeled platelets was tested by monitoring thrombin and PAF induced aggregation.

LiCl (10 mM) was added 5 min before the start of incubations. WEB 2086 (100 μL), dissolved in ethanol/water (1:1, v/v) was added 10 min before the start of incubation (10 min, 37°C) by adding the agonist. After stimulation, the tubes were sealed and shaken vigorously.

Analysis of IP-species was performed according to Berridge *et al.* (17) with some modifications. The incubation was stopped by adding 2 mL of methanol/chloroform (1:1, v/v). For better separation of the layers, an additional 700 μL of both H_2O and $CHCl_3$ was added, followed by centrifugation for 15 min at 3500 rpm. The aqueous layer was removed for anion exchange chromatography on columns filled with Dowex 1-X8 slurry (1:1, v/v, with H_2O; 1 mL Dowex slurry per column). Two mL of aqueous phase was loaded on one column and washed once with 2 mL H_2O and twice with 1.0 mL of 5 mM Na tetraborate in 60 mM ammonium formate. Inositol phosphate (IP) species were eluted in three steps. The first step used 1 mL followed by twice 0.5 mL of 5 mM Na tetraborate in 150 mM ammonium formate. The second step used 2 mL of 0.1 mM formic acid in 400 mM ammonium formate; and the final step used 2 mL of 0.1 M formic acid in 1 M ammonium formate. Ten mL of scintillation fluid (Quickszint 2000, Zinsser, Frankfurt, Germany) per 0.5 mL eluent were added and radioactivity was quantified in a liquid scintillation counter.

Data are presented as mean ± S.E. of individual experiments from different blood donors as indicated. Statistical significance was calculated by paired Student's *t*-test.

RESULTS

Stimulation of human platelets by PAF resulted in a concentration dependent increase of intracellular concentrations of IP_3, IP_2 and IP_1.

In Figure 1, a typical experiment of PAF (0.3–100 nM) induced IP_1 production is shown. PAF at 1 μM did not increase IP_1 production further. The line was calculated by means of an iterative program of "coupled mass equilibria" using a one-compartment model (18). The effective PAF concentration for half the maximum IP_1 production was EC_{50} 1.2 ± 0.3 nM (mean of 4 experiments ± SEM), with a maximum IP_1 production of up to 2.5 fold the IP_1 basal levels.

Figure 2 shows the relative increases in the IP species IP_3, IP_2 and IP_1 10 min after 100 nM PAF stimulation. Due to the inhibition of IP_1 metabolism by 10 mM lithium and the incubation time of 10 min, IP_1 was predominantly formed. Therefore, IP_1 levels were used as an indicator of IP_3 production throughout the results reported. PAF (100 nM) stimulated IP_1 concentration to 167 ± 47% of control (mean of 9 experiments ± SEM).

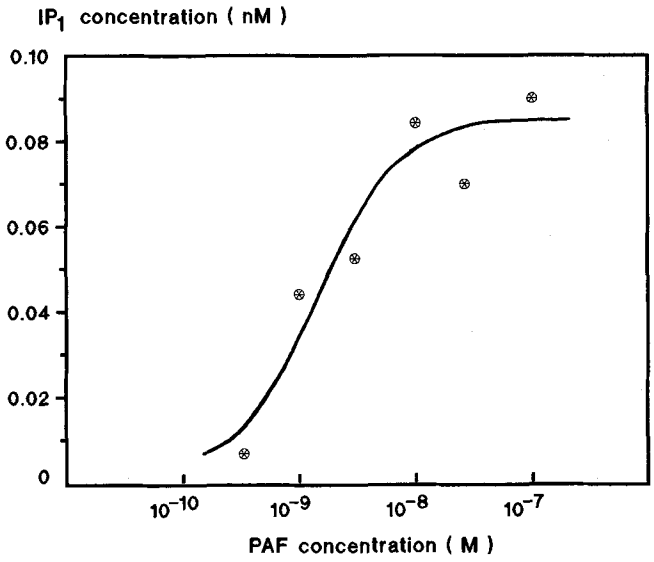


FIG. 1. Stimulation of IP_1 generation by PAF in human platelets. [^{3}H]Inositol-labeled human platelets (10^9/mL) were stimulated with increasing concentrations of PAF (0.3–100 mM). Inositol phosphates were extracted and fractionated by Dowex 1-X8 ion exchange chromatography. IP_1 concentration was calculated from the radioactivity of the IP_1 peak. Each point represents the mean of duplicate determinations in a typical experiment. The line was calculated by the program "coupled mass equilibria".

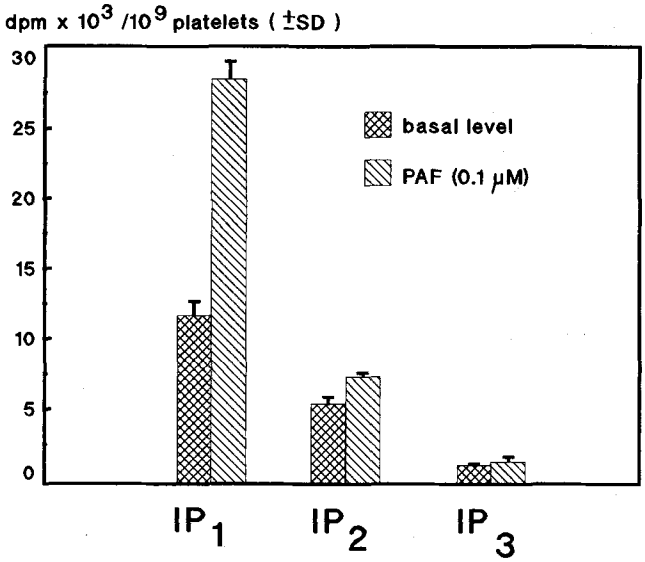


FIG. 2. Levels of the inositolphosphates IP_1, IP_2 and IP_3 induced by PAF in human platelets. Increase in inositol-1-phosphate (IP_1), inositol-1,4-bisphosphate (IP_2) and inositol-1,4,5-trisphosphate (IP_3) determined 10 min after 0.1 μM PAF stimulation in the presence of Li^+. The bars represent the mean of duplicate determinations ± S.E. in a typical experiment.

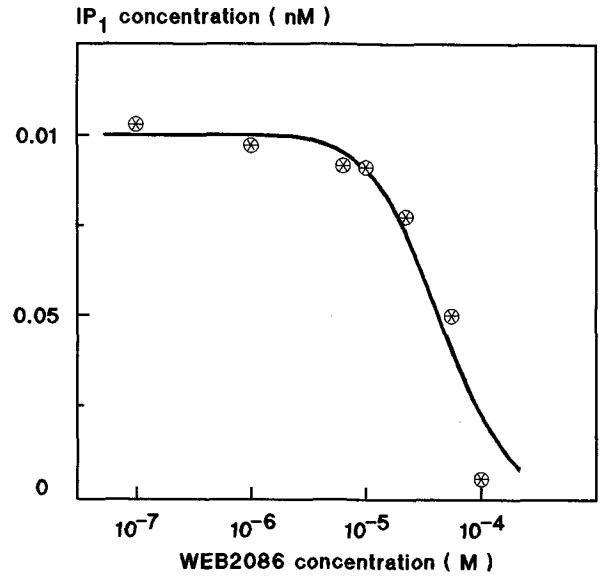


FIG. 3. Inhibition of PAF stimulated IP_1 production by WEB 2086 (0.1-100 μM). Each point of the WEB 2086 inhibition curve of 100 nM PAF stimulated IP_1 production represents the mean of triplicate determinations with SD less than 15%. WEB 2086 (100 μM) suppressed IP_1 generation to control levels. The line was fitted by the program "coupled mass equilibria". Considering the EC_{50} of 1.2 nM for PAF in a one-compartment model, a K_i value of 620 nM for WEB 2086 inhibition was calculated.

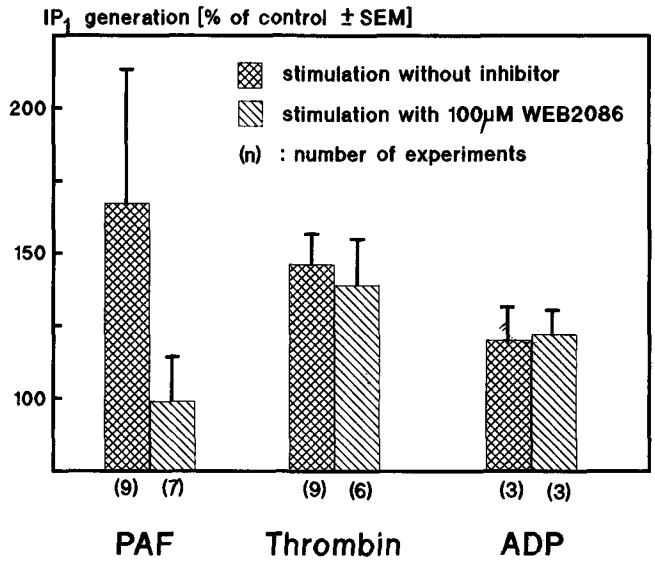


FIG. 4. Selectivity of WEB 2086 for PAF induced IP_1 generation in human platelets. WEB 2086 significantly inhibits PAF (100 nM) stimulated IP_1 generation ($p<0.01$), while thrombin stimulation (0.05 IU/mL) and ADP stimulation (1 μM) are not influenced. Baseline IP_1 levels (control) were set at 100% of IP_1 production.

Addition of the hetrazepinoic PAF antagonist WEB 2086 inhibited PAF induced IP_1 generation in a dose-dependent manner. WEB 2086 (100 μM) significantly blocked PAF mediated PIP_2 turnover of PLC, lowering IP_1 to the basal 100% level of controls (99 ± 16% of control, n=7, $p<0.05$).

In Figure 3, the inhibition of IP_1 production by increasing concentrations of WEB 2086 (1 μM-100 μM) is demonstrated in a typical experiment. Each point represents the mean of three independent determinations. The line was calculated by iterative fitting of data with a one-compartment model ("coupled mass equilibria" program). The concentration of WEB 2086 for half the maximum inhibition (IC_{50}) of IP_1 formation was 32 ± 12 μM (mean ± SEM, n=3). This corresponds to a calculated K_i value of 0.62 ± 0.23 μM for WEB 2086 inhibition, taking into consideration the intrinsic activity of PAF (EC_{50} 1.2 nM) in this system.

If WEB 2086 acts by binding selectively to the PAF receptor, platelet activation as measured by IP_3 production should not be influenced through mediators other than PAF using the same signaling pathway. Therefore, platelets were activated by thrombin and ADP (see Fig. 4). Using 1 μM ADP, only minor responses in IP_1 production were found in about half of the experiments (120 ± 11% of control, n=3). WEB 2086 (100 μM) did not influence this weak ADP stimulation (122 ± 9% of control, n=3).

In contrast to ADP, thrombin (0.05 IU/mL) consistently activated human platelet IP_1 production (146 ± 11% of control, n=9) to a degree comparable to 100 nM PAF stimulation. However, in contrast to PAF stimulation, thrombin stimulation of IP_1 generation was not changed by 100 μM WEB 2086 (139 ± 16% of control, n=6).

DISCUSSION

The results presented here confirm previous reports (for a review, see ref. 19) demonstrating the increase in intracellular inositol phosphate species (IP_1, IP_2, IP_3) after PAF stimulation of human platelets. IP generation reflects the activation of phospholipase C triggered by PAF binding to the PAF receptor and does not seem to be mediated by additional mediators (8).

The inositol-1,4,5-trisphosphate (IP_3) releasing effect of PAF was dose-dependently inhibited by WEB 2086. The relatively low potency of WEB 2086 (IC_{50} 32 μM) found for inhibition of PAF induced IP_1 generation, when compared with the K_i = 15 nM for [^{3}H]PAF displacement from the PAF receptor, may be due to the relatively high concentration of PAF (100 nM) used to assure maximum IP stimulation. Considering the high activity of PAF (EC_{50} 1.2 nM), however, a K_i value of 620 nM was calculated for WEB 2086 inhibition, which is only one order of magnitude higher than the inhibition constant derived from binding data. This difference is also found for the agonistic PAF comparing the EC_{50} 1.2 nM from platelet activation with K_D 0.07 nM from binding experiments (13). This may reflect the efficacy of coupling of receptor occupation with second messenger, *i.e.*, IP_3 production *via* G proteins. The inhibitory effect of WEB 2086 seems to be specific for PAF, since WEB 2086 did not influence thrombin or ADP stimulated IP production. Thrombin has been reported to use a similar transmembrane signalling pathway like PAF, as demonstrated by the heterologous desensitization of PAF stimulation by thrombin pretreatment (20). Additionally, thrombin seems

to use the same pool of PIP_2 as does PAF (9). Thus, when PAF and thrombin are using the same second messenger pathway, WEB 2086 specificity must be attributed to its specific interaction with the PAF receptor.

Further support for the specificity of WEB 2086 for PAF induced platelet activation is provided by *in vitro* platelet aggregation, where aggregation induced by thrombin, ADP, collagen or serotonin was not inhibited by WEB 2086 (11). Thus, we conclude that WEB 2086 inhibits PAF action at the level of the PAF receptor. This view is strongly supported by the direct binding experiments with [^{3}H]WEB 2086 (13,14) demonstrating high affinity (K_i 15 nM) and competitive binding of [^{3}H]WEB 2086 to PAF receptor sites on human platelets.

Additional support is provided by the report of Shukla (21), demonstrating the specific effects of the PAF antagonist CV 3988 on PAF induced IP_1, IP_2 and IP_3 generation in rabbit platelets. Simon *et al.* (22) reported similar effects of the PAF antagonist BN 52021 in human platelets. They, however, measured phosphatidic acid production by ^{32}P-labeling instead of IP generation. Phosphatidic acid is formed from diacylglycerol (DAG), which (like IP_3) is the product of PLC action on PIP_2. DAG is thought to activate protein kinase C(PKC) (23). A large proportion of diacylglycerol formed upon PLC action is immediately converted to phosphatidic acid. However, unwanted incorporation of ^{32}P into ATP and then into phosphatidic acid during the labeling procedure as described by Simon *et al.* (22) seems possible. These processes may produce [^{32}P]phosphatidic acid in addition to phosphorylation of the DAG arising from hydrolysis of phosphoinositides. There may also be competing fates of diacylglycerol disturbing its correlation to PLC activity.

Recent observations reported by Uhing *et al.* (24), using murine peritoneal macrophages, suggest that PAF can stimulate the accumulation of DAG derived from sources other than phosphatidylinositol and that this occurs subsequently to the activation of protein kinase C. This additional effect of PAF further complicates the interpretation of phosphatidic acid levels as a measure of PLC activation described by Simon *et al.* (22).

Thus, WEB 2086 is the first PAF antagonist reported to inhibit human platelet IP production as monitored by direct measurement of IP levels after PAF challenge.

In a recent paper by Barzaghi *et al.* (25), PAF induced production of phosphoinositols in U937 cells was shown to be inhibited by the PAF receptor antagonist CV 3988. This demonstrates that CV 3988, like WEB 2086, interacts with a PAF receptor in an example of a nucleated human cell.

The possible clinical relevance of PAF receptor blockage on human platelets is high-lighted by results obtained by Block *et al.* (26) which demonstrated increased PAF sensitivity in platelets of asthmatic patients as measured by a pronounced increase in phosphatidylinositol turnover in response to PAF. This finding, among others (for a review, see ref. 27) suggests that PAF plays a role in asthma and that PAF antagonists (like WEB 2086) may be useful in the therapy of this and other PAF dependent diseases.

In conclusion, it appears that WEB 2086 selectively inhibits the signal transduction evoked by PAF stimulation as measured at the level of phospholipase C. This inhibition of PAF stimulated PIP_2hydrolysis, but not thrombin- and ADP-stimulated PIP_2hydrolysis, supports PAF receptor antagonism as the mechanism of action of WEB 2086. WEB 2086 does not directly interfere with the G protein/phospholipase C mediated signaling pathway of platelet activation. WEB 2086 thus promises to represent a useful tool for investigating the role of PAF in physiology and pathophysiology.

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Phorbol Ester Stimulates PAF Synthesis *via* the Activation of Protein Kinase C in Rat Leukocytes[1]

Masahiko Hayashi, Yohsuke Imai and Sachiko Oh-ishi*
Department of Pharmacology, School of Pharmaceutical Sciences, Kitasato University, Tokyo 108, Japan

When rat pleural mononuclear leukocytes were stimulated with 1 μM phorbol myristate acetate (PMA), platelet-activating factor (PAF)-like activity was detected in the supernatant and the cellular fractions of the incubation mixture, as measured by rabbit platelet aggregation. C_{16}PAF activity peaked at 30 min in both fractions. Acetyltransferase activity in the microsomal fraction of the stimulated cells also increased rapidly and showed a peak at 10 min. A protein kinase C inhibitor, staurosporine, and an inhibitor of phospholipase A_2, *p*-bromophenacylbromide, inhibited stimulated PAF formation in both fractions. Staurosporine also inhibited PMA induced acetyltransferase activity. The data suggest that PMA stimulates PAF synthesis by the remodeling pathway in rat pleural cells through activation of both phospholipase A_2 and acetyltransferase, and that the acetyltransferase, in turn, may be activated through activation of protein kinase C.
***Lipids 26,* 1054–1059 (1991).**

Platelet-activating factor (PAF), chemically identified as 1-*O*-alkyl-2-acetyl-*sn*-glycero-3-phosphocholine (1–3), was shown to be secreted from antigen-stimulated, IgE-sensitized basophils (4). PAF is also formed by various tissues, and by inflammatory cells such as polymorphonuclear neutrophils and monocytes (for reviews, see refs. 5,6). PAF exerts a variety of biological effects, including increased vascular permeability (7), hypotension (3), bronchoconstriction (8), platelet aggregation (4) and leukocyte activation (6,9). These activities are closely related to inflammatory and allergic reactions.

In a previous study (10) on rat pleurisy induced by phorbol myristate acetate (PMA), we found that a specific PAF antagonist, CV-3988, suppressed pleural exudation. In addition, PAF-like activity was observed in the exudate. Both these observations suggested that PAF may be involved in this type of rat pleurisy.

In the present study, the activation of PAF synthesizing enzymes in intact cells, which were stimulated with tumor-promoting phorbol ester, was examined. We found that stimulation of PAF formation by PMA occurs through the activation of acetyltransferase and that this change in activity most likely results from the phosphorylation of the enzyme by protein kinase C.

MATERIALS AND METHODS

Animals. Male Sprague-Dawley rats (7–8 weeks-old, Japan SLC, Hamamatsu, Japan) were used for the isolation of pleural cells, and male Japanese white rabbits (Doken, Ibaragi, Japan) were used for the preparation of washed platelets for PAF assay.

Agents. 1-*O*-Hexadecyl/octadecyl-2-acetyl-*sn*-glycero-3-phosphocholine (C_{16}/C_{18}PAF, Bachem Feinchemikalien, Bubendorf, Switzerland) were gifts from Funakoshi Pharmaceutical Co. (Tokyo, Japan). CV-3988 (*rac*-3-(*N*-octadecylcarbamoyloxy)-2-methoxypropyl-2-thiazolioethyl phosphate) and AA-861 (2-(12-hydroxydodeca-5,10-diynyl)-3,5,6-trimethyl-1,4-benzoquinone) were kindly supplied by Takeda Chemical Industries. Ltd. (Osaka, Japan). Diltiazem, (3-(acetyloxy)-5-[2-(dimethylamino)-ethyl]-2,3-dihydro-2-(4-methoxyphenyl)-1,5-benzothiazepin-4(5H)-one hydrochloride) was a gift from Tanabe Pharmaceutical Co. (Osaka, Japan). Staurosporine was a gift from Dr. S. Ōmura, Kitasato Institute, Tokyo, Japan. Indomethacin, phorbol myristate acetate (PMA), phorbol-12,13-didecanoate (PDD), acetyl-CoA, adenosinetriphosphate (ATP), diolein (DG), alkaline phosphatase (from *E. coli.*, Type III-S), C_{16}lysoPAF and phospholipase A_2 (*Naja naja* venom) were purchased from Sigma Chemical Co. (St. Louis, MO). Phosphatidylcholine (PC), lysophosphatidylcholine (LPC) and sphingomyelin (SM) (Serdary Laboratories, London, Ontario, Canada) were from Funakoshi Pharmaceutical Co., and W-7 and H-7 from Seikagaku Industries (Tokyo, Japan). [^{3}H]C_{16}PAF (40 Ci/mmol) and [^{3}H]acetyl-CoA (3.7 Ci/mmol) were from New England Nuclear (Boston, MA) and Amersham Japan (Tokyo, Japan), respectively. *p*-Bromophenacylbromide (BPB) and the lactate dehydrogenase (LDH) ultraviolet (UV) test kit were from Tokyo Kasei (Tokyo, Japan) and Boehringer-Mannheim (Mannheim, Germany), respectively.

Preparation of pleural mononuclear cells. Rats under light ether anesthesia were killed by exsanguination, and pleural cells were collected by washing the pleural cavity twice, each time with 7 mL of Ca^{2+}/Mg^{2+}-free Hanks balanced salt solution (HBSS(−)). Collected cells were pooled, and washed twice with HBSS (−). The cell suspension (4 mL) was applied onto a Ficoll-Paque gradient (Pharmacia, Uppsala, Sweden) (2 mL of 90% Ficoll-Paque diluted with saline and 2 mL of Ficoll-Paque). After centrifugation at 450 × *g* for 25 min at 22°C, the mononuclear cell fraction was found at the 90–100% Ficoll-Paque interface. This fraction contained more than 90% mononuclear cells with better than 95% viability as judged by trypan blue exclusion. The cells were washed twice and resuspended in HBSS (−).

Stimulation of mononuclear cells. Mononuclear cells (1×10^7/0.7 mL HBSS (−)) were initially incubated at 37°C for 10 min in the presence or absence of inhibitors and then Ca^{2+} (final 1.3 mM) was added to the medium.

[1] Based on a paper presented at the Third International Conference on Platelet-Activating Factor and Structurally Related Alkyl Ether Lipids, Tokyo, Japan, May 1989.

*To whom correspondence should be addressed at Department of Pharmacology, School of Pharmaceutical Sciences, Kitasato University, 5-9-1 Shirokane, Minato-ku, Tokyo 108, Japan.

Abbreviations: ATP, adenosinetriphosphate; BPB, *p*-bromophenacylbromide; DG, diolein; DTT, dithiothreitol; HBSS, Hanks balanced salt solution; HPLC, high-performance liquid chromatography; LDH, lactate dehydrogenase; LPC, lysophosphatidylcholine; lysoPAF, 1-*O*-alkyl-*sn*-glycero-3-phosphocholine; PAF, platelet-activating factor, 1-*O*-alkyl-2-acetyl-*sn*-glycero-3-phosphocholine; PC, phosphatidylcholine; PDD, phorbol-12,13-didecanoate; PMA, phorbol myristate acetate; PMN, polymorphonuclear leukocyte; PS, phosphatidylserine; SM, sphingomyelin; TLC, thin-layer chromatography; UV, ultraviolet.

After 10 min, PMA at various concentrations was added to the medium, and the mixture was incubated for 30 min. The reaction was stopped by the addition of ice-cold HBSS (−) (2 vol) followed by centrifugation at 1600 × *g* for 10 min at 4°C. PAF activities in the supernatant and pellet were extracted separately by the method of Bligh and Dyer (11), and the PAF assay was carried out as previously described (10). The viability of the cells throughout the experiment was monitored by measuring LDH release.

Preparation of microsomal fraction. Mononuclear cells (not treated with PMA) were suspended in 20 mM Tris-HCl (pH 7.4) buffer containing 0.25 M sucrose and 1 mM DTT and were sonicated three times for 20 sec in an ice bath. The homogenate was centrifuged at 500 × *g* for 10 min at 4°C. The resulting supernatant was centrifuged at 15,000 × *g* for 15 min at 4°C. The supernatant was centrifuged at 105,000 × *g* for 60 min at 4°C. The 105,000 × *g* pellet (microsomal fraction) was resuspended in 20 mM Tris-HCl buffer containing 0.25 M sucrose and 1 mM dithiothreitol (DTT) (pH 7.4), and was used as the enzyme source of acetyltransferase. The cytosol fraction was used as the source of calcium/phospholipid dependent protein kinase (protein kinase C). Protein was quantitated by the method of Lowry *et al.* (12).

In other experiment, PMA stimulated cells were homogenized by sonication with 2 mL of 0.25 M sucrose, 20 mM Tris-HCl, 10 mM EGTA, 5 mM mercaptoethanol and 50 mM NaF (pH 7.4), according to the method of Lenihan and Lee (13). The homogenate was fractionated by the three centrifugation steps noted above. The microsomal fraction was used as the enzyme source of acetyltransferase.

Experiments with microsomal fractions. The microsomal fractions were incubated as described below and the incubation mixture was centrifuged at 105,000 × *g* to obtain the microsomal fraction for the acetyltransferase assay.

Phosphorylation/dephosphorylation of acetyltransferase was examined by the modified method of Lenihan and Lee (13). The stimulated microsomal fraction was resuspended in 20 mM Tris-HCl buffer containing 0.25 M sucrose and 1 mM DTT (pH 7.4). An aliquot of the cell suspension (100–120 μg protein) was incubated for 10 min at 25°C in the presence or absence of alkaline phosphatase (*E. coli.*, 10 units) in 100 mM Tris-HCl (pH 8.5, final volume 300 μL). The treated microsomal fraction was collected by centrifugation and resuspended as described above to assess acetyltransferase activity.

Assay of acetyltransferase activity. Microsomes obtained from leukocytes after various treatments were assayed for acetyltransferase as described by Wykle *et al.* (14) and Ninio *et al.* (15). Microsomes (50–100 μg/mg protein) and C_{16}lysoPAF (final 40 μM) were preincubated in an aliquot of 100 mM Tris-HCl buffer (pH 6.9) for 5 min at 37°C. The reaction was started by the addition of acetyl-CoA (final 200 μM) containing 0.5 μCi [^{3}H]acetyl-CoA and was continued for 10 min at 37°C in a final volume of 0.2 mL. Samples without lysoPAF served as blanks. The reaction was stopped by the addition of 1.25 mL of methanol containing 2% acetic acid and [^{14}C]PAF (3000 cpm, to assess recovery), and the mixture was extracted. The residue was dissolved in 30 μL of chloroform/methanol (1:1, v/v) containing authentic PAF and lysoPAF (5 μg each) as carrier and then applied onto a silica gel thin-layer plate (Kieselgel 60, Merck, Darmstadt, Germany). The plate was developed with chloroform/methanol/water (65:40:8, v/v/v). The zones corresponding to the authentic PAF and lysoPAF standards were scraped off and extracted according to Bligh and Dyer (11). The radioactivity was counted in a liquid scintillation counter. Characterization of ^{3}H-labeled products as PAF was by washed platelet aggregation assay as described below. The amount of ^{3}H-labeled PAF produced is expressed as nmole/min/mg protein and is corrected for recovery and after subtraction of blanks.

Extraction and measurement of PAF. PAF from media and cells was extracted and separated as previously reported (10). Briefly, [^{3}H]C_{16}PAF (about 4000 cpm) was added as internal standard to assess recovery, and PAF was extracted with chloroform containing 2% acetic acid by a modified Bligh and Dyer method (11). The combined chloroform layer was loaded onto a Sep-Pak silica cartridge (Waters, Milford, MA). The PAF fraction was eluted with 11.4 mL of chloroform/methanol/water (1:2:0.8, v/v/v) and reextracted according to Bligh and Dyer (11).

The final residue was dissolved in 50 μL of hexane/isopropanol (1:2, v/v) for high performance liquid chromatography (HPLC) using a Shimadzu LC-3A model system (Kyoto, Japan) fitted with a μ Bondasphere silica column (3.9 × 150 mm, Waters). The column was eluted with hexane/isopropanol/water (100:110:20, v/v/v) at a rate of 0.4 mL/min and the eluent was monitored at 206 nm. The fractions corresponding to authentic PAF were collected.

PAF activity was measured by aggregation of washed rabbit platelets as described previously (10), according to the method by Benveniste *et al.* (4). The amount of PAF was corrected for recovery.

Statistics. Statistical evaluation was performed by Student's t-test for unpaired values. Differences with $p<0.05$ were considered significant.

RESULTS

The molecular species of PAF synthesized by rat mononuclear leukocytes were analyzed by HPLC. As shown in Figure 1A, hexadecyl and octadecyl PAF migrated between SM and LPC, and all the fractions were separated from each other. Platelet-aggregating activity was found in the fraction corresponding to authentic C_{16}PAF, but not in the C_{18}PAF fraction, or other fractions, when the leukocyte sample was processed on HPLC (Fig. 1B). The results indicate that essentially the only molecular PAF species synthesized by rat pleural mononuclear cells was C_{16}PAF. Therefore, in the following experiments PAF content in the samples was expressed as ng of C_{16}PAF equivalent/10^7 cells.

The time course of PAF production and release in response to PMA was also examined. As shown in Table 1, PAF contents in both the medium and in cellular fractions increased with time and reached a maximum after 30 min.

Activation of intracellular acetyltransferase by PMA was examined in rat mononuclear cells. Acetyltransferase activity increased with time and reached a peak at 10 min when the cells were incubated with PMA (1 μM). The peak activity (1.74 ± 0.28 nmol/min/mg protein) was about 3 times the resting activity (Fig. 2A). A close correlation

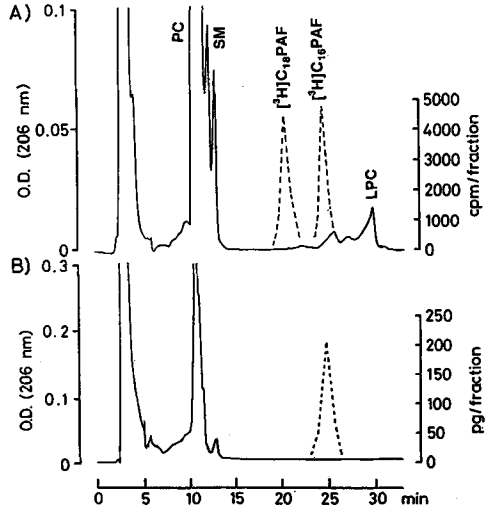


FIG. 1. HPLC profiles of authentic phospholipids, PAF (A) and extracted samples (B). (A) Authentic materials, 5 μg of phosphatidylcholine (PC), 20 μg of sphingomyelin (SM), 150 μg of lysophosphatidylcholine (LPC) and [^{3}H]labeled C_{16}/C_{18}PAF (5000 cpm, respectively) were subjected to HPLC. (B) A typical elution pattern of a sample from the cells stimulated by PMA (1 μM). Broken line shows platelet-aggregating activity.

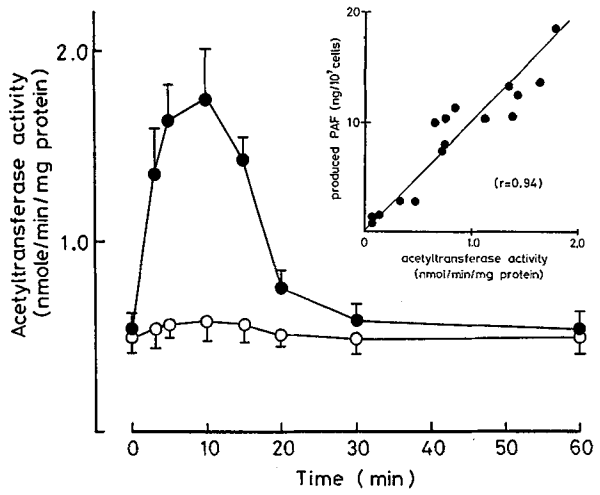


FIG. 2. Time course of induced acetyltransferase activity and its relationship with PAF production in PMA stimulated mononuclear cells of rat. Mononuclear cells were incubated with (●) or without (○) 1 μM PMA. At the times indicated, the cells were sonicated, and the acetyltransferase activities were assayed as described in the text. Inset: Relationship between PAF production and acetyltransferase activity in the cells stimulated with PMA (1 μM).

TABLE 1

Time Course of PAF Production and Release Resulting from PMA Stimulation by Pleural Mononuclear Cells[a]

Time (min)	n	ng/10^7 cells (C_{16}PAF equivalent)			
		Control		PMA (1 μM)	
		Medium	Cellular	Medium	Cellular
0	4	0.31 ± 0.19	1.05 ± 0.36	0.47 ± 0.18	0.96 ± 0.36
5	4	0.97 ± 0.48	1.76 ± 0.05	0.31 ± 0.04	1.96 ± 0.40
15	4	0.63 ± 0.02	1.93 ± 0.23	0.82 ± 0.07	4.93 ± 0.41
30	9	0.82 ± 0.17	2.93 ± 0.38	2.36 ± 0.31	10.24 ± 0.81
60	4	0.95 ± 0.52	2.29 ± 0.29	1.66 ± 0.09	3.89 ± 0.64
120	4	0.89 ± 0.46	1.19 ± 0.34	1.79 ± 0.40	2.84 ± 0.20

[a]Mononuclear cells (10^7 cells/mL) were incubated at 37°C with or without 1 μM PMA. At the times indicated, the reaction was terminated by centrifugation at 4°C, and PAF was immediately extracted from the supernatant (medium) and cellular fraction (cellular), respectively, as described in the text. Each value represents the mean ± SEM.

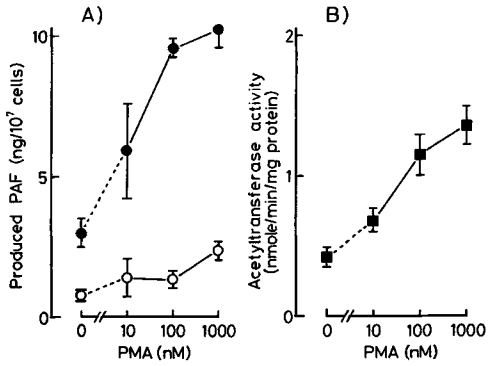


FIG. 3. Effect of PMA on PAF production (A) and acetyltransferase activity (B) in rat mononuclear leukocytes. After 10-min preincubation with Ca^{2+} (final 1.3 mM), the mononuclear cells were incubated with various concentration of PMA for 30 min to determine PAF production (A) or for 10 min for assay of acetyltransferase activity (B). PAF contents in the cellular fraction (●), PAF contents in the medium (○), and acetyltransferase activity (■) are the mean ± SEM from 4–9 experiments.

(r = 0.94) between PAF formation and acetyltransferase activity was observed in response to PMA (10–1000 nM) (Fig. 2B). In contrast, CDP choline cholinephosphotransferase activity, the *de novo* PAF synthesizing enzyme, was not changed by PMA stimulation (data not shown).

PAF production and acetyltransferase activation were dose dependent as shown in Figure 3. When incubated with PMA at concentrations from 10–1000 nM, rat mononuclear cells produced PAF dose-dependently; significant PAF release was observed at 1000 nM PMA (Fig. 3A). The acetyltransferase activity was also dose-dependently increased in response to PMA (Fig. 3B). However PAF induced, increased activity of acetyltransferase was not seen when PDD was used instead of PMA (data not shown).

The effects of some inhibitors on PMA-induced PAF production were examined in hope to shed light on the mechanism of PAF production (Table 2). Pretreatment of mononuclear cells with *p*-bromophenacylbromide (BPB) (50 μM) completely suppressed the increase in both production and release of PAF in response to PMA. Indomethacin, a cyclooxygenase inhibitor, and AA-861, a 5-lipoxygenase inhibitor, did not affect intracellular PAF production, and partially, but not significantly, reduced the release of PMA into the medium. These data suggest that PMA-induced PAF production may occur through the activation of phospholipase A_2 but that prostaglandins and leukotrienes are not involved in the formation of PAF in this cell system. Diltiazem, a Ca^{2+} channel blocker, and W-7, Ca^{2+}/calmodulin inhibitor, did not affect the production and release of PAF induced by PMA.

TABLE 2

Effects of Various Inhibitors on PAF Production and Release by Mononuclear Cells with PMA[a]

Treatment		n	ng/10^7 cells (C_{16}PAF equivalent) Medium	Cellular
PMA (1 μM)		5	2.42 ± 0.41	10.03 ± 0.67
+ BPB	50 μM	4	0.04 ± 0.04	0.57 ± 0.24[b]
+ indomethacin	50 μM	4	1.53 ± 0.26	9.00 ± 0.96
+ AA-861	10 μM	4	1.38 ± 0.31	9.62 ± 1.55
+ diltiazem	100 μM	4	1.80 ± 0.32	8.75 ± 0.50
+ W-7	10 μM	5	1.03 ± 0.36	8.76 ± 0.49
+ staurosporine	1 μM	5	0.89 ± 0.37[c]	1.62 ± 0.46[b]

[a]Inhibitors were added to the medium 20 min before the PMA stimulation. The reaction was terminated by centrifugation (1200 × *g*, 5 min, 4°C) at 30 min after PMA addition. PAF was extracted and assayed as described in the text. Each value represents the mean ± SEM.

[b,c]Statistically significant differences ($p<0.05$, $p<0.01$, respectively) from control value (PMA 1 μM).

In contrast, staurosporine, a potent protein kinase C inhibitor, caused a significant reduction of both PAF production and release. As shown in Figure 4A, increased production of PAF in mononuclear cells upon stimulation by PMA was dose-dependently inhibited by staurosporine (10–1000 nM). Increased activity of acetyltransferase was also dose-dependently inhibited by staurosporine (Fig. 4B). These data suggest that protein kinase C is involved in PMA induced PAF formation in rat mononuclear cells.

The effect of PMA on PAF production was further studied by examining the microsomal preparation from unstimulated cells (Table 3). The microsomal fraction (105,000 × *g* pellet) showed the highest acetyltransferase activity as compared to the cytosolic fraction (105,000 × *g* supernatant) or the mitochondrial fraction (15,000 × *g* pellet). When only PMA was added to the microsomal

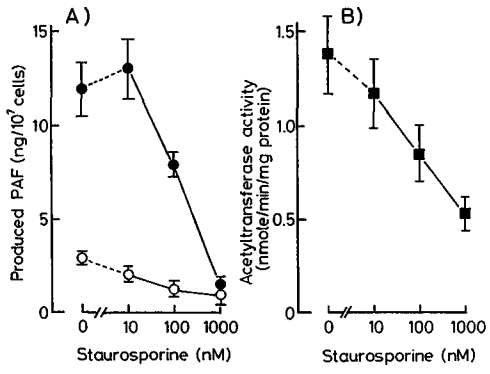


FIG. 4. Inhibitory effect of staurosporine on PMA stimulated PAF production and acetyltransferase activity. Mononuclear cells (10^7/mL) were incubated for 20 min in the presence or absence of staurosporine (final 10–1000 nM) and then incubated with PMA (1 μM) for 30 min for determination of PAF (A) or for 10 min for assay of acetyltransferase activity (B). Open circles (○) or closed circles (●) indicate released PAF or cellular PAF, respectively and closed squares (■) indicate acetyltransferase activity. Each point represents the mean ± SEM from 4–9 experiments.

TABLE 3

Effect of PMA on Acetyltransferase Activity in the Microsomal Fraction from Normal Cells[a]

Treatment	Acetyltransferase activity (nmol/min/mg protein)
105,000 × *g* ppt (microsome)	0.392 ± 0.024
105,000 × *g* sup (cytosol)	0.082 ± 0.008
Microsome + PMA (1 μM)	0.444 ± 0.031
Microsome + 105,000 × *g* sup	0.452 ± 0.102

[a]The microsomal fraction (105,000 × *g* pellet, 50–60 μg protein) or the cytosolic fraction (105,000 × *g* supernatant, 200–300 μg protein) was incubated in the presence of PMA (1 μM). Each value represents the mean ± SEM (n = 4).

fraction, no increase in acetyltransferase activity was observed, which suggests that PMA does not directly activate this enzyme. The data indicate that most of the enzyme activity is found in the microsomal fraction, and suggest that the activation of acetyltransferase takes place through the action of protein kinase C upon stimulation with PMA.

As shown in Table 4, acetyltransferase activity increased in the incubation mixture of the microsomal fraction with Ca^{2+} and/or PS, PMA or diolein in the presence of the cytosolic fraction, magnesium acetate (Mg^{2+}) and ATP. However, a more pronounced enhancement of the activity was observed when the mixture of Ca^{2+}, phosphatidylserine (PS) and diolein or Ca^{2+}, PS and PMA was incubated. The cytosolic fraction was used as the source of protein kinase C.

The effect of alkaline phosphatase treatment on acetyltransferase stimulation was also examined (Fig. 5). We found that the acetyltransferase activity induced by PMA or diolein was significantly reduced by treatment with 10 units of alkaline phosphatase. The data suggests that phosphorylation of the enzyme is necessary for activation of the acetyltransferase.

The effect of various inhibitors on microsomal acetyltransferase was then examined (Table 5). BPB inhibited the stimulation of acetyltransferase activity, while indomethacin (30 μM), AA-861 (10 μM), diltiazem (30 μM)

TABLE 4

Induction of Acetyltransferase Activity in the Microsomal Fraction from Normal Cells[a]

Treatment	Acetyltransferase activity (nmol/min/mg protein)
Ca^{2+}	0.526 ± 0.111
Ca^{2+} + PMA (100 nM)	0.892 ± 0.226
Ca^{2+} + DG (0.8 μg)	0.954 ± 0.194
Ca^{2+} + PS (5 μg)	1.056 ± 0.133
Ca^{2+} + PS + PMA	1.782 ± 0.266
Ca^{2+} + PS + DG	1.801 ± 0.234

[a]The microsomal fraction (50–60 μg) and the cytosol fraction (200 μg), plus magnesium acetate (10 mM) and ATP (5 mM) were mixed in a reaction tube at 0°C. After 10 min incubation at 25°C in the presence of the components indicated, acetyltransferase activity in the microsomal fraction was assayed as described in the text. Each value represents the mean ± SEM (n = 4).

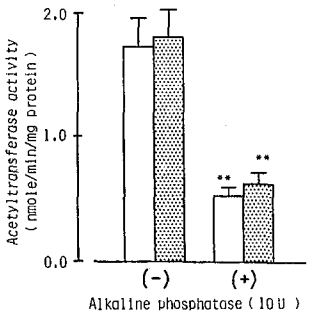


FIG. 5. Effect of treatment of alkaline phosphatase on the increased activity of acetyltransferase induced by PMA or diolein (DG). After incubation with Ca^{2+}, PS, and PMA (100 nM, open column) or with Ca^{2+}, PS, and DG (0.8 μg, dotted column) in the presence of other factors, microsomes were collected by centrifugation at 105,000 × *g* for 60 min at 4°C. The microsomal fraction was incubated for 10 min with or without alkaline phosphatase (10 units). After centrifugation at 105,000 × *g* for 60 min, acetyltransferase activity in the microsomal fraction was assayed as described in the text (n = 4–5). ** indicates statistically significant differences (p<0.01) relative to untreated groups (—).

TABLE 5

Effects of Various Inhibitors on PMA Induced Acetyltransferase Activity[a]

Treatment		Acetyltransferase activity (nmol/min/mg protein)
PMA (100 nM)		1.373 ± 0.161
+ indomethacin	30 μM	1.380 ± 0.206
+ AA-861	10 μM	1.195 ± 0.192
+ BPB	10 μM	0.512 ± 0.094[b]
+ W-7	10 μM	0.745 ± 0.139
+ diltiazem	30 μM	1.377 ± 0.112
+ H-7	10 μM	0.468 ± 0.064[b]
+ staurosporine	1 nM	1.328 ± 0.146
+ staurosporine	10 nM	0.783 ± 0.105
+ staurosporine	100 nM	0.283 ± 0.061[b]

[a]The microsomal fraction (50 μg protein) was incubated together with cytosolic fraction (200 μg protein), plus ATP (5 mM), magnesium acetate (10 mM), calcium chloride (2.5 mM), PS (5 μg) and PMA (100 nM) in the presence or absence of the indicated inhibitors. After 10 min incubation at 25°C, the reaction was terminated at 0°C. After centrifugation of 105,000 × *g* for 60 min, acetyltransferase activity in the microsomal fraction was assayed as described in the text.

[b]Indicates statistically significant difference (p<0.01) from control value (PMA 100 nM). Each value represents the mean ± SEM (n = 4–5).

and W-7 (10 μM) did not change the activity of the enzyme. In contrast, the protein kinase C inhibitors, staurosporine and H-7, significantly reduced enhanced activity of the enzyme. These data suggest the involvement of protein kinase C in the activation of acetyltransferase upon PMA stimulation in this cell free system.

DISCUSSION

As previously reported (10), rat mononuclear cells produce PAF in response to PMA stimulation. The major molecular PAF species produced in rat leukocytes in the present study was identified as C_{16}PAF, using HPLC (Fig. 1). This result is consistent with other reports that C_{16}PAF is predominantly synthesized in various types of rabbit and human cells (16,17).

As shown in Table 1, peak levels of PAF in the incubation medium and in various cellular fractions were reached 30 min after PMA stimulation, while peak activity of acetyltransferase was reached at 10 min (Fig. 2). The time lag between acetyltransferase activity and PAF levels would represent the time needed for the accumulation of PAF in the cells. Similar time lags were reported by other authors (18,19). However, acetyltransferase activity and PAF production may vary markedly between various cell types and stimuli used. The precise mechanism of this will need to be clarified in future study.

The pathways of PAF biosynthesis in various cells have been extensively studied. Two different pathways have been recognized: the *de novo* pathway through cholinephosphotransferase (20), and the remodeling pathway *via* phospholipase A_2 and acetyltransferase. In the remodeling pathway, PAF is formed by a two-stage process in which 1-*O*-alkyl-2-acyl-*sn*-glycero-3-phosphocholine is enzymatically degraded by phospholipase A_2 to form lyso-PAF which is then acetylated by a specific lysoPAF:acetyl-CoA acetyltransferase (14,15,21,22).

The results we obtained on rat mononuclear cells suggest that PAF synthesis in response to PMA takes place mainly through the remodeling pathway. A controversy still exists concerning the *de novo* pathway in PMA-induced PAF synthesis in human polymorphonuclear leukocyte (PMN) (23). However, we have not been able to pinpoint an involvement of the *de novo* pathway in rat monocytes in response to PMA, since CDP choline cholinephosphotransferase activity was not changed when rat monocytes were incubated with PMA (data not shown). Moreover, our results suggest an involvement of phospholipase A_2 and of acetyltransferase in this system. Acetyltransferase activity in the cells increased prior to PAF levels in response to PMA. In addition, both PAF levels and acetyltransferase activity were lowered by staurosporine pretreatment, suggesting that the acetyltransferase plays a major role in PAF production. The close relationship between PAF levels and acetyltransferase activity (r = 0.94) also indicates that PMA stimulation acts on this pathway.

The microsomal acetyltransferase preparation was activated by PMA or diolein provided sufficient amounts of cofactors were present (Table 4). Acetyltransferase activity was suppressed by staurosporine, a known and potent inhibitor of protein kinase C (24,25). Protein kinase C can be directly activated by diacylglycerol which can, in turn, be produced by signal-induced inositol phospholipid turnover (26); PMA can substitute for diacylglycerol to activate protein kinase C (27). Thus, protein phosphorylation induced by this kinase might be necessary for the activation of acetyltransferase in response to PMA. The phosphorylation of acetyltransferase from rat spleen (28), parotid gland (29) and human polymorphonuclear leukocytes (30) has been reported, and was ascribed to cyclic AMP- or calmodulin-dependent protein kinase, rather than protein kinase C. The reason of this discrepancy is presently not known, but may be due in part to the different enzyme sources studied.

As for the activation of phospholipase A_2 by PMA, we previously showed that prostaglandin production in rat

pleural cells occurs in response to PMA (31). Levine and Ohuchi (32) reported that phorbol esters stimulate prostaglandin synthesis through the activation of phospholipase A_2 in an MDCK cell line. In the present work we found that BPB reduced PAF production (Table 2), even though BPB may not be a specific inhibitor for phospholipase A_2. The results suggest that phospholipase A_2 is also activated by PMA in rat monocytes.

Recent reports showing that protein kinase C is involved in the production of PAF, leukotrienes and prostaglandins upon stimulation with PMA or ionophore A23187 (19,33, 34) are in line with our data. We therefore conclude that the stimulatory effect of PMA on PAF production involves stimulation of protein kinase C which induces activation of phospholipase A_2 as well as of acetyltransferase.

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Tumor Necrosis Factor Release by Human Monocytes Stimulated with Platelet-Activating Factor[1]

N.M. Ruis, J.K. Rose and F.H. Valone*
Department of Medicine, Veterans Administration Medical Center, San Francisco, California 94121

Platelet-activating factor (PAF) is a low molecular weight phospholipid which enhances human monocyte cytotoxicity for tumor cells. In the current studies, the capacity of PAF to stimulate release of tumor necrosis factor α (TNFα) by human monocytes was assessed. PAF induced maximal TNFα synthesis 2–3 hr after monocyte stimulation as assessed by dot blotting of cell-associated TNFα using polyclonal anti-TNF antibody. Maximal net release of TNFα occurred 5–16 hr after monocyte stimulation, as assessed by a specific ELISA. Dose-response studies revealed that a maximal two- to three-fold increase in release of TNFα occurs at 10–100 pM PAF. LysoPAF and the optical isomer of PAF did not stimulate release of TNFα, suggesting that stimulation is mediated by specific PAF receptors. Scatchard analysis of [^{3}H]PAF binding to monocyte membranes revealed 651 ± 495 binding sites/monocyte with a Kd of 4.7 ± 4.2 × 10^{-10}M. PAF is a structurally unique activator of monocytes whose interactions with TNFα and other cytokines may be critical to host defense against tumors.
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Platelet-activating factor (1-*O*-alkyl-2-acetyl-*sn*-glycero-3-phosphocholine; PAF) is a novel phospholipid which is a potent mediator of allergic and inflammatory diseases (1,2). PAF is synthesized and released after immunological activation of numerous cells including endothelial cells, neutrophils, monocytes and large granular lymphocytes (3–9). The name "platelet-activating factor" is clearly a misnomer as PAF activates numerous cells including monocytes, lymphocytes and neutrophils (3, 10–14). PAF stimulates several monocyte/macrophage functions which may be critical to host immunity against tumors, including adherence or aggregation (10), chemotaxis (11), release of reactive oxygen products (10,15) and release of the immunomodulatory cytokine IL-1β (16,17). PAF also directly enhances monocyte cytotoxicity (18). Pretreatment of human monocytes with 1 pM–1 nM PAF for 18 hr increases monocyte killing of tumor target cells. Several observations suggest that PAF increases monocyte cytotoxicity primarily by increasing release of tumor necrosis factor α (TNFα): PAF only stimulates monocyte killing of TNF-sensitive target cells and cell killing by PAF-treated monocytes is inhibited by specific antisera to TNFα. The experiments presented in this communication directly demonstrate that PAF stimulates human monocytes to release TNFα.

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*To whom correspondence should be addressed at Dept. of Medicine, Veterans Administration Medical Center, 4150 Clement Street, San Franciso, CA 94121.

Abbreviations: BSA, bovine serum albumin; enantioPAF, 3-*O*-hexadecyl-2-acetyl-*sn*-glycero-1-phosphocholine; IFNγ, γ Interferon; lysoPAF, 1-*O*-hexadecyl-*sn*-glycero-3-phosphocholine; PGE_2, prostaglandin E_2; MTT [3-(4,5-dimethylthiazol)2-yl)-2,5-diphenyltetrazolium bromide]; PAF, platelet-activating factor, 1-*O*-hexadecyl-2-acetyl-*sn*-glycero-3-phosphocholine; and TNFα, tumor necrosis factor α.

MATERIALS AND METHODS

PAF, lysoPAF and enantioPAF (Bachem, Torrance, CA), recombinant human TNFα and IFNγ (Genzyme Corp., Boston, MA), rabbit antisera to recombinant human TNFα (10^5 neutralizing units/mL; Genzyme Corp.), [^{3}H]PAF (1-*O*-[1′,2′-^{3}H]alkyl-; 51 Ci/mmol) (New England Nuclear, Boston, MA), radioimmunoassay kit for PGE_2 (Advanced Magnetics, Cambridge, MA), WEHI 164.13 cells (gift of Dr. Lois Epstein, University of California, San Francisco, CA) and cell culture media (University of California Cell Culture Facility, San Francisco, CA) were obtained as noted. All other chemicals were obtained from Sigma Chemical Co. (St. Louis, MO).

Quantitation of TNFα release. Monocytes were prepared from normal human donors and suspended at 1 × 10^6/mL in RPMI 1640 containing 0.25% (w/v) endotoxin-free bovine serum albumin (BSA) as described (3,18). One mL of cells was mixed with buffer, PAF, IFNγ or PAF analogs. After incubation for 30 min at 37°C in an atmosphere of 5% CO_2 and 95% air, 0.1 mL of low endotoxin fetal calf serum (Hyclone Laboratories, Logan, UT) was added to each tube. Serum was added after the 30 min to allow monocytes adequate exposure to PAF before PAF was degraded by serum acylhydrolases. The incubation was then continued for 30 min to 18 hr. TNFα release into culture supernates was quantified by cytotoxicity assay using WEHI 164 clone 13 cells (19). Serial two-fold dilutions of culture supernates or recombinant human TNFα were made in 96-well microtiter plates. WEHI 164.13 cells were pretreated with 1 μg actinomycin D/mL for 2 hr at 37°C in RPMI 1640 containing 7% fetal bovine serum. Twenty thousand cells were then added to each well in a final volume of 0.1 mL and incubated 18 hr at 37°C in an atmosphere of 5% CO_2 and 95% air. Viable cell number was determined by uptake of the dye MTT (20). One unit of TNFα was defined as the quantity of material which decreased cell number by 50%. In some cases TNFα released into culture fluids was quantified by a commercial ELISA (T-Cell Science, Cambridge, MA). The ELISA is specific for bioactive human TNFα and is not affected by denatured TNFα, lymphotoxin (TNFβ), Interleukin 1 or Interleukin 2.

Cell-associated TNFα protein (precursor and biologically active product) were quantified by dot blotting using specific rabbit antisera to recombinant human TNFα. Monocytes were treated with buffer or PAF, sedimented by centrifugation and resuspended at 1 × 10^6/mL in lysis buffer containing 1 μM pepstatin, 100 μM phenylmethylsulfonylfluoride, 10 mM EDTA, and 1 μM leupeptin in phosphate buffered saline. Aliquots of 5 × 10^5, 2.5 × 10^5, 1.25 × 10^5 and 0.625 × 10^5 cells were dotted onto nitrocellulose paper (BioRad, Richmond, CA) using a BioRad dot apparatus. The paper was blocked by incubation overnight in 5% non-fat dry milk in phosphate buffered saline and then incubated with rabbit anti-recombinant human TNFα at 1/200 dilution for 3 hr. Biotinylated goat anti-rabbit antibodies (Zymed, South

San Francisco, CA) at 1/2000 dilution was incubated with the paper for 2 hr, followed by washing and incubation with 10 microCi [^{125}I]strepavidin (Amersham, Arlington Heights, IL) for 2 hr. Bound radioactivity was quantified by autoradiography. Between each step, the nitrocellulose paper was washed extensively with 5% non-fat dry milk in phosphate buffered saline. Controls which were dotted on each paper were unstimulated cells and 10-1000 units recombinant, human TNFα.

PAF binding to monocyte membranes. Monocytes were treated with 5 mM diisopropylfluorophosphate and disrupted by nitrogen cavitation (21). After removing nuclei and large debris by sedimentation at 500 × *g* for 15 min the membranes were recovered by centrifugation at 30,000 × *g* for 30 min and resuspended at 10^7 cell equivalents/mL in RPMI 1640 containing 0.25 g/dl bovine albumin. Membrane protein recovery was 5.2 ± 3.3 mg/10^7 monocytes (mean ± SD, n = 3). One mL of membranes was incubated with one pmol [^{3}H]PAF (50,000 CPM) without or with inhibitors of PAF binding for 2 hr at 4°C. The membranes were sedimented by centrifugation at 30,000 × *g* for 15 min, after which unbound PAF in the supernates and bound PAF in the pellets was quantified by liquid scintillation counting. Specific binding was calculated as [^{3}H]PAF bound in the absence of inhibitors minus [^{3}H]PAF binding in the presence of a 10,000-fold molar excess of unlabeled PAF. Binding was subjected to Scatchard analysis as described (22).

RESULTS

Release of TNFα. In dose-response studies, PAF induced TNFα release by human monocytes with a peak effect at 10-100 pM PAF (Table 1). Maximal PAF-induced TNFα release was two- to three-fold greater than spontaneous TNFα release by monocytes incubated with buffer alone. Spontaneous TNFα release varied from 47 to nearly 300 units/10^6 monocytes. This wide variation resulted in wide standard errors in mean release of TNFα by the nine donors. Net TNFα release by PAF was comparable to net release by IFNγ, which was 232 ± 147 and 305 ± 241 units/10^6 monocytes for 10 and 100 units/mL, respectively (mean ± SD, n = 4).

Specific antibodies to TNFα were used to confirm that the cytotoxic factor present in monocyte supernates was TNFα. Aliquots of eight monocyte supernates containing 1-361 units TNF activity were incubated with non-specific antisera or 1,000 neutralizing units of antisera to recombinant human TNFα for 2 hr at 4°C, after which cytotoxic activity was assessed. In each case, the antibodies completely eliminated cytotoxic activity of the monocyte supernates (Table 2).

In order to confirm that enhanced release of TNFα is a specific effect of PAF, mediated by a PAF receptor, the capacity of the inactive PAF analog, lysoPAF, and PAF's unnatural optical isomer, enantioPAF, to stimulate TNFα release was examined. Neither lysoPAF (n = 4) nor entantioPAF (n = 2) stimulated release of TNFα above spontaneous release by unstimulated monocytes (Table 1).

The time course of TNFα synthesis and release induced by PAF was determined. Monocytes were incubated with 10 pM PAF or vehicle control for 1, 2, 3, 5 or 16 hr and sedimented by centrifugation. The quantity of TNFα released into culture media was determined by ELISA, and the quantity of cell-associated TNFα was determined by dot blotting. In two experiments, mean net TNFα release was 0 at 1 hr, 166 pg/10^6 cells at 3 hr, 206 pg/10^6 cells at 5 hr, and 200 pg/10^6 cells at 16 hr. Dot blotting revealed rapid appearance of cell-associated TNFα protein (Fig. 1). Negligible quantities of TNFα were detected in unstimulated cells; maximal cell-associated TNFα occurred 2-3 hr after cell stimulation, and minimal TNFα was detectable 5-16 hr after cell stimulation.

PGE_2 inhibits cytokine release by monocytes (23). As PAF stimulates PGE_2 release by monocytes under some circumstances (12), it was of interest to determine if PGE_2 release accounted for the failure of high concentrations of PAF to stimulate TNFα release. PGE_2 was quantified in culture supernatants from two of the experiments shown in Table 1. PGE_2 release by control monocytes exposed to buffer for 18 hr was 98 and 74 pg/mL in the two experiments. Incubation with 10 fM to 1 μM PAF for 18 hr did not increase PGE_2 release above control levels.

TABLE 1

Stimulation of TNF Release by PAF, LysoPAF and EnantioPAF

Stimulus concentration[a]	Net tumor necrosis factor release		
	PAF	LysoPAF	EnantioPAF
10^{-15}M	12 ± 82[b]		
10^{-13}M	93 ± 126[c]		
10^{-12}M	137 ± 142[d]	36 ± 161	0
10^{-11}M	235 ± 188[d]	46 ± 120	0
10^{-10}M	214 ± 316	−16 ± 61	28
10^{-9}M	126 ± 237	−52 ± 243	68
10^{-8}M	83 ± 179[c]	26 ± 159	68

[a]One × 10^6 monocytes/mL were incubated with buffer or the indicated stimulus for 18 hr, after which TNFα release into the supernatants was quantified.

[b]Mean (± SD) net TNFα release; n = 9 (PAF), n = 4 (lysoPAF), n = 2 (enantioPAF). Spontaneous release of TNFα by monocytes incubated with buffer was 158 ± 124 units/10^6 monocytes (mean ± SD, n = 9).

[c,d]Compared to buffer-stimulated monocytes $P < 0.05$ (c) or $P < 0.02$ (d) (paired t-test). PAF also increased release of TNFα significantly within each experiment at the 100 fM-10 pM concentrations ($p < 0.05$), log rank test.

TABLE 2

Inhibition of Cytotoxic Activity by Specific Antisera to TNFα

Stimulus[a]		TNF (Units/10^6 Monocytes) Anti − TNF − Antibody	
		−	+
Buffer		67	0
PAF	10^{-12}M	81	0
	10^{-11}M	361	0
	10^{-10}M	280	0
	10^{-9}M	227	0

[a]Monocytes were incubated with buffer or PAF for 18 hr. The supernatants were harvested, treated with non-specific antisera or antisera to human TNFα and then tested for TNF activity. This experiment is representative of two experiments performed using supernatants from monocytes from different donors.

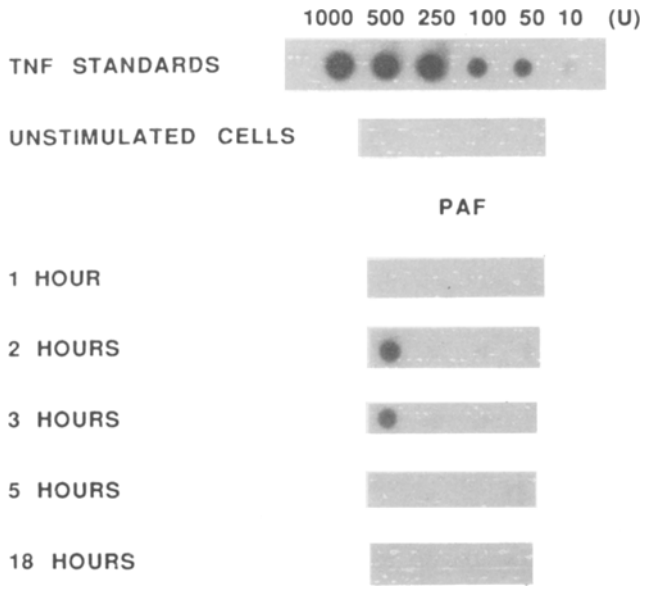


FIG. 1. Dot blot of monocyte-associated TNFα. Each time point shows blots of 1/2, 1/4, 1/8 or 1/16 dilutions of 1×10^6 monocytes treated with buffer or 10 pM PAF for the indicated times.

TABLE 3

Specificity of PAF Binding by Human Monocyte Membranes

Binding inhibitor[a]		% Inhibition of specific [^{3}H]PAF binding
Buffer		0
PAF	10^{-9}M	69 ± 22[b]
PAF	10^{-8}M	87 ± 20
PAF	10^{-7}M	100 ± 0
LysoPAF	10^{-7}M	29 ± 21
LysoPAF	10^{-6}M	45 ± 24
LysoPAF	10^{-5}M	65 ± 21
EnantioPAF	10^{-5}M	35 ± 13
Kadsurenone	10^{-6}M	21 ± 29
Kadsurenone	10^{-5}M	70 ± 23
Kadsurenone	10^{-4}M	85 ± 25
BN52021	10^{-4}M	98 ± 3

[a]Membranes from 10^7 human monocytes were incubated with 1 pmole [^{3}H]PAF (approximately 50,000 CPM) without or with the indicated binding inhibitor. After 2 hr, [^{3}H]PAF binding was quantified.

[b]Mean (± SD) percent inhibition of specific [^{3}H]PAF binding (n = 3–10). Total [^{3}H]PAF binding in the absence of inhibitors was 2825 ± 1476 CPM (6.1 ± 2.6%) (mean ± SD, n = 10). Specific [^{3}H]PAF binding (total binding minus [^{3}H]PAF binding in the presence of 10,000 pmoles unlabeled PAF) was 899 ± 651 CPM (1.6 ± 0.7%).

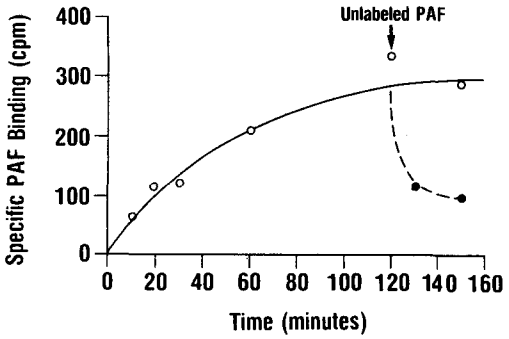


FIG. 2. Kinetics of [^{3}H]PAF binding association and dissociation from human monocyte membranes. Each point is the mean of three determinations using monocyte membranes from three donors.

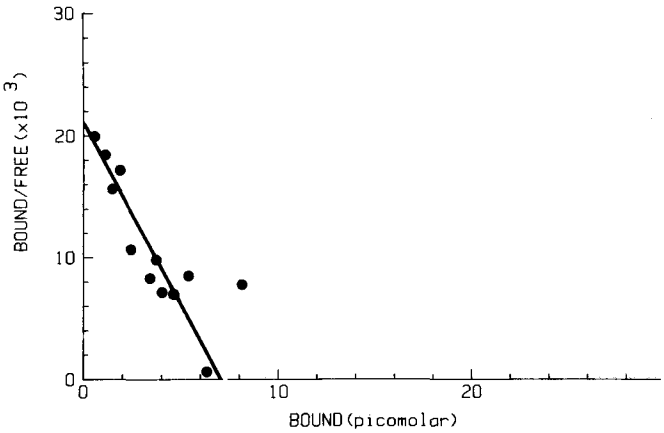


FIG. 3. Scatchard plot of PAF binding to monocyte membranes. The plot is representative of six plots prepared from binding studies with membranes from six donors. The binding data were fit to models composed of one or two specific binding sites plus a non-specific binding component. The plot for the one receptor model is shown.

Characterization of monocyte receptors for PAF. The observation that optimal TNFα release occurred at very low PAF concentrations (10–100 pM) suggested that human monocytes have unusually high affinity receptors for PAF which mediate TNFα release. So, PAF binding to monocyte membranes was examined. Analysis of the rate of binding revealed that specific PAF binding was maximal within 2 hr of incubation (Fig. 2). Specific binding was fully reversible, as shown by addition of a 1,000-fold molar excess of unlabeled PAF, which completely dissociated [^{3}H]PAF previously bond to the monocyte membranes. To examine the structural specificity of binding the capacity of PAF analogs and PAF binding antagonists to inhibit [^{3}H]PAF binding to monocyte membranes was determined (Table 3). The relatively inactive PAF analogs lysoPAF and enantioPAF were 10,000-fold less potent than unlabeled PAF for inhibiting [^{3}H]PAF binding. The capacity of the specific antagonists of PAF binding, BN52021 and kadsurenone, to inhibit PAF binding to monocyte membranes was tested. Both antagonists inhibited [^{3}H]PAF binding at concentrations which inhibit PAF binding to receptors on other cells.

PAF receptor number and binding affinity were determined by quantifying binding of 0.1–3 picomoles [^{3}H]PAF both alone and in the presence of 1 picomole to 1 nanomole unlabeled PAF. The binding data was subjected to Scatchard analysis using a computer program which uses the least squares method to fit the data to binding models composed of either one or two specific receptors plus a non-specific binding component (22,24).

The single receptor model fit the binding data best and revealed 651 ± 495 binding sites/monocyte with a dissociation constant of 4.7 ± 4.2 × 10^{-10}M (mean ± SD, n = 6) (Fig. 3). The binding data was also consistent with two PAF receptors: Receptor #1, 95 ± 80 sites/monocyte, Kd = 1.1 ± 1.4 × 10^{-10}M; receptor #2, 1035 ± 563 sites/monocyte, Kd = 1.9 ± 1.6 × 10^{-9}M. The second receptor model does not fit the binding data as well as the first receptor model, but the difference was not statistically significant.

DISCUSSION

Previous studies demonstrated that PAF enhances human monocyte cytotoxicity for tumor target cells (18). Indirect evidence suggested that PAF increases monocyte cytotoxicity by inducing release of TNFα. The current experiments directly demonstrated that PAF stimulates human monocytes to release TNFα with peak release at 10-100 pM PAF (Table 1). These concentrations of PAF are within the range of PAF concentrations detected *in vivo* during immunological reactions, so these *in vitro* observations are likely to be relevant to *in vivo* processes (25,26). It is likely that PAF stimulates TNFα synthesis rather than stimulating release of pre-existing TNFα in the monocyte preparations. Dot blotting of cell-associated TNFα did not demonstrate pre-existing TNFα, whereas PAF induced the appearance of TNFα 2-3 hr after monocyte activation (Fig. 1). PAF induced a two- to three-fold increase in TNFα release as compared to control monocytes incubated with buffer. TNFα release by 10 pM PAF is comparable to TNFα release by 10 U IFNγ/mL. Higher concentrations of IFNγ induce more TNFα release, whereas higher concentrations of PAF induce less TNFα release. High dose inhibition is frequently observed with PAF and the mechanisms are unknown. Our data indicate that high dose inhibition does not result from release of PGE_2 by the monocytes.

Bonavida *et al.* (27) reported recently that PAF stimulates release of TNFα by human monocytes. Compared to our study, those investigators found that smaller quantities of TNF were released and much higher concentrations of PAF were required to stimulate release. Methodological differences may account for the different results. Those investigators exposed the monocytes to PAF in media containing serum, so it is probable that much of the PAF was rapidly metabolized by serum acetylhydrolases. Higher PAF concentrations would be required to stimulate TNFα synthesis under those conditions than in the serum-free system used initially by us. Spontaneous and stimulated release of TNFα is higher in our study. This may result from contamination of our monocyte preparations by endotoxin concentrations or forms which are not detected by the Limulus assay (28). This possibility is supported by the recent observation that combinations of PAF and endotoxin stimulate release of TNFα by alveolar macrophages, whereas PAF alone was minimally effective (29).

Analysis of PAF binding to monocyte membranes reveals that models of binding consisting of either one or two specific receptors both fit the data. The high affinity PAF receptor in either model could account for TNFα release by 10 pM PAF. This high affinity receptor resembles other PAF receptors in that it neither binds (Table 3) nor is activated (Table 1) by lysoPAF and enantioPAF (24). There is no functional evidence for a second PAF receptor in monocytes and the one receptor model fits the binding data better than the two receptor model. Thus, current data indicate that monocytes have only one receptor for PAF.

PAF is a phospholipid and thus represents a novel class of monocyte activators which are structurally distinct from protein cytokines. However, there are complex, incompletely defined interactions between PAF and the cytokines TNFα, IL-1β and IFNγ in the regulation of human monocyte function and the immune response. PAF stimulates monocytes to release TNFα (Table 1) and IL-1β (16,17). In turn, TNFα, IL-1β and IFNγ stimulate monocytes to synthesize and release PAF in quantities sufficient to stimulate monocytes (3). These observations reveal a positive feedback loop in PAF and cytokine synthesis by monocytes which may be important to amplification of the immune response. PAF and TNFα have similar toxicities after administration *in vivo* (1,2,30). Thus, uncontrolled release of these toxic products because of the positive feedback loop could result in severe tissue damage. In this regard, it is noteworthy that specific PAF antagonists decrease bowel necrosis induced by intravenous administration of TNFα to rats (31). Further studies are required to fully elucidate the interactions among PAF, TNFα and IL-1β and to determine if these interactions can be manipulated to improve host immunity to tumors or to diminish the toxicity of immunotherapy.

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Guinea Pig Bone Marrow Cells Treated with Platelet-Activating Factor Generate Factor(s) Which Affects Their DNA Synthesis and Microbicidal Activity[1]

Ichiro Kudo, Toshiyuki Kato[2], Hidetoshi Hayashi[3], Ryohei Yanoshita[4], Koichi Ikizawa[5], Hiroko Uda and Keizo Inoue*

Faculty of Pharmaceutical Sciences, University of Tokyo, Hongo, Bunkyo-ku, Tokyo 113, Japan

We have previously reported that platelet-activating factor (PAF) induces proliferation and microbicidal activity of guinea pig bone marrow cells. In the present study, we have found that the conditioned medium of PAF- or nonmetabolizable PAF agonist-treated guinea pig bone marrow cells augmented DNA synthesis and induced microbicial activity of bone marrow cells. A PAF specific antagonist, CV-6209, inhibited generation of the active conditioned medium by PAF. Addition of the PAF antagonist only partially suppressed the augmentative effect of the active conditioned medium on DNA synthesis; this is consistent with the fact that, because of the rapid breakdown, no appreciable amount of PAF remained in the conditioned medium of PAF-treated cells. Although mouse bone marrow cells did not respond to PAF unlike guinea pig cells, their DNA synthesis was significantly enhanced by the conditioned medium of PAF-treated guinea pig bone marrow cells. Thus, some newly generated factor(s) distinct from the originally inoculated PAF seemed to modulate the bioactions of PAF on bone marrow cells. An appreciable amount of PAF was produced by calcium ionophore-treated guinea pig bone marrow cells. These findings indicate that PAF synthesized in guinea pig bone marrow cells induces generation in the cells of some factor(s) which affects proliferation or microbicidal activity.

***Lipids 26,* 1065–1070 (1991).**

Platelet-activating factor (PAF) is considered a potent mediator in various pathological and physiological reactions (1). Evidence has also been accumulating that PAF is involved in the regulation of cellular immune response (2). For instance, large granular lymphocytes were shown to release PAF on activation (3). Varying results concerning the effect of exogenous PAF on lymphocyte proliferation have been presented. Thus, Rola-Pleszczynski *et al.* (4) reported that PAF inhibited the proliferation of human peripheral blood lymphocytes. Patrignani *et al.* (5) reported that PAF caused a dose-dependent increase of prostaglandin E_2 and leukotriene B_4 synthesis in peripheral blood mononuclear cells. It was also shown that PAF partially down-regulated expression of the CD2 and CD3 antigens on human peripheral T cells (6).

The role of PAF in proliferation and induction of differentiation of immature leukocytes, however, has not been fully elucidated. Berdel *et al.* (7) showed that intravenous injection of bone marrow macrophages pretreated with ET18-OMe (1-*O*-octadecyl-2-*O*-methyl-*rac*-glycero-3-phosphocholine), a weak PAF agonist, inhibited the development of metastasis of 3-Lewis lung carcinoma in C57B1/6mice. ET18-OMe and related compounds were shown to induce cell differentiation *in vitro* of human and mouse myeloid cells (8), although the mechanism did not seem to involve PAF-specific binding sites. We suggested that the antitumor activity of synthetic alkyl ether lipid analogs might be mediated, at least in part, through *in vivo* activation of bone marrow macrophages (9). We also found PAF-specific binding sites on bone marrow cells of guinea pigs (10). Since it was widely accepted that PAF-responsive cells are activated through binding of PAF to specific PAF binding sites, we searched for functional alteration in PAF-treated bone marrow cells. We have found that PAF induces a microbicidal activity against *Candida parapsilosis* (10) and augments cell proliferation (11). The conditioned medium of bone marrow cells incubated in the presence of PAF was also shown to enhance DNA synthesis in another culture of bone marrow cells (11). In the studies mentioned below, evidence was obtained that PAF-induced proliferation and microbicidal activity are mediated by some newly generated factor(s), distinct from PAF itself, in bone marrow.

MATERIALS AND METHODS

Chemicals. Fetal calf serum (FCS) was obtained from HyClone Laboratories (Logan, UT) and used after heat inactivation. RPMI-1640 medium was from MA Bioproducts (Walkersville, MD). [*Methyl*-1′,2′-^{3}H]thymidine (specific activity, 112.7 Ci/mmol) was from the Radiochemical Centre, Amersham, UK; essentially fatty acid-free bovine serum albumin (BSA) was obtained from Sigma Chemical Co. (St. Louis, MO). PAF, 1-*O*-octadecyl-2-dimethylcarbamoyl-*sn*-glycero-3-phosphocholine (Compound I) (12), and CV-6209 (13) were provided by Takeda Chemical Industries, Ltd. (Osaka, Japan). FR900452 (14) was kindly provided by Fujisawa Pharmaceutical Co. Ltd. (Osaka, Japan).

Animals and preparation of bone marrow cells. Female Hartley guinea pigs (body weight 250–500 g), Japanese white rabbits, and ICR mice were obtained from the Japan Bio-supply (Tokyo, Japan). Bone morrow cells were prepared from femurs of guinea pigs as described previously (10).

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[2] Present address: Shionogi Research Laboratories, Shionogi & Co., Ltd., Osaka 553, Japan.

[3] Present address: Faculty of Pharmaceutical Sciences, Nagoya City University, Nagoya 467, Japan.

[4] Present address: Technical R & D Division, Terumo Corporation, Kanagawa 259-01, Japan.

[5] Present address: Biological Research Laboratory, Taiho Pharmaceutical Corporation Ltd., Tokushima 771-01, Japan.

*To whom correspondence should be addressed.

Abbreviations: BSA, bovine serum albumin; FCS, fetal calf serum; PAF, platelet-activating factor.

Preparation of conditioned medium. The conditioned medium was obtained by centrifugation after cultivation of bone marrow cells (2×10^6 cells/ml) in RPMI1640 medium containing 10% FCS in the presnce of PAF for indicated periods. After repeated dialysis (four times) against RPMI 1640 medium containing 0.1% BSA, the medium was sterilized by filtration using a cellulose acetate membrane filter (pore size, 0.20 μm, Corning).

Assay for [^3H]thymidine incorporation. Bone marrow cells (5×10^4 cells) were seeded in flat-bottomed, 96-well plates with 100 μl of RPMI 1640 medium containing 20% FCS. After one day of incubation at 37°C under a 5% CO_2 atmosphere, conditioned medium (50 μl) was added. Eight hours before completion of the incubation, 0.5 μCi of radioactive thymidine was added. Radioactivity of the acid-insoluble fractions of cells was counted (15). Activation index, representing a relative potency to induce DNA synthesis, was calculated as described previously (11).

Assay for microbicidal activity. Microbicidal activity of bone marrow cells was determined by the methods described previously (10). *Candida parapsilosis* were used as target cells. In brief, bone marrow cells (1×10^5 cells) were seeded in flat-bottomed, 96-well plates with 0.2 ml of the RPMI 1640 medium with 10% FCS. After one day of incubation at 37°C under a 5% CO_2 atmosphere, cells were treated with PAF or the conditioned medium, and then infected with 10 μl of two-fold serially diluted *Candida parapsilosis* suspensions. After incubation for 48 hr at 37°C under a 5% CO_2 atmosphere, outgrowth of *Candida parapsilosis* cells in each well was determined under a microscope. Microbicidal activity of bone marrow cells (the maximum number of microbes killed, MNMK) was evaluated as the maximum number of microbes added to a well where the growth was completely suppressed.

RESULTS

Generation of conditioned medium affecting DNA synthesis. The conditioned medium of guinea pig bone marrow cells was prepared after incubation of cells in the presence of 10^{-5} M PAF and added to another culture of bone marrow cells. The conditioned medium appreciably enhanced DNA synthesis of the second culture of bone marrow cells (Table 1, exp. 1). The conditioned medium of the control culture did not have any significant effect (data not shown). The augmentative activity of the conditioned medium was not dialyzable and the effect was dose-dependent (Fig. 1A). In an experiment, 15 nmole of PAF was added to 1.5 ml of a culture of bone marrow cells in the medium containing 20% FCS, and the degradation of PAF during incubation was examined by bioassay using rabbit platelets (12). Only 2.2 nmole of PAF was recovered just after the start of incubation, and no appreciable amount of PAF was detected in the conditioned medium after incubation for 2 hr or longer, indicating that exogenously-added PAF was rapidly degraded, most probably by serum (16) or cellular (17) PAF acetylhydrolase.

A strong PAF agonist, Compound I, showed more potent activity in the generation of active conditioned medium than PAF itself; the conditioned medium prepared from cells cultivated in medium containing 10^{-10} M Compound I exhibited an augmentative activity comparable to that with 10^{-5} M PAF (Table 1, exp. 2). When guinea pig bone marrow cells were incubated with a PAF-specific antagonist, CV-6209 and subsequently treated with PAF, no augmentative activity was found in the conditioned medium (Table 1, exp. 3).

TABLE 1

Enhancement of DNA Synthesis by Conditioned Medium[a]

	Conditioned medium from bone marrow cells treated with		Acid-insoluble radioactivity (dpm)[b]
Exp. 1	Control		1,203 (1.0)[e]
	PAF	10^{-6} M	981 (0.8)
		10^{-5} M	4,712 (3.9)
Exp. 2	Control		455 (1.0)
	PAF	10^{-5} M	2,275 (5.0)
	Compound I	10^{-11} M	4,065 (8.9)
		10^{-10} M[c]	4,161 (9.1)
Exp. 3	Control		1,491 (1.0)
	PAF	10^{-5} M	6,084 (4.1)
	CV-6209 10^{-7} M[d] + PAF	10^{-5} M	2,263 (1.4)

[a] The conditioned medium was obtained by centrifugation and dialysis after cultivation of guinea pig bone marrow cells (2×10^6 cells/ml) in the presence of the indicated dose of PAF or Compound I for 15 hr. Bone marrow cells from another guinea pig were incubated in the presence of the conditioned medium for 56 hr. DNA synthesis of the bone marrow cells was examined as described in Materials and Methods.

[b] Results are mean values from triplicate cultures.

[c] After extensive dialysis, approximately 1/100 of the originally inoculated Compound I was detected in the conditioned medium (data not shown), indicating that the potent effect observed is due to newly generated factors.

[d] Thirty minutes prior to treatment with PAF, a PAF antagonist, CV-6209, was added to the culture.

[e] Numbers in parentheses represent values of activation index.

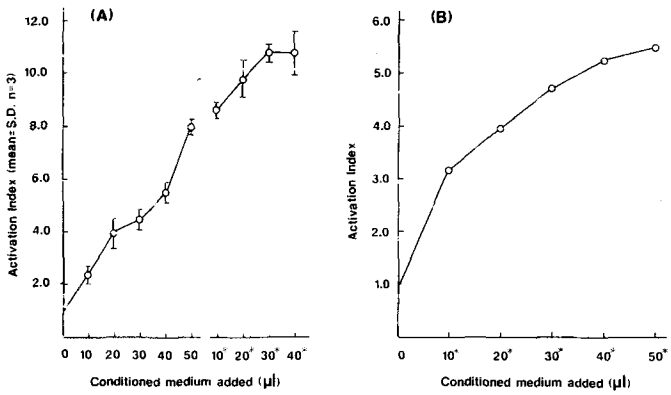


FIG. 1. Dose-dependent enhancement of DNA synthesis by conditioned medium. The conditioned medium was obtained by centrifugation and dialysis after cultivation of guinea pig bone marrow cells (2×10^6 cells/ml) in the presence of 10^{-5} M PAF for 15 hr. The various doses of conditioned medium or concentrated conditioned medium (*, one-fifth of the volume) were added to another culture of bone marrow cells of guinea pig (A), or mouse (B) for 72 hr. DNA synthesis was examined as described in Materials and Methods.

TABLE 2

Effect of Heat Treatment on the Conditioned Medium[a]

	Acid-insoluble radioactivity (dpm)[b]		
	Before heat treatment	After heat treatment	Control medium
Exp. 1	28,467 ± 4,439 (4.9)[c]	11,965 ± 759 (2.1)	5,790 ± 59 (1.0)
Exp. 2	5,937 ± 453 (5.3)	3,694 ± 178 (3.3)	1,112 ± 17 (1.0)
Exp. 3	17,961 ± 3,321 (4.1)	12,743 ± 1,246 (2.9)	4,337 ± 788 (1.0)

[a]The conditioned medium was prepared by centrifugation and dialysis after cultivation of guinea pig bone marrow cells in the presence of 10^{-5}M PAF for 8 hr (Exps. 1 and 2) or 15 hr (Exp. 3). The conditioned medium was then heated at 80°C for 15 min. The augmentative effect on DNA synthesis was then examined as described in Materials and Methods.

[b]Results are mean values ± S.D. from triplicate cultures.

[c]Numbers in parentheses represent values of activation index.

TABLE 3

Augmentation of DNA Synthesis in Bone Marrow Cells from Various Species by PAF or the Conditioned Medium[a]

		Acid-insoluble radioactivity (dpm)[b] of bone marrow cells treated with			
Species		PAF 10^{-5}M	Compound I 10^{-9}M	Conditioned medium from PAF-treated bone marrow cells	None
Mouse	Exp. 1	1,676 ± 392 (0.81)[c]	1,900 ± 163 (0.91)	6,877 ± 543 (3.31)	2,078 ± 53 (1.0)
	Exp. 2	N.D.[d]	N.D.	5,682 ± 910 (2.73)	2,078 ± 53 (1.0)
Rabbit	Exp. 1	97,626 ± 5,600 (2.20)	97,118 ± 13,694 (2.19)	91,875 ± 9,352 (2.07)	44,311 ± 3,864 (1.0)
	Exp. 2	14,305 ± 1,555 (2.13)	34,548 ± 2,396 (5.16)	N.D.	6,695 ± 484 (1.0)
Guinea pig	Exp. 1	31,927 ± 2,864 (5.76)	N.D.	23,194 ± 1,569 (4.18)	5,544 ± 465 (1.0)

[a]The conditioned medium was prepared by centrifugation and dialysis after cultivation of guinea pig bone marrow cells were cultivated in the presence of 10^{-5}M PAF for 5 hr. Bone marrow cells (2×10^6 cells/ml) from various species were treated with PAF, Compound I or the conditioned medium for 72 hr. DNA synthesis was examined as described in Materials and Methods.

[b]Results are mean values ± S.D. from triplicate cultures.

[c]Numbers in parentheses represent values of activation index.

[d]N.D. = not determined.

The augmentative effect of PAF on DNA synthesis of guinea pig bone marrow cells was inhibited by PAF-specific antagonists, CV-6209 or FR900452, in a dose-dependent manner, as reported previously (11). We found that the treatment of bone marrow cells with PAF-specific antagonists suppressed the augmentative effect of the conditioned medium, although the inhibitory effects were much weaker than those observed in the case of directly PAF-induced effects (Fig. 2). The activity decreased by approximately 30-60% when the conditioned medium was treated at 80°C for 15 min (Table 2).

Effect on bone marrow cells from various species. As in the case of guinea pig, cell proliferation was observed when rabbit bone marrow cells were cultured in medium containing PAF (Table 3). A similar augmentative effect was observed using a strong PAF agonist, Compound I. On the other hand, bone marrow cells prepared from ICR mouse did not respond to PAF or Compound I (Table 3). Other mouse strains (C3H/HeN, CDF1) were tested, but no significant effect of PAF has been observed so far (data not shown). In spite of the nonresponsiveness, mouse bone marrow cells carried specific binding sites for PAF (Fig. 3). The Kd value (6.1×10^{-10} M) and number of binding sites (4950) per cell were almost identical with those of guinea pig bone marrow cells (10).

The conditioned medium generated from PAF-treated guinea pig bone marrow cells was able to augment DNA synthesis in rabbit and mouse bone marrow cells as well (Table 3). The conditioned medium enhanced DNA synthesis of mouse bone marrow cells in a dose-dependent manner (Fig. 1B).

Induction of microbicidal action. The conditioned medium was prepared and added to another bone marrow cell culture. After three days of incubation, cells were

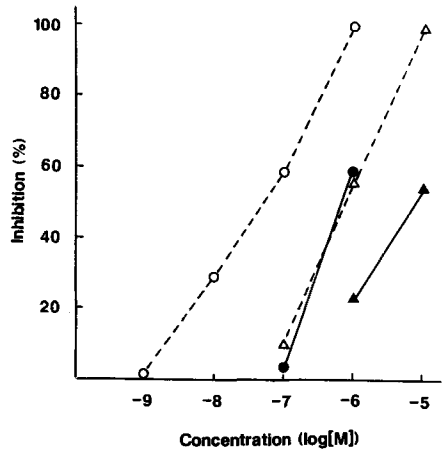


FIG. 2. Inhibition of augmentation of DNA synthesis by PAF antagonists. The conditioned medium was obtained from cultures of PAF-treated bone marrow cells. CV-6209 (○,●) or FR900452 (△,▲) was added to bone marrow cell culture 30 min prior to treatment with 10^{-5} M PAF (○,△) or 50 μl of conditioned medium (●,▲) for 72 hr. The DNA synthesis was examined as described in Materials and Methods.

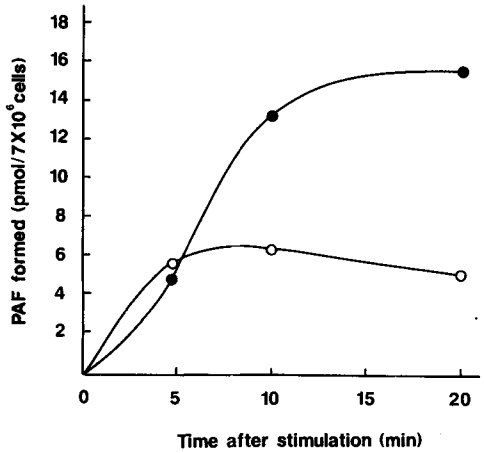


FIG. 4. Production of PAF by A23187-stimulated guinea pig bone marrow cells. Guinea pig bone marrow cells (7×10^6 cells/mL) were treated with (●) or without (○) phenylmethanesulfonyl fluoride (1 mM) for 10 min. After washing, cells were stimulated with A23187 (5 μg/ml) for the indicated periods. PAF was isolated by thin-layer chromatography and quantified by bioassay using rabbit platelets (12).

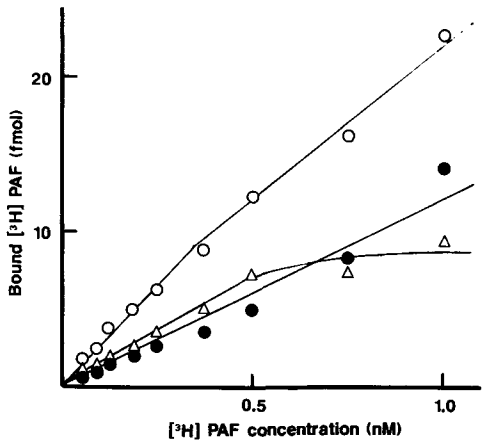


FIG. 3. Binding of PAF to murine bone marrow cells. [^{3}H]PAF binding to bone marrow cells was measured by the method described previously (10). Total binding, ○; nonspecific binding, ●; specific binding, △.

TABLE 4

Microbicidal Activity of Guinea Pig Bone Marrow Cells Treated with Conditioned Medium

Addition	Maximum number of microbes killed[a]		
	Exp. 1	Exp. 2	Exp. 2
Conditioned medium[b]			
Nontreated cells	74	10	10[d]
PAF(10^{-5}M)-treated cells	1,184	1,316 (82)[c]	1,316[d]
PAF(10^{-6}M)-treated cells	590	N.D.[e]	N.D.
PAF(10^{-6}M)	1,184	2,632	82[d]
BSA(0.25%) solution	37	82	82[d]

[a] Candidacidal activities of guinea pig bone marrow cells were determined as described in Materials and Methods.

[b] The conditioned medium was prepared after cultivation of guinea pig bone marrow cells for 20 hr in the absence or presence of PAF (10^{-5} M or 10^{-6} M).

[c] Conditioned medium heated at 80°C for 15 min.

[d] Bone marrow cells were treated with PAF antagonist, CV-6209 (10^{-6}M) 30 min prior to addition of the conditioned medium or PAF solution. Candidacidal activities were then determined.

[e] N.D. = not determined.

infected with *Candida parapsilosis* at various doses. After further incubation for two days, viable microbes present in each culture well were monitored. When bone marrow cells were cultivated in medium containing the conditioned medium, microbes were killed more effectively than in the control culture (Table 4, exp. 1). The effect was abolished when the conditioned medium was preincubated at 80°C for 15 min (Table 4, exp. 2). The PAF antagonist did not affect the microbicidal activity induced by the conditioned medium while, as reported previously (10), it inhibited that induced by PAF itself (Table 4, exp. 2).

Production of PAF in calcium ionophore-treated bone marrow cells. Stimulation of guinea pig bone marrow cells by addition of 2.5 μM calcium ionophore (A23187) led to the formation of a PAF-like substance when evaluated by bioassay using rabbit platelets (Fig. 4). The following results supported the idea that this substance is actually PAF: i) Treatment of cells with phenylmethanesulfonyl fluoride, a known inhibitor of PAF acetylhydrolase (18), prior to calcium ionophore stimulation increased the amount of PAF synthesized (Fig. 4); ii) a specific PAF antagonist, CV-6209, inhibited platelet activation (data not shown); and iii) treatment with porcine pancreatic phospholipase A_2 (19) diminished the activity (data not shown).

DISCUSSION

The conditioned medium of PAF-treated guinea pig bone marrow cells augmented the DNA synthesis of another culture of guinea pig bone marrow cells. We have

previously shown that PAF affects the proliferation of one or more classes of guinea pig bone marrow cells (11). PAF-specific antagonists inhibited the PAF-induced augmentation of DNA synthesis, as well as the induction of active conditioned medium. However, the proliferative activity detected in the conditioned medium did not appear to be due to exogenously added PAF, since after incubation for a short period at 37°C, no appreciable amount of PAF remained in the conditioned medium. The exogenously added PAF was most probably degraded to inactive lysoPAF during incubation by serum PAF acetylhydrolase, which is present in the culture medium. Thus, the augmentative effect of PAF on DNA synthesis seemed to be mediated through some factor(s) newly generated in the conditioned medium by PAF-treated bone marrow cells. The active conditioned medium appears to be produced through a specific mechanism via binding of PAF to receptors presnt on some cell population of bone marrow cells.

The augmentative effect of PAF on DNA synthesis in bone marrow cells was not always observed when various experimental animals were used. Cells from guinea pig or rabbit respond to PAF, while cells from mouse do not. Murine platelets do not respond to PAF because of the lack of binding sites on the cell surface (20). The nonresponsiveness to PAF of murine bone marrow cells, however, must have another reason, since specific and saturable binding sites were observed. The fact that the conditioned medium of PAF-treated guinea pig bone marrow cells augmented DNA synthesis of murine bone marrow cells further supported the hypothesis that an active factor(s) is generated.

In addition to the augmentative effect on DNA synthesis, the conditioned medium of PAF-treated guinea pig bone marrow cells induced the microbicidal action of cells against *Candida parapsilosis*, indicating that the active conditioned medium induces a functional alteration in bone marrow cells. It is noteworthy that a simple treatment of bone marrow cells with a recombinant human G-CSF did not cause any functional alteration (data not shown). We have observed slightly different characteristics of the two bioactions of the conditioned medium. The induction of microbicidal activity was not affected by a PAF-specific antagonist, though the antagonist did partially inhibit the augmentation of DNA synthesis. Heat treatment of the active conditioned medium totally abolished the activity to induce the microbicidal action. On the other hand, heat-treated conditioned medium still caused an augmentation of DNA synthesis, although the effect was rather weak. These differences would indicate an involvement of different factors in these two reactions. More than one factor might be produced.

One of the most important questions is to identify the active factor(s) present in the conditioned medium. Several groups have reported the production of cytokines by monocytes on stimulation with PAF (21). However, no information concerning the production of cytokines by bone marrow cells is available so far. We have observed several unique characteristics of the conditioned medium, such as a partial resistance to heat treatment and cross reactivity between species. In our preliminary experiments, a tumor necrosis factor-like activity and an interferon-like activity were detected in the conditioned medium of PAF-treated guinea pig bone marrow cells. However, in a preliminary experiment, recombinant mouse tumor necrosis factor or γ interferon failed to show an appreciable effect on bone marrow cells (data not shown). It therefore seems unlikely that the proliferative and induced microbicidal activities of PAF are wholly dependent upon these well-characterized cytokines.

It should be noted that the concentration of PAF (10^{-5} M) used in this experiment was high as compared to the expected level of PAF *in vivo* (22). Even so, we venture to emphasize the importance of the present observations for the following reasons. First, poor activity of PAF toward bone marrow cells could be due to the rapid degradation to inactive lysoPAF catalyzed by serum acetylhydrolase (17). Compound I, which is resistant to such metabolic inactivation, showed a relatively high activity. The activity of Compound I to induce functional alterations in bone marrow cells was similar to the relative potency to activate peritoneal macrophages or platelets (12). Second, on stimulation of bone marrow cells with calcium ionophore, an appreciable amount of PAF was produced. *In vivo*, PAF may modulate the fate and functions of bone marrow cells under conditions where it is produced and is delivered without degradation to the target cells. The relevance of the present study to the physiological and/or pathological roles of PAF *in vivo* needs to be further clarified.

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Effect of Platelet-Activating Factor on Tumor Necrosis Factor-Induced Superoxide Generation from Human Neutrophils. Possible Involvement of G Proteins[1]

Pierre Braquet[a,*], David Hosford[a], Philipe Koltz[b], Jean Guilbaud[b] and Monique Paubert-Braquet[b]
[a]Institute Henri Beaufour, 92350 Le Plessis-Robinson and [b]Clinical Research Unit Burn Center, 92141 Clamart, France

The effect of platelet-activating factor (PAF) and of two specific PAF antagonists on tumor necrosis factor (TNF) induced superoxide production by human polymorphonuclear neutrophils (PMN) was examined. PAF alone (0.1 pM to 0.1 nM) failed to evoke superoxide production; however, when PAF was added for 10 min to cells upon prior incubation with 10 ng/mL TNF for 50 min, superoxide production was significantly enhanced as compared to that induced by TNF alone. Maximum amplification (+30%) was obtained with 10 pM PAF; however, the effect was completely abolished by two structurally unrelated PAF antagonists, BN 52021 and BN 52111. The antagonists also decreased by 25% the superoxide production elicited solely by TNF, implicating the involvement of endogenous PAF in this process. Pretreatment of the PMN with either pertussis or cholera toxin attenuated the PAF amplified superoxide production in TNF stimulated cells, suggesting that G proteins sensitive to these toxins may be involved in the mechanisms controlling amplification.
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The normal bactericidal response of human polymorphonuclear neutrophils (PMN) involves cell stimulation, followed by a respiratory burst which results from the activation of NADPH oxidase. This multicomponent electron transport chain transfers electrons from intracellular NADPH to extracellular oxygen, reducing molecular oxygen to superoxide. The latter product is rapidly converted to hydrogen peroxide and toxic free radicals (1). Activated PMN also release lysosomal proteases and adhere to the surface of endothelial cells leading to microvascular injury characteristic of pathologies such as shock, asthma, ischemia and graft rejection. All these processes may be partially mediated by dysregulation of PMN function.

Among the various agonistic mediators released into the inflammatory microenvironment, which are capable of modulating PMN activity, are platelet-activating factor (PAF) and tumor necrosis factor (TNF). PAF, structurally characterized as 1-*O*-alkyl-2-acetyl-*sn*-glycero-3-phosphocholine, is a potent autacoid implicated in a diverse range of human pathological conditions (2). This alkyl phospholipid is now known to be produced by, and to act on, a variety of cell types including eosinophils, monocytes, macrophages, platelets, endothelial cells and PMN. PAF is a potent chemotactic agent for neutrophils, inducing superoxide release, aggregation and degranulation of these cells. *In vivo*, the mediator evokes pulmonary sequestration of neutrophils, hypotension, bronchoconstriction, increased vascular permeability, and plasma extravasation (2).

TNF is a polypeptide cytokine produced primarily by monocytes and macrophages in response to endotoxin and other immune and inflammatory stimuli (3). Large doses of TNF mimic the symptoms of endotoxic shock, such as circulatory collapse, hemorrhage and end-organ failure in a variety of species, and can lead to death (4). Similarly to PAF, TNF induces superoxide production by PMN and causes adherence of this cell type to the endothelium (5).

Although it is well established that endothelial cells produce PAF when stimulated with agonists such as thrombin, Camussi *et al.* (6) have shown that TNF *in vitro* can induce endothelial cells to synthesize PAF, the majority of which remains associated with the cells. Furthermore, Bonavida *et al.* (7) have reported that PAF can activate peripheral blood derived monocytes to produce and secrete TNF. In addition to eliciting the production of each other from cell types intimately involved in inflammatory reactions, at very low concentrations both PAF and TNF can also "prime" PMN to respond in an enhanced manner to subsequent agonistic stimuli. Following PAF (8) or TNF (9) priming, amplified PMN responses, including aggregation, adhesiveness, superoxide production and elastase release, have been observed using formyl-methionyl-leucyl-phenylalanine (FMLP) as inducing agonist.

The mechanisms involved in PAF and cytokine priming and amplification processes remain unclear. However, interaction of PAF with an undefined receptor protein may result in transmembrane signalling *via* the activation of a pertussis toxin sensitive guanyl nucleotide regulatory protein, Gi, involving GTP hydrolysis (9-11). Phospholipase C may then be stimulated, and phosphodiesterase-mediated cleavage of inositol phospholipids may induce Ca^{2+} mobilization from internal pools. Thus, activation of the Gi subunit by PAF or cytokines may lead to both inhibition of the catalytic unit of adenylate cyclase and activation of the phosphatidylinositol cycle (reviewed in ref. 12).

A positive self-generating feedback process may become operative between TNF and PAF, and PAF associated to the endothelium may prime or amplify PMN responses elicited by TNF and other agonists. The present study was therefore undertaken to examine the effect of PAF on TNF induced superoxide production by human PMN, and to define whether or not structurally unrelated specific PAF antagonists could modulate superoxide release elicited by these two mediators. The effect of bacterial toxins from

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*To whom correspondence should be addressed at the Institut Henri Beaufour, 17, avenue Descartes, F-92350 Le Plessis Robinson, France.

Abbreviations: ADP, adenosine-5'-monophosphate; BN 52021, ginkgolide B; BN 52111, 2-heptadecyl-2-methyl-4-[5-(pyridinium-pentyl-carbonyloxymethyl)]-1,3-dioxolane bromide; BSA, bovine serum albumin; FMLP, formyl-methionyl-leucyl-phenylalanine; GTP, guanosine-5'-triphosphate; NADPH, nicotinamide adenine dinucleotide, reduced form; PAF, platelet-activating factor; PBS, phosphate-buffered saline; PMN, polymorphonuclear neutrophils; TNF, tumor necrosis factor.

Vibrio cholerae and *Bordetella pertussis* on PAF modulation of TNF induced superoxide release was studied as well.

MATERIALS AND METHODS

Materials. PAF, FMLP, cytochrome C, phosphate buffered saline (PBS) and sodium chloride were obtained from Sigma Chemical Co. (St. Louis, MO). Ficoll Paque was purchased from Pharmacia (Uppsala, Sweden). Tumor necrosis factor was supplied by Genentech (San Francisco, CA). The PAF antagonists, BN 52021 (ginkgolide B), a 20-carbon cage terpene, and BN 52111, which is 2-heptadecyl-2-methyl-4-[5-(pyridinium-pentylcarbonyloxymethyl)]-1,3-dioxolane bromide, were produced by IHB Research Labs (Le Plessis-Robinson, France). Cholera and pertussis toxins were obtained from Institute Merieux (Chabonnieres les Bains, France).

Preparation of PMN. Blood was centrifuged at 1000 × *g* for 25 min. The platelet-rich plasma was discarded, and the PMN fraction was obtained following 6% dextran-500 sedimentation, centrifugation over Ficoll Paque, and ammonium chloride (160 mM) plus Tris (170 mM) treatment. PMN were then resuspended in a Ca^{2+}-free standard phosphate-buffered medium (PBS) containing (mM): Na^+, 146; K^+, 4; Cl^-, 142; HPO_4^{2-}, 2.5; $H_2PO_4^-$, 0.5; Mg^{2+}, 1; glucose, 10; (pH 7.4; determined osmolarity N 284 mOsM). Some experiments were performed in the same PBS containing 1mM Ca^{2+} to determine the effect of external Ca^{2+} on TNF induced superoxide generation. PMN were counted electronically using a Coulter Counter. The final preparation contained less than 5% lymphocytes and 15% platelets. Trypan blue staining revealed 95–99% viable cells.

TNF/PAF amplification and priming experiments. Priming and amplification are defined as follows: In a given cell system, for two effectors, A and B, inducing, respectively, the effects E_A and E_B, priming is the process when A added by itself at a subthreshold concentration at a given time induces no effect (E_A=0); however, when A is given prior to B the final effect is greater than E_B. Amplification is a process when A added alone at a given time prior to B applied at a subthreshold concentration (*i.e.*, B=0) results in a final effect greater than E_A.

TNF was freshly diluted with 1% bovine serum albumin (BSA), and the reaction was carried out in a Ca^{2+}-free phosphate-buffered saline (PBS). Cytochrome C (75 μM) was added to 2 × 10^6 cells/mL at 37°C. TNF was then added to the medium either at a concentration of 10 ng/mL (amplifcation experiments) or at very low concentrations (priming experiments), and the cells were incubated for 1 hr. After the incubation, superoxide production was determined as described below. PAF (10^{-16} to 10^{-8} M) was added 10 min before the end of the reaction to examine its potential amplifying effect. The 1-hr incubation was terminated by centrifugation for 7 min at 4°C. The supernatant was removed for measurement of cytochrome C reduction. When PAF antagonists, or pertussis or cholera toxins were used, they were added at a concentration of 10^{-6} M simultanuously with TNF at the start of the incubation period.

Results are expressed in nanomoles of superoxide anion following the equation:

$$\text{Concentration of } O_2^{\cdot-} = \frac{\text{Absorbance}}{21.1} \times 1000 \text{ nM} O_2^{\cdot-} = O_2^{\cdot-}/2 \times 10^6 \text{ cells/hr,}$$

where 21.1 μM^{-1} cm^{-1} = the absorption molecular coefficient (Σ).

Results are expressed as mean ± standard error of the mean (SEM), and differences from controls were statistically analyzed by the unpaired Student's *t*-test.

RESULTS

TNF induced superoxide production. In Ca^{2+}-free buffer, PMN (2 × 10^6 cells/mL) were incubated for 1 hr at TNF concentrations ranging from 0.01 ng/mL to 1000 ng/mL. A dose-response relationship was observed between TNF concentration and superoxide generation. The maximum response was obtained with 10 ng/mL TNF where the $O_2^{\cdot-}$ production was 4.45 ± 0.67 nmoles $O_2^{\cdot-}/2 \times 10^6$ cells/mL/hr. Thus, this concentration of TNF was selected for further studies on PMN with PAF. The presence of Ca^{2+} in the buffer significantly enhanced the superoxide production induced by TNF. At the lowest concentration (0.01 ng/mL) the free radical production became significant, and at 10 ng/mL the maximum $O_2^{\cdot-}$ production of 15.10 ± 1.36 nmoles $O_2^{\cdot-}/2 \times 10^6$ cells/mL/hr was obtained. Superoxide production was also dependent on cell number. As already shown, with 2 × 10^6 cells/mL, the TNF (10 ng/mL) induced superoxide generation was 4.45 ± 0.67 nmoles/hr, which was considered adequate to detect any modulatory effect of PAF on superoxide production. Doubling the cell number increased the superoxide release only by 35%.

To determine the time-course of TNF induced superoxide generation in the Ca^{2+}-free buffer, we measured the difference between the basal PMN $O_2^{\cdot-}$ production (control, cells not stimulated with TNF) and that obtained after stimulation of PMN with 10 ng/mL TNF for 3 hr at 37°C. Superoxide generation by TNF stimulated cells increased after 15 min, reached a maximum value at 90 min, and then gradually declined. An incubation period of 1 hr was selected for studies on the effect of PAF, as this marked the onset of a relatively stable period of maximal superoxide production in the presence of TNF.

PAF induced superoxide production. In Ca^{2+}-free buffer, PMN were incubated at various concentrations of PAF (10^{-16} M to 10^{-8} M) for periods of between 5 min to 3 hr. No significant superoxide production was elicited by PAF at any of the concentrations examined, except for 10^{-8} M PAF, which elicited the release of a marginally detectable amount (less than 1 nmole/hr) of superoxide (Fig. 1). PAF alone did not affect superoxide production regardless of incubation time or the cell number used.

PAF amplification of TNF induced superoxide production. As shown in Figure 1, when PAF (10^{-16} to 10^{-8} M) was incubated for 10 min with neutrophils which had been previously exposed to 10 ng/mL TNF for 1 hr in Ca^{2+}-free buffer, $O_2^{\cdot-}$ production was significantly amplified. Maximum amplification of TNF induced $O_2^{\cdot-}$ production was elicited by 10^{-12} M PAF, which increased superoxide release by 25–30% relative to that induced by TNF alone after 1 hr. In this system, when PAF was added before TNF at the start of the PMN incubation, no amplification was observed. Furthermore, the amplification effect required the intact PAF molecule because its biologically

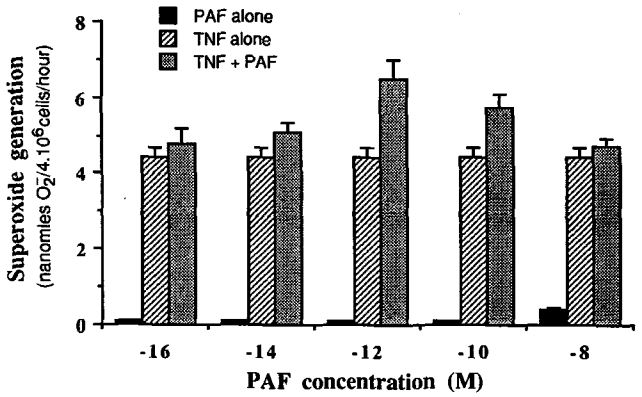


FIG. 1. PAF amplification of TNF induced superoxide generation in human neutrophils. A concentration of 10 ng/mL TNF was used and superoxide production was recorded after an incubation time of 1 hr. When PAF and TNF were used in combination, PAF was introduced 10 min before the 1 hr incubation with TNF. Values are means ± SEM, n=10. Amplification by PAF of TNF induced responses were statistically significant ($p < 0.05$) at 10^{-12} and 10^{-10} M PAF.

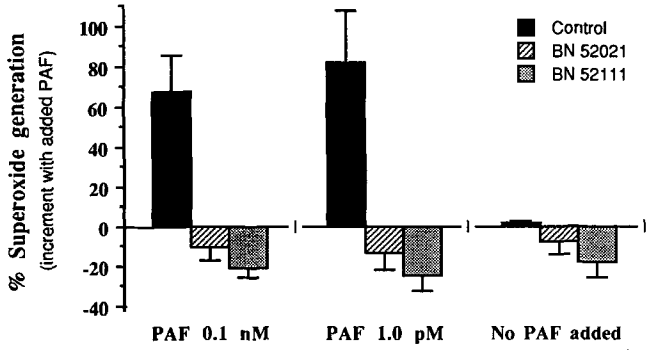


FIG. 2. Effect of two chemically unrelated PAF antagonists on PAF amplified TNF (10 ng/mL) induced superoxide production in human neutrophils. Results show means ± SEM, n=5; both PAF antagonists were used at 10^{-6} M and induced a statistically significant inhibition of PAF amplification of TNF induced superoxide production ($p < 0.05$).

inactive precursor and metabolite, lysoPAF, had no effect on TNF induced $O_2^{\cdot-}$ production. Cells in some experiments were washed twice with Ca^{2+}-free buffer to remove excess TNF before addition of PAF to determine whether the amplifying effect of the phospholipid mediator on superoxide release was dependent on the continuous presence of exogenous TNF during the incubation with PAF. PAF still amplified superoxide release when TNF was removed. In addition, the effect of PAF was found to result from an enhanced rate of superoxide release rather than from a prolongation of a relatively short oxidative response induced by PAF.

Inhibition of PAF amplification by PAF antagonists. The effects of two structurally unrelated specific antagonists on PAF amplification of TNF induced superoxide production were examined, namely, ginkgolide B (BN 52021) (10) and BN 52111 (13). These two antagonists inhibit [^{3}H]PAF binding to its rabbit platelet membrane receptor with IC_{50} values of 0.25 μM and 35 nM, respectively. As shown in Figure 2, both PAF antagonists added at a concentration of 1 μM concomitantly with TNF to the PMN at the beginning of the incubation in Ca^{2+}-free buffer completely inhibited the amplification of superoxide production induced by subsequent addition of 10^{-10} M PAF (left-hand graph) or 10^{-12} M PAF (middle graph). The inhibition of superoxide release was greater than the amplification effect of PAF indicating that the antagonists partially inhibited the superoxide production induced by TNF alone. This was confirmed by examining the effect of the two antagonists on TNF induced superoxide production in PMN which received no exogenous PAF. Figure 2 (right-hand graph) clearly shows that BN 52021 and BN 52111 inhibited TNF induced superoxide release by 8-25%. BN 52111 was the more potent antagonist.

Effect of pertussis and cholera toxin on PAF amplification of TNF induced superoxide production. The individual effects of pertussis and cholera toxin on PAF amplification of TNF induced superoxide production were examined. In addition, the effect of PAF on neutrophils preincubated in Ca^{2+}-free medium with the toxins and TNF was studied. The effect of incubation with PAF (10^{-10}, 10^{-12} M) for 10 min on neutrophils which had been previously incubated with pertussis toxin (1 μg/mL) and TNF (10 ng/mL) for 50 min is shown in Figure 3. Pertussis toxin alone released superoxide anion and significantly increased the effect of TNF. However, when PAF was added to neutrophils preincubated with pertussis toxin and TNF, no amplification of superoxide generation was observed. Instead, superoxide production decreased concentration-dependently by addition of PAF with 10^{-12} M, causing the greatest reduction (Fig. 3).

As shown in Figure 4, cholera toxin alone (0.1 or 1 μg/mL) was not capable of eliciting $O_2^{\cdot-}$ generation. When neutrophils were treated concomitantly with this toxin and TNF, there was a slight decline in the generation of

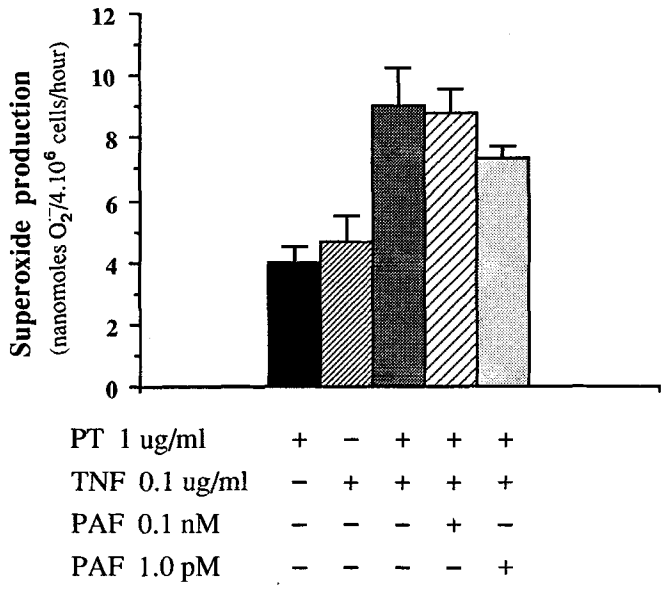


FIG. 3. Effect of pertussis toxin (PT) on PAF amplification of TNF induced superoxide production. PAF was introduced for 10 min before the end of 1 hr incubation with TNF. Pertussis toxin was added to the cells at the beginning of incubation with TNF, and significantly reduced superoxide production induced by 0.1 μg/mL TNF plus 1.0 pM PAF ($p < 0.05$).

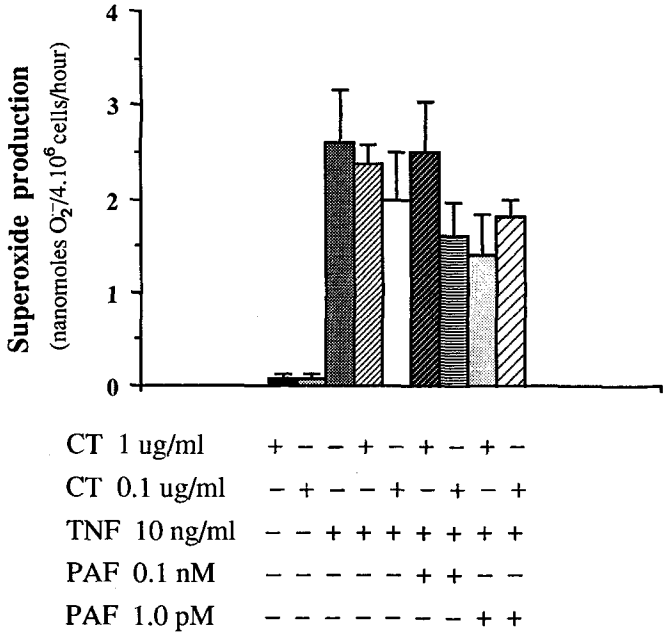


FIG. 4. Effect of cholera toxin (CT) on PAF amplication of TNF induced superoxide production. PAF was introduced 10 min before the end of the 1 hr incubation with TNF. Cholera toxin was added to the cells at the beginning of the incubation with TNF and showed a tendency to reduce superoxide generation induced by TNF and PAF.

superoxide as compared to that induced by TNF alone. When PAF (10^{-10} or 10^{-12} M) was added to the neutrophils one hr after initiation of the incubation with cholera toxin and TNF, alterations in superoxide generation depended on both the concentration of the mediator and the toxin. However, in all cases except for 10^{-10} M PAF and 1 μg/mL cholera toxin, $O_2^{\cdot-}$ production decreased when compared to that induced by cholera toxin and TNF. Thus, the amplication of TNF induced superoxide production induced by PAF was inhibited in the presence of cholera toxin, although, in contrast to pertussis toxin, cholera toxin itself was unable to elicit superoxide release and partially suppressed that induced by TNF.

TNF priming of PAF induced superoxide production. When neutrophils were incubated in Ca^{2+}-free medium for 1 hr with 0.01 and 0.1 ng/mL of TNF which were unable to elicit any detectable superoxide release by itself, the higher of these two concentrations was capable of priming the cells for $O_2^{\cdot-}$ release induced by addition of 10^{-10} M PAF for 10 min, while the other concentrations of PAF tested were ineffective. Similar to the result obtained for PAF amplification of the TNF induced response, even marginal superoxide production induced by a low concentration of TNF (1 ng/mL) was amplified when PAF was added 10 min before the end of the incubation.

DISCUSSION

Stimulation of PMN with various agonists leads to a respiratory burst which results in superoxide release, degranulation, and cell adhesion. Both TNF and PAF are modulators of the activity of PMN and play a crucial role in inflammation and host defense. In our system, TNF induced a time- and dose-dependent production of superoxide from normal human PMN. PAF alone (10^{-16} M to 10^{-8} M) caused no significant release of superoxide from the cells, regardless of cell number or incubation time. However, when PAF was added 10 min prior to the end of the 1 hr incubation period with TNF, the superoxide production of the cells was significantly enhanced in comparison with that found in cells incubated with TNF alone. Maximum amplification of superoxide production was observed at a concentration of 10^{-12} M PAF.

Several other investigators have reported PAF and TNF interactions *in vitro*. Valone and Epstein (14) have recently demonstrated that PMN stimulated with TNF are able to produce PAF in a biphasic fashion. The autacoid represented in the early peak (1–2 hr) is largely retained intracellularly, whereas the majority of PAF in the late peak (6–8 hr) is released into the medium. Studies by Rola-Pleszczynski (15) also have shown that priming of human PMN for 18 hr at various concentrations of PAF (0.1 fM to 1 μM) markedly enhanced subsequent TNF production in response to interleukin I. Maximum priming activity was observed at 1 pM to 1 nM PAF, which resulted in a 2–3-fold increase in TNF production.

In the present study, PAF had no effect on $O_2^{\cdot-}$ release when it was added to the cells before TNF at the start of the incubation period, and had only a relatively small amplifying effect when it was added before 40 min or after 2 hr (data not shown). The fact that PAF could not influence superoxide production alone and that its more pronounced amplifying effect occurred within the period when TNF induced $O_2^{\cdot-}$ release was maximal indicates that PAF augments TNF dependent $O_2^{\cdot-}$ generation even when TNF produces maximal superoxide output. Although the mechanism remains undefined, $O_2^{\cdot-}$ generation elicited solely by TNF may involve the production and release of endogenous PAF.

The results obtained with PAF antagonists support this hypothesis. The two structurally unrelated antagonists, BN 52021 and BN 52111, not only completely abolished the amplifying effect of PAF, but also partially inhibited the superoxide production evoked by TNF alone. The degree of this inhibition was related to the potency of the compound in antagonizing [^{3}H]PAF binding. The extremely potent PAF related dioxolane-derived antagonist, BN 52111 (IC_{50} 35 nM), reduced TNF induced $O_2^{\cdot-}$ release by 20%, while the less potent ginkgolide PAF antagonist, BN 52021 (IC_{50} 0.25 μM), reduced superoxide production by only 8%. This strongly suggests that TNF elicits PAF production in PMN and that this *de novo* synthesized PAF contributes to the TNF induced $O_2^{\cdot-}$ production *via* a receptor mediated process. Interestingly, a recent study by Sun and Hsueh (16) has also shown that a PAF antagonist inhibits TNF induced effects. These authors found that *in vivo*, the PAF antagonist, SRI 63-119, prevents TNF induced bowel necrosis in the rat.

PAF may have no amplifying effect when added before TNF and only a small effect when added before or after the period of maximum TNF induced $O_2^{\cdot-}$ release, because the phospholipid mediator has a short half-life in the cells. Neutrophils rapidly metabolize PAF to lysoPAF by means of an acetylhydrolase (17), although the precise half-life of PAF in our system remains to be determined. Other *in vivo* studies have found that PAF does not induce amplification when administered before TNF. Heuer *et al.* (18) have shown that pretreatment of mice with

either *S. typhosa* endotoxin or TNF significantly enhanced the mortality induced by PAF. Mortality occurred at doses of PAF, endotoxin or TNF which, given alone, did not significantly affect survival rate; however, the enhancing effect was not observed when PAF was given prior to TNF.

Our results suggest that pertussis toxin-sensitive G proteins are involved in superoxide production. Pretreatment of PMN with the toxin inhibited the amplification of TNF induced superoxide production by low doses of PAF. Recently, TNF has been shown to modulate the activity of guanylate cyclase and adenylate cyclase in human PMN, and to induce an increase in GTP binding and GTPase activity in HL-60 promyelocytic leukemic cells. GTP binding and GTPase activity are sensitive to pertussis toxin (19). The toxin also inhibits TNF induced cytotoxicity in mouse L296 fibroblasts, suggesting the involvement of a GTP binding protein in the TNF induced signalling process. In regard to the increased formation of cAMP in human neutrophil responses (20), one may speculate that the inhibition could at least in part be due to PAF induced amplification. Cholera toxin which induces cAMP production *via* activation of a G_s regulatory subunit, or phosphodiesterase inhibitors such as isobutyl methyl xanthine, antagonize the enhancement by PAF of TNF induced $O_2^{\cdot-}$ production. In addition, incubation of human PMN with TNF inhibits the alkalinization response to PAF, while preincubation with TNF results in an increase in the magnitude of the calcium transients that follow stimulation of the cells with PAF (unpublished observations). Such a phenomenon has already been observed in similar experiments using granulocyte monocyte colony stimulating factor as priming agent (21–23).

The mechanism of the amplification (and priming for low doses of TNF) may involve a modification of the functional state of certain subtypes of G protein, and/or a modulation of intracytosolic cAMP levels related to a decrease in calcium signal threshold. Finally, a similar priming phenomenon has also been found with endotoxin (24), which has been shown to prime PMN for enhanced production of superoxide upon subsequent stimulation, and that endotoxin priming induces a small but significant increase in intracellular PAF. Further stimulation of the primed PMN also produces a marked increase in cell-associated PAF as compared to controls (24). These data are consistent with results obtained in the present study, indicating that endogenous PAF production is an essential component of TNF induced superoxide generation, and suggests a role for the autacoid in the mediation of various agonist-induced cell responses.

In conclusion, an interaction between PAF and TNF is suggested to be involved in the modulation of neutrophil responses and consecutive inflammatory disorders. Although the biochemical mechanisms of priming and amplification remain to be defined, the present study shows that PAF plays an integral role in TNF induced superoxide production and suggests that this process involves several subtypes of G proteins. Furthermore, PAF antagonists may have some therapeutic value in treating conditions where this response is excessive and detrimental.

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Protein Kinase C is not Involved in the Desensitization of Platelet Activating Factor Receptor in Rabbit Platelets[1]

Lee-Young Chau
Institute of Biomedical Sciences, Academia Sinica, Nankang, Taipei 11529, Taiwan, Republic of China

Rabbit platelets pretreated with platelet activating factor (PAF) became refractory to further stimulation by PAF. The effect was specific for PAF. In this study, the alteration in the specific agonist binding to PAF receptor in platelets following desensitization was investigated. As revealed by the Scatchard analysis of radioligand binding data, the affinity for specific PAF binding to desensitized platelet membranes was substantially lowered as compared with that to control platelet membranes. Guanine nucleotide triphosphate, which was shown to decrease the affinity of specific PAF binding to platelet membranes, had less effect on the PAF binding affinity to the desensitized preparation. In platelets pretreated with phorbol 12-myristate-13-acetate, the binding affinity of PAF receptor remained unaltered. Pretreatment of platelets with 1-(5-isoquinolinesulphonyl)-2-methylpiperazine, a protein kinase C inhibitor, or neomycin, an inhibitor of the polyphosphoinositide breakdown, failed to prevent the reduction of specific PAF binding affinity following subsequent exposure to PAF. These results suggest that the agonist-induced desensitization of PAF receptor in rabbit platelets is independent of activation of protein kinase C.
Lipids 26, **1076–1079 (1991).**

Platelet activating factor (PAF) is a potent lipid mediator implicated in a variety of pathophysiological states (1). The biological effects of this agent on target cells and tissues are believed to be receptor-mediated. Specific high affinity binding sites for PAF have been identified on various cells and in membrane preparations (2–8). It has been observed that prolonged exposure of cells or tissues to PAF results in the loss of responsiveness to further stimulation by PAF (9–13), which was recognized as a "desensitization" phenomenon. In rabbit platelets, the PAF-induced desensitization was characterized as "homologous" since the desensitized platelets retained the ability to respond normally to other aggregatory agents (9,11,13). The underlying biochemical mechanism for this specific desensitization remains unclear. Recently accumulating evidence has suggested a role for protein kinase C in the down regulation of receptors which are coupled to the phosphatidylinositol signaling pathway (14–16), presumably through phosphorylation of the receptor proteins. Since in platelets pretreated with protein kinase C activator, such as PMA, the inositol phospholipid breakdown and other responses induced by PAF, thrombin and collagen were attenuated markedly (17–22), we asked whether protein kinase C could serve as feed-back regulator in the PAF-induced desensitization of platelets.

In the present study, we demonstrated the conversion of a high affinity state of specific PAF binding to a low affinity state in rabbit platelets following desensitization. Direct activation of protein kinase C by pretreating platelets with phorbol 12-myristate-13-acetate (PMA) did not significantly affect the specific PAF binding sites. Pretreatment of platelets with a potent protein kinase C inhibitor 1-(5-isoquinolinesulfonyl)-2-methylpiperazine (H7) (23), again did not prevent the conversion of the high affinity PAF binding sites to the low affinity binding sites following the prolonged incubation with PAF. Likewise, neomycin, which blocks the turnover of polyphosphoinositides (24), was shown to block the accumulation of inositolphosphates in PAF-activated platelets without affecting the desensitization of PAF receptor. These results indicate that protein kinase C is not implicated in the specific agonist-induced desensitization of PAF receptor in rabbit platelets.

MATERIALS AND METHODS

Materials. [^{3}H]PAF, 1-*O*-[^{3}H]alkyl-2-acetyl-*sn*-glycero-3-phosphocholine (specific activity, 179 Ci/mmol), myo-[^{3}H]inositol (specific activity, 19 Ci/mmol) and [^{3}H]inositolphosphate standards were purchased from Amersham International (Buckinghamshire, England). Unlabeled 1-*O*-hexadecyl-2-acetyl-*sn*-glycero-3-phosphocholine (C_{16} PAF), neomycin sulfate, H7 and guanine nucleotide triphosphate (GTP) were obtained from Sigma Chemical Co. (St. Louis, MO). Phorbol 12-myristate-13-acetate (PMA) was from Calbiochem (San Diego, CA).

Preparation of platelet membranes. Rabbit blood was drawn from an ear vein directly into 1/7 volume citrate-dextrose anticoagulant solution and centrifuged at 400 × *g* for 10 min. The platelet-rich plasma was removed and centrifuged at 900 × *g* for 20 min. The sedimented platelets were washed twice with Tyrode's buffer, pH 6.5, containing 0.1 mM EGTA [ethylene glycol *bis*(aminoethyl ether)*N,N,N',N'*-tetraacetic acid] and were finally resuspended in Tyrode's buffer, pH 7.2, at concentration of 1 × 10^9 platelets/mL. Unless otherwise stated, desensitization of platelets was conducted by adding 1 nM PAF into the platelet suspension and incubating at 37°C for 15 min. Bovine serum albumin (BSA) was then added to the platelet suspension at final concentration of 0.5% and the incubation was continued for an additional 2 min. Platelets were pelleted by centrifugation at 900 × *g* for 15 min and washed once with Tyrode's, pH 7.2, containing 0.1 mM EGTA. The control platelets were treated according to the same procedure except that PAF was omitted from the incubation. Platelets were finally resuspended in ice-cold 25 mM Tris-HCl, pH 7.4, containing

[1]Based on a paper presented at the Third International Conference on Platelet-Activating Factor and Structurally Related Alkyl Ether Lipids, Tokyo, Japan, May 1989.

Abbreviations: B_{max}, maximal binding site density; BSA, bovine serum albumin; EDTA, ethylenediaminetetraacetic acid; EGTA, ethylene glycol *bis*(aminoethyl ether)*N,N,N',N'*-tetraacetic acid; GTP, guanine nucleotide triphosphate; H7, 1-(5-isoquinoline sulfonyl)-2-methylpiperazine; HPLC, high performance liquid chromatography; IP_1, inositolmonophosphate; IP_2, inositolbisphosphate; IP_3, inositoltrisphosphate; K_d, dissociation constant; PAF, platelet activating factor; PMA, phorbol 12-myristate-13-acetate; PMSF, phenylmethylsulfonyl fluoride.

5 mM EDTA (ethylenediaminetetraacetic acid) and 0.1 mM phenylmethylsulfonyl fluoride (PMSF) at a concentration of 2×10^9 platelets/mL. Platelet membranes were prepared as described previously (25).

[^{3}H]PAF binding to platelet membranes. Platelet membrane proteins (15–20 μg) were incubated with [^{3}H]-PAF (50 fmol) and various amounts of unlabeled PAF (0–5000 fmol) in 0.5 mL of 25 mM Tris-HCl, pH 7.4, containing 0.1% BSA, 0.1M KCl and 10 mM $MgCl_2$ at room temperature for an hour. At the end of the incubation, membrane-bound [^{3}H]PAF was separated from free radioligand by vacuum filtration on glass fiber filters (Whatman GF/C) followed by rapid washing of the filters with 10 mL of ice-cold binding buffer. The radioactivity remaining on the filters was determined by liquid scintillation spectrometry. All assays were carried out in duplicate tubes. Specific binding was defined as the difference between the total binding and the non-specific binding which was obtained in the presence of 5000 fmol unlabeled PAF. When the incubation was carried out in the presence of [^{3}H]PAF alone, the specific binding activity accounted for 80% of the total binding activity. Analysis of both the filtrate and membrane-associated radiolabeled products by high performance liquid chromatography (HPLC) (25) indicated no degradation of [^{3}H]PAF under the conditions of the binding assay.

Preparation of [^{3}H]inositol-labeled platelets. [^{3}H]Inositol-labeled platelets were prepared according to the method of Morrison and Shukla (26). Prelabeled platelets were incubated with buffer alone, 10 mM neomycin, or 0.2 μM PMA, respectively, at 37°C for 15 min and then stimulated with 1 nM PAF at 37°C for 15 seconds. The reaction was terminated and inositolphosphates were extracted (26) and analyzed by HPLC (27). The extracts were injected onto a nucleosil SB-10 column (25 × 4.6 cm) equilibrated with water. The eluent was increased linearly to 0.7 M ammonium formate (adjusted to pH 3.7 with H_3PO_4) over 10 min. This concentration was held for an additional 2 min and then increased over 11 min to 1.1 M ammonium formate/H_3PO_4. The flow rate was 1.25 mL/min [^{3}H]IP_1, [^{3}H]IP_2 and [^{3}H]IP_3 standards were eluted at 10, 13, and 17 min, respectively (IP_1, inositolmonophosphate; IP_2, inositolbisphosphate; IP_3, inositoltrisphosphate).

RESULTS AND DISCUSSION

Rabbit platelets preincubated with 1 nM PAF at 37°C for 15 min became refractory to further stimulation with the same concentration of PAF (data not shown). The effect of desensitization on specific PAF binding to platelets was investigated. In this study, all the binding experiments were conducted with membrane preparations to avoid the complication of internalization of PAF by intact platelets (28). As shown in Figure 1, Scatchard analysis of the competition binding data revealed two PAF binding sites in rabbit platelets. As noted in previous observations (2,28,29), one of the binding sites is high affinity with low capacity, presumably representing the specific receptor binding for PAF; another type of binding site is low affinity with infinite capacity and is generally considered to be non-specific. Prolonged exposure of rabbit platelets to PAF resulted in a marked decrease in the affinity of the specific PAF binding sites from K_d = 0.78 nM to K_d = 2.73 nM (Fig. 1 and Table 1).

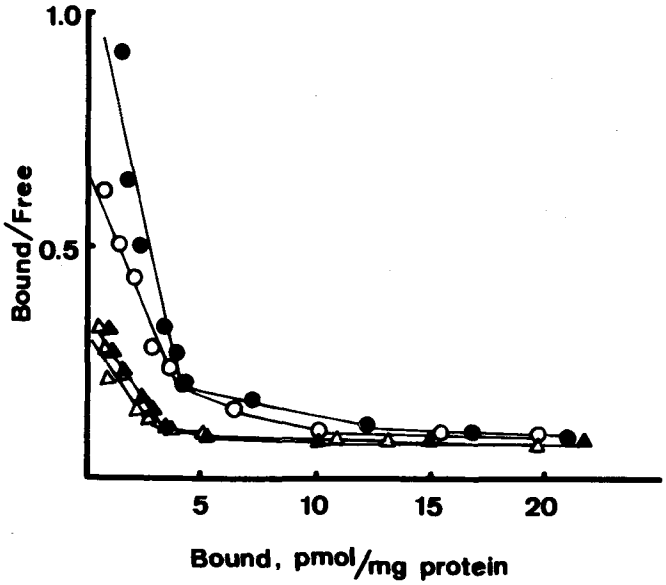


FIG. 1. Scatchard analysis of [^{3}H]PAF binding to control (circles) and desensitized (triangles) platelet membranes in the absence (•,▲) and presence (○,△) of GTP. Specific binding activity of high affinity binding sites was calculated by subtracting the nonspecific binding activity from the total binding.

The receptor density (B_{max}; B_{max} = maximal binding site density), however, was not significantly affected ($p > 0.05$). The observation of decreased agonist binding affinity to PAF-desensitized rabbit platelets was comparable to previous study on desensitized human platelets (11), and indicated the conversion of a high affinity state of the receptor to a low affinity state following agonist-induced desensitization. Recently, several lines of evidence supported the coupling of the PAF receptor to a regulatory GTP-binding protein (G-protein) which, in turn, mediated the PAF-induced polyphosphoinositide hydrolysis and inhibition of adenylate cyclase in platelets (30–32). A PAF-activated GTPase activity was demonstrated in platelet membranes (30–32). Furthermore, the specific PAF binding to rabbit platelet membranes was specifically regulated by guanine nucleotide triphosphate (GTP) (33). As illustrated in Figure 1, when the binding assay was carried out in the presence of 0.1 mM GTP, the binding affinity of the specific PAF binding to the control membranes was substantially reduced. The K_d was shifted to a value which was comparable to that of the desensitized membranes (Table 1). Although the affinity of the desensitized PAF binding sites was also further decreased by GTP, the effect was not statistically significant ($p > 0.05$). In accordance with the previous reports on the decreased PAF-induced GTPase activity in desensitized platelets (31,33), the reduced sensitivity of specific PAF binding to the modulation by GTP suggested the impairment of coupling of PAF receptor to a G-protein after desensitization.

To test whether protein kinase C—which is presumably activated following the hydrolysis of polyphosphoinositides—might be involved in the down regulation of PAF receptor, the effects of protein kinase C activator (PMA) and inhibitor (H7) on the specific PAF binding activity to platelet membranes were examined. As shown in Table 2, activation of rabbit platelets with 1 nM PAF resulted in a rapid accumulation of inositolphosphates.

TABLE 1

Effect of GTP[a] on Binding Parameters of PAF Receptor in Rabbit Platelet Membranes Before and After PAF-Induced Desensitization

	Control membranes		Desensitized membranes	
	−GTP	+GTP	−GTP	+GTP
K_d (nM)	0.78 ± 0.05[b]	2.60 ± 0.43[c]	2.73 ± 0.36[c]	3.77 ± 0.60
B_{max} (pmol/mg protein)	1.90 ± 0.08	1.93 ± 0.09	1.57 ± 0.33	1.46 ± 0.09

[a] [^{3}H]PAF binding assays were performed in the absence or presence of 0.1 mM GTP at room temperature for 1 hr as described in Materials and Methods.

[b] Data presented are the mean ± S.D. of three separate experiments. Significance was determined by paired Student's *t*-test.

[c] $P < 0.025$ for difference from value of control membranes (−GTP).

TABLE 2

Effects of PMA and Neomycin on PAF-Induced Formation of Inositolphosphates in Rabbit Platelets

Treatment[a]	[^{3}H]inositolphosphates/cpm (IP_1 + IP_2 + IP_3)
None	2430[b]
PAF	4515
PMA/PAF	2681
Neomycin/PAF	2491

[a] [^{3}H]Inositol-labeled rabbit platelets were pretreated with buffer alone, 0.2 μM PMA or 10 mM neomycin, respectively, at 37°C for 15 min and then stimulated with 1 nM PAF at 37°C for 15 seconds.

[b] Data shown is the representative of two separate experiments with similar results.

TABLE 3

Effects of H7, PMA and Neomycin on PAF-Induced Desensitization of PAF Receptor in Rabbit Platelet Membranes

Pretreatment	K_d (nM)	B_{max} (pmol/mg protein)
None	0.80 ± 0.24[c] (n=3)	1.87 ± 0.34 (n=3)
PAF	2.64 ± 0.32[d] (n=3)	1.73 ± 0.25 (n=3)
H7[a]	1.05 ± 0.19 (n=3)	1.70 ± 0.14 (n=3)
H7 + PAF	3.17 ± 0.5[d] (n=3)	1.62 ± 0.19 (n=3)
PMA[b] 0.25 μM	1.14 ± 0.26 (n=2)	2.05 ± 0.35 (n=2)
1.0 μM	1.13 ± 0.35 (n=2)	1.83 ± 0.12 (n=2)
Neomycin[a]	0.87 ± 0.28 (n=5)	1.60 ± 0.28 (n=5)
Neomycin + PAF	2.66 ± 1.0[d] (n=5)	1.92 ± 0.46 (n=5)

[a] Rabbit platelets were preincubated with 200 μM H7 or 10 mM neomycin at 37°C for 15 min prior to the desensitization with buffer alone or 1 nM PAF as described in Materials and Methods.

[b] Rabbit platelets were pretreated with 0.2 μM PMA or 1 μM PMA at 37°C for 15 min. Platelet membranes were then prepared and the [^{3}H]PAF binding activity was determined as described in Materials and Methods.

[c] Data presented are the mean ± S.D. of numbers of experiments indicated in parentheses.

[d] $P < 0.025$ by paired Student's *t*-test for difference from values of PAF-untreated membranes.

It was conceivable that the formation of the other product, diacylglycerol, would lead to the activation of the protein kinase C which, in turn, phosphorylated several platelet proteins and contributed in part to the aggregation of platelets. When rabbit platelets were preincubated with 200 μM H7 at 37°C for 15 min, the aggregation induced by 1 nM PAF was completely abolished (data not shown). Nonetheless, pretreatment of platelets with H7 followed by the prolonged incubation with the same concentration of PAF did not prevent the conversion of the high affinity PAF binding sites to the low affinity binding sites (Table 3), suggesting that protein kinase C is unlikely to be participating in the desensitization of PAF receptor. This was further confirmed by the experiment with PMA. When platelets were pretreated with PMA (0.2 μM) at 37°C for 15 min, the accumulation of inositolphosphates following PAF activation was inhibited (Table 2), indicating uncoupling of PAF receptor from the phospholipase C pathway by PMA treatment. However, the specific PAF binding to the PMA-pretreated membranes was not significantly altered as compared with that to control membranes (Table 3), again suggesting that the PAF receptor is not the site of inhibitory action of protein kinase C. Pretreatment of rabbit platelets with higher concentrations of PMA (1 μM) which would induce the aggregation of platelets to some extent still had no effect on PAF binding acativity to the platelet membranes (Table 3). In an attempt to further explore the relationship between the phosphoinositide turnover and the desensitization of PAF receptor following the activation of platelets by PAF, neomycin, which was known to inhibit the breakdown of polyphosphoinositides by binding to the phospholipid substrates (24), was also employed in present study. As revealed in Table 2, preincubation of platelets with 10 mM neomycin completely blocked the hydrolysis of the polyphosphoinositides in response to PAF stimulation. When neomycin-treated platelets were incubated subsequently with 1 nM PAF at 37°C for 15 min, the decrease in the affinity of specific PAF binding sites was still observed (Table 3), indicating that the desensitization of the PAF receptor is an event distinct from inositol phospholipid turnover.

Taken together, these results clearly demonstrated that protein kinase C is not involved in the agonist-induced desensitization of PAF receptor in rabbit platelets. Since pretreatment of platelet with PMA also resulted in the attenuation of the subsequent cellular events induced by

other aggregatory agents (17-22), it was conceivable that protein kinase C might alter the function of a common target coupling to various receptors. Nevertheless, it would then be puzzling that the protein kinase C activated in PAF-treated platelets did not cause the heterologous desensitization of platelets activated by other agents. More recently, a study (34) has demonstrated that PAF-induced desensitization of platelets also resulted in the decrease in the phosphorylation of several proteins, including a 40K protein which was known to be the target of protein kinase C in activated platelets (35). Whether the protein kinase C also became desensitized in PAF-pretreated platelets is an intriguing question to be answered. Although a recent study with human neutrophils demonstrated that the inhibition of PAF-induced Ca^{2+} transients, as well as degranulation, by a protein kinase C activator was correlated with the down regulation of high affinity PAF receptor (36), it is apparent that the actions of protein kinase C in various cell types are quite different. Previously, a distinct β-adrenergic receptor kinase was shown to be responsible for the homologous desensitization of β-adrenergic receptor (37). Whether a similar mechanism is involved in the agonist-induced desensitization of PAF receptor in rabbit platelets remains to be clarified.

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A Unique Pool of Free Arachidonate Serves as Substrate for both Cyclooxygenase and Lipoxygenase in Platelets[1]

Françoise Chevy, Claude Wolf and Odile Colard*
URA CNRS 1283, Département de Biochimie, Université Paris VI, 75571 Paris, France

Stimulation of platelets induces a rapid release of arachidonate from specific phospholipids and subsequent remodeling of arachidonate-containing phospholipids. This process is accompanied by transformation of released arachidonate by cyclooxygenase and lipoxygenase enzymes. We addressed the question of whether the cyclooxygenase and the lipoxygenase products originated from the same arachidonate-containing phospholipids. [^{14}C]Arachidonate prelabeled platelets were stimulated by thrombin or by ionophore A 23187. We monitored the cyclooxygenase pathway by following 12-hydroxy-5,8,10-heptadecatrienoic acid [12(S)-HHT] formation and the lipoxygenase pathway by following 12-hydroxy-5,8,10,14-eicosatetraenoic acid [12(S)-HETE] formation and compared specific activities. The data showed that the same pool of released arachidonate can be utilized by either cyclooxygenase or by lipoxygenase. Indeed, the specific activity of both products was identical when both enzymes were acting. Since cyclooxygenase was rapidly deactivated while lipoxygenase continued to be active, the specific activity of 12(S)-HETE became lower than the specific activity of 12(S)-HHT when large amounts of 12(S)-HETE were synthesized. Based on comparison of specific activity between phospholipids and oxygenated products, the pools of arachidonate-containing phospholipids involved in the synthesis of oxygenated products are dependent on the amount of arachidonate released.
***Lipids* 26, 1080–1085 (1991).**

When platelets are stimulated by thrombin or calcium ionophore A 23187, they release eicosanoids which are formed from arachidonate (AA) by initial action of cyclooxygenase and 12-lipoxygenase enzymes. Arachidonate is transformed by cyclooxygenase, *via* cyclic endoperoxides, primarily into thromboxane A_2 and 12-hydroxy-5,8,10-heptadecatrienoic acid [12(S)-HHT] (1–3), and by lipoxygenase into 12-hydroxy-5,8,10,14-eicosatetraenoic acid [12(S)-HETE] (4,5). The first step of the biosynthetic pathway of eicosanoids involves the release of arachidonate from membrane phospholipids (6,7).

The mechanisms involved in the stimulation of platelets by thrombin and by A 23187 are different. Thrombin stimulates polyphosphoinositide hydrolysis leading to intracellular calcium mobilization and protein kinase C activation (reviewed in refs. 8 and 9) whereas A 23187 stimulates the influx of extracellular calcium (10). The lipid changes induced by the two agonists are also different in platelets (11,12).

We have recently compared the specific activity of non-oxygenated arachidonate released from arachidonate radiolabeled platelets stimulated by thrombin to the specific activities of the different classes of phospholipids (13). In the present study, the specific activities of the products of the cyclooxygenase and lipoxygenase pathways released in response to stimulation by thrombin or by A 23187 were examined to determine whether cyclooxygenase and lipoxygenase derivatives originate from the same arachidonate-containing phospholipids. A similar arachidonate labeling strategy was used in both studies and consisted of short labeling periods to obtain a wide range of different specific activities in the phospholipid precursor pools.

MATERIALS AND METHODS

Materials. [1-^{14}C]Arachidonic acid (58 mCi/mmol) and calcium ionophore A 23187 were purchased from CEA (Paris, France) and Boehringer (Mannheim, Germany), respectively. Bovine serum albumin, bovine thrombin, 12-hydroxy-5,8,10-heptadecatrienoic acid [12(S)-HHT], arachidonic acid, phenacyl bromide and triethylamine were purchased from Sigma (St. Louis, MO). BW 755C was obtained from Wellcome Research Laboratories (Beckenham, Kent, U.K.). Gelatin used in Tyrode buffers was purchased from Merck (Darmstadt, Germany). All solvents were high-performance liquid chromatography (HPLC) grade (BDH Chemicals Ltd., Poole, U.K.). 12-Hydroxy-5,8,10,14-eicosatetraenoic acid [12(S)-HETE] was synthesized from arachidonic acid as described by Boynaems *et al.* (14), separated from other hydroxylated products, and repurified by HPLC. The 12(S)-HETE was identified by mass spectrometry. The two standards were quantified from their molar absorption coefficient at 235 nm in ethanol: E = 33400 cm^{-1} for 12(S)-HHT and E = 30500 cm^{-1} for 12(S)-HETE (15).

Methods. Rat platelets were isolated and washed as previously described (12). Washed platelets suspended in Tyrode buffer, pH 6.5, were labeled with arachidonate by incubating them for 30 min with [^{14}C]arachidonate (0.8 μCi/mL of platelets) bound to serum albumin. The labeled platelets were washed twice, resuspended in Tyrode buffer, pH 7.4, and allowed to stand for 20 min at 37°C before stimulation. Platelets were stimulated with 1 unit/mL of thrombin or with 1 μM Ca^{2+} ionophore A 23187. Reactions were stopped by addition of solvents.

Thin-layer chromatography (TLC) analysis of arachidonate derivatives. Chloroform/methanol (1:2, v/v) was added to the platelet suspension and lipids were extracted by the method of Bligh and Dyer (16) after acidification to pH 3.0 with acetic acid. The chloroform layer was dried under N_2, and the residue was spotted

[1]Based on a paper presented at the Third International Conference of Platelet-Activating Factor and Structurally Related Alkyl Ether Lipids, Tokyo, Japan, May 1989.

*To whom correspondence should be addressed at URA CNRS 1283, Biochimie, C.H.U. Saint-Antoine, 27 rue Chaligny, 75571 Paris, France.

Abbreviations: AA, arachidonate; GPC, *sn*-glycero-3-phosphocholine; GPE, *sn*-glycero-3-phosphoethanolamine; 12(S)-HHT, 12-hydroxy-5,8,10-heptadecatrienoic acid; 12(S)-HETE, 12-hydroxy-5,8,10,14-eicosatetraenoic acid; HPLC, high-performance liquid chromatography; TLC, thin-layer chromatography.

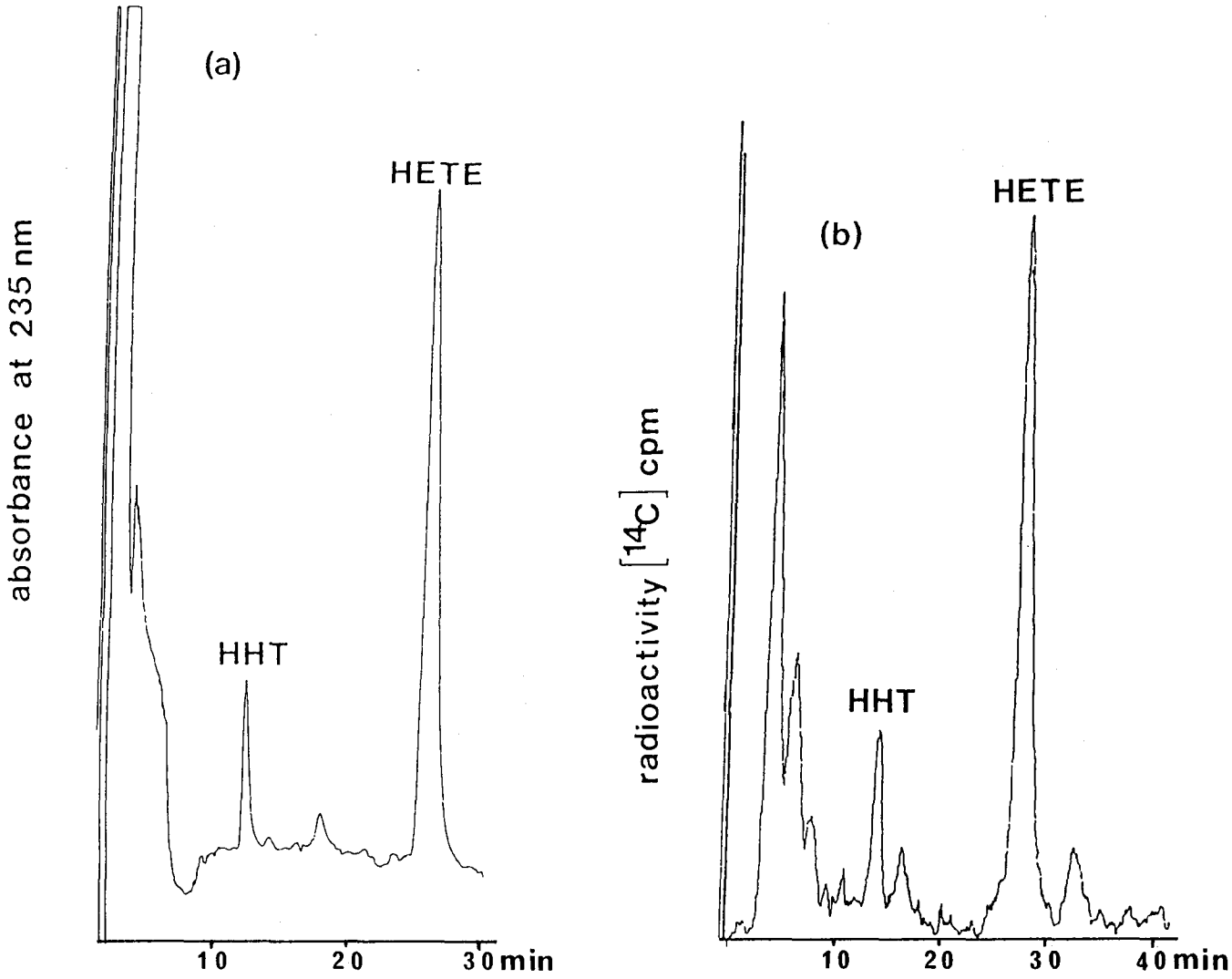


FIG. 1. HPLC analysis of 12(S)-HHT and 12(S)-HETE produced upon rat platelet stimulation. [1-^{14}C]Arachidonate prelabeled platelets (1 mL) were stimulated with A 23187 for 5 min. The reaction was stopped by adding 0.5 mL of methanol, and the mixture was centrifuged. The supernatant, acidified with acetic acid to pH 3.0, was directly injected onto a reverse phase C-18 column and analyzed by isocratic elution (1.2 mL/min) with methanol/water/acetic acid (74:26:1, v/v/v). Absorbance at 235 nm is shown in (a) and radioactivity counted on line is shown in (b).

onto silica gel thin-layer plates, and the plates were developed according to Croset *et al.* (17). A first elution with hexane/diethyl ether/acetic acid (60:40:1, v/v/v) separated arachidonate from 12(S)-HETE and 12(S)-HHT; prostaglandins and thromboxanes remained at the origin together with the phospholipids. A second elution with diethyl ether/methanol/acetic acid (90:1:2, v/v/v) separated thromboxane B_2 from the other compounds.

HPLC analysis of 12(S)-HHT and 12(S)-HETE. Arachidonate derivatives were separated by a slight modification of the method of Borgeat (18). Methanol (0.5 mL) was added to the prelabeled platelet suspension (1 mL). The mixture was allowed to stand for 30 min over ice and was centrifuged at 1600 × *g* for 15 min. The supernatant, which was acidified to pH 3.0 with 5 μL acetic acid, was directly injected into a reverse phase C-18 column (250 × 4 mm, 10 μm particles, Merck). The compounds were eluted with methanol/water, (74:26, v/v; pH 3.0) at a flow rate of 1.2 mL/min. The column was then washed with methanol and re-equilibrated with the elution mixture for 15 min before the next injection. 12(S)-HHT and 12(S)-HETE were quantified by measuring UV absorbance at 235 nm after calibration with standards. The radioactivity corresponding to 12(S)-HHT and 12(S)-HETE was counted on line following HPLC separation, as shown in Figure 1. Alternatively, fractions corresponding to 12(S)-HHT and 12(S)-HETE were collected, and the radioactivity was counted in a scintillation counter. The specific activities of the two hydroxylated compounds were expressed as dpm/nmol.

RESULTS

Preliminary experiments were carried out to determine the different eicosanoids synthesized by rat platelets, and their amounts were compared to total arachidonate re-

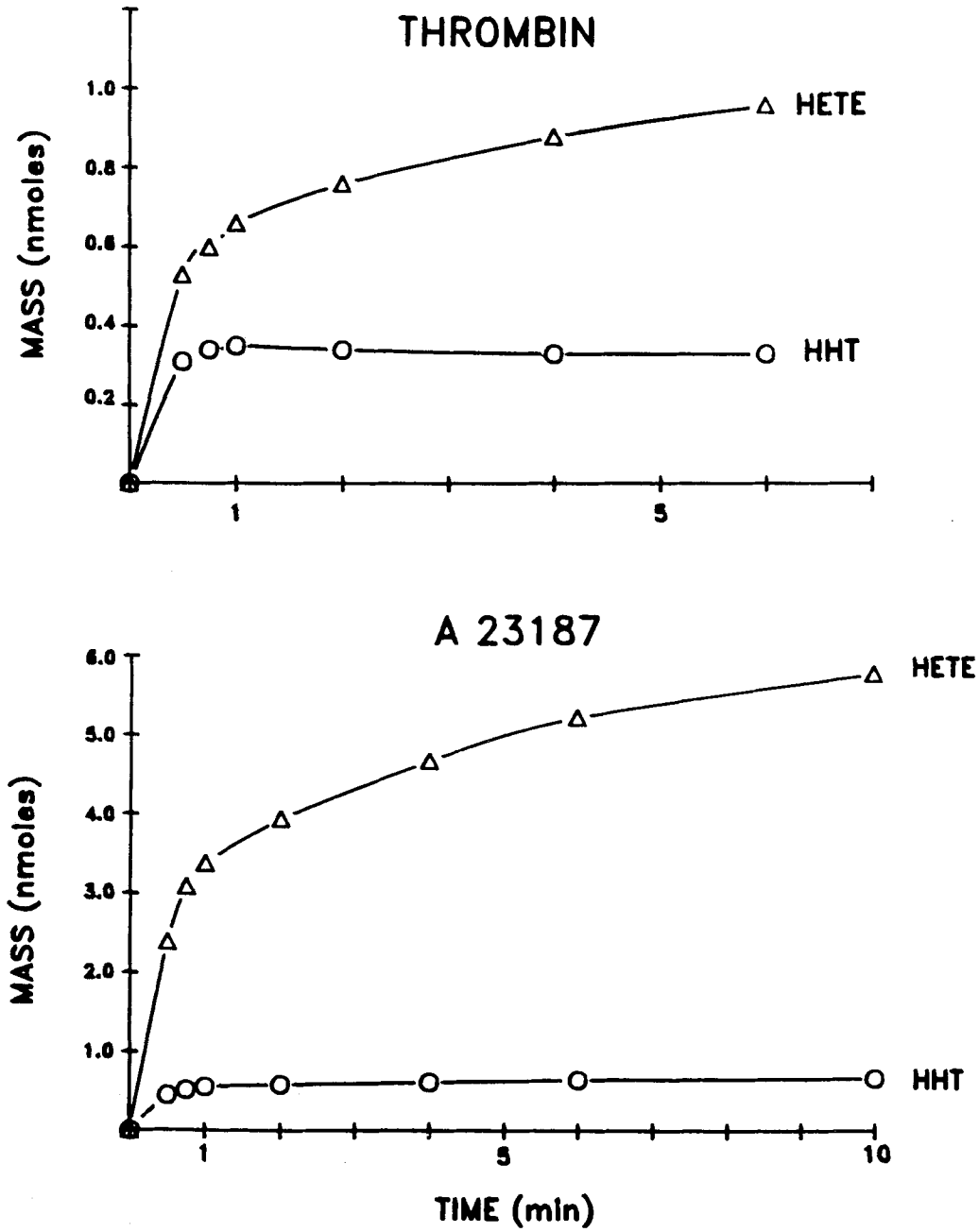


FIG. 2. Time dependence of 12(S)-HHT and 12(S)-HETE synthesis following platelet stimulation. The platelet suspension was challenged with 1 unit/mL of thrombin or with 1 μM A 23187 as described in Materials and Methods. 12(S)-HHT and 12(S)-HETE, separated by HPLC as in Figure 1, were quantified based on their absorbance at 235 nm. The data are from one platelet preparation and are representative of three other experiments.

leased from phospholipids. [^{14}C]Arachidonate prelabeled platelets were stimulated with thrombin or with Ca^{2+} ionophore A 23187; then the lipids were extracted, and the arachidonate derivatives were separated from arachidonate and from phospholipids by TLC. In the solvent system used four main radioactive fractions were detected whose R_f values corresponded to those of 12(S)-HHT, arachidonic acid, 12(S)-HETE and thromboxane B_2 (19). The non-oxygenated [^{14}C]arachidonate represented only 10.3 ± 2.9% of the total radioactively labeled arachidonate released from the phospholipids, whether the platelets were stimulated with thrombin or with A 23187. Therefore, most of the arachidonate released from phospholipids was transformed by the cyclooxygenase pathway leading to thromboxane B_2 and 12(S)-HHT, or by the lipoxygenase pathway leading to 12(S)-HETE.

The specific activities of 12(S)-HETE and of 12(S)-HHT were compared to determine whether biosynthesis of cy-

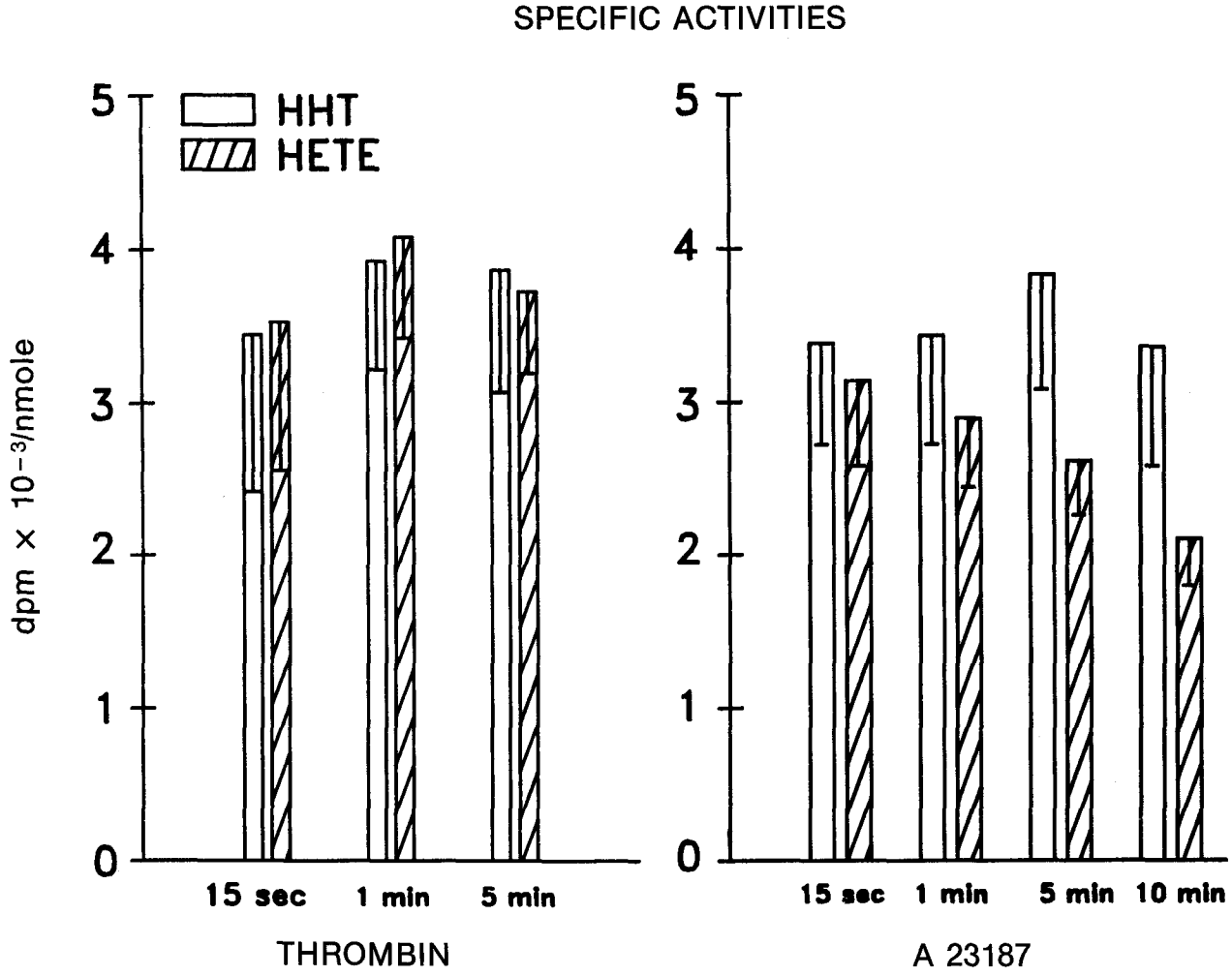


FIG. 3. Specific activities of 12(S)-HHT and 12(S)-HETE as a function of stimulation time. Prelabeled platelets were treated as in Figure 2. The fractions corresponding to 12(S)-HHT and 12(S)-HETE were collected and counted. The data are the mean ± SE of four different experiments.

clooxygenase and lipoxygenase products was linked to specific phospholipid pools. Figure 1 shows a typical HPLC separation of 12(S)-HHT and 12(S)HETE derived from the acidified supernatant of stimulated platelets; radioactivity and absorbance patterns are shown in a and b, respectively.

The kinetics of 12(S)-HHT and 12(S)-HETE synthesis upon stimulation are shown in Figure 2. The production of 12(S)-HHT almost reached the maximum as early as 30 seconds after stimulation with thrombin or with A 23187. In contrast, 12(S)-HETE synthesis increased rapidly until 1 min, and then at a slower rate up to 10 min. The amounts of the two compounds formed upon stimulation of platelets with A 23187 were always higher than upon stimulation with thrombin. In addition, the ratios between 12(S)-HHT and 12(S)-HETE differed depending on the stimulus. The amount of 12(S)-HETE was two-fold the amount of 12(S)-HHT following 5 min of platelet stimulation by thrombin; and six-fold the amount upon stimulation by A 23187.

The specific activities of 12(S)-HHT produced following stimulation with thrombin and A 23187 were not significantly different and did not vary until 10 min (Fig. 3). The specific activities of 12(S)-HETE and 12(S)-HHT were identical after thrombin stimulation and after 15 seconds of A 23187 stimulation. In contrast, the specific activity of 12(S)-HETE was significantly lower than that of 12(S)-HHT after 1 min of A 23187 stimulation, and continued to decrease until 10 min.

The decrease in the specific activity of 12(S)-HETE appeared to be correlated with massive formation of 12(S)-HETE as shown in Figure 4. When the amount of 12(S)-HETE produced was lower than 2 nmol/10^9 platelets, its specific activity was not significantly different from 12(S)-HHT, and this is consistent with the relatively low amount of 12(S)-HETE synthesized when platelets were

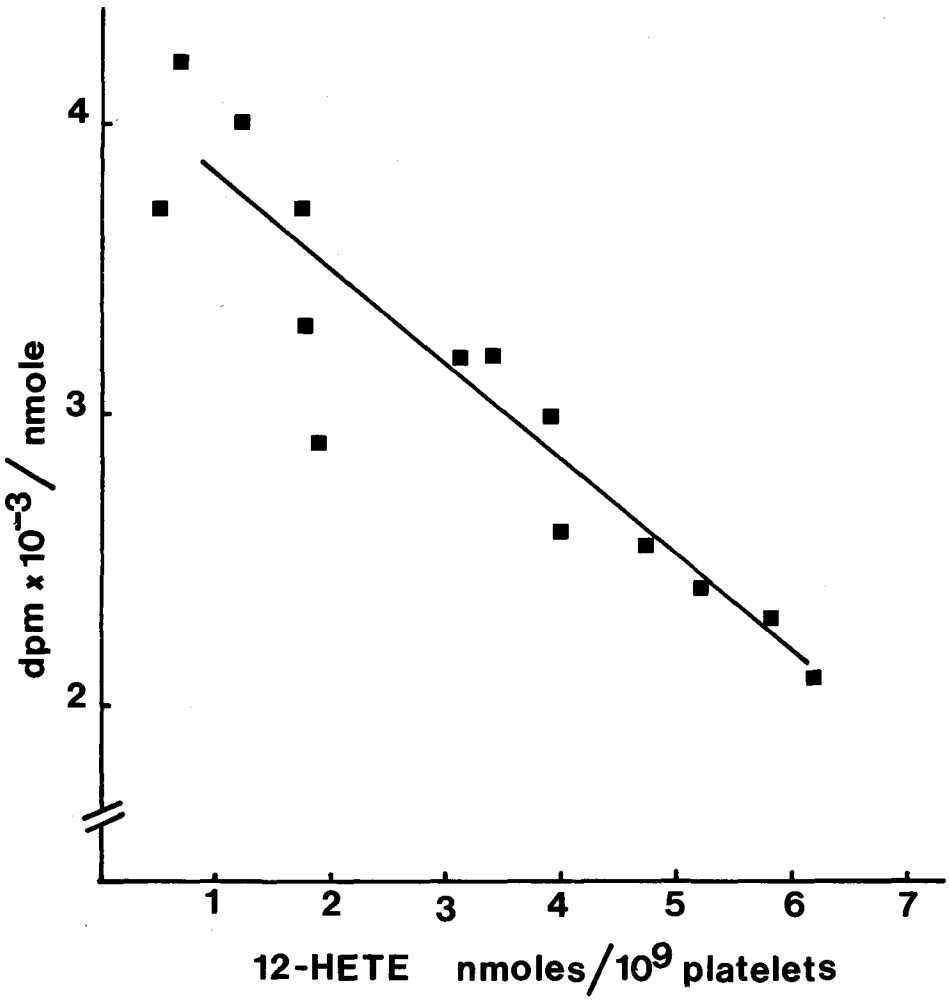


FIG. 4. Correlation between the amount of 12(S)-HETE synthesized and its specific activity. The specific activity of 12(S)-HETE, following stimulation with thrombin or A 23187, is reported as a function of the mass production of 12(S)-HETE. The values are from one experiment.

stimulated with thrombin. The amount of 12(S)-HETE produced upon A 23187 stimulation was higher than 3 nmol/10^9 platelets. Thus, the specific activities of 12(S)HHT and 12(S)-HETE produced after 1 min of stimulation with the ionophore differed.

DISCUSSION

We had previously observed that the specific activity of total arachidonate released by rat platelets stimulated with thrombin remained unchanged for 5 min (13). The total arachidonate was measured in the presence of BW 755C, an inhibitor of both cyclooxygenase and lipoxygenase in platelets. In the present work, the specific activities of 12(S)-HHT and of 12(S)-HETE were compared to determine whether the arachidonic acid available for the cyclooxygenase or for the lipoxygenase originated from the same pool of arachidonate-containing phospholipids. 12(S)-HHT is a side product of the cyclooxygenase reaction and absorbs in the UV range. Its release has been shown to parallel the release of thromboxane B_2 in human platelets (20). 12(S)-HETE is the major product of the lipoxygenase pathway in platelets and also absorbs in the UV range. The specific activities of the two compounds can be determined after HPLC separation from the same sample.

Our data show that the same pool of arachidonate released from platelet phospholipids appears to be available for the synthesis of both compounds. There was no significant difference between the specific activities of 12(S)-HHT and 12(S)-HETE when platelets were stimulated with thrombin for 5 min or with ionophore A 23187 up to 30 seconds. This greatly favors the hypothesis that the cyclooxygenase and lipoxygenase pathways are not preferentially linked to a particular pool of arachidonate-containing phospholipids, although cyclooxygenase activity is found in the intracellular membrane system (21,22), while lipoxygenase activity is mainly located in the cytosol (23,24). In addition, the hypothesis of a unique pool of arachidonate available for cyclo-

oxygenase and lipoxygenase is consistent with the increased synthesis of lipoxygenase derivatives when inhibitors of the cyclooxygenase pathway are used (25).

In accordance with the self-deactivation of cyclooxygenase observed by others (26), the maximum biosynthesis of 12(S)-HHT was reached rapidly, whereas 12(S)-HETE biosynthesis increased more steadily. 12(S)-HETE biosynthesis proceeded at a higher rate following A 23187 stimulation than following thrombin stimulation. The specific activity of 12(S)-HETE decreased and was significantly lower than the specific activity of 12(S)-HHT 1 min after platelets were challenged with ionophore A 23187. Then, a decrease in the specific activity of 12(S)-HETE was observed when cyclooxygenase was no longer active and when 12(S)-HETE was synthesized by lipoxygenase in large amounts. Moreover, the decrease in the specific activity of 12(S)-HETE correlated with the amount of 12(S)-HETE released. This may very well be related to the recruitment of phospholipid pools with slower and slower turnovers.

We have previously shown that the specific activity of total arachidonic acid released when platelets were stimulated with thrombin corresponded to the specific activity of alkyl-arachidonoyl-GPC (13). In contrast with other cells where only a fraction of the released arachidonate is transformed (27-29), most of the arachidonate released from rat platelets, as well as from human platelets (20), was transformed into oxygenated derivatives. Moreover, under conditions where a small amount of arachidonate was released (stimulation by thrombin or a short challenge by A 23187), 12(S)-HHT and 12(S)-HETE had identical specific activities. Therefore, the specific activity should correspond to the specific activity of alkyl-arachidonoyl-GPC. Chilton (29) also observed such a correlation between the specific activity of alkyl-acyl-GPC and leukotriene B_4 in human neutrophils in which only 10-30% of the arachidonate released after challenge by A 23187 was converted to leukotrienes. However, alkyl-arachidonoyl-GPC cannot account for the total amount of the arachidonate released after thrombin stimulation; diacyl-GPE, following arachidonate transfer from diacyl-GPC, is probably also involved in supplying the arachidonate available for the synthesis of oxygenated derivatives (13). Moreover, the present results suggest that hydrolysis of pools of arachidonate-containing phospholipids with a lower specific activity, such as alkenyl-acyl-GPE, has to occur and does account for the decrease in the specific activity of 12(S)-HETE when large amounts of arachidonate were released. This is in accordance with the hydrolysis of diacyl-GPE and alkenyl-acyl-GPE observed upon A 23187 stimulation of endothelial cells labeled at equilibrium with arachidonate (27).

In summary, depending on the stimulus used and the amount of arachidonate released, different pools of arachidonate-containing phospholipids may be mobilized. The arachidonate originating from the initial pool can be oxygenated by both cyclooxygenase and lipoxygenase. Further pools with a lower specific activity are mobilized after cyclooxygenase is self-deactivated but lipoxygenase continues to synthesize 12(S)-HETE.

ACKNOWLEDGMENTS

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REVIEW

Biotransformation of Alkylglycerols in Plant Cell Cultures: Production of Platelet Activating Factor and Other Biologically Active Ether Lipids[1,2]

Helmut K. Mangold, Shashank S. Apte[3] and Nikolaus Weber*
Federal Center for Lipid Research, Institute for Biochemistry and Technology, H.P. Kaufmann-Institute, D-4400 Münster, Germany

Plant cells in culture are capable of incorporating exogenous 1-*O*-alkyl-*sn*-glycerols into various neutral and ionic ether lipids. 1-*O*-Alkyl-2-acyl-*sn*-glycero-3-phosphocholines, the major class of compounds thus formed, are used for the preparation of platelet activating factor (PAF) in high yields. Similarly, the prochiral 2-*O*-alkyl-*sn*-glycerols are transformed to chiral 2-*O*-alkyl glycerophospholipids from which compounds can be obtained that exhibit antiviral activity in plant and animal cells. Reaction of 1-*O*-alkyl-2-acyl-*sn*-glycero-3-phosphocholines with phospholipase D in the presence of ethanolamine leads to 1-*O*-alkyl-2-acyl-*sn*-glycero-3-phosphoethanolamines, which serve as starting material for the preparation of 1-*O*-alkyl-2-acyl-*sn*-glycero-3-phospho-(*N*-acyl)ethanolamines, compounds known to have antitumor activity.
***Lipids* 26, 1086–1092 (1991).**

Plant cells in suspension culture rapidly absorb lipophilic compounds from the medium and incorporate them into lipid constituents of various organelles. This forms the basis for the use of plant cell cultures in the biotechnological production of fairly complex lipids from simple precursors.

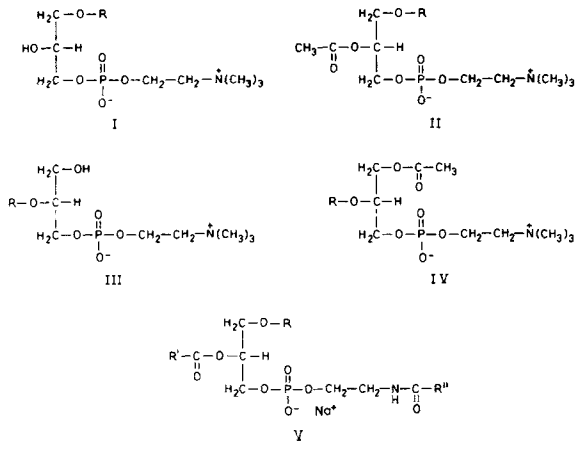
Ether lipids that do not occur in plants can be prepared by biotransformation of synthetic alkylglycerols in cell suspension cultures of rape (*Brassica napus*), soya (*Glycine max*) and other plants. 1-*O*-Alkyl-2-acyl-*sn*-glycero-3-phosphocholines, the major lipids formed by incubating *rac*-1(3)-*O*-alkylglycerols with such cell suspension cultures, serve as starting materials for the semisynthesis of some ether lipids of great current interest and high value. A few "unnatural" ether lipids that are biologically active can be prepared similarly from 2-*O*-alkyl-1-acyl-*sn*-glycero-3-phosphocholines, compounds formed by incubating 2-*O*-alkyl-*sn*-glycerols with cell suspension cultures of higher plants.

The present review summarizes our recent studies which have led to the production of a series of biologically active ether lipids whose structures are shown in Scheme 1. In addition, this article includes some pertinent experimental procedures.

The isolation of ionic ether lipids from plant cell cultures can be a rather tedious task as these substances occur predominantly in the cell membranes. Thus, the amounts of biologically active ether lipids that can be obtained by semisynthesis following this technique are typically small. Plant cell cultures are nevertheless of great value, especially if radioactively labelled chiral compounds are to be prepared. We venture to predict that in the future, immobilized plant cells as well as isolated enzymes will be found useful in the preparation of ether lipids in larger amounts.

PLANT CELL CULTURES

The techniques used in work on plant cell cultures resemble those employed in microbiology (1,2), they are easy to learn (3,4). Cells or sterile sections of plant tissue are incubated on a sterile solid medium contain-



SCHEME 1. Chemical structures of classes of ether phospholipids that were prepared using photomixotrophic cell suspension cultures of rape. I, 1-*O*-Alkyl-*sn*-glycero-3-phosphocholines (lysoPAF); and II, 1-*O*-alkyl-2-acetyl-*sn*-glycero-3-phosphocholines (PAF); III, 2-*O*-alkyl-*sn*-glycero-3-phosphocholines, positional isomers of lysoPAF; and IV, 2-*O*-alkyl-1-acetyl-*sn*-glycero-3-phosphocholines, positional isomers of PAF; V, 1-*O*-alkyl-2-acyl-*sn*-glycero-3-phospho-(*N*-acyl)ethanolamines (plasmanyl-(*N*-acyl)-ethanolamines, PNAE); R = alkyl; R'-CO-O- = acyl.

[1]Based on a paper presented at the Third International Conference on Platelet-Activating Factor and Structurally Related Alkyl Ether Lipids, Tokyo, Japan, May 1989.
[2]Dedicated to Professor Morris Kates, Ottawa, on the occasion of his retirement.
[3]Permanent address: Kakatiya University, College of Pharmaceutical Sciences, Warangal-506 009 (AP), India.
*To whom correspondence should be addressed at H.P. Kaufmann-Institute Piusallee 68, D-4400 Münster, Germany.

Abbreviations: EDTA, ethylenediaminetetraacetic acid; lysoPAF, 1-*O*-alkyl-*sn*-glycero-3-phosphocholines; PAF, platelet activating factor, 1-*O*-alkyl-2-acetyl-*sn*-glycero-3-phosphocholines; PNAE, plasmanyl-(*N*-acyl)ethanolamines, 1-*O*-alkyl-2-acyl-*sn*-glycero-3-phospho-(*N*-acyl)ethanolamines; TLC, thin-layer chromatography.

ing agar, sucrose as the major source of 'organic carbon', small proportions of auxins plus a number of inorganic salts as sources of nitrogen and phosphorus as well as trace elements (5,6).

Plant cells grown on a solid medium form a callus which is divided every four to six weeks, and each of the resulting parts is placed on fresh medium. Alternatively, aliquots of such callus cultures can be transferred into liquid media in which they grow as cell suspension cultures under continuous shaking. Aliquots of these cultures are transferred to fresh medium every two to three weeks.

Heterotrophic callus cultures and cell suspension cultures use organic compounds such as sucrose as sources of carbon; they are grown in the dark, the latter under shaking. Photomixotrophic callus cultures and cell suspension cultures use both organic compounds and carbon dioxide as sources of carbon; they require light, and the suspension cultures must be shaken. Photoautotrophic cell suspension cultures use carbon dioxide as the sole source of carbon; they require light and continuous shaking.

LIPIDS IN PLANT CELL CULTURES

The lipid contents, the proportions of lipid classes as well as their fatty acid patterns depend predominantly on the mode of carbon supply, the light conditions, and the age of the cultures (7–9).

Heterotrophic cell suspension cultures contain lipids in amounts between 15 and 50 mg/g dry weight. Photosynthetically active cultures have much larger amounts of lipids than heterotrophic ones. Thus, photoautotrophic cell suspension cultures contain lipids in amounts between 50 and 120 mg/g dry weight.

The lipid classes found in plant cell cultures are the same as those occurring in various parts of intact plants. In the exponential phase of growth, the total lipids of heterotrophic cell suspension cultures consist predominantly of phospholipids. After one week, diacylglycerophosphocholines, diacylglycerophosphoethanolamines, and diacylglycerophosphoglycerols occur at levels of about 3, 1.5 and 0.1 mg/g dry weight. Glycolipids are found in even smaller amounts. In the stationary phase, i.e., after two to four weeks of growth, sterols and complex lipids containing sterols predominate. In the exponential phase of growth, the total lipids of photosynthetically active cell suspension cultures produce much larger amounts of phospholipids and glycolipids than those of heterotrophic ones. After one week, photoautotrophic cell suspension cultures contain diacylglycerophosphocholines, diacylglycerophosphoethanolamines, and diacylglycerophosphoglycerols at levels of about 8, 4, and 2 mg/g dry weight. Glycolipids are found in even larger amounts. In the stationary phase, i.e., after two to four weeks of growth, sterols and complex lipids containing sterols predominate.

The major constituent fatty acid of the various acyl lipids in heterotrophic cell suspension cultures is linoleic acid, whereas in photomixotrophic and photoautotrophic cultures linolenic acid predominates.

BIOTRANSFORMATION OF ALKYLGLYCEROLS IN PLANT CELL CULTURES

Cell suspension cultures of different plants are capable of incorporating exogenous 1-*O*-alkyl-*sn*-glycerols as well as 2-*O*-alkyl-*sn*-glycerols into more complex compounds, especially phospholipids, although ether lipids do not occur in cells of higher plants. The proportions of complex lipids in the cultures is not markedly influenced by incubating them with such precursors. The proportions of various lipid classes also remain about the same as shown in Figure 1. Yet, most classes of glycerolipids isolated from cells that had been incubated with alkylglycerols consist of two subclasses, the endogenous neutral and ionic 1,2-diacylglycerolipids and the corresponding 1-*O*-alkyl-2-acyl- or 2-*O*-alkyl-1-acylglycerolipids, respectively.

The major neutral ether lipids formed by incubating cell suspension cultures with 1-*O*-alkyl-*sn*-glycerols (11–14) or 2-*O*-alkyl-*sn*-glycerols (14,15) are the various alkylacylglycerols, whereas alkyldiacylglycerols are found in trace amounts. The predominant components are ionic lipids, however, especially the 1-*O*-alkyl-2-acyl-*sn*-glycero-3-phosphocholines and 2-*O*-alkyl-1-acyl-*sn*-glycero-3-phosphocholines, respectively (Scheme 2). Alkylacylglycerophosphoethanolamines and other ether phospholipids are formed in much smaller proportions.

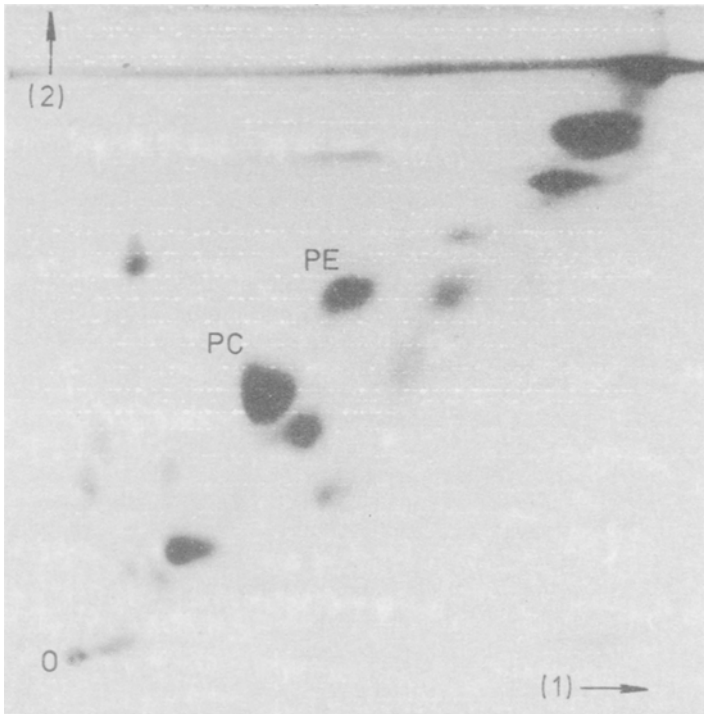
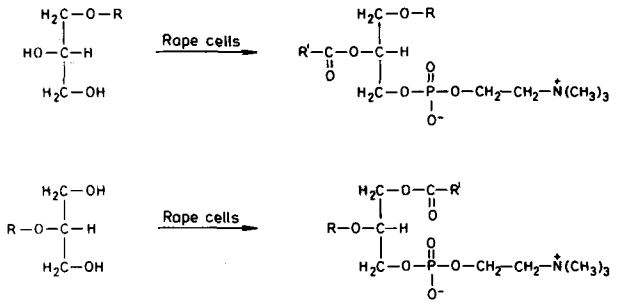


FIG. 1. Thin-layer chromatogram of the total lipids extracted from a photomixotrophic cell suspension culture of rape that had been incubated with *rac*-1(3)-*O*-hexadecylglycerol for three days. Adsorbent: Silica gel H (Merck). Solvents: Chloroform/methanol/conc. ammonia (13:7:1, v/v/v) (1st direction); acetone/ethanol/acetic acid/water (5:2:1:1, v/v/v/v) (2nd direction) (10). Fractions were detected by charring after spraying the plate with chromic-sulfuric acid solution. The cell suspension culture was 10 days old, its chlorophyll content was 1.0 mg/g dry weight. PC, diradylglycerophosphocholines; PE, diradylglycerophosphoethanolamines.



SCHEME 2. Preparation of starting material for the semisynthesis of biologically active ether lipids: Biotransformation of *rac*-1(3)-*O*-alkylglycerols to 1-*O*-alkyl-2-acyl-*sn*-glycero-3-phosphocholines and of 2-*O*-alkyl-*sn*-glycerols to 2-*O*-alkyl-1-acyl-*sn*-glycero-3-phosphocholines using photomixotrophic cell suspension cultures of rape (R = alkyl; R'-CO-O- = acyl).

When cell suspension cultures are incubated with various *rac*-1(3)-*O*-alkylglycerols, the cells specifically utilize the "natural" 1-*O*-alkyl-*sn*-glycerols from such racemic mixtures for the formation of phospholipids—though at different rates—whereas the "unnatural" 3-*O*-alkyl-*sn*-glycerols are not incorporated (12,14). It is remarkable that plant cells in suspension culture can even transform a 2-*O*-alkyl-*sn*-glycerol, i.e., a prochiral compound, into optically active 2-*O*-alkyl glyceroether lipids (14,15), as illustrated in Scheme 2.

In work aimed at the isolation of biologically active ether lipids, photomixotrophic cell suspension cultures are preferred over heterotrophic ones mainly because of their much higher lipid contents, and they are preferred over photoautotrophic as well as heterotrophic cultures because they grow much faster than the latter two. Data on the incorporation of alkylglycerols into alkylacylglycerols, the major neutral ether lipids, and alkylacylglycerophosphocholines, by far the predominant ionic ether lipids of photomixotrophic cell suspension cultures of rape, *B. napus*, are presented in Table 1.

The extent of biotransformation of 1-*O*-alkyl-*sn*-glycerols to more complex ether lipids is obviously dependent on both chain length and degree of unsaturation of their alkyl chains (14:0 ~ 18:1 > 16:0 > 18:0) (14,16) (Fig. 2). The rates of incorporation of individual 1-*O*-alkyl-*sn*-glycerols from an equimolar mixture of four saturated *rac*-1(3)-*O*-alkylglycerols reflect the substate specificities of enzymes involved in the biosynthesis of various classes of ether lipids (13).

The results of quantitative determination of 1-*O*-alkyl-2-acyl-*sn*-glycero-3-phosphocholines and the stereospecific distribution of acyl moieties in these lipid classes of rape cell cultures reveal that 1-*O*-alkyl-2-acyl-*sn*-glycero-3-phosphocholines can replace the corresponding natural membrane lipids (i.e., 1,2-diacyl-*sn*-glycero-3-phosphocholines) to an appreciable extent (16,17).

In photomixotrophic rape cells, complex ether lipids are remarkably stable; dehydrogenation of alkyl moieties to 1-alkenyl moieties does not occur and enzymatic cleavage of the ether bond, which leads to the formation of fatty acids, is insignificant (14,16).

Rape cells in suspension culture incorporate up to 3 mg alkylglycerol/g wet weight under the experimental conditions specified in the following procedure.

Incorporation of alkylglycerols into the lipids of rape cells in suspension culture. Procedure (14). Photomixotrophic cell suspension cultures of rape (*B. napus*) are propagated in modified MS-medium (6) containing 1×10^{-6} M 2,4-dichlorophenoxyacetic acid, 1×10^{-6} M 6-benzylaminopurine and 1×10^{-6} M gibberellic acid. The cultures are shaken under continuous illumination at 25°C. After 10 to 14 days, rape cells are collected on sintered glass funnels and washed briefly with distilled water. An aliquot of the cells is used for the determination of dry weight. After drying at 110°C for 3 hr, 10 g wet cells yield about 0.9 g of dry material.

A solution of 25 mg 1-*O*-alkyl-*sn*-glycerol (or 50 mg of *rac*-1(3)-*O*-alkylglycerol or 2-*O*-alkyl-*sn*-glycerol) in 0.125–0.25 mL ethanol is added to about 50 g photomixotrophic rape cells (wet weight) in 150 mL MS-

TABLE 1

Alkylacylglycerols and Alkylacylglycerophosphocholines Isolated from Photomixotrophic Rape Cells Grown in Suspension Culture After Three Days Incubation with Various *rac*-1(3)-*O*-Alkylglycerols or a 2-*O*-Alkyl-*sn*-Glycerol (14)

	Ether lipids isolated[a] (mg/kg wet weight)		
Alkyl chains	Alkylacylglycerols	Alkylacylglycero-phosphocholines[b]	Yield[c] %
1-*O*-Tetradecyl	82	190	8
1-*O*-Hexadecyl	86	96	6
1-*O*-Octadecyl	43	80	4
1-*O*-[(Z)-9'-Octadecenyl]	64	233	9
2-*O*-Hexadecyl	73	95	5

[a]In addition, small amounts of 1-*O*-alkyl-2,3-diacyl-*sn*-glycerols, 1-*O*-alkyl-2-acyl-*sn*-glycero-3-phosphoethanolamines and 1-*O*-alkyl-2-acyl-*sn*-glycero-3-phosphoglycerols were isolated.

[b]Calculated from the content of 1-*O*-alkyl-*sn*-glycero-3-phosphocholines or 2-*O*-alkyl-*sn*-glycero-3-phosphocholines derived by alkaline hydrolysis from total diradylglycerophosphocholines of rape cells after incubation with *rac*-1(3)-*O*-alkylglycerols or a 2-*O*-alkyl-*sn*-glycerol, respectively.

[c]On the basis of the amount of *sn*-1 or *sn*-2 isomer in the substrate added. Chiral 1-*O*-alkyl-*sn*-glycerols are incorporated in substantially higher yields.

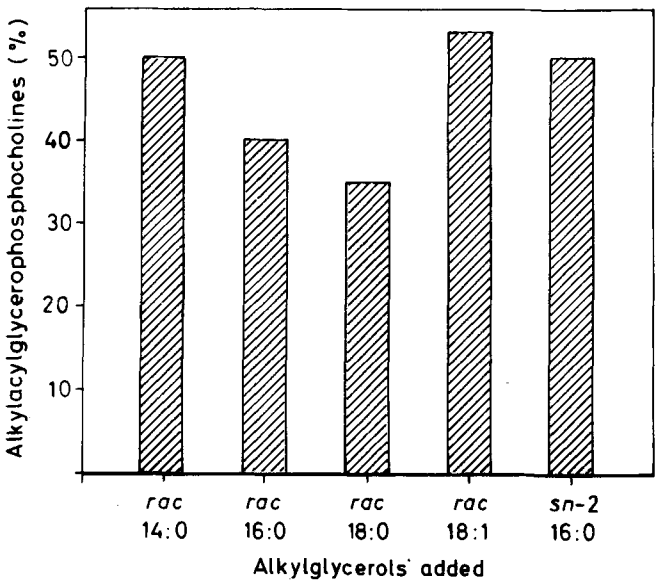


FIG. 2. Proportions of alkylacylglycerophosphocholines in the total diradylglycerophosphocholines of photomixotrophic rape cells grown in suspension culture after three days incubation with various *rac*-1(3)-*O*-alkylglycerols or a 2-*O*-alkyl-*sn*-glycerol (16).

medium on days 7, 8, and 9 (each) of growth. On day 10 the cells are collected by suction on a sintered glass funnel and washed briefly with distilled water. The lipids are extracted with hot isopropanol and chloroform/methanol following an established procedure (18).

The total lipids, about 0.4 g/100 g wet cells, are fractionated by TLC on silica gel H with chloroform/methanol/water (65:25:4, v/v/v). The neutral lipids and the diradylglycerophosphocholines are each eluted with chloroform/methanol/water (5:10:4, v/v/v) (19) and purified by repeated chromatography under the previously described experimental conditions. Chiral purity of the final product is best checked by enzymatic hydrolysis with phospholipase A_2 in combination with TLC analysis (20); yields are given in Table 1.

SEMISYNTHESIS OF ALKYLGLYCEROPHOSPHOCHOLINES (I,III) AND ALKYLACETYLGLYCEROPHOSPHOCHOLINES (II,IV)

The biotransformation of synthetic *rac*-1(3)-*O*-alkylglycerols in cell suspension cultures of rape yields stereochemically pure 1-*O*-alkyl-2-acyl-*sn*-glycero-3-phosphocholines having a defined alkyl chain. These substances are eminently suitable as starting materials for the preparation of 1-*O*-alkyl-*sn*-glycero-3-phosphocholines (lysoPAF) (I) and 1-*O*-alkyl-2-acetyl-*sn*-glycero-3-phosphocholines (PAF) (II). Similarly, the biotransformation of synthetic 2-*O*-alkyl-*sn*-glycerols in cell suspension cultures of rape yields stereochemically pure 2-*O*-alkyl-1-acyl-*sn*-glycero-3-phosphocholines having a defined alkyl chain. These substances are suitable as starting materials for the preparation of 2-*O*-alkyl-*sn*-glycero-3-phosphocholines (III) and 2-*O*-alkyl-1-acetyl-*sn*-glycero-3-phosphocholines (IV). The specific optical rotations of various lysoPAF and PAF prepared by semisynthesis from ether lipids of rape cell cultures are given in Table 2.

TABLE 2

Specific Optical Rotations[a] of Ether Lipids Isolated from Rape Cell Suspension Cultures and Subsequently Derived by Alkaline Hydrolysis or Hydrolysis Followed by Acetylation (14)

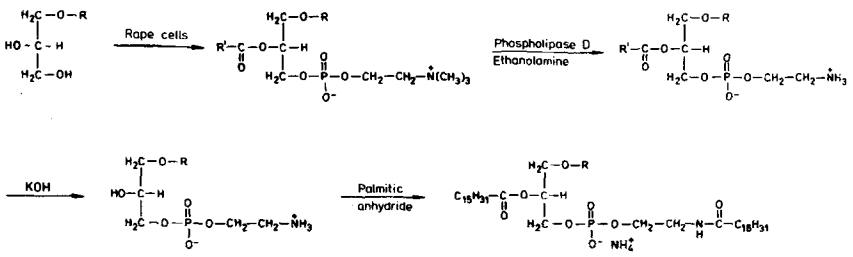
1-*O*-Alkyl-*sn*-glycero-3-phosphocholines (lysoPAF) (I) or 2-*O*-Alkyl-*sn*-glycero-3-phosphocholines (III)	
Alkyl chain	$[\alpha]_D^{20}$
1-*O*-Tetradecyl	−6.0
1-*O*-Hexadecyl	−5.8
1-*O*-Octadecyl	−5.3
1-*O*-[(*Z*)-9′-Octadecenyl]	−5.3
2-*O*-Hexadecyl	+6.6
1-*O*-Alkyl-2-acetyl-*sn*-glycero-3-phosphocholines (PAF) (II) or 2-*O*-Alkyl-1-acetyl-*sn*-glycero-3-phosphocholines (IV)	
Alkyl chain	$[\alpha]_D^{20}$
1-*O*-Tetradecyl	− 1.1
1-*O*-Hexadecyl	− 1.1
1-*O*-Octadecyl	− 1.5
1-*O*-[(*Z*)-9′-Octadecenyl]	− 1.1
2-*O*-Hexadecyl	+13.5

[a]Optical rotations of alkylglycerophosphocholines were determined in chloroform/methanol (1:1, v/v), those of alkylacetylglycerophosphocholines in chloroform (c = 1).

The use of cell suspension cultures of higher plants is particularly rewarding in the preparation of chiral ether lipids labelled with an isotope. 1-*O*-[1′-^{14}C]Hexadecyl-2-acetyl-*sn*-glycero-3-phosphocholine, for example, can be obtained in an overall yield of 50–60%, based on [1-^{14}C]palmitic acid used as starting material for the synthesis of 1-*O*-[1′-^{14}C]hexadecyl-*sn*-glycerol (21). The specific activity of this precursor is not diminished during its biotransformation in cell suspension cultures of rape because the cells do not contain ether lipids that would lead to isotope dilution. Similar results are obtained in the preparation of 2-*O*-[1′-^{14}C]hexadecyl-1-acetyl-*sn*-glycero-3-phosphocholine (15). Radioactively labelled alkylacylglycerols isolated as by-products can be used for the preparation of additional alkylacylglycerophosphocholines.

The simple procedure described below leads in excellent yields to chemically pure and biologically highly active compounds.

Preparation of lysoPAF (I) and PAF (II). Procedure (14). Diradylglycerophosphocholines, 160 mg (~0.2 mmol) isolated from photomixotrophic rape cells that had been incubated with an alkylglycerol are dissolved in 10 mL of chloroform/methanol (2:1, v/v) and deacylated by alkaline hydrolysis with 5 mL of 0.33 N methanolic potassium hydroxide solution for 1 hr at room temperature. The reaction mixture is neutralized by addition of 3 mL of ethyl formate, stirred for 15 min at room temperature, and diluted with 8 mL of chloroform/methanol (2:3, v/v). After the addition of 6 mL of water, the chloroform phase is collected, the aqueous-alcoholic phase is extracted with two portions of 10 mL chloroform each, and the chloroform solutions are



SCHEME 3. Preparation of 1-*O*-alkyl-2-palmitoyl-*sn*-glycero-3-phospho-(*N*-palmitoyl)ethanolamines (PNAE) by incubating photomixotrophic cell suspension cultures of rape with *rac*-1(3)-*O*-alkylglycerols followed by enzymatic and chemical reactions. R = Tetradecyl, hexadecyl or (*Z*)-9-octadecenyl; PNAE containing a 1-*O*-octadecyl moiety is obtained by catalytic hydrogenation of the corresponding unsaturated compound; R'-CO-O- = acyl; $C_{15}H_{31}$ CO-O- = palmitoyl.

combined. The chloroform is distilled off in a rotary evaporator and traces of water are removed by repeated distillation with chloroform/methanol (2:1, v/v) and chloroform. The residue, dissolved in chloroform/methanol (2:1, v/v) is fractionated by TLC on silica gel H with chloroform/methanol/water (10:5:1, v/v/v). The fraction of lysophospholipids ($R_f \sim 0.2$) is eluted from the adsorbent with chloroform/methanol/water (1:2:0.8, v/v/v); yield of alkylglycerophosphocholine ~ 95%.

After evaporation of the solvents in a rotary evaporator, an aliquot of 50 mg alkylglycerophosphocholine is acetylated by reaction with 2 mL of acetic anhydride/dichloromethane (1:10, v/v) containing 10 mg of 4-(*N',N'*-dimethylamino)pyridine for 1 hr at room temperature. The resulting alkylacetylglycerophosphocholine is purified by TLC on silica gel H with chloroform/methanol/acetic acid/water (14:7:1:1, v/v/v/v). The desired fraction ($R_f \sim 0.2$) is eluted with chloroform/methanol/water (1:2:0.8, v/v/v); yield of alkylacetylglycerophosphocholine (PAF) ~ 95%. The biological activities, defined as 50% of the maximum aggregation of rabbit platelets, were found to be: 1-*O*-tetradecyl PAF, $6.6 \cdot 10^{-11}$ M; 1-*O*-hexadecyl PAF, $2.2 \cdot 10^{-11}$ M; 1-*O*-octadecyl PAF, $5.7 \cdot 10^{-11}$ M; 1-*O*-[(*Z*)-9'-octadecenyl] PAF, $2.6 \cdot 10^{-11}$ M.

SEMISYNTHESIS OF ALKYLACYLGLYCEROPHOSPHO-(*N*-ACYL)ETHANOLAMINES (V)

Stereochemically pure 1-*O*-alkyl-2-acyl-*sn*-glycero-3-phosphocholines having a defined alkyl chain are suitable as starting material for the preparation of 1-*O*-alkyl-2-acyl-*sn*-glycero-3-phospho-(*N*-acyl)ethanolamines (V). The sequence of reactions leading from *rac*-1(3)-*O*-alkylglycerols to the desired compounds is shown in Scheme 3.

The biotransformation of *rac*-1(3)-*O*-alkylglycerols in photomixotrophic cell suspension cultures of rape under the previously described experimental conditions yields 1-*O*-alkyl-2-acyl-*sn*-glycero-3-phosphocholines. The latter substances are converted to 1-*O*-alkyl-2-acyl-*sn*-glycero-3-phosphoethanolamines by means of phospholipase D from *Streptomyces chromofuscus* in presence of ethanolamine (22,23). Mild alkaline hydrolysis of the ethanolamine phospholipids thus obtained and the fraction isolated from the total lipids of rape cells followed by acylation of the intermediate lyso compounds, e.g., with palmitic anhydride, yields 1-*O*-alkyl-2-acyl-*sn*-glycero-3-phospho-(*N*-acyl)ethanolamines in which both the *O*-acyl and the *N*-acyl moieties consist of palmitoyl groups.

The procedure described below leads to chemically pure and biologically highly active compounds.

Preparation of PNAE (V). Procedure (24). Diradylglycerophosphocholines, 40 mg (~ 0.05 mmol), isolated from photomixotrophic rape cells that had been incubated with an alkylglycerol are dissolved in 1 mL of chloroform. This solution is combined with a solution of ethanolamine, 150 mg (~ 2.4 mmol) in 3 mL of 0.1 M Tris-HCl buffer, pH 8.0, containing 40 mmol calcium chloride. A solution of 100 U of phospholipase D of *S. chromofuscus* (Boehringer Mannheim, Mannheim, Federal Republic of Germany) in 1 mL of Tris-HCl buffer is added; the two-phase system is shaken vigorously in a Vortex mixer for 30 seconds and then magnetically stirred in a water bath at 35°C. After 2 hr, the base exchange reaction is stopped by the addition of 2 mL 0.1 M Na_2EDTA, the mixture is diluted with 10 mL of chloroform and 7 mL of methanol, and after shaking the chloroform layer is taken off. The aqueous layer is washed twice with 10 mL of chloroform (each) the chloroform layers are combined, and the solvents distilled off in a rotary evaporator. The residue is dissolved in chloroform/methanol (2:1, v/v), and the mixture of diradylglycerophosphoethanolamines and unchanged diradylglycerophosphocholines is resolved by TLC on silica gel H with chloroform/methanol/water (65:25:4, v/v/v). The two fractions are recovered, each, by elution from the adsorbent with chloroform/methanol/water (1:2:0.8, v/v/v). The unreacted diradylglycerophosphocholines of several batches are combined and subjected to the enzymatic reaction once more. Overall yields of diradylglycerophosphoethanolamines range from 65–80%.

The diradylglycerophosphoethanolamines are deacylated by alkaline hydrolysis with 0.33 N methanolic

potassium hydroxide solution and isolated as described in the previous procedure.

About 5 mg (~ 10 μmol) of alkylglycerophosphoethanolamine thus obtained is dissolved in 2 mL of dry chloroform, and 75 mg (0.15 mmol) of palmitic anhydride plus 5 mg of 4-(*N'*,*N'*-dimethylamino)pyridine are added. The solution is shaken in a water bath at 50°C for 8 hr. After evaporation of the chloroform in a rotary evaporator, the desired PNAE (V) is purified by TLC on silica gel H with chloroform/methanol/conc. aqueous ammonia (40:10:1, v/v/v) and eluted from the adsorbent with chloroform/methanol/water (1:2:0.8, v/v/v); yields range from 50–60%. Specific optical rotations of the ammonium salts were determined in chloroform/methanol (1:1, v/v; c = 1):1-*O*-tetradecyl-2-palmitoyl PNAE, $[\alpha]_D^{20}$ + 1.8°; 1-*O*-hexadecyl-2-palmitoyl PNAE, $[\alpha]_D^{20}$ + 1.7°; 1-*O*-octadecyl-2-palmitoyl PNAE, $[\alpha]_D^{20}$ + 1.5°; 1-*O*-[(*Z*)-9'-octadecenyl]-2-palmitoyl PNAE, $[\alpha]_D^{20}$ + 1.5°. 1-*O*-Octadecyl-2-palmitoyl PNAE was prepared by hydrogenation of 1-*O*-[(*Z*)-9'-octadecenyl]-2-palmitoyl PNAE in methanol using a PtO_2 catalyst.

DISCUSSION

Past efforts to produce specific lipids by means of plant cell cultures have not been very successful. Evidently, cell cultures do not contain all the constituents of various parts of a plant from which they are derived. They are capable, however, of transforming certain exogenous precursors to more complex compounds. Thus, as we have shown, plant cells in culture stereospecifically incorporate alkylglycerols into both neutral and ionic ether lipids even though such compounds do not occur in cultured plant cells.

The stereochemically pure alkylacylglycerophosphocholines and -ethanolamines that can be isolated from cell suspension cultures of various plants after incubation with alkylglycerols constitute ideal starting materials for the semisynthesis of various biologically active ether lipids, some of which are difficult to prepare by total synthesis (25). The ether lipids derived from such alkylacylglycerophospholipids by deacylation and reacylation or acetylation are individual compounds. In contrast, ether lipids obtained by semisynthesis from lipid constituents of microorganisms and animal tissues are mixtures of compounds differing in chain length and degree of unsaturation of their alkyl moieties.

Identity and purity of the compounds we obtained using plant cell suspension cultures for the preparation of the chiral starting materials were established by their rates of migration in a number of chromatographic systems, their IR spectra and specific optical rotations as well as chemical and enzymatic reactions (26). The biological effects of these compounds were tested in cooperation with other laboratories.

The activity of PAF obtained by our fairly simple procedure is as high as that of synthetic preparations, or even higher (21). To the best of our knowledge, PAF is the first lipid that could be produced economically using plant cell cultures; this applies particularly to radioactively labelled species. The same is true for ionic ether lipids having the alkyl chain in position 2 of the glycerol backbone. Some of the latter compounds are of interest as antiviral agents (27,28). PNAE, a class of ether lipids exhibiting antineoplastic activity *in vitro* and *in vivo*, has been isolated from degenerating chick embryonal tissues (29). A set of compounds we prepared includes PNAE having a 1-*O*-tetradecyl, 1-*O*-hexadecyl, 1-*O*-octadecyl, or 1-*O*-[(*Z*)-9'-octadecenyl] chain in position 1, an *O*-palmitoyl moiety in position 2 and an *N*-palmitoyl moiety at the polar head group. All of these substances exhibit selective cytolytic effects in human tumor cells (J. Kára, *et al.*, unpublished data).

We found that photomixotrophic cell suspension cultures of rape incorporate exogenous 1-*O*-(1'-alkenyl)-*sn*-glycerols into various classes of 1-*O*-(1'-alkenyl)-2-acyl-*sn*-glycerophospholipids, but yields are low (14). Thus far, we neither succeeded in using cell suspension cultures to effect the biotransformation of *rac*-1(3)-*O*-alkyl-2-*O*-methylglycerols to the corresponding diradylglycerophosphocholines—antineoplastic agents of great current interest (30,31)—nor were we able to incorporate *rac*-1(3)-*S*-alkylglycerols or *rac*-1(3)-*S*-alkyl-2-*O*-methylglycerols into photomixotrophic rape cells.

Algae grow much faster and contain considerably larger proportions of lipids than cell cultures of higher plants. Therefore, we investigated whether *Chlorella fusca*, a green alga, can be used to effect the biotransformation of alkylglycerols to alkylacylglycerophospholipids (32). We found that *C. fusca* is indeed able to utilize synthetic *rac*-1(3)-*O*-alkylglycerols as precursors of neutral and ionic ether lipids, yet the rates of incorporation of alkylglycerols into the algal cells—as observed in tissue slices of higher plants as well (33)—are much lower than in cell suspension cultures of rape and soya.

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REVIEW

Paf-Acether in Human Skin[1]

Yves Denizot[a,2], Laurence Michel[b], Jacques Benveniste[a,*], Alain Meybeck[c], Yolène Thomas[a] and Louis Dubertret[b]

[a]INSERM U.200, 92140 Clamart, [b]INSERM U.312, 92010 Créteil, and [c]LVMH Recherche, 92704 Colombes, France

Paf is a phospholipid mediator present in human skin which induces inflammatory events, such as neutrophil infiltration and increased vascular permeability. Recent data suggest that cutaneous cells, such as fibroblasts and keratinocytes, produce paf and that paf is released during allergic cutaneous reactions. It is tempting to speculate that paf may contribute to the development of various skin disorders with acute and chronic skin inflammation. Paf antagonists may help in bringing answers to this hypothesis and may offer new prospects for the treatment of cutaneous inflammatory diseases.
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Inflammatory reactions commonly observed in human skin have largely been attributed to the action of lipid and peptide mediators, including arachidonic acid metabolites and paf-acether. Paf-acether (paf) first described as platelet-activating factor (1) and later identified as 1-*O*-alkyl-2-*O*-acetyl-*sn*-glycero-3-phosphocholine (2-4) is one of the most potent mediators of acute allergic and inflammatory reactions (5,6). Paf is synthesized by a wide variety of human inflammatory cells including neutrophils, monocytes, macrophages, platelets, eosinophils, and vascular endothelial cells (7).

Paf may also play an important role in the development and maintenance of cutaneous inflammation and in the pathogenesis of inflammatory dermatoses as it produces inflammatory responses in the skin and has been identified in lesional tissue (8,9). For instance, paf has been found in psoriatic scales (10) and has been shown to be associated with primary acquired cold urticaria (11). In human skin, paf is a potent *in vivo* agonist capable of inducing inflammatory events. When locally applied onto human dermis, it increases vascular permeability and neutrophil infiltration (9). When injected intracutaneously, paf elicits a wheal-and-flare response with a perivascular neutrophil infiltrate in normal individuals (8), whereas in allergic patients, a typical hypersensitivity cutaneous reaction occurs associated with an intense eosinophil infiltration (12). Such paf-induced eosinophil recruitment is similar to that induced by antigen in atopic individuals.

By using a skin chamber technique *in vivo*, we recently showed that prolonged contact of allergen with the dermis of atopic volunteers induces appearance of paf and intense eosinophil recruitment (13,14). We also observed that, in response to antigen, paf precursors are released in skin chamber fluids from pollen-sensitive patients. In such patients, paf and its precursors appeared during the late phase of the reaction and persisted for at least 18 hr after the allergen had been removed. The cellular origin of paf is not clear. Inflammatory cells, such as eosinophils, migrating to the inflammatory site in response to the allergen, may produce paf (12,15). Resident cutaneous cells may also synthesize paf. We recently demonstrated that at least two populations of cutaneous cells, one the fibroblasts in the dermis (16), and the other the keratinocytes in the epidermis (17), produce paf in response to calcium ionophore. Addition of lyso paf-acether (lyso paf) to fibroblast cultures enhanced paf synthesis two-fold. By contrast, addition of lyso paf to cultured keratinocytes did not modify paf production. However, acetyl-coenzyme A (acetyl-CoA) did enhance epidermal paf production five-fold. During inflammatory reactions, platelets or neutrophils may carry exogenous lyso paf and/or acetate donors to fibroblasts or epidermal cells, thus increasing the production of paf by these cells.

At the present time, the physiologic signals that induce fibroblasts or epidermal cells to produce paf are unknown. One possibility is that paf production is induced by cytokines present in the skin such as interleukin 1 (IL1). Epidermal cells secrete IL1 in large amounts (18), and IL1 induces paf synthesis in endothelial cells (19) and monocytes (20).

CONCLUSION

The ability of human epidermal cells and human dermal fibroblasts to generate paf provides additional evidence that paf is a mediator of diverse cellular origin including cells not classically designed as proinflammatory. Paf production by skin may contribute to the development of acute inflammation, to the late allergic response, and to the pathophysiology of various inflammatory dermatoses including psoriasis and cold urticaria.

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[1]Based on a paper presented at the Third International Conference on Platelet-Activating Factor and Structurally Related Alkyl Ether Lipids, Tokyo, Japan, May 1989.

[2]Present address: C.J.F. INSERM 88-03, Faculté de Médecine, 2 rue du Docteur Marcland, 87025 Limoges, France.

*To whom correspondence should be addressed at INSERM U.200, 32 rue des Carnets, 92140 Clamart, France.

Abbreviations: Acetyl-CoA, acetyl-coenzyme A; IL1, interleukin 1; lyso paf, lyso paf-acether, 1-*O*-alkyl-*sn*-glycero-3-phosphocholine; paf, paf-acether, 1-*O*-alkyl-2-*O*-acetyl-*sn*-glycero-3-phosphocholine.

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Chemical Synthesis and Physiological Activity of Sulfonium Analogues of Platelet Activating Factor[1]

Morris Kates[a,*], George A. Adams[b], Merle L. Blank[c] and Fred Snyder[c]

[a]Department of Biochemistry, University of Ottawa, Ottawa, Ontario K1N 9B4, [b]University of Ottawa Heart Institute, Ottawa Civic Hospital, Ottawa, Ontario K1Y 4E9, Canada and [c]Medical and Health Sciences Division, Oak Ridge Associated Universities, Oak Ridge, Tennessee 37831

Phosphatidylsulfocholine (PSC), the sulfonium analogue of phosphatidylcholine (PC), occurs naturally in some diatoms. The replacement of the $-\overset{+}{N}(CH_3)_3$ group by a $-\overset{+}{S}(CH_3)_2$ results in an increase in the polar head group size in PSC relative to that of PC, consistent with the observed increase in permeability of PSC bilayers towards urea. It was of interest to see whether replacement of the $-\overset{+}{N}(CH_3)_3$ group in platelet activating factor (PAF) by an $-\overset{+}{S}(CH_3)_2$ group leads to any change in platelet aggregation or other physiological activity. Synthesis of the sulfonium analogue of PAF was carried out by suitable modifications of known procedures. The PAF-sulfonium analogue was found to have almost the same platelet aggregating activity as PAF itself, in the concentration range 1–20 μM, but a much lower activity in the range 0.01–1 μM. The analogue had little or no effect on the platelet aggregation activity of PAF when added in the concentration range 0.01–1 μM and had about half the hypotensive activity of PAF towards hypertensive CDF male rats. The sulfonium analogue, however, was much more cytotoxic to HL-60 cells than PAF itself, in the concentration range 0–15 μM; replacement of the acetate group by a benzyl group increased the cytotoxicity to the level of that of the methoxy analogue of PAF. Thus, replacement of the $-\overset{+}{N}(CH_3)_3$ group by a $-\overset{+}{S}(CH_3)_2$ group in the polar head group region of PAF results in a relatively small change in its platelet aggregation activity and a decrease in its hypotensive activity, but greatly increases its antitumor activity.

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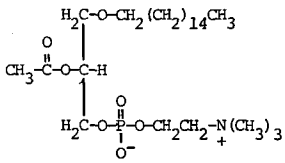
Since the discovery and structure elucidation of platelet activating factor (1-*O*-alkyl-2-acetyl-*sn*-glycero-3-phosphocholine; PAF) considerable information has become available concerning the structure-function relationships of this potent bioactive phospholipid (1,2). The structural features of the PAF molecule shown to be essential for its biological activity (see reviews, refs. 1 and 2) are: i) an ether-linked alkyl group (usually 16:0 or 18:0) at the *sn*-1 position; ii) an acetyl or propionyl group at the *sn*-2 position; and iii) a phosphocholine or phosphoquaternary base group at the *sn*-3 position of the glycerol moiety (Fig. 1).

The effect on PAF activity of replacing the trimethyl ammonium $[-\overset{+}{N}(CH_3)_3]$ group by a dimethyl sulfonium $[-\overset{+}{S}(CH_3)_2]$ group (see Fig. 1) has not previously been studied. When such a replacement is performed on phosphatidylcholine (PC) to form phosphatidylsulfocholine (SC) (3), liposomes of the latter show only minor changes in thermal properties as compared to PC liposomes (4), but the PSC liposomes have appreciably higher permeabilities than PC liposomes in the presence or absence of cholesterol (5,6). Such an effect may be due to the larger volume and lower surface charge density of the sulfocholine group compared to the choline group.

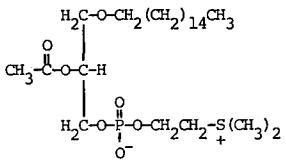
We have therefore synthesized the sulfonium as well as the 2-*O*-benzyl sulfonium analogues of PAF (Fig. 1) by the procedure shown in Scheme 1 and examined the effect of these changes on platelet aggregation, hypotensive activity and cytotoxicity to tumor cells.

MATERIALS AND METHODS

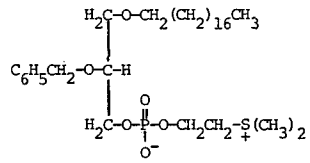
Materials. 1-*O*-Hexadecyl-*sn*-glycerol, 1-*O*-octadecyl-*sn*-glycerol and their racemates were purchased from Sigma Chemical Co. (St. Louis, MO). *Bis*(trichloroethyl)chlorophosphate and triisopropylbenzenesulfonylchloride (TPS) were obtained from Aldrich Chemical Co. (Milwaukee, WI). Sulfocholine tosylate or iodide were synthesized as described elsewhere (3). Authentic PAF (synthetic 1-*O*-hexadecyl-2-acetyl-*sn*-glycero-3-phosphocholine) was purchased from Sigma Chemical Co., or from Serdary Research Labs Inc. (London, Ontario, Canada, beef heart preparation). All solvents were glass distilled before use. Pyridine was dried over fresh KOH pellets and used without distillation.



PAF



7



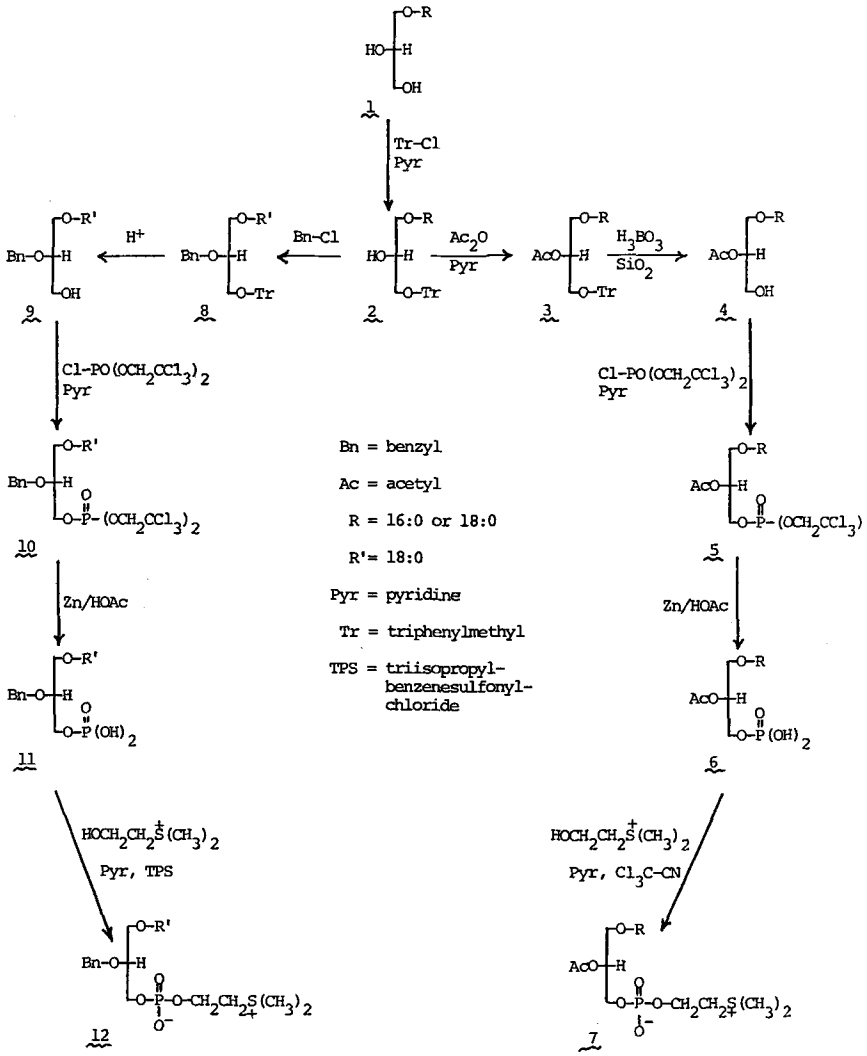
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FIG. 1. Chemical structures of PAF (1-*O*-hexadecyl-2-acetyl-*sn*-glycero-3-phosphocholine), and PAF sulfonium analogues, 7 (1-*O*-hexadecyl-2-acetyl-*sn*-glycero-3-phosphosulfocholine), and 12 (1-*O*-octadecyl-2-*O*-benzyl-*sn*-glycero-3-phosphosulfocholine).

[1]Based on a paper presented at the Third International Conference on Platelet-Activating Factor and Structurally Related Alkyl Ether Lipids, Tokyo, Japan, May 8–12, 1989.

*To whom correspondence should be addressed.

Abbreviations: FAB-MS, fast-atom bombardment mass-spectrometry; GPC, glycerophosphocholine; GPSC, glycerophosphosulfocholine; NMR, nuclear magnetic resonance; PC, phosphatidylcholine; PSC, phosphatidylsulfocholine; TLC, thin-layer chromatography; TMS, tetramethylsilane; TPS, triisopropylbenzenesulfonyl chloride; trityl, triphenylmethyl.



SCHEME 1. Procedures for chemical synthesis of PAF sulfonium analogues.

Chromatography. Thin-layer chromatography (TLC) was carried out on silica gel G precoated plates (Whatman, 20 × 20 cm; 0.25 mm thick for analytical and 0.75 mm thick for preparative use). Solvent systems used were: A, chloroform/methanol/28% NH_4OH (65:35:5, v/v/v); B, chloroform/methanol/water (65:25:4, v/v/v); C, petroleum ether/diethyl ether (70:30, v/v); and D, chloroform/methanol (45:5, v/v).

Physical measurements. ^1H NMR (nuclear magnetic resonance) spectra of $CDCl_3$ solutions of the compounds were measured on a Bruker CXP-300 spectrometer, and chemical shift values (δ) are given as ppm relative to tetramethylsilane (TMS). Positive fast atom bombardment (FAB) and chemical ionization (CI, in source, diethyl ether) mass spectra were obtained on a VG-7070E mass spectrometer.

PAF activity measurement. Platelet aggregating activity was measured in an aggregometer (Payton Instruments, Toronto, Canada) on platelet rich plasma or washed platelets (7) diluted in autologous plasma at a cell concentration of 3×10^8 cells/mL; maximum extent of aggregation during 4 min was measured. Solutions of PAF or PAF analogs in 0.9% sodium chloride were tested against a blank of 0.9% sodium chloride alone over the concentration range 0.001 to 100 μM, and compared with aggregation produced by a standard 0.005% collagen solution.

Hypotensive activity waas measured by recording changes in arterial blood pressure in an 11-month-old CDF male rat after intravenous injection of 10 pmole of PAF or PAF analogs, as described elsewhere (8).

Cytotoxicity of PAF or PAF analogs was assayed as described previously (9), using cultured HL-60 cells grown in serum-free RPMI 1540 medium (9) and measuring cell viability by trypan blue exclusion (10).

Chemical synthesis of PAF analogues (see Scheme 1) *1-O-Hexadecyl-2-acetyl-sn-glycero-3-phosphate* (6). 1-*O*-Hexadecyl-*sn*-glycerol (1; R = 16:0) was tritylated with trityl chloride in anhydrous pyridine as described previously (11), and the product was freed from most of the contaminating tritanol by trituration with low-boiling petroleum ether to yield the 1-*O*-hexadecyl-3-trityl derivative (2, R = 16:0); TLC (solvent C) R_f = 0.57 (still contaminated with some tritanol, tritane and ditrityl ether). The crude trityl derivative 2, without further purification, was acetylated (12) with a ten-fold excess of acetic

anhydride/anhydrous pyridine (1:2, v/v) at 50°C for 2 hr to yield the 1-*O*-hexadecyl-2-acetyl-3-trityl-*sn*-glycerol (3, Rf = 0.88 in solvent C). The latter was then detritylated by passage of its solution in petroleum ether through a silicic acid/boric acid (10:1, w/w) column and elution with petroleum ether/diethyl ether (3:1, v/v), according to the method of Buchnea (13). The resulting product, 1-*O*-hexadecyl-2-acetyl-*sn*-glycerol (4), gave a single spot on TLC (petroleum ether/diethyl ether, 3:2, v/v; R_f = 0.20), corresponding to a 1,2-diacylglycerol standard; only low amounts of the faster-moving (R_f = 0.23) 1,3-isomer could be detected; yield, 70–80% from 3. ^{1}H NMR ($CDCl_3$): 0.86 (terminal CH_3, 3H), 1.24 (alkyl CH_2, ω-1 to C-3, 26H), 1.56 (alkyl C-2, 2H), 2.08 (minor), 2.10 (major) (acetyl CH_3, 3H); 2.48 (C-O$\underline{H}$); 3.42 (O-C$\underline{H}_2$ alkyl, 2H); 3.50 (C$\underline{H}_2$-O, *sn*-1 glycerol, 2H), 3.60 (C$\underline{H}_2$-OH, *sn*-3 glycerol, 2H), 4.95 (C$\underline{H}$-O, *sn*-2 glycerol, 1H) ppm. CI-MS: 359 (M + 1), 341 (M + 1 − H_2O); calcd. for $C_{21}H_{72}O_4$, M = 358.

Compound 4 (R = 18:0) was synthesized in the same way starting from 1 (R = 18:0); yields, TLC mobilities, and NMR spectra were essentially identical to 4 (R = 16:0); CI-MS: 387 (M + 1), 369 (M + 1 − H_2O); calcd. for $C_{23}H_{46}O_4$, M = 386.

Compound 4 (0.17 g, 0.49 mmol) was then reacted with *bis* (trichloroethyl)chlorophosphate (0.2 g, 0.5 mmole) in anhydrous pyridine (2.6 mL) at room temperature, by the procedure described previously (14) and the blocked phosphorylated product (5) was isolated by acidic Bligh-Dyer extraction (12,14); yield of crude 5, 0.46 mmole (95%); TLC (solvent D) R_f = 0.89 (contaminated with some tritylated material). The crude product 5 (0.46 mmole) was then deblocked by reaction with Zn powder (0.27 g) in a mixture of chloroform (2.5 mL) and glacial acetic acid (4 mL) at room temperature for 1 hr, as described elsewhere (14). The product, 1-*O*-hexadecyl-2-acetyl-*sn*-glycero-3-phosphate (6), was isolated by acidic Bligh-Dyer extraction and purified by repeated acetone precipitation of the ammonium salt (12,14); yield, 0.15 g (0.32 mmole, 70%); TLC (solvent A) R_f = 0.08 (P-positive) (12). ^{1}H NMR ($CDCl_3$): 0.86 (terminal CH_3, 3 H), 1.23 (alkyl CH_2, ω-1 to C-3, 26 H), 1.50 (alkyl C-2, 2H), 2.08 (acetyl CH_3, 3 H), 3.39 (O-CH_2 alkyl, 2 H), 3.54 (CH_2-, *sn*-1 glycerol, 2 H), 3.90 (CH_2-O-P, *sn*-3 glycerol, 2 H), 5.13 (CH-O, *sn*-2 glycerol, 1 H) ppm. Anal. Calcd. for monoammonium salt monohydrate $C_{21}H_{46}O_7NP{\cdot}H_2O$ (MW 473.59): C, 53.26; H, 10.22; N, 2.96; Found: C, 52.36; H, 10.03; N, 3.17. Negative FAB-MS (mass spectrometry): 437 $(M - NH_3)^-$, 395 (M − NH_3 − acetyl)$^-$; calcd. for $C_{21}H_{46}O_7NP$, M=455. The racemic form of compound 6 (R = 16:0) was synthesized by the same route as for the enantiomeric form (Scheme 1), but starting from *rac*-1-hexadecylglycerol (1, R = 16:0).

The enantiomeric and racemic forms of compound 6 (R = 18:0) were also synthesized by the same route (Scheme 1), but starting from 1-*O*-octadecyl-*sn*-glycerol or *rac*-1-octadecylglycerol (1, R = 18:0), respectively. Yields, TLC mobilities and NMR spectra were identical or very similar to those of 6 (R = 16:0).

*1-O-Hexadecyl-2-acetyl-*sn*-glycero-3-phosphosulfocholine (7; R = 16:0). Compound* 6 (77 mg, 0.16 mmole) was converted to the free acid form by acid Bligh-Dyer extraction (12), mixed with sulfocholine tosylate (0.45 g, 1.6 mmole) and dried overnight *in vacuo* over phosphorus pentoxide; anhydrous pyridine (10 mL) was added and the mixture was stirred at 50°C until clear (1 hr). Trichloracetonitrile (3 mL) was added and stirring was continued at 50°C for 48 hr, following the procedure of Ali and Bittman (15). The clear, dark-colored reaction mixture was brought to dryness under reduced pressure and the residual dark brown oil was partitioned by the acidic Bligh-Dyer procedure (12) to remove pyridine and other water-soluble material. The chloroform phase was concentrated to dryness under reduced pressure and the residual oil was dissolved in methanol/water (95:5, v/v; 2 mL) and passed through a column of mixed-bed ion-exchange resin (Bio-Rad AG 501-X8(D), Bio-Rad Laboratories, Richmond, CA; 9 g], equilibrated in the same solvent. The column was washed with a total of 10 mL of methanol/water (95:5, v/v). Alternatively, treatment with the ion-exchange resin can be done batchwise with filtration and washing of the resin on a sintered glass filter. The combined column washings were diluted with appropriate amounts of chloroform and water to form a two phased mixture (chloroform/methanol/water, 1:1:0.9, v/v/v), and the chloroform phase was diluted with benzene and brought to dryness under reduced pressure; yield 13.3 mg of crude product 7; TLC (solvent A) R_f = 0.18; contains unidentified fast-moving contaminants (R_f = 0.8), but is free of starting material 6.

Further purification was achieved by chromatography of crude 7 (13 mg) on a column of silicic acid (Bio-Sil A, 100–200 mesh; 2.5 g) eluted in succession with 10 column volumes each of chloroform, chloroform/methanol (9:1), chloroform/methanol (1:1) and methanol. The PAF analogue 7 appeared in the chloroform/methanol (1:1) and methanol eluates together with some unidentified contaminants. Pure compound 7 was finally obtained by preparative TLC in solvent A and elution from the silica with chloroform/methanol/water (1:2:0.8, v/v/v), followed by Bligh-Dyer partition (12); yield, 3.4 mg (4%); TLC (solvent A) R_f 0.18 (P-positive) (12); ^{1}H NMR ($CDCl_3$) (Fig. 2), 0.86 (terminal CH_3, 3H), 1.22 (alkyl CH_2-, ω-1 to C-3, 26 H), 1.50 (alkyl C-2, 2 H), 1.78 (H_2O), 2.05 (acetyl CH_3, singlet, 3 H), 3.15 (-$\overset{+}{S}(CH_3)_2$, singlet, 6 H), 3.39 (alkyl CH_2O-, 2 H), 3.55 (CH_2 *sn*-1 glycerol, 2 H), 3.67 (CH_2S, 2 H), 3.94 (CH_2-O-P,*sn*-3 glycerol, 2 H), 4.30 (CH_2-O-P-,sulfocholine, 2 H), 5.12 (-C$\underline{H}$-O-acetyl, 1 H) ppm. Positive FAB-MS: 527 $(M + 1)^+$, 485 $(M + 2 - CH_3CO)^+$; 341 (M + 2 − phosphosulfocholine)$^+$; (calcd. for $C_{25}H_{51}O_7PS$, M = 526).

The 18:0 homologue of compound 7 (R = 18:0) was synthesized by the same procedures as for the 16:0 homologue, but starting from compound 6 (R = 18:0); yields, TLC mobilities and NMR spectra were essentially the same as for 7 (R = 16:0). Positive FAB/MS: 555 $(M+1)^+$, 513 $(M+2-CH_3CO)^+$; 369 (M+2−phosphosulfocholine)$^+$; (calcd. for $C_{27}H_{55}O_7PS$, M = 554).

*1-O-Octadecyl-3-benzyl-*sn*-glycero-3-phosphate* (11). 1-*O*-Octadecyl-*sn*-glycerol (1, R= 18:0) was tritylated as described previously (11) and the crude product, 1-*O*-octadecyl-3-trityl-*sn*-glycerol (2, R = 18:0) was isolated as described above for (2, R = 16:0); TLC (solvent C) R_f = 0.59. Without further purification, the crude trityl derivative was converted to the 1-*O*-octadecyl-2-benzyl-3-trityl-*sn*-glycerol (8) by reaction with benzyl chloride in dry benzene in presence of powdered KOH, as described elsewhere (16); TLC (petroleum ether/diethyl ether, 3:2,

v/v) R_f = 0.67 (crude **8** contains some tritanol and dibenzyl octadecylglycerol). The crude product (**8**) was detritylated by hydrolysis in methanol/water/conc. HCl (18:1:1, v/v/v) and the 1-*O*-octadecyl-2-benzyl-*sn*-glycerol (**9**) was isolated and purified by chromatography on a column of silicic acid as described previously (16); TLC (petroleum ether/diethyl ether, 3:2, v/v) R_f = 0.35. ^{1}H NMR ($CDCl_3$): 0.86 (terminal CH_3, 3H), 1.22 (alkyl CH_2, ω-1 to C-3,30H), 1.52 (alkyl C-2, 2H), 2.15 (C-OH); 3.42 (O-CH_2 alkyl, 2H), 354 (CH_2, *sn*-1 glycerol, 2H), 3.64 (CH_2OH, *sn*-3 glycerol, 2H), 3.75 (CH-O, *sn*-2 glycerol, 1H) 4.65 (benzyl CH_2, 2H), 7.30 (aryl H, 5H) ppm. CI-MS: 435 (M+1), 417 (M+1−H_2O); 343 (M−benzyl); calcd. for $C_{28}H_{50}O_3$, M = 434.

Compound **9** (0.38 g, 0.87 mmole) was phosphorylated with *bis*(trichloroethyl)chlorophosphate (0.36 g, 0.95 mmole) in dry pyridine (3.5 mL), as described elsewhere (14), and the blocked phosphorylated product (**10**) was isolated by acidic Bligh-Dyer extraction (12,14); yield of crude **10**, 0.52 g (77%); TLC (solvent D) R_f =0.80 (P-positive) (12). The crude product **10**, without further purification, was dissolved in chloroform (3.5 mL) diluted with glacial acetic acid (6 mL) and deblocked by treatment with Zn powder (0.4 g) with stirring for 1 hr at room temperature (14). The product 1-*O*-octadecyl-2-benzyl-*sn*-glycero-3-phosphate (**11**) was isolated by acidic Bligh-Dyer extraction and purified by repeated acetone precipitation of the ammonium salt (12,14); yield 92 mg (25%); TLC (solvent A) R_f =0.05 (P-positive) (12). ^{1}H NMR ($CDCl_3$): 0.86 (terminal CH_3, 3 H), 1.22 (alkyl CH_2, ω-1 to C-3, 30 H), 1.50 (alkyl C-2, 2H), 3.36 (alkyl CH_2-O-, 2 H), 3.52 (CH_2-, *sn*-1 glycerol, 2 H), 3.74 (H_2O), 3.96 (CH_2-O-P, *sn*-3 glycerol, 2 H), 4.65 (benzyl CH_2, 2 H), 7.30 (aryl H, 5 H) ppm. ^{13}C NMR ($CDCl_3$): 14.0 (terminal CH_3) 22.6 (ω-1 CH_2), 26.0 (alkyl C-2), 29.6 (alkyl CH_2-, ω-3 to C-3), 32.0 (ω-2 CH_2), 65.0 (CH_2-O-P), 70.5, 71.5 (CH_2-O-CH_2), 77.0 (CH-O-), 128 (phenyl C) ppm. Negative FAB-MS: 513 (M−2NH_4+1)$^-$; 423 (M−2NH_3−benzyl)$^-$; calcd. for $C_{28}H_{57}O_6N_2P$, M = 548.

*1-O-Octadecyl-2-benzyl-*sn*-glycero-3-phosphosulfocholine* (**12**). Compound **11** (73.6 mg, 0.14 mmole) was converted to the free acid form (**12**) which was reacted with anhydrous sulfocholine iodide (61.2 mg, 0.17 mmole) and TPS (45 mg, 0.15 mmole) in anhydrous pyridine (27 mL) with stirring at room temperature for 48 hr. After addition of water (2.5 mL) to hydrolyze any unreacted TPS, the reaction mixture was diluted with benzene and brought to dryness under reduced pressure, and the residue was partitioned by the acidic Bligh-Dyer procedure (12) to remove water-soluble material. The chloroform phase was concentrated *in vacuo* and the residual oil was dissolved in 5 mL of methanol/water, 95:5 (plus 0.1 mL of chloroform) and passed through a column of mixed-bed ion exchange resin [Bio-Rad AG 501-X8 (D), 10 g], followed by elution with 25 mL of methanol/water/chloroform (95:5:5, v/v/v) to remove final traces of starting material **11**, the treatment with ion exchange resin was repeated. The combined eluates were then diluted with appropriate amounts of chloroform and water to form a two-phased mixture and the chloroform phase was diluted with benzene and brought to dryness *in vacuo*. The product **12** (13 mg), now free of compound **11** but still contaminated with fast-moving impurities, was finally purified by repeated acetone precipitation from chloroform solution (0.2–0.3 mL) with 3.5 mL of acetone in the cold. The precipitate of compound **12** now gave only a single spot (P-positive) (12) on TLC; yield, 6.3 mg (7.5%); TLC (solvent A) R_f = 0.25 (standard PAF had R_f = 0.18). ^{1}H NMR ($CDCl_3$): 0.86 (terminal CH_3, 3H), 1.23 (alkyl CH_2, 30 H), 1.55 (alkyl C-2, 2 H), 2.44 (H_2O), 3.06 (−$S(CH_3)_2$, 6 H), 3.42 (alkyl CH_2-O-, 2 H), 3.54 (*sn*-1 glycerol CH_2, 2 H), 3.78 (CH_2S, 2 H), 3.93 (CH_2-O-P, *sn*-3 glycerol, 2 H), 4.19 (CH_2-O-P, sulfocholine, 2 H), 4.68 (Ph-CH_2, 2 H), 7.30–7.34 (aryl H, 5 H) ppm. Positive FAB-MS: 603 (M+1)$^+$; 513 (M+2−$CH_2C_6H_5$); Negative FAB-MS: 587 (M−CH_3)$^-$; 539 [M−$S(CH_3)_2$−1]$^-$; 513 [M+1−$CH_2CH_2S(CH_3)_2$]$^-$; calcd. for $C_{32}H_{59}O_6PS$, M = 602.

RESULTS AND DISCUSSION

Chemical synthesis. Synthesis of the sulfonium analogue of PAF (**7**) was carried out by the following steps (see Scheme 1): i) Tritylation of *rac*- or *sn*-1-*O*-hexadecyl (or octadecyl) glycerol (**1**) to form the respective 3-trityl derivative (**2**), as described previously (11); ii) acetylation of compound **2**, as described elsewhere (12), to form the respective 1-alkyl-2-acetyl-3-trityl-glycerol; iii) detritylation of compound **3** on a column of boric acid/silicic acid by the procedure of Buchnea (13) to form the 1-alkyl-2-acetyl-glycerol (**4**) with little contamination from the isomeric 1-alkyl-3-acetyl-glycerol; iv) Phosphorylation of compound **4** with *bis*(trichloroethyl)chlorophosphate, as described previously (14), to give the 1-alkyl-2-acetyl-glycerol-3-*bis*(trichloroethyl)phosphate (**5**), followed by removal of the trichloroethyl groups by treatment with Zn in chloroform/glacial acetic acid (14) to give the 1-alkyl-2-acetyl-glycero-3-phosphate (**6**)—the latter compound was isolated as the ammonium salt in TLC- and isomerically-pure form, as confirmed by NMR and FAB-MS; v) reaction of **6** with sulfocholine-*p*-toluenesulfonate in dry pyridine in presence of trichloracetonitrile by the procedure of Ali and Bittman (15) or in the presence of TPS (3), to give the expected PAF sulfonium analogue, 1-alkyl-2-acetyl-glycero-3-sulfocholine (**7**).

The 2-*O*-benzyl sulfonium analogue (**12**) was synthesized by essentially the same route, but starting from 1-alkyl-2-benzylglycerol (**9**), which was prepared by benzylation of 1-alkyl-3-tritylglycerol (**2**) followed by detritylation of compound **8**, as described previously (16).

The sulfonium analogues **7** and **12** were obtained in rather low yields (4% and 8%, respectively), due mostly to the poor yield of the sulfocholine condensation reaction (see Scheme 1) and the large losses incurred in isolation and purification of the final products **7** and **12**. Much higher yields have been reported for the synthesis of bulky head group analogues of phosphatidylcholine using trichloracetonitrile as condensing agent (15) and for synthesis of sulfonium analogues of phosphatidylcholine using TPS as condensing agent (3). The low yields with the PAF analogues **7** and **12** may perhaps be attributed to unexplained low reactivity of the 1-alkyl-2-acetyl- or benzyl-glycerophosphates. It is of interest that Bittman *et al.* (17) reported high yields of PAF analogues by carrying out the phosphorylation and base condensation steps in one step, using $POCl_3$ and choline *p*-toluenesulfonate. It might be worthwhile to apply such a strategy to the synthesis of sulfonium analogues of PAF.

However, both PAF analogues 7 and 12 were obtained in TLC- and isomerically-pure form and were shown by NMR (Fig. 2) and mass spectrometry to have the respective PAF sulfonium structure. The ^1H NMR spectrum of 7 (Fig. 2B) showed signals for the 1-*O*-alkylglycerol group (δ, 0.86, 1.22, 1.50, 3.39 and 3.55 ppm), the glycero-3-phosphate group (δ 3.94 ppm), the dimethylsulfonium group [δ 3.15 ppm singlet; cf. the $N(CH_3)_3$ signal in PAF at δ 3.4 ppm; Fig. 2A], the phosphosulfocholine group (δ 4.30 and 3.67) and the 2-acetylglycerol group (δ 2.05 ppm, singlet, and 5.12 ppm), consistent with the structure 1-*O*-hexadecyl-2-acetyl-*sn*-glycero-3-phosphosulfocholine (7). The positive FAB-MS spectrum showed a mass peak $(M+1)^+$ at *m/z* 527 (calcd. M = 526) and peaks at *m/z* 485 and 341 attributed to the loss of the acetyl group $(M-acetyl)^+$, and the phosphosulfocholine group, respectively, as expected for compound 7.

The ^1H NMR spectrum of compound 12 (Fig. 2C) showed the same signals as for compound 7, except for the replacement of the acetyl signal by signals attributed to the benzyl group (δ 4.6 and 7.3 ppm), consistent with the structure 1-*O*-octadecyl-2-benzyl-*sn*-glycero-3-phosphosulfocholine. The positive FAB-MS spectrum showed a mass peak $(M+1)^+$ with *m/z* 603 (calcd. M = 602) and a peak with *m/z* 513 $(M+2-benzyl)^+$, as expected for compound 12; negative FAB-MS showed a peak for $(M-CH_3)^-$ at *m/z* 587, and peaks at *m/z* 539 and 513 due to the loss of $S(CH_3)_2$ and $CH_2CH_2S(CH_3)_2$, respectively.

Platelet aggregating activity. The effect of the sulfonium analogue of PAF (7) on platelet agregation using either washed platelets or platelet-rich plasma, compared with the effect of PAF, is shown in Figure 3 and Table 1. The sulfonium analogue 7 had essentially the same platelet aggregating activity as PAF itself, in the concentration range 1 to 20 μM, with either platelet preparation, but with washed platelets, analogue 7 had a much lower activity than PAF in the concentration range 0.1 to 1 μM. No activity was observed either with PAF or the analogue 7 below 0.01 μM. Analogue 7 also had little or no inhibiting effect, in the concentration range 0.001 to 1 μM, on the platelet aggregating activity of PAF (Table 1).

Hypotensive activity. Intravenous injection of 10 pmole of PAF into an eleven-month-old CDF rat lowered the mean arterial blood pressure (99 mm Hg) an average of 45%; normal pressure was restored after 2.1 min. By comparison, the PAF sulfonium analogue 7, under the same conditions, lowered the blood pressure to the extent of 35% and returned to normal in 1.5 min (56% of the hypotensive activity of PAF) (Table 2). In contrast, the benzyl sulfonium analogue 12 had no effect at all on the mean arterial pressure, up to a dose of 1 nmole injected intravenously.

Cytotoxicity to HL-60 tumor cells. The sulfonium analogue 7 of *sn*-3 PAF showed a moderate cytotoxic effect (LD_{50} = 11 μM) towards HL-60 cells in the concentration range 0–20 μM (Fig. 4). In the same concentration range and under the same conditions, *sn*-3 PAF itself showed little or no cytotoxicity, as reported previously (9). In contrast, the 2-*O*-benzyl sulfonium analogue (12) was highly toxic to HL-60 cells in the concentration range 0–10 μM (LD_{50} = 4.5 μM), almost as toxic as the 2-*O*-methoxy PAF analogue (LD_{50} = 3.5 μM) (Fig. 4); the high cytotoxicity of the latter has been reported previously (9,18,19).

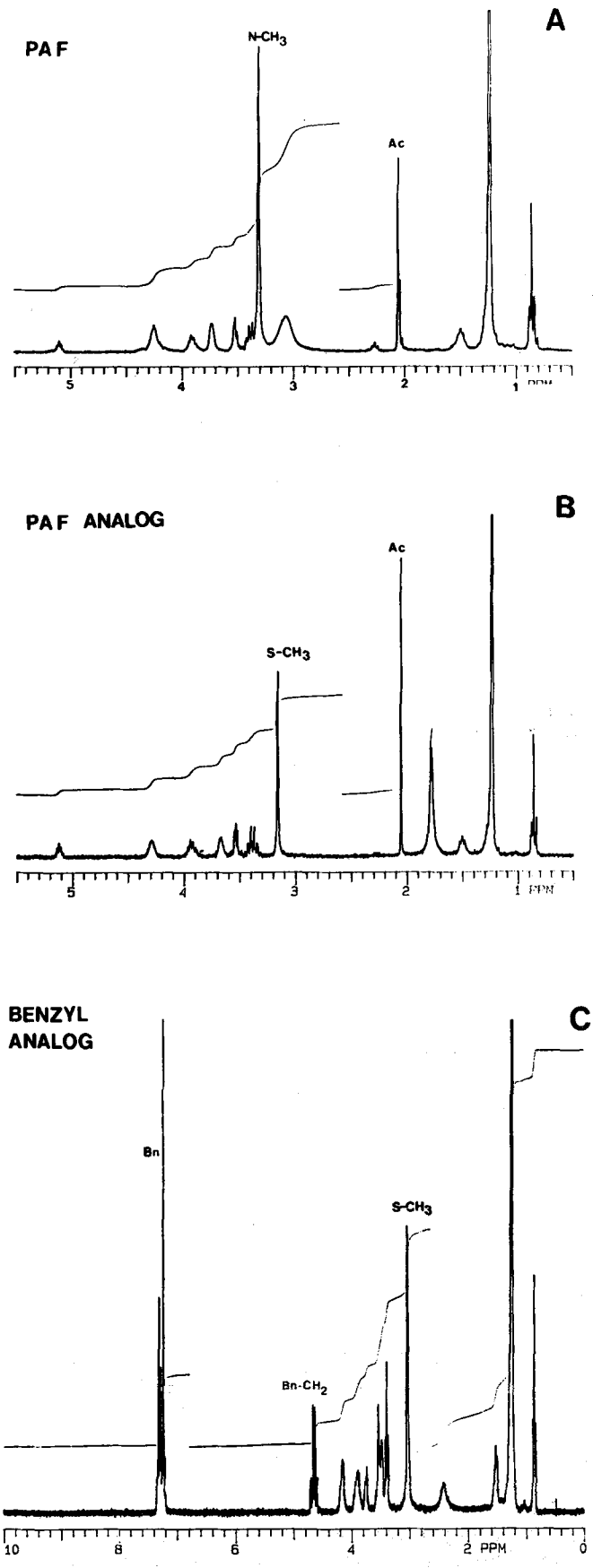


FIG. 2. ^1H NMR spectra in $CDCl_3$ of: A, authentic PAF (Serdary); B, PAF sulfonium analogue 7; and C, PAF sulfonium analogue 12.

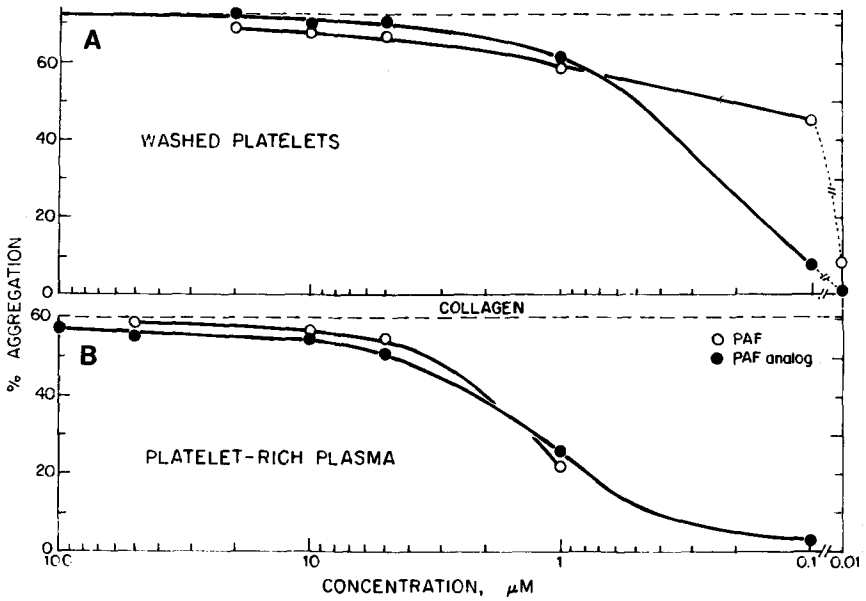


FIG. 3. Platelet aggregating activity of PAF (Sigma) and PAF sulfonium analogue 7 towards: A, washed human platelets; and B, human platelet-rich plasma.

TABLE 1

Effect of Sulfonium Analogue of PAF on Platelet Aggregating Activity of PAF[a]

Substance tested	Concentration PAF μM	Concentration PAF analogue μM	Platelet aggregation %
PAF (Sigma)	20	0	77
	1	0	73
PAF sulfonium analogue **7**	0	20	79
	0	2	75
	0	1	73
	0	0.1	24
	0	0.01	0
	0	0.001	0
PAF plus sulfonium analogue **7**	1	1	68
	1	0.1	69
	1	0.01	68
	1	0.001	75

[a]Platelet aggregation was measured as described in the text under Materials and Methods; 0.9% NaCl blanks gave zero % aggregation and a 0.005% collagen control gave 80% aggregation. The data in this table are independent of those in Figure 3.

TABLE 2

Hypotensive Activities of PAF and PAF Analogues[a]

Phospholipid tested	Amount injected pmole	Hypotensive activity
PAF: 1-hexadecyl-2-acetyl-*sn*-GPC	10	95 ± 13 (n=3)
PAF analogue **7**: 1-hexadecyl-2-acetyl-*sn*-GPSC	10	53 ± 8 (n=6)
PAF analogue **12**: 1-octadecyl-2-benzyl-*sn*-GPSC	1000	0 (n=4)

[a]Samples were injected into an 11-month-old normotensive CDF male rat (normal control mean blood pressure, 99 mm Hg). Hypotensive activity, defined as maximum % decrease in pressure × time (min) for blood pressure to return to normal, was measured as described elsewhere (8). Values reported are averages ± S.D. based on three or more determinations.

The results presented show that replacement of the quaternary $\overset{+}{N}(CH_3)_3$ group in the polar head group of PAF by a sulfonium $\overset{+}{S}(CH_3)_2$ group has little effect on the platelet aggregating activity of PAF and does not impart any detectable antagonistic activity towards the platelet aggregating activity of PAF (Fig. 3, Table 1). This finding would suggest that replacing the choline group of PAF by a sulfocholine group, which has a larger volume and resulting lower surface charge density than the choline group (4-6), does not significantly influence the binding of PAF to the appropriate receptor sites involved in the platelet aggregating process. Such a replacement appears to have an effect on platelet aggregating activity equivalent to changing the trimethyl ammonium group of PAF to a diethyl amino group (1,2); it is noteworthy that an increase in the bulkiness of the quaternary ammonium group to a diethyl methyl ammonium group doubles the platelet aggregating activity of PAF, but that a further increase to a triethyl ammonium group abolishes this activity (1,2).

However, replacement of the choline group of PAF by a sulfocholine group does have a lowering effect on the hypotensive action of PAF, by a factor of 2 (Table 2); replacement of the 2-acetyl group in sulfonium PAF by a benzyl group abolished the hypotensive action (Table 2), as similar replacement in PAF itself also results in no detectable hypotensive activity (8).

Of great interest was the fact that the sulfonium analogue **7** was much more cytotoxic to HL-60 cells than PAF itself, and that the 2-*O*-benzyl sulfonium analogue

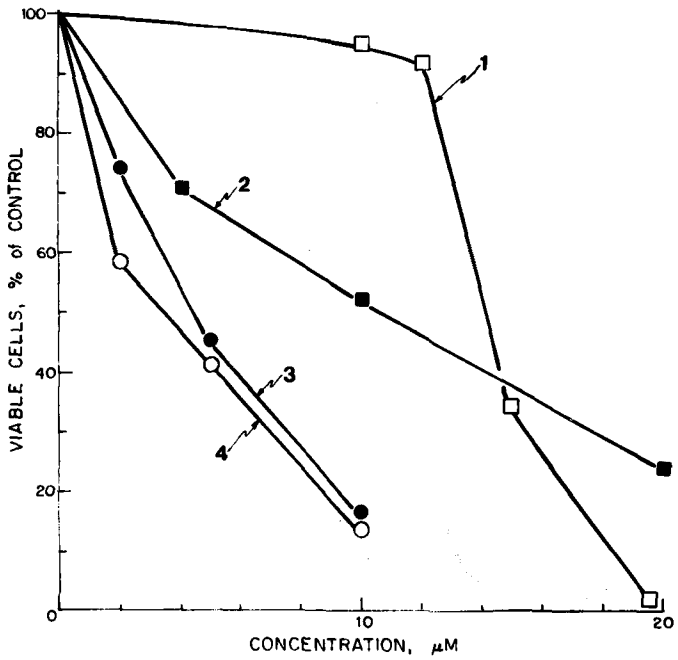


FIG. 4. Cytotoxic activity of PAF (Sigma) and PAF analogues towards HL-60 cells. 1, *sn*-3 PAF; 2, PAF sulfonium analogue 7; 3, PAF 2-*O*-benzyl sulfonium analogue 12; 4, 2-*O*-methyl analogue of PAF. Data for *sn*-3 PAF are taken from reference 9. Values plotted are means of quadruplicate measurements with mean deviations of 1-7%.

12 was much more toxic than analogue 7 (Fig. 4), but comparable to that of the 2-methoxy analogue of PAF (9,18,19). The increased cytotoxic effect of analogue 7 may be attributable to the presence of the sulfonium group, which has been shown previously to impart cytotoxicity to the sulfonium analogues of phosphatidylcholine (20,21). The further increase in cytotoxicity imparted by replacing the 2-acetyl group by a 2-benzyl group cannot be explained by the present data; further studies along the lines used for the 2-methoxy and 2-acetamide analogues (9,18,19) are necessary to establish structure-cytotoxicity relationships for the 2-benzyl analogue 12.

The sulfonium analogues of PAF thus have some interesting physiological properties that may be useful in the design of drugs for various pharmaceutical purposes. Further study of the properties of sulfonium analogues of PAF should be of interest.

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Effect of Platelet-Activating Factor on Lipoprotein Lipase and Blood Lipids[1]

Keiji Mimura*, Susumu Yukawa, Yoshio Mori, Kazuya Okada, Masatoshi Mune, Osamu Nishikawa, Akira Hibino, Miyahiko Sonobe, Tetuya Goto and **Hiroshi Nomoto**
Third Department of Internal Medicine, Wakayama Medical University, Wakayama 640, Japan

We investigated the effect of platelet-activating factor (PAF) and of the PAF specific antagonist CV-6209 on plasma lipid metabolism, and particularly on post-heparin plasma lipolytic activity in male Wistar rats. Lipoprotein lipase (LPL) activity was enhanced by intravenous injection of PAF before intravenous injection of heparin when the PAF dose was low (0.2 μg/kg). PAF activated hepatic triacylglycerol lipase (HTGL) activity dose-dependently. Plasma triacylglycerols (TG) significantly decreased with the activation of LPL and/or HTGL. Plasma total cholesterol (TC) and phospholipid (PL) levels decreased at a low dose of PAF (0.2 μg/kg), but increased when higher doses were used. The PAF antagonist CV-6209 partially reversed the PAF induced effects on HTGL, TC and PL. ***Lipids 26,*** **1102–1107 (1991).**

Platelet-activating factor (PAF), a biologically active phospholipid (PL), induces a variety of effects, including platelet activation (1), hypotension (2), vasopermeability (3), and increased intestinal movement (4). PAF's involvement in a variety of physiological or pathologic processes has attracted much attention. PAF appears to play a role in differentiation and proliferation of undifferentiated cells of bone marrow (5,6) as well as in such processes as implantation of fertilized ovum (7), parturition (8), and maturation of fetal lung (9). In addition, PAF has been shown to elicit pathologic changes including inflammation (10), bronchial asthma (11), nephropathy (12), collagen diseases (13), anaphylactic shock (14), and ulceration (15). Involvement of PAF in energy metabolism is little understood, and it has only been reported that PAF accelerates glycogenolysis in the liver (16). The effect of PAF on lipid metabolism has also remained unclear.

In the present study, we investigated the effects of PAF and of a PAF antagonist on plasma lipid metabolism, and particularly on post-heparin plasma lipolytic activity *in vivo.*

MATERIALS AND METHODS

PAF (1-*O*-hexadecyl-2-acetyl-*sn*-glycero-3-phosphocholine, purity 98%, Sigma Chemical Co., St. Louis, MO) and a PAF antagonist, CV-6209 (17) (provided by Takeda Chemical Industry, Osaka, Japan) were dissolved in isotonic saline containing 0.25% defatted bovine serum albumin (Fraction V, Sigma). Male Wistar rats, each weighing 300 g, were separated into 5 groups (A–E) after 12-hr fasting, anesthetized with sodium pentobarbitol (50 mg/kg body wt i.p.), and blood samples were taken (Fig. 1). In group A, heparin, 100 units/kg, (Novo Industries, Copenhagen, Denmark) was administered intravenously, followed by blood sampling 10 min after heparin administration (n = 5). In group B, heparin (100 units/kg) was administered intravenously, then PAF (1 μg/kg) was administered intravenously 5 min after heparin administration, followed by blood sampling 5 min later (n = 4). In Group C, PAF (0.2 μg/kg) was administered intravenously to 4 animals, 0.5 μg/kg to 4 animals, 1.0 μg/kg to 5 animals, and 1.5 μg/kg to one animal. Five min later, heparin, 100 units/kg, was intravenously administered to all subgroups, followed by blood sampling 10 min later. In Group D, PAF, (1 μg/kg), was administered intravenously and blood samples were drawn 15 min later (n = 5). In Group E, CV-6209 at 0.001, 0.01, and 0.1 mg/kg was administered intravenously to 1, 3, and 2 animals, respectively, and PAF (1 μg/kg) was administered intravenously 5 min later. Heparin (100 units/kg) was administered intravenously 5 min after PAF administration, and 10 min later blood samples were taken. Plasma of the latter blood samples from each group was used to determine post-heparin plasma lipolytic activity, consisting of lipoprotein lipase (LPL) and hepatic triacylglycerol lipase (HTGL) activities, following the method of Hernell *et al.* (18). LPL activity was obtained by subtraction of HTGL activity from total post-heparin plasma lipolytic activity. Levels of total protein, albumin, total cholesterol (TC), triacylglycerols (TG), PL, free fatty acids (FFA), high density

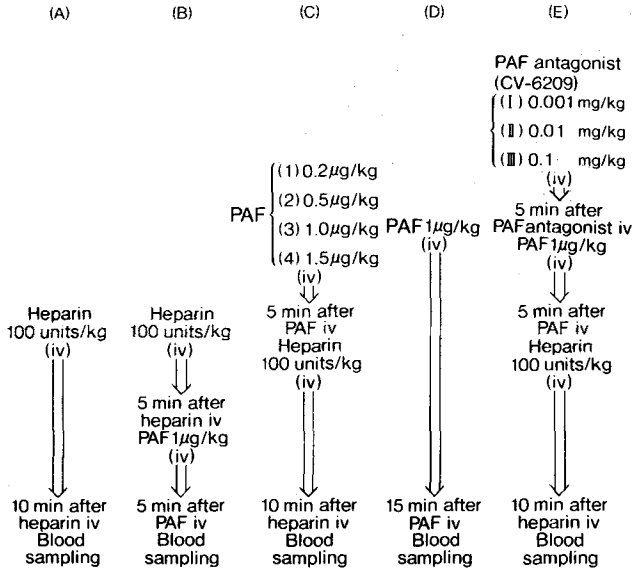


FIG. 1. Experimental design. After 12-hr fasting, blood samples were taken and the protocol shown was followed.

[1]Based on a paper presented at the Third International Conference on Platelet-Activating Factor and Structurally Related Alkyl Ether Lipids, Tokyo, Japan, May 1989.

*To whom correspondence should be addressed.

Abbreviations: FFA, free fatty acids; HTGL, hepatic triacylglycerol lipase; LDL, low density lipoprotein; LPL, lipoprotein lipase; MDA, malondialdehyde; PAF, platelet-activating factor; PL, phospholipid; TC, total cholesterol; TG, triacylglycerols; VLDL, very low density lipoprotein.

lipoprotein (HDL) cholesterol, blood urea nitrogen (BUN), and malondialdehyde (MDA) (MDA with Lipoperoxide Test Kit, Wako Chemical Industry, Osaka, Japan) (19) were measured in all blood samples. We then compared the results from each group.

Statistical analysis. Student's t-test for unpaired values was used to compare different groups. Student's t-test for paired values was used in experiments comparing the changes before and after each treatment. Differences were considered significant at $p \leq 0.05$. Each value is reported as mean ± SD.

RESULTS

Post-heparin plasma lipolytic activity was 17.3 ± 0.7 and 17.8 ± 2.5 μmol FFA/mL/hr in Groups A and B, respectively, with no significant difference between both groups. In group C, post-heparin plasma lipolytic activity was 26.4 ± 0.3 in those animals given PAF at 0.2 μg/kg, 22.6 ± 1.1 in those given 0.5 μg/kg, 23.4 ± 1.3 in those given 1.0 μg/kg, and 24.0 in one animal given 1.5 μg PAF/kg. The respective subgroups which had received 0.2, 0.5 and 1.0 μg PAF/kg showed a significant increase in post-heparin plasma lipolytic activity compared to Group A. The post-heparin plasma lipolytic activity increased by 53, 31 and 35%, respectively. The subgroup given the lowest dose of PAF (0.2 μg/kg) showed the highest post-heparin plasma lipolytic activity. In Group D, no appreciable post-heparin plasma lipolytic activity (1.1 ± 0.4) was observed as no heparin was administered. In Group E, the post-heparin plasma lipolytic activity was 24.6 in one animal given CV-6209 at 0.001 mg/kg, 24.2 ± 3.5 in three given 0.01 mg/kg, and 20.0 ± 2.3 in two given 0.1 mg/kg. As compared to the subgroup of Group C, given PAF at 1.0 μg/kg, no appreciable difference in post-heparin plasma lipolytic activity was noted in two subgroups of Group E given CV-6209 at 0.001 and 0.01 mg/kg; a significantly smaller decrease of 15.6% was seen in a subgroup given CV-6209 at 0.1 mg/kg (Fig. 2).

LPL activity was 6.1 ± 2.6 μmol FFA/mL/hr in Group A and 3.9 ± 1.5 in Group B. Group B had a lower LPL activity than did Group A, but the difference was not significant. In group C, LPL activity was 11.7 ± 1.2 in those animals treated with PAF at 0.2 μg/kg, 7.3 ± 1.7 in those treated with 0.5 μg/kg, 7.8 ± 1.2 in those treated with 1.0 μg/kg, and 9.1 in one animal treated with 1.5 μg/kg. Only a subgroup given the lowest dose of PAF (0.2 μg/kg) had a significant increase in LPL activity (91.8%) compared to Group A. In Group D, no appreciable LPL activity (0.6 ± 0.5) was observed. In Group E, LPL activity was 8.2 in one animal given CV-6209 at 0.001 mg/kg, 10.4 ± 3.1 in three given 0.01 mg/kg, and 7.4 ± 1.4 in two given 0.1 mg/kg. As compared to the subgroup of Group C, given 1.0 μg PAF/kg, no appreciable difference in LPL activity was noted in the three subgroups of Group E (Fig. 3).

HTGL activity was 11.2 ± 2.1 μmol FFA/mL/hr in group A and 13.9 ± 1.6 in Group B. Group B had a higher HTGL activity than did Group A, but the difference was not significant. In Group C, HTGL activity was 14.7 ±

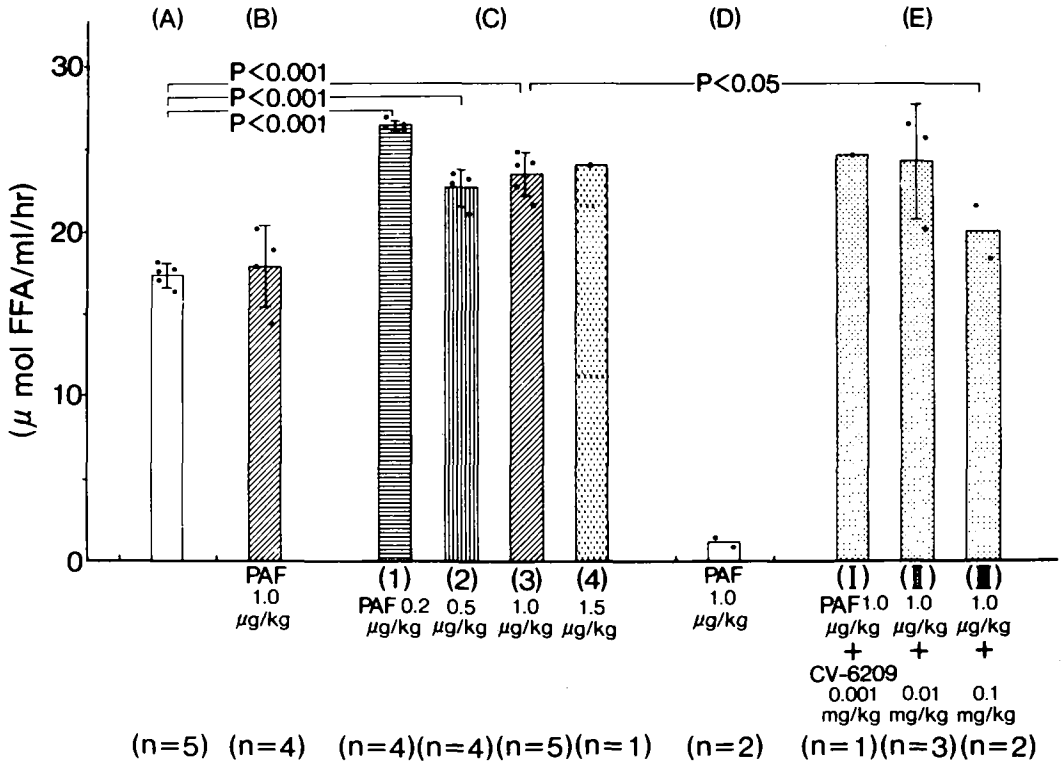


FIG. 2. Effect of PAF and PAF antagonist CV-6209 on post-heparin plasma lipolytic activity. Post-heparin plasma lipolytic activity was determined after each treatment, as shown in Figure 1. Each value represents the mean ± SD.

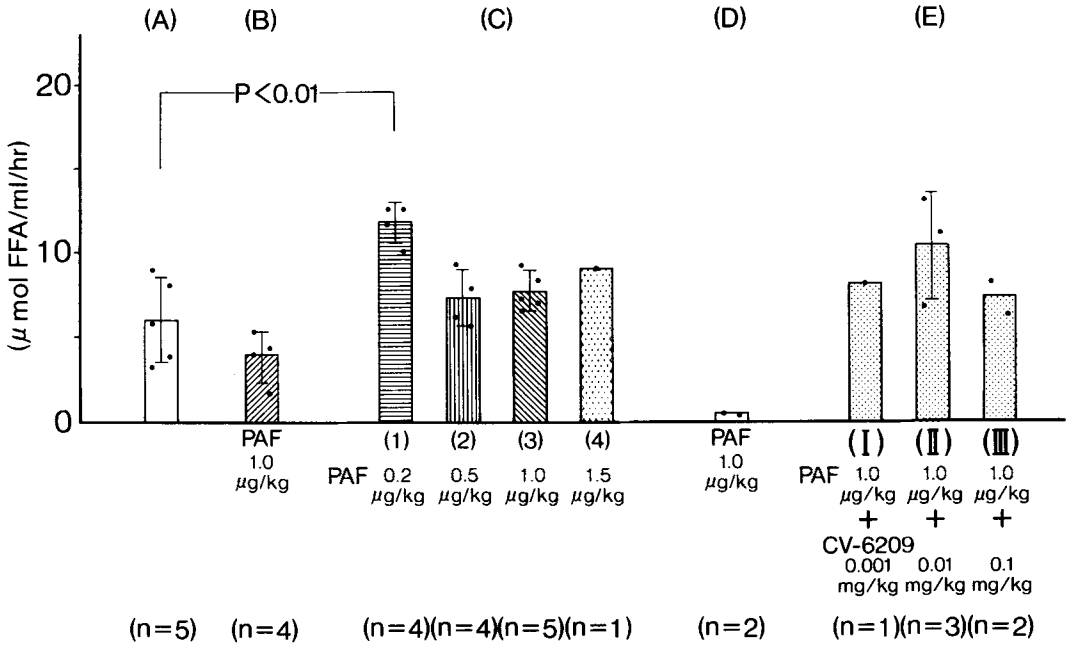


FIG. 3. Effect of PAF and PAF antagonist CV-6209 on plasma LPL activity. LPL activity was determined after each treatment, as shown in Figure 1. Each value represents the mean ± SD.

1.2 in the animals treated with 0.2 μg PAF/kg, 15.3 ± 1.1 in those treated with 0.5 μg/kg, 15.6 ± 1.2 in those treated with 1.0 μg/kg, and 14.9 in one animal treated with PAF at 1.5 μg/kg. Three subgroups treated with PAF at 0.2, 0.5, or 1.0 μg/kg had a significant increase in HTGL activity compared to Group A, with the activity being increased by 31, 37 and 39%, respectively. A dose-dependent increase was noted in these subgroups. In Group D, as in post-heparin plasma lipolytic activity, HTGL activity (0.6 ± 0.5) was barely observed. In Group E, HTGL activity was 16.4 in one animal treated with CV-6209 at 0.001 mg/kg, 13.8 ± 0.8 in three treated with 0.01 mg/kg, and 12.6 ± 0.9 in two treated with 0.1 mg/kg. Compared to a subgroup of Group C treated with 1.0 μg PAF/kg, a subgroup of Group E treated with CV-6209 at 0.1 mg/kg showed a 19.2% decrease (Fig. 4).

Group A showed a significant decrease in the plasma levels of TG, TC and PL after treatment. In Group B, the TG level was significantly decreased, while the TC level was significantly increased, and the PL level was higher. In Group C, there was a significant decrease in TG level. The TC level decreased in the subgroup treated with PAF at 0.2 μg/kg, while the other subgroups showed increased levels. A tendency toward a decrease in PL levels was noted only in a subgroup treated with 0.2 μg PAF/kg. In Group D, the TG level was significantly decreased, while TC and PL levels increased. In Group E, the TG, TC and PL levels significantly decreased (Figs. 5–7). FFA increased in all groups. HDL cholesterol increased in all groups other than Group D, with a significant increase being offered in Group E. There were no appreciable changes in MDA and other parameters tested (data not shown).

DISCUSSION

We have demonstrated that LPL activity is enhanced by intravenous injection of PAF, but only when the dose is low, 0.2 μg/kg. The fact that the activation of LPL was noted only at the time of PAF injection and before heparin injection could imply that PAF may induce some changes in LPL that exists on the surface of capillary endothelial cells and thus may stimulate its activation. It is generally thought that LPL on the surface of vascular endothelial cells originates from parenchymal cells, such as fat and cardiac muscle cells (20). Recently, a high level of LPL activity in arteriosclerotic lesions has also been reported (21,22) and attention has been focused on the release of LPL in macrophages (23). LPL may also be of importance in foam cell formation (24). However, there appear to be no receptors for PAF on rat macrophages (25); hence increased LPL is not likely to be of macrophage origin.

Several factors that affect LPL activity have been reported. Feeding and insulin increase LPL activity in adipose tissue (20,26,27). In contrast, fasting and glucagon increase LPL in the heart muscle (26,28). In macrophages, endotoxin (lipopolysaccharide) (29) and dexamethason (30) inhibit LPL secretion, while inflammation (31) and very low density lipoprotein (VLDL) (24) increase LPL secretion. However, the mediators and hormones which increase LPL secretion of macrophages are not known. It would be of interest to examine whether PAF enhances the activity of LPL in macrophages of animals which have PAF receptors on macrophages, such as human, rabbit and guinea pig.

In the present study, a marked increase in post-heparin lipolytic activity, especially LPL activity, was noted in a

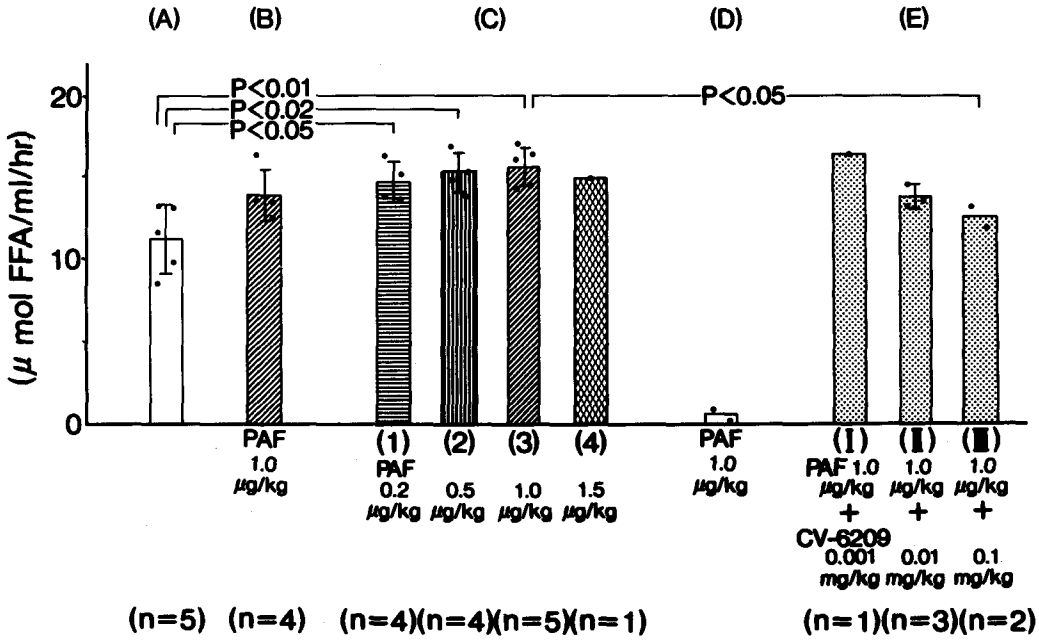


FIG. 4. Effect of PAF and PAF antagonist CV-6209 on plasma HTGL activity. HTGL activity was determined after each treatment, as shown in Figure 1. Each value represents the mean ± SD.

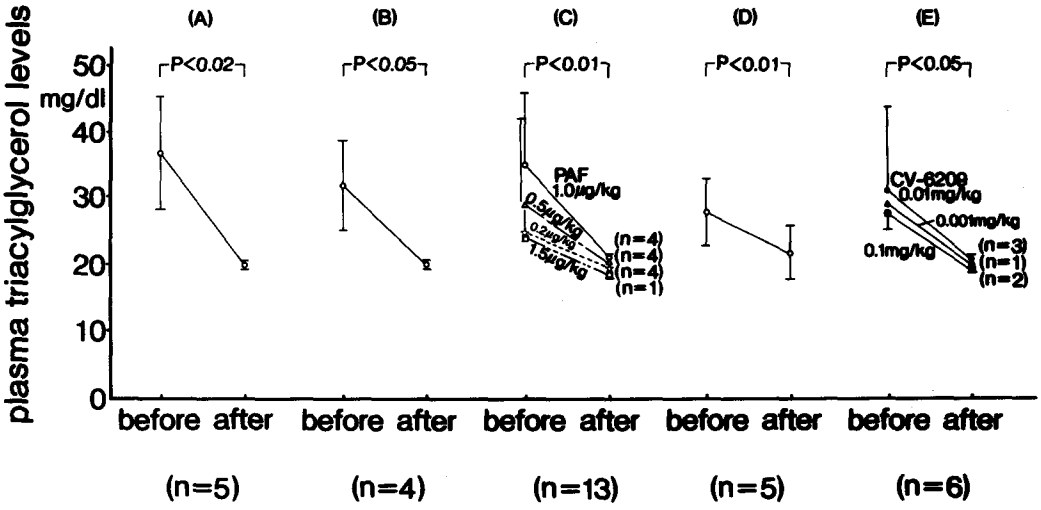


FIG. 5. Effect of PAF and PAF antagonist CV-6209 on plasma triacylglycerol levels. Plasma triacylglycerol levels were measured before and after each treatment, as shown in Figure 1. Each value represents the mean ± SD.

subgroup of Group C which had been treated with the lowest dose of PAF (0.2 μg/kg). In this subgroup, plasma levels of TC and PL tended to decrease, in contrast to those of other subgroups treated with higher doses of PAF. The decreases seem to be associated with accelerated transport of lipids to cells. In this subgroup, PAF could thus be involved in regulating the supply of energy. In the other subgroups, the levels of HTGL and plasma TC were increased, while the PL level remained unchanged, suggesting that lipid transport was impaired.

PAF may also play a pathological role in respect to arteriosclerosis, as recent *in vitro* studies have demonstrated that low density lipoprotein (LDL) modified by LPL or HTGL is taken up by macrophages more readily than is native LDL (32).

PAF activates HTGL activity dose-dependently. Thus, PAF may be able to affect the energy metabolism in the liver by modulating HTGL. It has been reported that hepatic cells have PAF receptors (33). Because HTGL activity was partially inhibited by CV-6209, which is

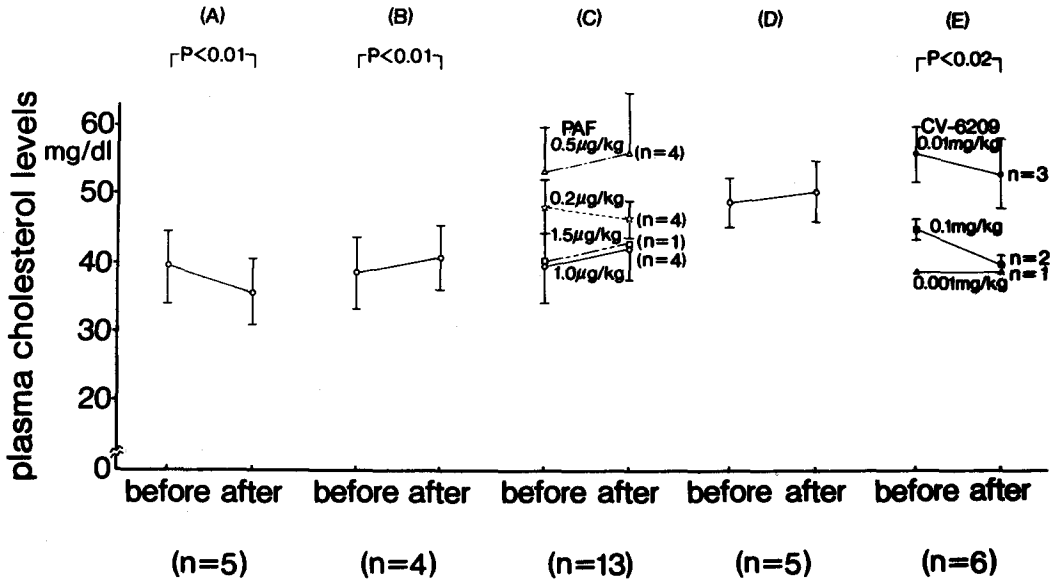


FIG. 6. Effect of PAF and PAF antagonist CV-6209 on plasma cholesterol levels. Plasma cholesterol levels were measured before and after each treatment, as shown in Figure 1. Each value represents the mean ± SD.

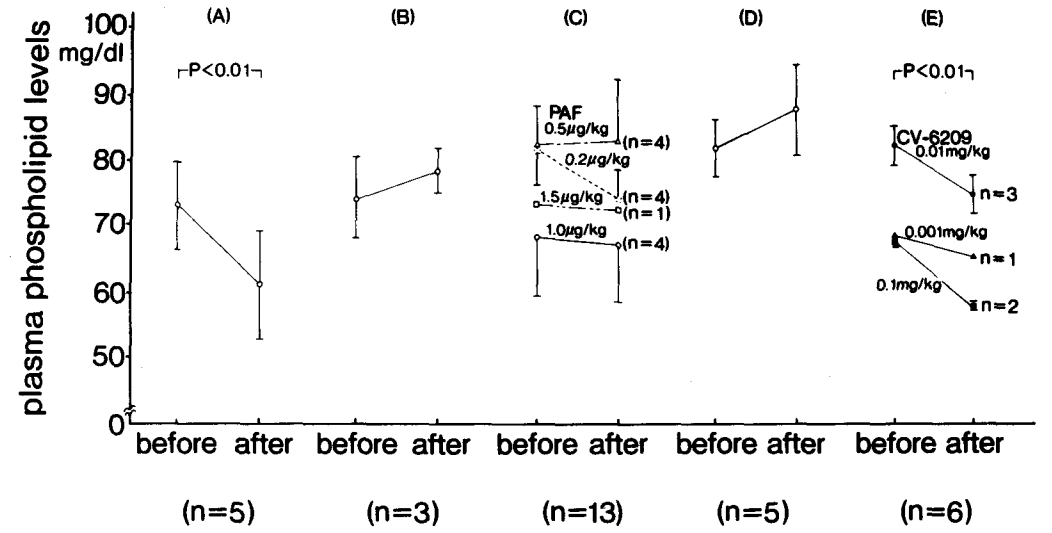


FIG. 7. Effect of PAF and PAF antagonist CV-6209 on plasma phospholipid levels. Plasma phospholipid levels were measured before and after each treatment, as shown in Figure 1. Each value represents the mean ± SD.

a specific PAF receptor antagonist, the observed activation of HTGL may indeed be mediated *via* PAF receptor.

Increased levels of TC and PL were noted after pretreatment with higher doses of PAF. These increases were significantly inhibited after administration of the PAF antagonist, which again suggests that the effect is mediated by PAF. However, the definite mechanism involved remains unknown.

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Effect of Platelet-Activating Factor on Cortisol and Corticosterone Secretion by Perfused Dog Adrenal[1]

Tadaomi Aikawa[a,*], Taeko Hirose[a], Itsuro Matsumoto[a], Toshiko Morikawa[a], Toshio Shimada[a], Yumi Mine[a], Yoshiki Tsujimoto[b] and Yoshiro Tsuji[b]

Departments of [a]Physiology and [b]Pediatrics, Nagasaki University School of Medicine, Nagasaki 852, Japan

Administration of platelet-activating factor (PAF) to perfused adrenal increased cortisol and corticosterone secretion. With hexadecyl PAF (C_{16}PAF; 1-*O*-hexadecyl-2-acetyl-*sn*-glycero-3-phosphocholine), the increase was significant at 1 nM and maximal at 10 nM. The responses to 10 nM octadecyl PAF (C_{18}PAF; 1-*O*-octadecyl-2-acetyl-*sn*-glycero-3-phosphocholine) were one fourth of those to 10 nM C_{16}PAF. The addition of C_{16}PAF to dispersed adrenal cells significantly increased cortisol and corticosterone production at 0.1 nM and 10 nM, respectively. C_{16}PAF was about 1000 times more potent than histamine on a molar basis in respect to cortisol response in both perfused adrenal and dispersed adrenal cells. The results suggest that PAF induces cortisol release from dog adrenal.

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Platelet-activating factor (PAF), which was discovered in 1972 (1), is a phospholipid mediator involved in anaphylaxis and inflammation. PAF is produced by a variety of cell types and causes platelet aggregation, neutrophil activation, increased vascular permeability, bronchoconstriction, decreased cardiac output, and anaphylactic hypotension (2–4).

Glucocorticoids are known to suppress various inflammatory and immune responses. This can mainly be attributed to the fact that glucocorticoids depress the effect of certain mediators and cytokines (5) and induce phospholipase A_2 inhibitory protein (lipocortin), which affects the arachidonic acid cascade and release of the PAF precursor, lysoPAF (6,7).

In the present study we found that PAF is a potent stimulator of adrenal cortex in dogs.

MATERIALS AND METHODS

Materials. Collagenase (CLSPA), trypsin (TRL) and lima bean trypsin inhibitor were obtained from Cooper/Worthington (Freehold, NJ). Deoxyribonuclease and bovine serum albumin (BSA, fraction V) were purchased from Sigma (St. Louis, MO). 1-*O*-Hexadecyl- and 1-*O*-octadecyl-2-acetyl-*sn*-glycero-3-phosphocholine (C_{16}PAF, C_{18}PAF) and 1-*O*-hexadecyl-*sn*-glycero-3-phosphocholine (C_{16}lyso-PAF) were purchased from Bachem (Bubendorf, Switzerland). Synthetic adrenocorticotropic hormone 1-24 (ACTH, Cortrosyn) was obtained from Organon (Boxtel, Holland). Krebs-Ringer bicarbonate glucose solution (KRBG) contained (in mM): 121 NaCl, 2.37 KCl, 1.19 $MgSO_4 \cdot 7H_2O$, 1.19 KH_2PO_4, 2.54 $CaCl_2 \cdot 2H_2O$, 25 $NaHCO_3$, and 10 glucose.

Perfusion procedure. Adult male mongrel dogs (7.8–13.6 kg) were used. The left adrenal of hypophysectomized-nephrectomized dogs was perfused *in situ* with KRBG containing 0.2% BSA as described earlier (8,9). Briefly, perfusion medium was introduced, through the right renal artery, into an isolated segment of the aorta, from which the left adrenal arteries arise, and was collected from the cannula in the left lumboadrenal vein after passing through the adrenal gland. The perfusion started 4 hr after the end of hypophysectomy and nephrectomy. The right renal artery was cannulated, and the cannula was connected to the long arm of a silicon T-tube (Phicon No. 5, Fuji Systems, Tokyo, Japan), which was covered with a water jacket maintained at 39°C by circulating water from a constant temperature bath by means of a Perista mini-pump (SJ-1211, Atto, Tokyo, Japan). One short arm of the T-tube was connected to a large beaker and the other short arm was attached to a small beaker. Both beakers were placed in a water bath maintained at 39°C. KRBG in the beakers was gassed with 95% O_2/5% CO_2. PAF dissolved in saline containing 0.2% BSA was added to the small beaker. The aorta just below the celiac trunk was clamped with a hemostatic forceps to stop the blood flow after the KRBG began to flow from the large beaker to the left adrenal. The silk thread that had been passed around the left lumboadrenal vein was ligated to direct the adrenal venous flow toward the exterior. Approximately 40 min after the perfusion started, PAF was administered over a 10-min period. The perfusate was collected, for 1 min, at 5 min before, and 2.5, 5, 10, 15 and 20 min after the start of PAF infusion. Constant flow was maintained by changing pump speed during the experiment. Outflow after 10 or 100 nM C_{16}PAF infusion did not change by increasing inflow rate by +15%. The weight of 22 left adrenals infused with various amounts of C_{16}PAF was 599 ± 26 mg (means ± S.E.). Adrenal flow rates at 5 min before, and 5 and 20 min after the start of C_{16}PAF infusion were 503 ± 27, 484 ± 29, and 505 ± 29 μL/100 mg adrenal weight/min (means ± S.E., n=22), respectively. Completeness of hypophysectomy was checked by inspection of the brain at the end of the experiment and by the cortisol level at 35 min after the start of the perfusion.

Dispersed adrenal cells. Dispersed cells were prepared by the method of Hirose *et al.* (10) with some modification. The adrenal glands were cut into small pieces. The minced tissue was incubated in 2.5 mL KRBG containing collagenase (150 U/mL), 0.005% deoxyribonuclease and 4% BSA for 30 min at 38°C under an atmosphere of 95% O_2/5% CO_2 with stirring. The supernatant was

[1]Based on a paper presented at the Third International Conference on Platelet-Activating Factor and Structurally Related Alkyl Ether Lipids, Tokyo, Japan, May 1989. The present data were also reported at the VIIth International Congress on Hormonal Steroids, Madrid, Spain, September, 1986 (*J. Steroid Biochem.* 25, 76S, 1986, Abstract).

*To whom correspondence should be addressed.

Abbreviations: ACTH, adrenocorticotropic hormone; BSA, bovine serum albumin; C_{16}lysoPAF, 1-*O*-hexadecyl-*sn*-glycero-3-phosphocholine; C_{16}PAF, 1-*O*-hexadecyl-2-acetyl-*sn*-glycero-3-phosphocholine; C_{18}PAF, 1-*O*-octadecyl-2-acetyl-*sn*-glycero-3-phosphocholine; KRBG, Krebs-Ringer bicarbonate glucose solution; lysoPAF, 1-*O*-alkyl-*sn*-glycero-3-phosphocholine; PAF, platelet-activating factor, 1-*O*-alkyl-2-acetyl-*sn*-glycero-3-phosphocholine.

discarded and the tissue fragments were rinsed with KRBG containing 4% BSA. The tissue fragments were incubated with collagenase for 1 hr in the same manner as described above. After rinsing with KRBG, 3 mL KRBG containing 0.01% trypsin was added. The mixture was incubated with stirring for 10 min at 38°C under an atmosphere of 95% O_2/5% CO_2, and the supernatant was separated. Trypsin treatment was repeated eight times. Pooled supernatants were filtered through siliconized gauze and centrifuged at 100 × *g* for 30 min at 4°C. The sedimented cells were resuspended in KRBG containing 0.5% BSA, 0.1% lima bean trypsin inhibitor and 7.65 mM calcium, and the concentration of cells was adjusted to approximately 3 × 10^5 cells/mL. Incubations were carried out with shaking for 2 hr at 38°C under an atmosphere of 95% O_2/5% CO_2. Both media and cells were extracted with methylene chloride.

In each preparation, viability of adrenal cells was >88% as assayed by trypan blue exclusion. Cortisol and corticosterone production upon stimulation with 10 nM C_{16}PAF or 10 ACTH (ng/mL), for 2 hr was twice that after 1 hr.

Biochemical analysis. The perfusate was centrifuged and the supernatant was extracted with methylene chloride. Cortisol and corticosterone in the extracts were analyzed by fluorometric assay subsequent to thin-layer chromatography (11), and secretory rates were calculated by multiplying steroid concentration (ng/mL) and adrenal flow (mL/100 mg adrenal weight/min). Steroid production by the adrenal cells was expressed as ng/10^5 cells.

Histamine was measured by single isotope enzymatic assay (12) following column chromatography. Half a milliliter of perfusate was deproteinized with 1 mL of 1 M $HClO_4$ and centrifuged. The supernatant was neutralized and was applied to a Dowex 50 × 4 column (H^+ form, buffered with 0.1 M sodium phosphate buffer, pH 6.5, 8 × 15 mm). Impurities were washed off with 3 mL of 0.1 M sodium phosphate buffer, 3 mL of distilled water, and 3 mL of 1 M HCl. Finally, histamine was eluted with 3 mL of 4 M HCl. The frozen eluate was lyophilized and dissolved in 50 μL of 0.002 M HCl. For the assay of histamine, 10 μL *S*-adenosyl-L-methionine:histamine *N*-methyltransferase (EC 2.1.1.8) was incubated at 37°C for 90 min with 20 μL of the sample or the histamine standard (3–50 pmol), 0.25 μCi *S*-adenosyl-L-[*methyl*-^{3}H]methionine (500 Ci/mmol, NEN, Boston, MA) and a sufficient amount of 0.1 M sodium phosphate buffer, pH 7.9, to make a final volume of 40 μL. The reaction was terminated by the addition of 20 μL of 0.8 M $HClO_4$ containing 1-methylhistamine (5 μg/20 μL). After the addition of 2 N NaOH saturated with NaCl, the radiolabeled methylhistamine was extracted with 5 mL of chloroform. The extract was washed with 2 mL of 2 N NaOH, transferred to counting vials, evaporated to dryness, and assayed for radioactivity.

Statistical analyses were by paired and unpaired Student's *t*-test.

RESULTS AND DISCUSSION

In the present studies we found that PAF acts directly on adrenocortical cells, and facilitates synthesis and release of glucocorticoids. PAF has been shown to cause platelet aggregation at subnanomolar concentrations in several species (2,13). In the present study, the effective dose of C_{16}PAF for cortisol response in the perfused adrenal and isolated cells were 1 nM and 0.1 nM, respectively (Fig. 1, Table 1). We previously reported that the effective doses of histamine for cortisol response in the perfused adrenal and isolated cells were 1 μM and 0.1 μM, respectively (9,14). C_{16}PAF was about 1000 times more potent than histamine on a molar basis in regard to cortisol response in the dog. The increment of the cortisol secretion rate at 5 min after the onset of continuous infusion of 10 nM C_{16}PAF was 5 times that of 10 μM histamine (9) and about half that of 100 pg/mL ACTH (data not shown). The results suggest that PAF is a potent stimulator of the adrenal cortex and that the induction of cortisol release may be one of pathophysiological actions of PAF in the dog.

TABLE 1

Effect of C_{16}PAF on Cortisol and Corticosterone Production by Dog Adrenal Cells[a]

	Cortisol	Corticosterone
Vehicle	23.3 ± 1.2	8.1 ± 0.7
C_{16}PAF		
0.1 nM	32.4 ± 2.9[c]	9.9 ± 1.3
1 nM	36.6 ± 2.8[d]	10.7 ± 1.5
10 nM	38.1 ± 4.0[c]	13.1 ± 1.7[b]
100 nM	38.0 ± 2.8[d]	15.1 ± 2.0[c]
1000 nM	37.6 ± 3.9[c]	14.6 ± 1.8[c]
ACTH 10 ng/mL	170.4 ± 15.8[d]	76.5 ± 9.4[d]

[a]Data shown are means ± S.E. (ng/10^5 cells/2 hr; n=10).

[b]$P < 0.05$, [c]$P < 0.01$, [d]$P < 0.001$, significantly different when compared to vehicle by unpaired *t*-test.

The secretory responses of cortisol and corticosterone to 10 nM C_{18}PAF were one fourth of those to 10 nM C_{16}PAF (Fig. 1). Infusion of 10 nM C_{16}lysoPAF into the perfused adrenal did not induce any change in steroid response. This suggests that the acetyl moiety in the 2 position and the length of alkyl side chain in the 1 position affect the induction of cortisol response as they affect platelet activation in rabbits (15).

Recently, we found that the infusion of histamine into the canine adrenal perfused with artificial medium induced marked increases in the secretory rates of aldosterone, cortisol and corticosterone (9), and that the infusion of 200 μg/mL of compound 48/80 caused a marked release of histamine from mast cells in the adrenal cortex, followed by steroid release (data not shown). Therefore, the histamine concentration of the perfusate was measured before and after the infusion of 10 nM C_{16}PAF over a 10-min period. Histamine values were 82 ± 34 at 5 min before, 48 ± 12 at 0 min, 45 ± 12 at 1 min, 43 ± 16 at 2.5 min, 45 ± 13 at 5 min, 39 ± 10 at 10 min, 42 ± 10 at 15 min, and 40 ± 11 pmol/mL (means ± S.E., n=3) at 20 min after the start of PAF infusion, respectively. Administration of 10 nM C_{16}PAF to the perfused adrenal did not induce any change in histamine levels. The addition of 200 μg/mL of compound 48/80 to the isolated cells had no effect on cortisol and corticosterone production (data not shown). It appears likely that the action of PAF on the perfused adrenal and on isolated cells is independent of histamine release.

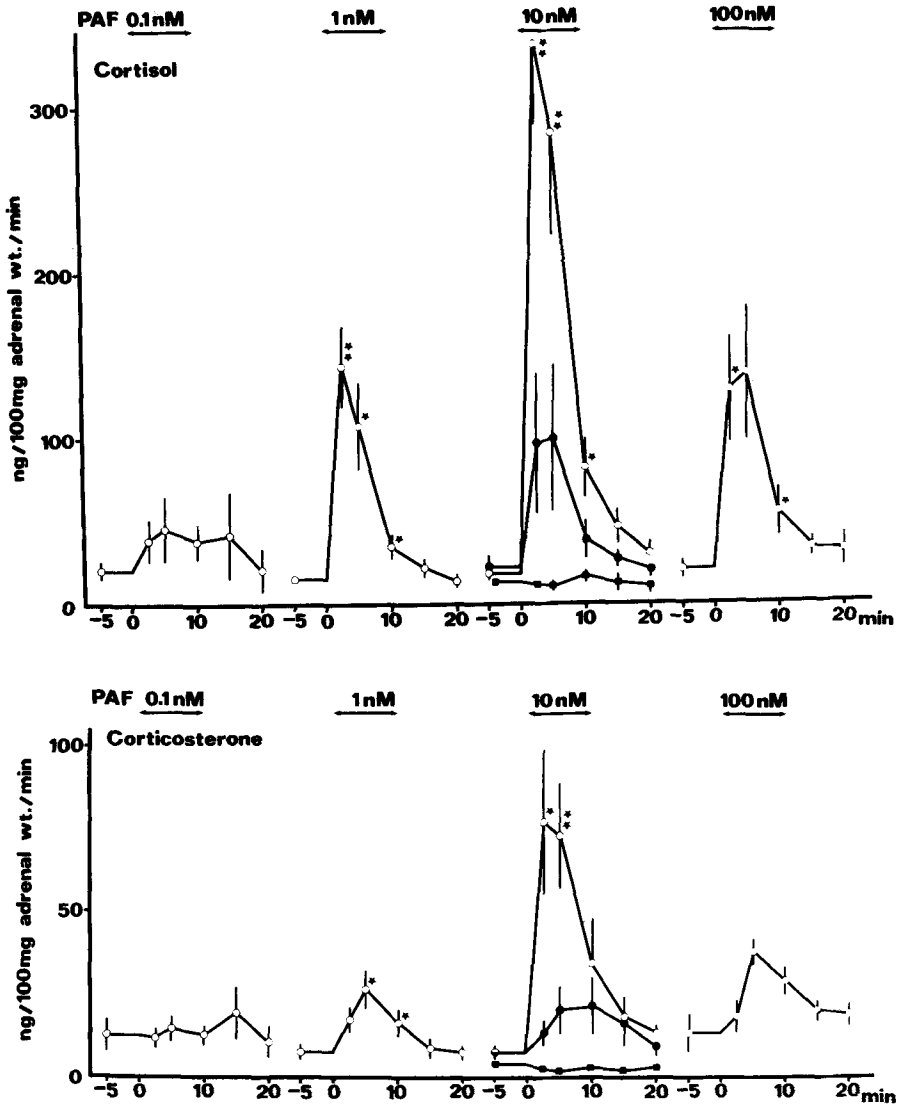


FIG. 1. Secretory responses of cortisol and corticosterone to increasing amounts of C_{16}PAF. The response to 10 nM C_{16}PAF (open circle) were compared to the responses to 10 nM C_{18}PAF (closed circle) and 10 nM C_{16}lysoPAF (closed square). The infusion of agents over 10 min started 40 min after the onset of perfusion. Values are means ± S.E. (0.1, 1 and 10 nM C_{16}PAF, n=6; 100 nM C_{16}PAF, n=4; 10 nM C_{18}PAF and 10 nM C_{16}lysoPAF, n=5). *, $p<0.05$; **, $p<0.01$; significantly different when compared to control values at 5 min before PAF infusion by paired *t*-test.

Cortisol and corticosterone response of the perfused adrenal to 10 nM C_{16}PAF was much higher than that to 10 μM histamine (9), while cortisol and corticosterone production by dispersed cells in response to 10 nM C_{16}PAF was comparable to that to 10 μM histamine (14). The response of the perfused adrenal to 100 nM C_{16}PAF was less pronounced than that to 10 nM. Conversely, steroid production of the cells in response to C_{16}PAF reached a plateau at 10 nM and 100 nM for cortisol and corticosterone, respectively. Infusion of 10 nM or 100 nM C_{16}PAF into the perfused adrenal evoked a slight decrease in outflow. PAF at high concentration may cause increased vascular permeability and vasoconstriction in the adrenal. The possibility cannot be excluded that the marked response to 10 nM C_{16}PAF and sub-maximal response to 100 nM C_{16}PAF in the perfused adrenal are modified by the action of PAF on various cells other than adrenocortical cells.

PAF, a phospholipid mediator of inflammation and allergy, is produced by a variety of cell types such as neutrophils, basophils, eosinophils, monocytes, macrophages, mast cells, mesangial cells, endothelial cells and platelets. It still remains to be determined whether or not the PAF which is released from various cell types at inflammatory or allergic loci into the systemic circulation and/or the PAF locally released from the cells of the adrenal cortex cause the glucocorticoid release. Glucocorticoids are known to suppress various inflammatory and immune responses. Glucocorticoids depress the actions of certain chemical mediators and cytokines (5) and inhibit

vascular permeability induced by PAF, histamine and leukotriene C_4 (16). In addition, glucocorticoids induce phospholipase A_2 inhibitory protein (lipocortin), which affects the arachidonic acid cascade and the release of the PAF precursor, lysoPAF (6,7). Hence, glucocortoid release induced by PAF may result in the inhibition of the action and in the generation of chemical mediators, including PAF itself, in inflammatory or allergic loci.

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Tandem Mass Spectrometry of Negative Ions from Choline Phospholipid Molecular Species Related to Platelet Activating Factor[1]

J.A. Zirrolli, K.L. Clay and **R.C. Murphy***
Department of Pediatrics, National Jewish Center for Immunology and Respiratory Medicine, Denver, Colorado 80206

Fast atom bombardment mass spectrometry of choline phospholipids produces negative ions characteristic of the intact molecule and tandem mass spectrometry of collision-induced decomposition of M-15 anions characterizes both the identity and substituent position of radyl groups. Certain choline phospholipid molecular species which may be of special interest in the generation of platelet activating factor contain a highly unsaturated fatty acyl substituent at *sn*-2 and an ether radyl group at *sn*-1; other choline phospholipid molecular species which contain esterified arachidonic acid are of interest as potential sources of arachidonate for eicosanoid biosynthesis. Collisional activated decomposition of 1-hexadecanoyl-2-arachidonoyl-*sn*-glycerophosphocholine produce abundant carboxylate anions at *m/z* 303 (arachidonate) and *m/z* 255 (hexadecanoate) in a ratio of 3:1, diagnostic for the *sn*-2 arachidonoyl position. The ether analog, 1-*O*-hexadecyl-2-arachidonoyl glycerophosphocholine, produces only one collision-induced dissociation ion at *m/z* 303 and no product ions corresponding to the ether substituent at *sn*-1. Molecular weight information from the M-15 ion combined with the CID generated carboxylate anions completely characterize these important phospholipids.

Precursor ion studies of M-15 anions from glycerophosphocholine lipids indicate that this ion is derived directly from a unique adduct ion formed by attachment of the molecular species to a matrix alkoxide ion, neutralizing the positive charge of the quaternary choline nitrogen. Decomposition of this adduct ion yields a methylated matrix molecule and the nominal M-15 ion.
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Considerable advances have been made in understanding the complex roles that phospholipids play in cell biology. The recognition of platelet activating factor as an important mediator of numerous intra- and intercellular events has significantly altered the view that phospholipids are inert or inactive constituents of cellular membranes. Therefore, the need to characterize not only phospholipid classes present in cellular membranes, but also molecular species within each class has emerged as an important analytical requirement.

A wide variety of analytical techniques is available for the characterization of phospholipid molecular species. The most traditional approach has involved isolation and purification of classes followed by enzymatic or chemical degradation in order to identify radyl substituents at *sn*-1 and *sn*-2 (1). It is difficult to precisely assign substituents at *sn*-1 and *sn*-2 with a chemical degradative strategy and, therefore, the use of rather specific enzymes, e.g. phospholipase A_2, have been required to ascertain radyl group substituent position (2). Another approach has involved diglyceride analysis following phospholipase C hydrolysis (3), and molecular species information has been reported in several systems (4). Choline phospholipids have been studied by position ion electron ionization mass spectrometry; however, determination of the radyl position has been rather difficult (5). Field desorption mass spectrometry enabled characterization of the intact molecular weight, but lacked specific information concerning the substituents at *sn*-1 or *sn*-2 (6). Chemical ionization mass spectrometry has been useful in providing molecular species information (7); however, numerous abundant ions not related to radyl group position are produced in the isobutane (8) and ammonia (7) chemical ionization mass spectra of phosphatidylcholine species. These ions make mixture analysis somewhat difficult and only a few classes of phospholipids are amenable to such analyses. The most successful technique has been fast atom bombardment mass spectrometry (9) which has been applied to the study of intact choline phospholipids with investigation of both positive (10) and negative ions (11).

Tandem mass spectrometry has been shown to be a useful technique for the structure elucidation of phosphatidylserine and phosphatidylcholine molecular species through analysis of negative ions generated by fast atom bombardment (FAB) (11). The position ion tandem mass spectrometric behavior of PAF has recently been reported (12) and is substantially different than that described in this report. Phosphatidylcholines do not produce negative ions characteristic of the intact molecule; however, three abundant ions are observed at high mass corresponding to $[M\text{-}CH_3]^-$, $[M\text{-}HN^+(CH_3)_3]^-$ and $[M\text{-}CH{=}CH\text{-}HN^+(CH_3)_3$ corresponding to the loss of 15, 60, and 86 atomic mass units from the molecualr weight of the phosphocholine. Five different molecular species of phosphatidylcholines have been studied and each one of these produced a characteristic pattern by tandem mass spectrometry, notably the decomposition of the precursor $[M\text{-}CH_3]^-$ ion yielding a product carboxylate anion corresponding to each ester at *sn*-1 and *sn*-2. More interestingly, a substantial difference in the abundance of the carboxylate anions was reported for the radyl group de-

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*To whom correspondence should be addressed at Dept. of Pediatrics, National Jewish Center for Immunology and Respiratory Medicine, 1400 Jackson St., Denver, CO 80206.

Abbreviations: B/E, product ion linked scans where the mass spectrometer magnet is scanned while holding the ratio of the magnet field (B) to electric sector voltage (E) constant; B^2/E; precusor ion linked scans where the mass spectrometer magnet is scanned while holding the ratio of the square of the magnetic field (B) to electric sector voltage (E) constant; CID, collision-induced decomposition; FAB, fast atom bombardment; GPCho, glycerophosphocholine.

pending upon its substitution position (11). The group at *sn*-1 was two to three times less abundant than the carboxylate anion corresponding to the radyl group from *sn*-2. Analysis of choline phospholipid molecular species containing highly unsaturated fatty acyl substituents have not been reported by this technique, nor species with ether radyl groups at *sn*-1. Since these molecular species are of substantial interest in the generation of platelet activating factor, these compounds were the subject of the present study.

EXPERIMENTAL PROCEDURES

All fast atom bombardment mass spectra and collision-induced decomposition (CID) spectra were obtained on a VG7070E double focusing mass spectrometer (VG Instruments, Altrincham, U.K.) equipped with a commercial FAB probe. The capability for linked scan, B/E [product ion linked scans where the mass spectrometer magnet is scanned while holding the ratio of the magnet field (B) to electric sector voltage (E) constant] and B^2/E [precursor ion linked scans where the mass spectrometer magnet is scanned while holding the ratio of the square of the magnetic field (B) to electric sector voltage (E) constant] for product ion and precursor ion experiments, was possible with this instrumentation. The instrument was equipped with a standard VG FAB source (VG Instruments) and Ion Tech saddle field ion gun (Ion Tech, Teddington, U.K.). The ion gun was operated at 6–8 KV using xenon as the particle source. The instrument was operated at 6 KV accelerating voltage in the negative ion mode and the sample was placed in the matrix which was ether glycerol or 3-nitrobenzyl alcohol.

Collision-induced decomposition spectra were obtained in the first field region by adjusting the pressure of the collision gas (helium) until a 50% reduction of the abundance of the selected ion was observed. Product ions formed in this process were detected by B/E scanning and 5–20 scans were typically signal averaged for each spectrum. Signal averaging was accomplished with a Teknivent (Teknivent, St. Louis, MO) data system. Precursor ions were studied by focusing the production of interest with the collision gas pressure set as for product ion formation (*vide supra*) and scanning the instrument in a B^2/E mode.

Materials. 1-*O*-Hexadecanoyl-2-arachidonoyl-*sn*-glycero-3-phosphocholine was obtained from Avanti Polar Lipids (Birmingham, AL) and 1-*O*-hexadecyl-2-arachidonoyl-*sn*-glycero-3-phosphocholine was obtained from Biomol Laboratories (Philadelphia, PA).

RESULTS AND DISCUSSION

Carboxylate Anions. The negative ions obtained by fast atom bombardment mass spectrometry of 1-hexadecanoyl-2-arachidonoyl-*sn*-glycero-3-phosphocholine are shown in Figure 1A. As previously described by Jensen, Tomer and Gross (11), there is no molecular anion observed at *m/z* 781, but rather ions at M-15 (*m/z* 766, loss of methyl), M-60 [*m/z* 721, loss of $HN^+(CH_3)_3$), and M-86 [*m/z* 695, loss of CH=CH-

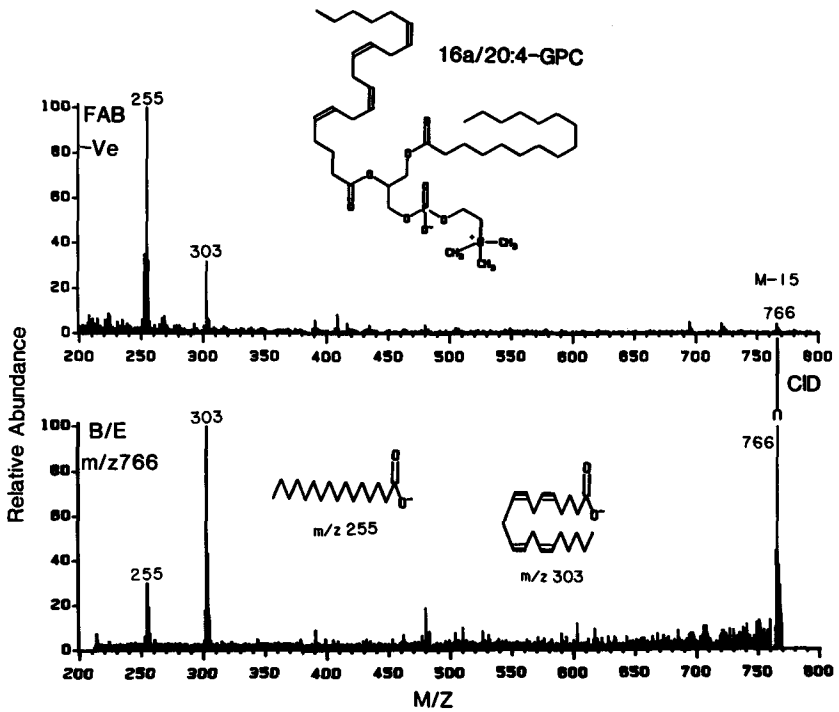
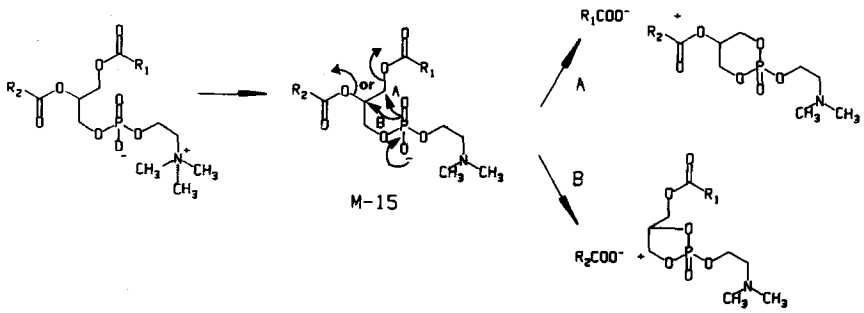


FIG. 1. A, fast atom bombardment mass spectrum of 1-hexadecanoyl-2-arachidonoyl-*sn*-glycero-3-phosphocholine using glycerol as matrix; B, collision-induced decomposition of the M-15 ion at *m/z* 766 and linked scan (B/E) analysis.

$N^+(CH_3)_3$]. Also present were two abundant carboxylate anions at *m/z* 255 and 303, corresponding to the hexadecanoyl and arachidonoyl carboxylate anions, respectively. Figure 1 does not indicate other ions present above *m/z* 800 which were less than 1% abundant on the scale indicated. These source-formed FAB ions were subjected to collision-induced decompositions in the first field free region, and the products derived from *m/z* 766 are shown in Figure 1B. Two abundant ions were observed, again the carboxylate anions at *m/z* 255 (hexadecanoyl carboxylate anion) and *m/z* 303 (arachidonoyl carboxylate anion).

The ratio of the abundances of the ions at *sn*-2/*sn*-1 was approximately three (Fig. 1B), which was previously observed for other diacyl species of phosphatidylserine and phosphatidylcholine analyzed by tandem mass spectrometry (11). This work extends the analysis of diacyl molecular species of phosphatidylcholine and further confirms the ability to assign the acyl groups at *sn*-1 and *sn*-2 based upon the relative abundance of the carboxylate anions. It is of interest to note a reversal of the abundances of carboxylate anions observed in direct FAB analysis (source ions) where the ions corresponding to the radyl group at *sn*-1 were more abundant than those ions arising from the radyl group at *sn*-2 (arachidonate). A possible explanation for the reversed ion ratio for these carboxylate anions following collision-induced decomposition



SCHEME 1

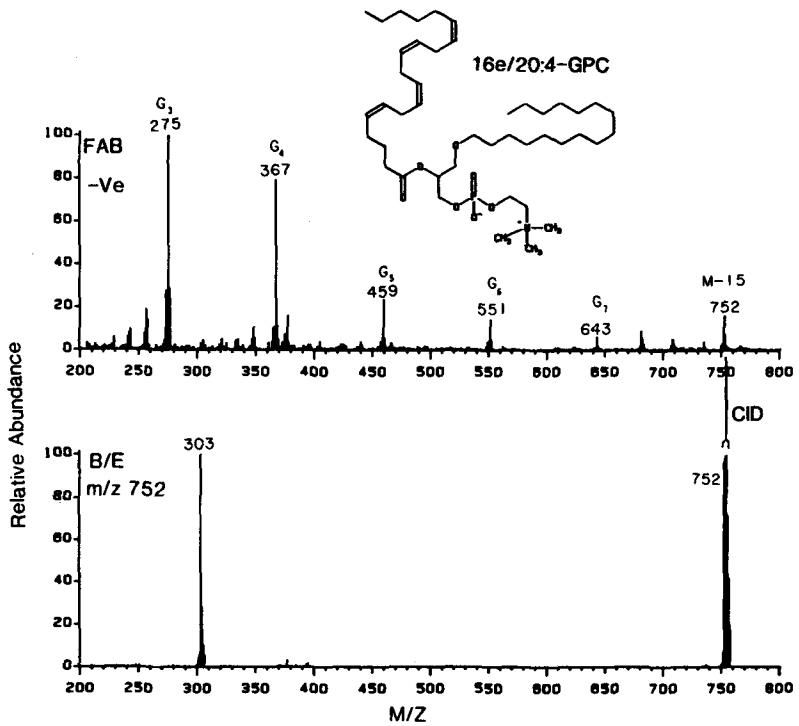


FIG. 2. A, fast atom bombardment mass spectrum of 1-*O*-hexadecyl-2-arachidonoyl-*sn*-glycero-3-phosphocholine using glycerol as matrix, B, collision-induced decomposition of the M-15 ion at *m/z* 752 and linked scan (B/E) analysis.

might be related to the mechanism of ion formation. As shown in Scheme 1, the formation of the M-15 ion would most likely have the site of charge localization on the phosphate moiety. This anionic site can attack the carbon atoms either at *sn*-1 or *sn*-2 by pathways A and B through a six- or five-membered ring transition state, respectively. In the gas phase it would appear that the transition state for the attack of the phosphate anion (formation of a cyclic phosphate ester and liberation of the carboxylate anion) appears to be more favored in a five-membered ring case as compared to the six-membered ring. It would appear that both the stability of the cyclic phosphate ester as well as the stability of the carboxylate anions are quite important in the competitive generation of these abundant anions. It has also been suggested that steric hindrance in the transition state plays an important role (13).

As shown in Figure 2A, the negative ions derived from the 1-*O*-alkyl choline phospholipid species are somewhat different. Fast atom bombardment of 1-*O*-hexadecyl-2-arachidonoyl-*sn*-glycero-3-phosphocholine yields abundant ions in the high molecular weight region at M-15, M-60, and M-86, characteristic of choline phospholipids. However, for this mass spectrum substantially less phospholipid was placed in the glycerol matrix, and the most abundant ions at lower masses arose from the glycerol cluster ions (*m/z* 275, 367, 459,

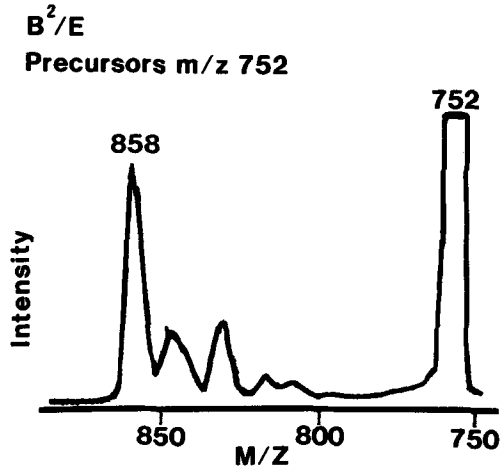
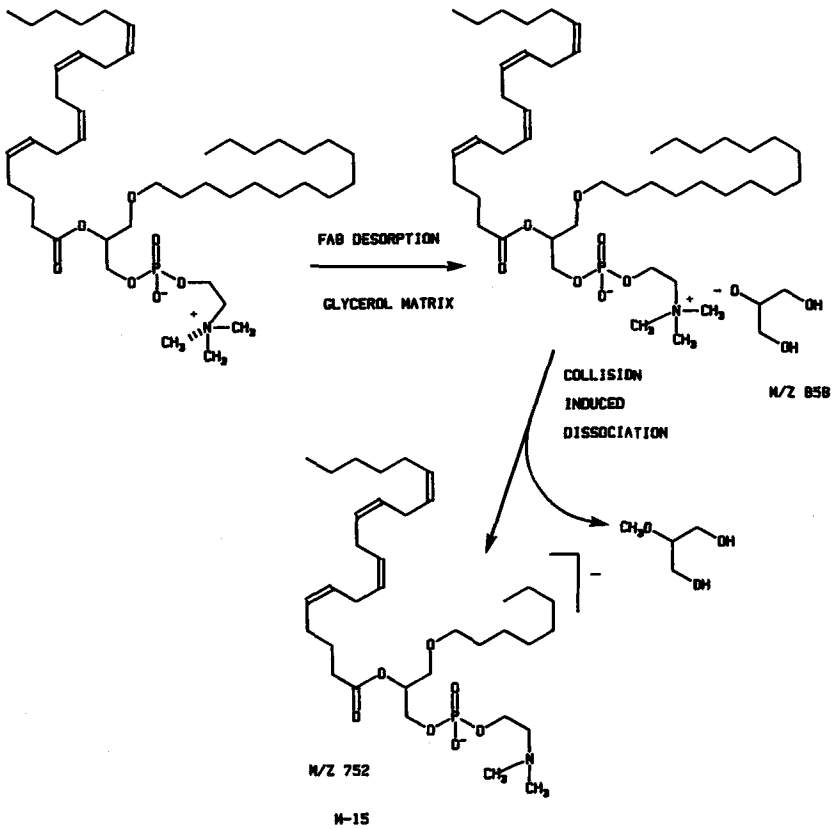


FIG. 3. B²/E linked scan experiment of *m/z* 752 (M-15) formed in the fast atom bombardment mass spectrometry of 1-*O*-hexadecyl-2-arachidonoyl-*sn*-glycero-3-phosphocholine. The observed precursor ions at *m/z* 858 suggest formation of the glycerol alkoxide adduct ion with the intact choline phospholipid.



SCHEME 2

551, and 643). Again, ions of less than a 1% abundance above *m/z* 800 are not indicated. The appearance of the abundant ions illustrates a fundamental problem with source-generated ions present in the direct fast atom bombardment mass spectrum. Ions could originate from substances other than the lipid, e.g., the matrix or impurities present in a phospholipid mixture. Collision-induced decomposition of the M-15 at *m/z* 752 (Fig. 2B) yielded essentially one ion, *m/z* 303 (the arachidonoyl carboxylate anion). No negative ions were observed resulting from charge retention on the alkyl ether substituent present at the *sn*-1 group. This is in keeping with the mechanism of formation of the ions illustrated in Scheme 1, where the stability of the carboxylate anion is an important facet in the collisional activation process and formation of carboxylate anions. Thus, alkyl ether choline phospholipids yield one carboxylate anion in accordance with only one ester moiety present and its position is at *sn*-2.

Origin of the M-15 ion. As shown in Scheme 1, the formation of these important carboxylate anions in the collision-induced decomposition of choline phospholipids relies on the generation of a N,N-dimethylamino moiety from the quarternary ammonium group in choline. Little is known concerning the mechanism by which such M-15 ions are formed. Linked scans were carried out to search for any precursor ion species for the M-15 ion in the gas phase. It is possible that the M-15 ion originated during the FAB process in the glycerol matrix, and thus demethylation would have taken place in the condensed phase. However, the B^2/E experiments revealed that there was a precursor ion for the M-15 ion for both the 1-*O*-hexadecyl-2-arachidonoyl molecular species and the diacyl molecular species as well. This ion from the 1-*O*-hexadecyl-2-arachidonoyl-*sn*-glycerophosphocholine appeared at *m/z* 858, corresponding to an attachment of a glycerol alkoxide anion to the choline phospholipid molecular species $[M + 91]^-$ (Fig. 3). When 3-nitrobenzyl alcohol was used for the fast atom bombardment matrix, an adduct ion was observed 152 daltons above the molecular weight of the choline phospholipid supporting formation of the alkoxide attachment ion from this matrix as well. Linked scans (B/E) for these adduct ions from each matrix revealed decomposition to the M-15 ions, suggesting that the demethylation process most likely involved simultaneous formation of the methyl ether of the matrix alcohol and a demethylated choline phosphate anion (M-15; Scheme 2). Since these adduct ions were observed in the B^2/E experiment, they were source-formed ions; however, it is possible that the demethylation process occurred to some extent in the condensed phase.

In summary, the tandem mass spectrometry of choline phospholipids which contain highly unsaturated acyl groups, such as arachidonic acid at the *sn*-2 group, provides useful information as to the radyl groups at *sn*- and *sn*-2. In the case of the 1-*O*-alkyl-2-acyl-glycerophosphocholines, a single acyl carboxylate anion is observed in the decomposition of the M-15 ion. The observation of this single carboxylate anion combined with the molecular weight of the molecule (deduced from the ions at M-15, M-60, and M-86), allows one to construct the radyl groups at *sn*-1 (an alkyl group) as well as *sn*-2 (the acyl group). Correspondingly for diacyl glycerophosphocholines, decomposition of the M-15 ion results in two carboxylate anions and from the ratio of these two products one can assign the fatty acyl substituents at *sn*-1 and *sn*-2. The formation of the carboxylate anion from the *sn*-2 group is preferred, most likely due to the stability of the five-membered ring cyclic phosphate ester as compared to the six-membered cyclic ester product. It is of interest that the formation of the M-15 ion—which is quite useful for molecular species analysis/arises from a demethylation reaction of a desorbed attachment ion of the phosphocholine with an ionized matrix molecule. This alkoxide ion from the matrix neutralizes the permanent positive charge on the quaternary nitrogen of the choline species, leaving a phosphate anion to direct acceleration into the mass spectrometer. Identical decomposition reactions were observed in a triple quadrupole mass spectrometer for these glycerophosphocholines which supports the charge-driven mechanisms outlined in Schemes 1 and 2 as being low energy processes.

ACKNOWLEDGMENT

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Determination of Platelet-Activating Factor by a Chemiluminescence Method and Its Application to Stimulated Guinea Pig Neutrophils[1]

Yukio Hasegawa*, Eiko Kunow, Junko Shindou and Hidetaka Yuki
Department of Clinical Chemistry, School of Pharmaceutical Sciences, Toho University, Chiba 274, Japan

A simple, rapid and sensitive chemiluminescence method has been developed to measure platelet-activating factor (PAF). Hydrogen peroxide generated from PAF, upon phospholipase D cleavage, by choline oxidase is determined as chemiluminescence by a luminol-microperoxidase system. The detection limit of PAF by this method is 5 pmol/tube. The method is reproducible with a 5.5% coefficient of variation at 10 pmol of PAF (n = 5). Lipids were extracted from guinea pig neutrophils after stimulation with cytochalasin B and *N*-formyl-methionyl-leucyl-phenylalanine, and PAF was isolated by high-performance liquid chromatography and determined by chemiluminescence measurements. The amount of PAF detected was 96.1 ± 39.7 (mean ± SD, n = 7) pmol/10^8 cells. This highly sensitive method could be useful for the determination of PAF generated under pathophysiological conditions.
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Platelet-activating factor (PAF, 1-*O*-alkyl-2-acetyl-*sn*-glycero-3-phosphocholine) is an important mediator of allergic and inflammatory reactions (1). A wide variety of cell types produce PAF in very low concentrations upon stimulation (2). Few structural characteristics of PAF exist which can be detected easily; thus a simple and sensitive method for the measurement of PAF is needed.

PAF is usually determined by bioassay of platelet aggregation, the release of 5-hydroxytryptamine (3), or by gas chromatography/mass spectrometry (GC/MS)(4,5). The bioassay is a very sensitive method that can detect even picogram quantities of PAF, but its accuracy is limited because the platelet-activating activity of PAF varies with the chain length of the alkyl ether moiety of the molecule, and by other variables. By comparison, GC/MS is specific, sensitive and accurate. However, it requires expensive equipment and multistep sample preparation procedures which are time-consuming.

Various chemiluminescent reactions have been used for the quantitative analysis of a variety of biological substances (6,7). In our present study, a simple, rapid, and sensitive chemiluminescence method has been utilized to determine choline-containing phospholipids. The method was applied to the measurement of PAF produced in stimulated guinea pig neutrophils. The method is based on the detection of choline liberated from PAF. Takayama *et al.* (8) have measured choline-containing phospholipids by an enzymatic method; however, this method is not sensitive enough to determine trace amounts of PAF. We applied chemiluminescence detection to the enzymatic assay by using chemiluminescent reagents instead of colorimetry. As seen in the sequence of reactions shown in Figure 1, PAF is hydrolyzed by phospholipase D (PLD), and the choline liberated is subsequently oxidized by choline oxidase (CO). The hydrogen peroxide produced is then determined as chemiluminescence by the luminol-microperoxidase (mPOD) system.

$$\text{PAF} + H_2O \xrightarrow{\text{PLD}} \text{1-}O\text{-alkyl-2-acetyl-}sn\text{-glycerophosphate} + \text{choline}$$

$$\text{choline} + 2O_2 + H_2O \xrightarrow{\text{CO}} \text{betaine} + 2H_2O_2$$

$$\text{luminol} + 2H_2O_2 + 2OH \xrightarrow{\text{mPOD}} \text{aminophthalate} + N_2 + 4H_2O + \text{light}$$

FIG. 1. The sequence of enzymatic and chemiluminescent reactions for determination of PAF.

MATERIALS AND METHODS

Chemicals. 1-*O*-Hexadecyl- and 1-*O*-octadecyl-2-acetyl-*sn*-glycero-3-phosphocholine (C_{16}PAF and C_{18}PAF) and 1-*O*-hexadecyl-*sn*-glycero-3-phosphocholine (LPAF) were products of Novabiochem (Laufelfingen, Switzerland). Phosphatidylcholine (PC), phosphatidylethanolamine (PE), and phosphatidylglycerol (PG), all from egg yolk; phosphatidylinositol (PI) from soybeans; and sphingomyelin from bovine brain were obtained from Sigma Chemical Co. (St. Louis, MO). Palmitoyl and stearoyl lysophosphatidylcholine (LPC), phospholipase D (PLD) from *Streptomyces chromofuscus,* choline oxidase (CO) from *Alcaligenes,* microperoxidase (mPOD, MP-11), *N*-formyl-methionyl-leucyl-phenylalanine (FMLP), cytochalasin B, and bovine serum albumin (BSA; essentially fatty acid-free) were purchased from Sigma. Luminol and Triton X-100 were obtained from Wako Pure Chemical Industries (Osaka, Japan). All other reagents used were reagent grade.

Chemiluminescence determination. Enzymatic reactions by PLD and CO were carried out according to a modification of the method of Takayama *et al.* (8). PAF, LPAF, PC or LPC (0.025–2.5 μM) was dissolved in 10 mM Tris-HCl buffer (pH 8.0) containing 1 mM $CaCl_2$ and Triton X-100 (0.1–2 g/L). The above solution (200 μL) was transferred to a microtube (5 × 50 mm), and 10 μL each of PLD (50 U/mL) and CO (30 U/mL) was added. The mixture was then incubated for 15 min at room temperature. Tris-HCl buffer (pH 8.0) used for dissolving the choline-containing lipids was also incubated with PLD and CO to serve as blank. After incubation, the hydrogen

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*To whom correspondence should be addressed at Department of Clinical Chemistry, School of Pharmaceutical Sciences, Toho University, Miyama-2-2-1, Funabashi-shi, Chiba 274, Japan.

Abbreviations: BSA, bovine serum albumin; CL, cardiolipin; CO, choline oxidase; FMLP, *N*-formyl-methionyl-leucyl-phenylalanine; GC/MS, gas chromatography/mass spectrometry; HPLC, high-performance liquid chromatography; LPAF, lyso platelet-activating factor, 1-*O*-alkyl-*sn*-glycero-3-phosphocholine; LPC, lysophosphatidylcholine; mPOD, microperoxidase; PAF, platelet-activating factor, 1-*O*-alkyl-2-acetyl-*sn*-glycero-3-phosphocholine; PC, phosphatidylcholine; PE, phosphatidylethanolamine; PG, phosphatidylglycerol; PI, phosphatidylinositol; PLD, phospholipase D.

peroxide generated in the reaction mixture was determined as chemiluminescence. A calibration curve was prepared for each choline phospholipid.

The chemiluminescence reaction was carried out according to a modification of the method of Schroeder *et al.* (9). Luminol was dissolved in 0.1–1.0 M sodium carbonate-sodium bicarbonate buffer (pH 8.6–12). Stock solution of 100 μM mPOD was prepared in 10 mM Tris-HCl buffer (pH 7.4) and diluted to 20 μM in the carbonate buffer just before use. The chemiluminescence reaction was initiated by adding 50 μL each of 0.1 mM luminol and 20 μM mPOD to the microtube in which hydrogen peroxide was generated by CO upon PLD cleavage. Upon vortexing for 3 sec, the microtube was immediately inserted into a cell holder cavity of the reaction chamber mounted on top of a photometer NU-620 with an integrated timer (Nition, Tokyo, Japan), and chemiluminescence (350–550 nm) was measured at room temperature. The chemiluminescence time course was simultaneously recorded. The chemiluminescence intensity was expressed as the amount of chemiluminescence integrated over 1 min.

Preparation of guinea pig neutrophils. Neutrophils were prepared from guinea pig (Hartley, 300–350 g, female) peritoneal exudates by intraperitoneal injection of 30–35 mL of 2% sodium caseinate solution 14–16 hr before peritoneal lavage, as described previously (10). The exudates were collected by washing the peritoneal cavity with 60 mL of phosphate buffered saline (pH 7.4) containing heparin (1 unit/mL). Cells were then centrifuged (400 × *g*, 5 min) and washed with the same buffer. Contaminated erythrocytes were eliminated by suspending the cells in 0.2% NaCl for 30 sec at 4°C, and by then returning the osmolarity to normal by addition of an equal volume of 1.6% NaCl. Cells were sedimented by centrifugation (400 × *g*, 5 min) and then suspended in phosphate buffered saline before use.

Production of PAF. Guinea pig neutrophils were adjusted to a concentration of 1×10^7 cells/mL in Hanks' balanced saline buffer (pH 7.4) containing 0.25% gelatin and 0.25% bovine serum albumin as described (11). Neutrophils (10^8 cells) were prewarmed for 5 min at 37°C, incubated with cytochalasin B (1 μg/mL) for 5 min at 37°C, and then challenged with 1 μM FMLP for 3 or 10 min. Phosphate buffered saline, the vehicle of FMLP and cytochalasin B, were added to another sample as control. After incubation, the cell suspensions were centrifuged and the pellets were used for lipid extraction.

Lipid extraction and purification of PAF. Lipids were extracted from the cell pellets by the method of Bligh and Dyer (12). The extracted lipids were dissolved in 200 μL of chloroform/methanol (1:1, v/v), and 100 μL of the solution was analyzed by high-performance liquid chromatography (HPLC) on a Senshu-pak silica 1151-N column (4.6 × 150 mm) fitted with a Shimadzu LC-3A pump (Osaka, Japan). The solvent(100 μL) used for dissolving the lipids was also applied to HPLC as blank. The column was eluted with chloroform/methanol/water (240:220:11, v/v/v) at a flow rate of 1 mL/min (elution system I) as described before (13). PAF was further purified by HPLC using a Wakosil (Osaka, Japan) 5 SIL column (4.5 × 150 mm), a Tosoh ultraviolet (UV)-8000 detector (Tokyo, Japan), a Tosoh CCPM dual pump, and a linear solvent gradient of n-hexane/isopropanol/water from 100:100:12 (v/v/v) to 100:100:18 (v/v/v) over 20 min at a flow rate of 1 mL/min (elution system II), as described before (14). One mL of eluate was collected per fraction, and then dried with a centrifugal evaporator (Taitec VC-360, Taiyo Scientific Industrial, Tokyo, Japan). The PAF residue from each fraction was dissolved in 400 μL of 10 mM Tris-HCl buffer (pH 8.0) containing 1 mM $CaCl_2$ and Triton X-100 (2 g/L), and an aliquot of above solution (200 μL) was transferred to a microtube (5 × 50 mm) for chemiluminescence measurements. The chemiluminescence intensity of blanks for each fraction was also determined. The intensities of the blanks were almost identical for all fractions and corresponded to about 10 pmol of C_{16}PAF. The chemiluminescence intensity attributed to PAF was obtained by subtracting the intensity of the corresponding blank. The amount of PAF per fraction was calculated from the calibration curve established for the C_{16}PAF standard. The total amount of PAF present was the sum of the values obtained for the individual fractions.

Recovery of PAF. C_{16}PAF standard (1 nmol) was added to the neutrophil (without stimulation) suspension just before lipid extraction. PAF was isolated by HPLC using elution systems I and II as described above, and determined by the chemiluminescence method. The neutrophil suspension with no addition of PAF was used as a control. The recovery of PAF was calculated from the chemiluminescence intensity obtained by subtracting the intensity of the control. The PAF standard (100 pmol) was also directly applied to HPLC using elution systems I and II, and the recovery of PAF by HPLC was determined.

RESULTS AND DISCUSSION

The chemiluminescence intensity of PAF by the luminol-mPOD system is a function of pH and concentration of the carbonate buffer, and of the concentrations of luminol and mPOD. Each of these variables was examined individually. The chemiluminescence intensity rose sharply with an increase in the pH of the carbonate buffer, and reached a plateau above pH 10.5. At pH 10.5, 95% of the chemiluminescence reaction was completed within one minute. Chemiluminescence intensity also increased with an increase of the concentration of the buffer; the maximum intensity was obtained at the final concentration of 330 mM. Optimal concentrations of luminol and mPOD added to the reaction mixture were 0.1 mM and 20 μM, respectively. Under optimized conditions, a linear relationship was obtained between chemiluminescence intensity and the amount of hydrogen peroxide produced. The intensity was linear with hydrogen peroxide concentrations in the range of 4 to 500 pmol of hydrogen peroxide (coefficient of variation, 2.8% at 12 pmol H_2O_2, n = 3).

To save incubation time, the hydrolysis of PAF by PLD and the oxidation by CO were initiated by the simultaneous addition of both enzymes to the reaction mixture. The reaction was virtually complete within 15 min, the time span that was chosen as the incubation time. The optimal concentrations of PLD and CO were 50 U/mL and 30 U/mL, respectively. The enzymatic reactions of PLD and CO were not affected by 0.1–2.0 g/L Triton X-100 or by the addition of other lipids such as PE, PG, PI or cardiolipin (CL) to the reaction mixture. The reaction mixture without PAF was used as the blank. The blank itself gave a comparatively high background, which corresponded to about 10 pmol of C_{16}PAF standard. This background is

likely to be due to contaminations in the enzyme preparations. The yield of hydrogen peroxide from PAF by PLD and CO was 92.9 ± 4.4% (mean ± SD, n = 4), based on the chemiluminescence intensity due to hydrogen peroxide theoretically generated from PAF. The yield of conversion of choline to hydrogen peroxide by CO was about 98.2 ± 3.7% (n = 3), based on the chemiluminescence intensity produced by a hydrogen peroxide standard.

Figure 2 shows the relationship between the chemiluminescence intensity and the increasing concentrations of PAF on a double log plot under the optimized conditions specified above for both the enzymatic and the chemiluminescent reactions. The chemiluminescence intensity increased in direct proportion to the amount of PAF present, within the range of 5 to 500 pmol. The coefficient of variation was 5.5% at 10 pmol of PAF (n = 5) indicating good reproducibility.

Determination of PAF by the present method is as sensitive as is GC/MS (4). In the chemiluminescence method, the impurities in both enzyme preparations affect chemiluminescence intensity as the reactions are performed in the same tube. Purification of the enzymes would be necessary to decrease background noise and to achieve lower PAF detection limits. Alternatively, utilizing a flow injection system equipped with columns of immobilized PLD and CO would eliminate the effects of impurities in the enzyme preparations and should provide a higher sensitivity for the determination of PAF. We have previously demonstrated the effectiveness of a flow system with immobilized enzymes which minimize the background of chemiluminescence for the determination of inorganic phosphate (15). The chemiluminescence intensities of PC, LPC and LPAF determined by this method were almost the same as those of equimolar PAF. The results suggest that the chemiluminescence method is a sensitive detection system for choline phospholipids, including PAF.

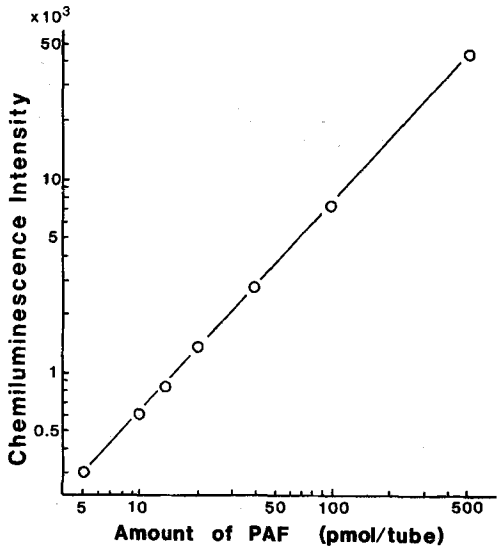


FIG. 2. Calibration curve for C_{16}PAF by the chemiluminescence method. C_{16}PAF (0.025–2.5 μM) was dissolved in 10 mM Tris-HCl buffer (pH 8.0) containing 1 mM $CaCl_2$ and Triton X-100 (2 g/L). Ten μL each of PLD (50 U/mL) and CO (30 U/mL) was added to 200 μL of the PAF solution. After incubation for 15 min, the chemiluminescence intensity was measured as described in Materials and Methods. Each point represents the mean of three experiments.

To investigate the applicability of the chemiluminescence method to biological samples, guinea pig peritoneal neutrophils were stimulated with FMLP and cytochalasin B, and the PAF produced was determined. PAF was separated from other choline phospholipids by HPLC using first the elution system I and then elution system II. Figure 3 shows the HPLC separation of choline phospholipid standards using elution system I. PAF was well separated from other choline phospholipids. C_{16}PAF and C_{18}-PAF were eluted in fraction 30–32 and fraction 32–34, respectively, between sphingomyelin and LPC. HPLC of the extracted lipids from the stimulated neutrophils was done as described above. PAF was not completely separated from the pronounced tailing of the sphingomyelin peak (Fig. 4); therefore, PAF was further purified

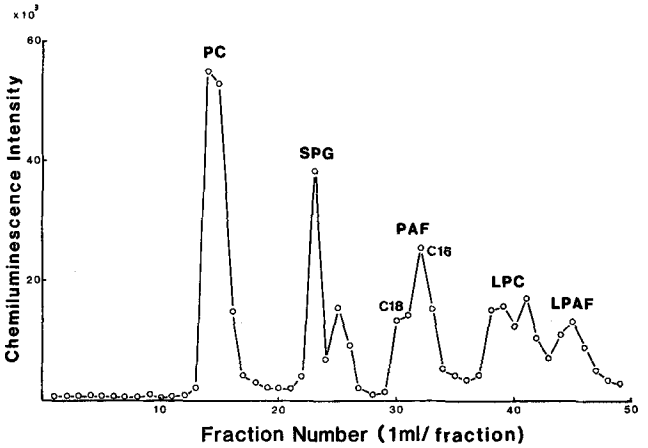


FIG. 3. HPLC separation of choline-containing phospholipid standards using elution system I. PC (2 nmol), sphingomyelin (1 nmol), C_{16}PAF (700 pmol), C_{18}PAF (300 pmol), palmitoyl LPC (500 pmol), stearoyl LPC (500 pmol) and LPAF (500 pmol) were dissolved in 100 μL of chloroform/methanol (1:1,v/v) and analyzed by HPLC with a Senshu-pak silica 1151-N column (4.6 × 150 mm). HPLC conditions were as described in Methods and Materials. One mL of eluate was collected in each fraction and dried. The dried lipids in each fraction were dissolved in 400 μL of 10 mM Tris-HCl buffer (pH 8.0) containing 1 mM $CaCl_2$ and Triton X-100(2 g/L), and 200 μL of the solution was used for measurement of the chemiluminescence intensity as described in Materials and Methods.

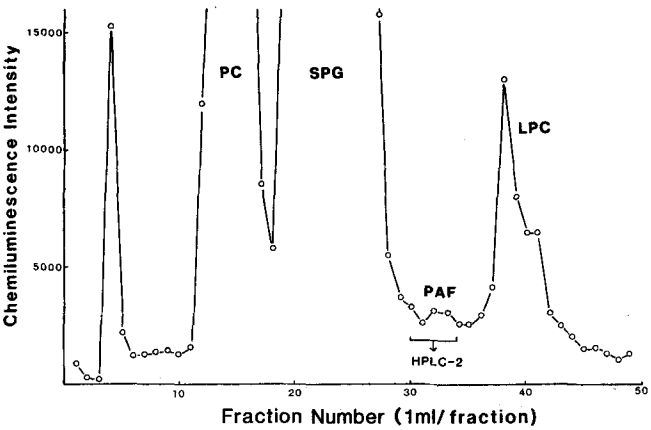


FIG. 4. HPLC separation of lipids extracted from stimulated guinea pig neutrophils using elution system I. Conditions for HPLC and for chemiluminescence measurements are the same as those shown in Figure 3. PAF contained in fraction 30–34 was further purified by HPLC using elution system II.

by HPLC using elution system II. Figure 5 shows the HPLC separation of the phospholipid standards using elution system II. PAF and sphingomyelin in HPLC fractions 30–34, using elution system I, were eluted separately (Fig. 6). Most of the PAF was eluted in the same fractions (fraction 25–27) as the C_{16}PAF standard. The amount of PAF produced upon stimulation by FMLP and cytochalasin B for 3 or 10 min was 41.8 ± 12.5 (mean ± SD, n = 3) or 96.1 ± 39.7 (n = 7) pmol/10^8 cells, respectively. The amounts of PAF in the control group, which was incubated for 3 or 10 min without stimulation, were 21.4 ± 8.1 and 40 ± 19.2 pmol/10^8 cells (n = 3), respectively. Relatively high levels of PAF were detected in the guinea pig neutrophils without stimulation. Approximately 65% of the PAF standard added to the neutrophils was recovered in the course of the isolation (n = 3). The recovery of PAF by HPLC, using elution system I, was 89.3 ± 6.1% (n = 3). Using elution system II, the recovery of PAF by HPLC was 76.8 ± 9.7% (n = 3). Ramesha and Pickett (16) reported that PAF determined by GC/MS in guinea pig peritoneal neutrophils stimulated with ionophore A23187 was 8.4 ng (about 15 pmol)/10^7 cells. A very high amount of PAF (5.2 ng/10^7 cells) was also detected in guinea pig neutrophils without stimulation. The major molecular species of PAF detected by GC/MS were C_{16}PAF and C_{17}PAF. The value of PAF determined by the chemiluminescence method in the neutrophils, stimulated with FMLP and cytochalasin B, was a little lower than that obtained by GC/MS in guinea pig neutrophils stimulated with ionophore A23187. Lynch and Henson (17) found that the quantity of PAF generated upon FMLP stimulation was less than that obtained upon stimulation with ionophore A 23187. Direct comparisons between the PAF amounts determined by the chemiluminescence method and those determined by GC/MS will be needed. PAF determined by GC/MS (18) and bioassay (19) in human neutrophils stimulated by ionophore A 23187 were 80 and 1000 pmol/10^7 cells. The reason for this large discrepancy between the values obtained by two methods is unknown. The levels of PAF detected in human neutrophils were somewhat higher than those determined by GC/MS or with the chemiluminescence method in stimulated guinea pig neutrophils. PAF detected by GC/MS in rabbit peripheral neutrophils stimulated with ionophore A 23187 was 40 ± 15 pmol/10^7 cells (20). The results suggested that only low levels of PAF occur in stimulated guinea pig peritoneal neutrophils, the reasons for which are not clear.

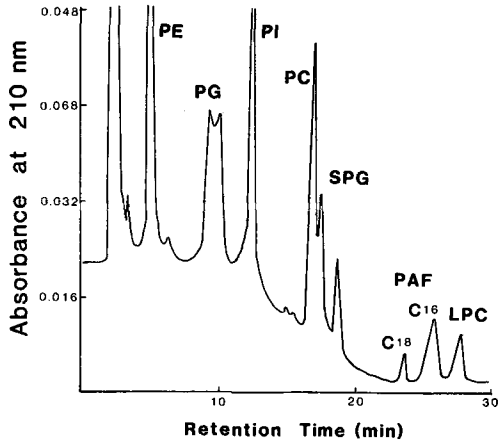


FIG. 5. HPLC separation of the phospholipid standards using elution system II. PE (25 μg), PG (11 μg), PI (25 μg), PC (25 μg), C_{18}PAF (100 nmol), C_{16}PAF (300 nmol) and palmitoyl LPC (250 nmol) were dissolved in 100 μL of n-hexane/isopropanol/water (100:100:12, v/v/v), and separated by HPLC using a Wakosil 5 SIL column (4.6 × 150 mm) and UV detection at 210 nm. HPLC conditions are those described in Materials and Methods.

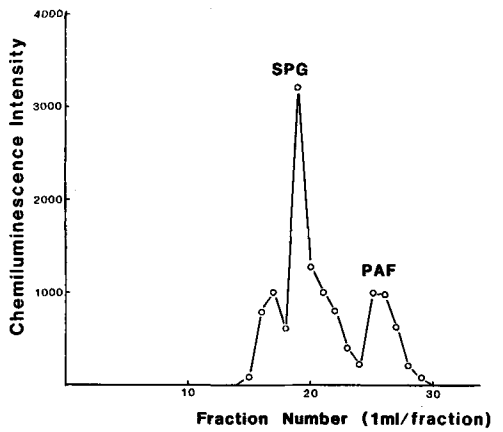


FIG. 6. HPLC separation of PAF and sphingomyelin using elution system II. PAF contained in HPLC fraction 30–34 using elution system I (Fig. 4) was dissolved in 100 μL of n-hexane/isopropanol/water (100:100:12, v/v/v) and separated from sphingomyelin by HPLC using elution system II. Conditions are the same as those shown in Figure 5, except for the chemiluminescence detection. The chemiluminescence measurements were carried out as described in Materials and Methods.

Many studies have shown the presence of different molecular species of PAF in various types of cells (20,21). Although in the present study we did not determine the molecular species of PAF, the retention time in HPLC suggests that most of the PAF produced in guinea pig neutrophils was C_{16}PAF. More accurate determination of the molecular species may be possible by reverse phase HPLC.

The proposed chemiluminescence method is simple, sensitive, and rapid, and does not require expensive instrumentation for the determination of choline-containing phospholipids. Our method is applicable to the determination of PAF produced in stimulated neutrophils, and may also be useful for studies of other choline phospholipids, including LPC and LPAF. The determination of PAF by flow injection analysis using immobilized PLD and CO columns is presently being investigated.

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Specific Binding of Antibodies to Platelet-Activating Factor (PAF) as Demonstrated by Thin-Layer Chromatography/Immunostaining[1]

Ken Karasawa, Noriko Satoh, Toshio Hongo, Yasuhito Nakagawa, Morio Setaka and Shoshichi Nojima*

Department of Membrane Biology, Faculty of Pharmaceutical Science, Teikyo University, Sagamiko, Kanagawa 199-01, Japan

The specificity of rabbit antibodies produced by injection of 1-*O*-(15'-carboxypentadecyl)-2-*N,N*-dimethylcarbamoyl-*sn*-glycero-3-phosphocholine bovine serum albumin (BSA) conjugates was examined by a thin-layer chromatography (TLC)/immunostaining method. Phosphatidylcholine (PC), lysophosphatidylcholine (lysoPC), lyso platelet-activating factor (lysoPAF), phosphatidylethanolamine (PE), phosphatidylglycerol (PG), phosphatidylserine (PS), sphingomyelin (SM), phosphatidylinositol (PI), phosphatidic acid (PA) and cardiolipin (CL) were not immunostained. Among several synthetic PAF-related compounds, the antibodies only bound to PAF agonists which have the activity to induce washed rabbit platelet aggregation. The results suggest that the binding sites of the antibodies on the PAF molecule are the acetyl group at the *sn*-2 position and the choline moiety at the *sn*-3 position of glycerol, both of which are essential for exerting the biological function of PAF and for binding to the PAF receptors located on cellular membranes.
***Lipids 26,* 1122–1125 (1991).**

1-*O*-Alkyl-2-acetyl-*sn*-glycero-3-phosphocholine (PAF) is a potent biologically active phospholipid that plays a major role in inflammatory reactions (1). We have recently reported the successful production of rabbit polyclonal antibodies to PAF by injection of a nonhydrolyzable synthetic PAF analogue conjugated to BSA with Freund's complete adjuvant (2). High specificity is required for the application of antibodies, e.g., as reagents for immunoassay or immunohistochemistry, or as tools for study of the receptor. A sensitive enzyme-immunochemical staining method has been used for detection of glycosphingolipids on silica gel thin-layer chromatography (TLC) (3). In this study we examined the specificity of rabbit antibodies to PAF by the TLC/immunostaining method and identified the binding sites for the antibodies on the PAF molecule.

MATERIALS AND METHODS

Materials. 1-*O*-Hexadecyl PAF, 1-*O*-octadecyl PAF and 1-*O*-hexadecyl lysoPAF were obtained from Bachem (Bubendorf, Switzerland). Dipalmitoyl phosphatidylcholine and rabbit γ-globulin, bovine serum albumin (BSA), polyvinylpyrrolidone (PVP) and 4-chloro-1-naphthol were from Sigma Chemical Co. (St. Louis, MO). Freund's complete adjuvant was from Difco Laboratories (Detroit, MI). Phosphatidylinositol (PI) (from yeast), phosphatidic acid (PA) (from egg yolk), sphingomyelin (from bovine brain) and 1-palmitoyl-*sn*-glycero-3-phosphocholine were a gift from Dr. M. Umeda (Univ. of Tokyo, Japan). Biotinylated goat anti rabbit-IgG (Ig, immunoglobulin) and avidin-biotinylated horseradish peroxidase complex were from Vector Laboratories Inc. (Burlingame, CA). Phosphatidylethanolamine (PE), phosphatidylglycerol (PG) and cardiolipin (CL) were prepared from *Escherichia coli* (4). Enantio-PAF (3-*O*-hexadecyl-2-acetyl-*sn*-glycero-1-phosphocholine) was from Nova Biochem (Laufelfingen, Switzerland). Polyisobutylmethacrylate was from Aldrich (Milwaukee, WI). Protein A-Sepharose CL-6B was from Pharmacia Fine Chemicals (Uppsala, Sweden).

Preparation of antibodies. 1-*O*-(15'-Carboxypentadecyl-2-*N,N*-dimethylcarbamoyl-*sn*-glycero-3-phosphocholine BSA conjugates were injected into rabbits according to the method described in our previous report (2). At one week after the booster injection, blood was drawn from the ear vein of the rabbit which had the highest titer of antibody activity. The IgG fraction was separated from antisera by protein A-Sepharose CL-6B column chromatography.

TLC/immunostaining. TLC/immunostaining was performed according Higashi *et al.*, with slight modifications (3). In brief, phospholipids were applied to a plastic-backed TLC plate (Polygram Sil G, Macherey-Nagel, W. Germany). The spotted TLC plate was dried and dipped into a hexane solution containing 0.4% polyisobutylmethacrylate for several seconds. After drying, the plate was soaked in PBS containing 1% ovalbumin and 1% PVP for 2 hr. After washing of the TLC plate with PBS, IgG from rabbit antiserum diluted with PBS containing 1% PVP (46.8 μg protein/ml) was applied to the TLC plate and incubated for 1 hr. After washing of the TLC plate with PBS, biotinylated goat anti-rabbit IgG diluted at 1:200 with PBS containing 1% PVP was applied to the plate and incubated for 30 min. The plate was again washed with PBS, and an aliquot of the avidin-biotinylated horseradish peroxidase complex diluted at 1:200 with PBS was applied. After incubation for 30 min, the TLC plate was washed thoroughly with PBS and then exposed for 15 min at room temperature, to 4-chloro-1-naphthol/hydrogen peroxide solution, which was freshly prepared before use by the method of Hawkes *et al.* (5). The enzyme reaction was stopped by washing the TLC plate with water/methanol (5:1, v/v). The immunostained TLC plate was allowed to dry and was scanned with a dual-wave-

*To whom correspondence should be addressed.

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Abbreviations: BSA, bovine serum albumin; CL, cardiolipin; Ig, immunoglobulin; PA, phosphatidic acid; PAF, platelet-activating factor; PBS, phosphate-buffered saline; PC, phosphatidylcholine; PE, phosphatidylethanolamine; PG, phosphatidylglycerol; PI, phosphatidylinositol; PS, phosphatidylserine; SM, sphingomyelin; TLC, thin-layer chromatography.

length TLC scanner, CS-910 (Shimadzu Co., Kyoto, Japan). The absorbance at 578 nm was measured in the transmittance mode. Peak areas were measured with a Chromatopack CR-1B integrator (Shimadzu Co., Kyoto, Japan).

Platelet aggregation. Washed rabbit platelets were prepared according to the method described by Pinckard *et al.* (6). Test compounds were solubilized in 0.15 M NaCl containing 2.5 mg/ml BSA. Platelet aggregation was monitored using an aggregometer (RAM-11, Rikadenki Kogyo, Co., Tokyo, Japan).

RESULTS

We were successful in applying the TLC/immunostaining method to the detection of very small amounts of PAF on a TLC plate, by introducing a fixation step with polyisobutylmethacrylate. Otherwise, PAF spotted on a TLC plate is easily lost from the plate during conventional TLC/immunostaining. This fixing method has previously been used in the TLC/immunostaining of glycosphingolipids (7). Figure 1 shows the results of TLC/immunostaining of PAF and other phospholipids. Both PAF and 1-*O*-hexadecyl-2-*N*,*N*-dimethylcarbamoyl-*sn*-glycero-3-phosphocholine were immunostained as blue-purple spots, but were not immunostained at all by IgG separated from preimmune rabbit serum. The antibodies did not bind to other naturally occurring phospholipids such as PC, PE, PG, CL, PA, sphingomyelin (SM), lysoPC and lysoPAF. The effect of fixation by polyisobutylmethacrylate on the lipid fractions on TLC plates was examined. Figure 2 shows that PAF, lysoPAF and DPPC are well fixed and stained with phosphomolybdic acid reagent, but only PAF was immunostained. Similar results were obtained with mouse liver lipids and mouse brain lipids. Also, in these cases, the lipids were not lost, but were well retained on TLC plates even after treatment with a polyisobutylmethacrylate solution, but only PAF was immunostained (data not shown).

Various amounts of PAF were applied onto a TLC plate and immunostained, and the plate was scanned with a densitometer (Fig. 3). As little as 1 pmol of PAF could be detected with a good signal-to-noise ratio (S/N > 2). An acceptable densitometric response was observed up to 125 pmol of PAF, although it deviated somewhat from linearity.

We studied the structure-immunoreactivity relationship of PAF and its analogues by the TLC/immunostaining method (Table 1). In regard to modification of the 1-*O*-alkyl group, no significant difference in immunoreactivity was recognized between C_{18}PAF (II) and C_{16}PAF (I). The acyl analogue of PAF [1-palmitoyl-2-acetyl-*sn*-glycero-3-phosphocholine (III)] reacted weakly as compared to PAF. This result showed that a change from the alkyl ether bond ($-CH_2-O-CH_2CH_2-$) to the acyl ester one ($-CH_2-OCO-CH_2-$) markedly decreased the cross-reactivity with the antibodies. The acetyl group at *sn*-2 was required for the binding of PAF to the antibodies, as lysoPAF (V) did not show any cross-reactivity. PAF analogues with substituents such as $(CH_3)_2NCOO-$ (VIII), CH_3CONH- (IX), CH_3COCH_2COO- (X) and $CH_3OCONH-$ (XI), in place of the acetyl group at *sn*-2 of the glycerol backbone,

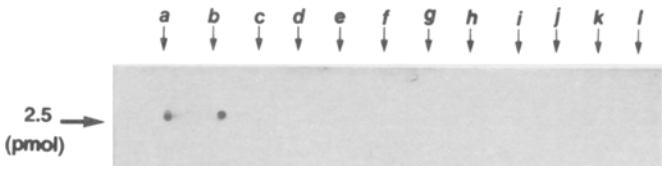


FIG. 1. TLC/immunostaining of PAF and other phospholipids. In this case, 2.5 pmol of each phospholipid was spotted on the TLC plate and immunostained as described in Materials and Methods. (a) 1-*O*-Hexadecyl PAF, (b) synthetic hapten (1-*O*-(15'-carboxypentadecyl)-2-*N*,*N*-dimethylcarbamoyl-*sn*-glycero-3-phosphocholine), (c) phosphatidylcholine, (d) lysophosphatidylcholine, (e) lysoPAF, (f) phosphatidylethanolamine, (g) phosphatidylserine, (h) phosphatidylglycerol, (i) phosphatidylinositol, (j) cardiolipin, (k) phosphatidic acid, and (l) sphingomyelin.

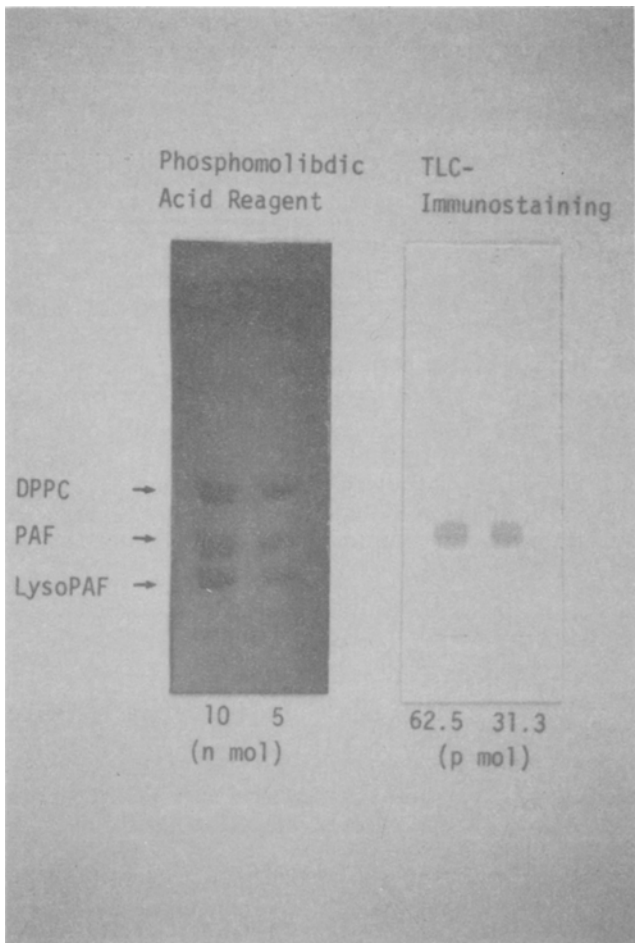


FIG. 2. Effect of fixation with polyisobutylmethacrylate on thin-layer chromatograms of DPPC, PAF and lysoPAF. Left panel: DPPC, 1-*O*-hexadecyl PAF and lysoPAF (10 or 5 nmol, respectively) were spotted on a plate and developed in $CHCl_3/CH_3OH$/water (65:35:6, v/v/v). After drying, the plate was treated with polyisobutylmethacrylate. The treated plate was dipped in phosphomolybdic acid reagent (10%, w/v, phosphomolybdic acid in ethanol) for a few seconds and heated at 120°C on a hot plate. Right panel: After DPPC, 1-*O*-hexadecyl PAF and lysoPAF (62.5 or 31.3 pmol, respectively) were spotted and developed in the same manner as described above (left panel), the plate was treated for immunostaining as described in Materials and Methods.

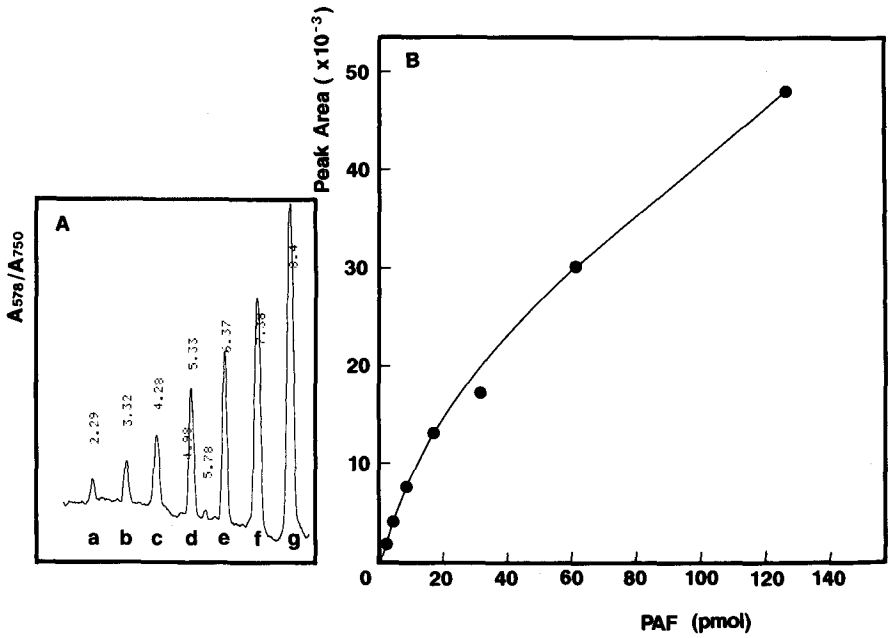


FIG. 3. TLC/immunostaining of PAF and densitometric scanning of the enzyme-immunostained spots on the chromatogram. A series of 1-*O*-hexadecyl PAF samples [(a) 0.98, (b) 1.95, (c) 3.90, (d) 7.81, (e) 15.6, (f) 31.3 and (g) 62.5 pmol] were spotted onto a TLC plate, immunostained, and the plate was scanned with a densitometer (see Materials and Methods). The integral densitometric response was plotted vs PAF concentration.

were immunostained to various extents. The D-isomer of PAF, the inactive enantiomer of natural PAF (IV), was not stained. A compound containing a morpholino group at *sn*-3 (XII) in place of the choline moiety was immunostained. All of the immunostained compounds had the activity to induce rabbit platelet aggregation, while the other compounds were neither immunostained nor had PAF agonist activity.

DISCUSSION

It has been reported already that antibodies to some phospholipids, such as CL (8), PI (9), polyphosphoinositides (10,11) and PS (12) are produced in rabbits and mice. The haptenic activity was enhanced by the addition of cholesterol and lecithin with an appropriate carrier protein such as methylated BSA. However, antibodies to PAF could not be produced by the injection of a mixture of PAF, lecithin, cholesterol and methylated BSA. We considered that this might be because PAF is easily removed from the lipid micelles, since serum proteins such as albumin have a high affinity for PAF (13,14). Further, PAF is rapidly degraded in plasma and tissues; $T_{1/2}$ of PAF in rabbit plasma is only 10 min (15). Therefore, we attempted to produce antibodies to PAF by injection of a nonhydrolyzable PAF analogue covalently attached to a carrier protein. Since 1-*O*-hexadecyl-2-*N*,*N*-dimethylcarbamoyl-*sn*-glycero-3-phosphocholine has almost the same activity as PAF and is resistant to acetylhydrolase (16), we synthesized a hapten bearing a carboxy group at the ω-position of the C-1 alkyl chain of this derivative and used the conjugate composed of this PAF analogue covalently attached to BSA as an immunogen. The antibodies to this immunogen showed high specificity for PAF.

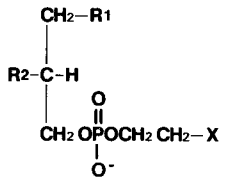
We also showed that the acetyl group at C-2 of PAF was distinctly recognized by the antibodies. In addition, we observed that rabbit antibodies to the antigen in which a monobutylcarbamoyl group was present at the *sn*-2 position of glycerol, in place of the dimethylcarbamoyl group, have higher affinity for the monobutylcarbamoyl derivative of PAF than for PAF (Karasawa, K., *et al*, unpublished data). We are now attempting to produce rabbit antibodies to immunogenic conjugates composed of synthetic haptens having acyl groups with different chain lengths or being deacylated at the *sn*-2 position of glycerol. A series of antibodies with different specificities for the acyl chain length at the *sn*-2 position of glycerol might be useful for analysis of each of these phospholipids in biological materials.

A unique feature of the antibodies is the recognition of the chirality of the *sn*-2 position of PAF. These antibodies had negligible cross-reactivity with D-PAF in TLC/immunostaining and radioimmunoassay (less than 0.047 %) (17). To our knowledge, this is the first report of an antibody that recognizes the stereospecificity at C-2 of phospholipids, although Umeda *et al.* (12) reported the stereospecific recognition of the polar head group in PS by mouse monoclonal antibodies.

Among the PAF-related compounds, only PAF agonists showed cross-reactivity with the antibodies. These results suggest that the binding sites of the antibodies on PAF involve at least part of the structure which is necessary for binding to PAF receptors. The existence

TABLE 1

Cross-Reactivity and Platelet Aggregating Activity of PAF-Related Phospholipids



	Compounds R1	R2	X	TLC/immunostaining[a]	Platelet aggregation[b]
I	$C_{16}H_{33}O$-	CH_3CO-	$-\dot{N}(CH_3)_3$	+	+
II	$C_{18}H_{37}O$-	CH_3CO-	$-\dot{N}(CH_3)_3$	+	+
III	$C_{15}H_{31}CO$-	CH_3CO-	$-\dot{N}(CH_3)_3$	+ [c]	–
IV	$C_{16}H_{33}O$-	CH_3CO- (D-isomer)	$-\dot{N}(CH_3)_3$	–	–
V	$C_{16}H_{33}O$-	OH-	$-\dot{N}(CH_3)_3$	–	–
VI	$C_{15}H_{31}CO$-	OH-	$-\dot{N}(CH_3)_3$	–	–
VII	$C_{15}H_{31}CO$-	$C_{15}H_{31}CO$-	$-\dot{N}(CH_3)_3$	–	–
VIII	$C_{18}H_{37}O$-	$(CH_3)_2NCO$-	$-\dot{N}(CH_3)_3$	+	+
IX	$C_{18}H_{37}O$-	CH_3CNH-	$-\dot{N}(CH_3)_3$	+	+
X	$C_{18}H_{37}O$-	CH_3CCH_2CO-	$-\dot{N}(CH_3)_3$	+	+
XI	$C_{18}H_{37}O$-	CH_3OCNH-	$-\dot{N}(CH_3)_3$	+	+
XII	$C_{18}H_{37}O$-	CH_3NHCO-		+	+

[a]Twenty-five pmol of each compound was spotted on the TLC plate and immunostained as described in Materials and Methods. Positive (+) compounds were detectable as blue-purple spots.

[b]Positive (+) compounds caused aggregation of washed rabbit platelets at 5×10^{-7} M (over 50% change was recorded in light transmission).

[c]Weakly positive in TLC/immunostaining.

of PAF receptors has been demonstrated in platelets (18), neutrophils (19) and other cells.

In the present study we showed that PAF can be quantitatively detected by the TLC/immunostaining method. This method has already successfully been employed to detect and estimate glycosphingolipid (e.g., tumor-associated Hanganuitziu-Deicher antigen) (20). We are now interested in the application of our PAF antibodies to biological samples. We developed the TLC/immunostaining method for measurement of PAF using C_{16}PAF as authentic standard, since C_{16}PAF is a predominant component in a number of biological materials (21). However, PAF in biological materials consist of various molecules species, such as C_{18}PAF, $C_{18:1}$PAF and others. In this respect, caution is required when the amount of PAF in biological samples is estimated by the TLC/immunostaining method, because C_{18}PAF is also cross-reacting with these antibodies.

In the previous report we described the substantial cross-reactivity between lysoPAF and PAF antibodies by ELISA inhibition assay (2). On the other hand, we have shown in this paper [and in an accompanying one (17)] that no cross-reactivity of lysoPAF with PAF antibodies could be found by either TLC/immunostaining and or radioimmunoassay (less than 0.025%). We cannot explain this discrepancy at the present time, but the inconsistent results may be due, in part, to the different immunochemical assay systems used. Anyway, antibodies which we used here for the present study do not cross-react at all with lysoPAF up to the amount of 125 pmol tested (unpublished results).

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Radioimmunoassay for Platelet-Activating Factor[1]

Ken Karasawa, Noriko Satoh, Toshio Hongo, Yasuhito Nakagawa, Morio Setaka and Shoshichi Nojima*
Department of Membrane Biology, Faculty of Pharmaceutical Science, Teikyo University, Sagamiko, Kanagawa 199-01, Japan

A radioimmunoassay (RIA) for measurement of platelet-activating factor (PAF) was developed. At a final antiserum dilution of 1:640, the lowest detection limit of PAF was 0.1 pmol (50 pg). The standard curve obtained was suitable for measurement of PAF in amounts ranging from 0.1 pmol to 30 pmol. The antiserum showed high specificity. Cross-reaction for lysoPAF, lysophosphatidylcholine and long-chain phosphatidylcholines was very low (less than 0.025%). 1-Palmitoyl-2-acetyl-*sn*-glycero-3-phosphocholine cross-reacted slightly (6.25%). PAF exogenously added to macrophage suspensions was quantitatively determined by RIA after solvent extraction and high-performance liquid chromatographic separation. RIA was also used to estimate PAF formation after stimulation of rabbit alveolar macrophages in suspension with calcium ionophore A23187.
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Platelet-activating factor [PAF; 1-*O*-hexadecyl (or octadecyl)-2-acetyl-*sn*-glycero-3-phosphocholine] is a potent lipid mediator of various inflammatory and allergic reactions (1). PAF administered *in vivo* elicits pathophysiological responses at low doses, and PAF antagonists are effective in several pathological animal models. Although quantitative estimation of PAF levels in various disease states is necessary for evaluation of its pathological role, very little information has been obtained about the amounts of PAF present in biological samples. The most commonly employed methods for measurement of PAF are the bioassay, based on aggregation or secretion of washed rabbit platelets (2), and gas chromatography-mass spectrometry (GC-MS) (3). The former is sensitive, but not sufficiently accurate, because the conditions used for platelet preparation affect reproducibility. The latter method yields structural information, but requires relatively large samples. Radioimmunoassay (RIA) is specific, sensitive and practical for routine measurements. The RIA method has been widely used for the quantitation of lipid mediators, such as prostaglandins and leukotrienes. Therefore, we have attempted to establish a RIA for PAF. We have already reported the preparation of rabbit antibodies by injection of a non-hydrolyzable synthetic PAF analogue conjugated to bovine serum albumin (BSA) with Freund's complete adjuvant (4). In the present study we developed a sensitive (0.1 pmol) and specific RIA for PAF using these antibodies.

MATERIALS AND METHODS

Materials. 1-*O*-Hexadecyl PAF and 1-*O*-hexadecyl lysoPAF were obtained from Bachem (Bubendorf, Switzerland). Dipalmitoyl phosphatidylcholine, rabbit γ-globulin, BSA and ionophore A23187 were from Sigma Chemical Co. (St. Louis, MO). Freund's complete adjuvant was from Difco Laboratories (Detroit, MI). Phosphatidylinositol (PI) (from yeast), phosphatidic acid (PA) (from egg yolk), sphingomyelin (SM) (from bovine brain) and 1-palmitoyl-*sn*-glycero-3-phosphocholine were a gift from Dr. M. Umeda (University of Tokyo, Japan). Phosphatidylethanolamine (PE), phosphatidylglycerol (PG), and cardiolipin (CL) were prepared from *Escherichia coli* (5). Enantio-PAF (3-*O*-hexadecyl-2-acetyl-*sn*-glycero-1-phosphocholine) was from Nova Biochem (Laufelfingen, Switzerland). 1-*O*-[^{3}H]Octad ecyl PAF (132 Ci/mmol) and ACS scintillation fluid were from Amersham International (Amersham, Buck, U.K.). Polyethylene glycol (#6000) was from Nakarai Chemicals (Kyoto, Japan). 1-Palmitoyl-2-acetyl-*sn*-glycero-3-phosphocholine was prepared by acetylation of 1-palmitoyl-*sn*-glycero-3-phosphocholine in pyridine and purified by thin-layer chromatography.

Preparation of Antibodies. 1-*O*-(15′-Carboxypentadecyl)-2-*N,N*-dimethylcarbamoyl-*sn*-glycero-3-phosphocholine/BSA conjugates were injected into a rabbit according to the method described in our previous report (4). At one week after the booster injection, blood was drawn from the ear vein of a rabbit which had a high titer of antibody activity. Antiserum was inactivated at 56°C for 30 min.

RIA. RIA with polyethylene glycol 6000 was performed by a slight modification of the method by Schiller *et al.* (6). Antiserum, radiolabelled PAF, standard PAF and other reagents were all prepared in phosphate-buffered saline (PBS) containing 0.05% Tween 20. The addition of Tween 20 to the assay buffer decreased both the non-specific binding of labelled PAF to γ-globulin and adsorption to the plastic test tubes. The non-specific binding of labelled PAF was less than 3% for the γ-globulin precipitation. 1-*O*-[^{3}H]Octadecyl PAF (0.1 mL, 30,000 dpm), and standard PAF (0.1 mL, 0–100 pmol) were added to plastic, micro test tubes (Eppendorf, Hamburg, FRG) with antiserum (0.25 mL) diluted to 1:320 (final concentration, 1:640). Then, 1% rabbit γ-globulin (0.05 mL) was added. The tubes were vortexed and incubated for 16 hr at 4°C. At equilibrium, the antibody-bound radiolabelled PAF and the free PAF were separated by precipitation of γ-globulin with 0.5 mL of a chilled (4°C) aqueous solution of 25%

*To whom correspondence should be addressed.
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Abbreviations: BSA, bovine serum albumin; CL, cardiolipin; GC-MS, gas chromatography-mass spectrometry; GPC, glycerophosphocholine; HPLC, high performance liquid chromatography; PA, phosphatidic acid; PAF, platelet-activating factor; PBS, phosphate-buffered saline; PC, phosphatidylcholine; PE, phosphatidylethanolamine; PG, phosphatidylglycerol; PI, phosphatidylinositol; PS, phosphatidylserine; RIA, radioimmunoassay; SM, sphingomyelin.

w/v polyethylene glycol, incubation for 1 hr at 4°C and centrifugation at 4°C for 10 min at 9,000 × *g* to compact the γ-globulin pellet. The supernatant was aspirated and discarded. The γ-globulin pellet was reconstituted in 0.5 mL of 0.5 N NaOH, and the sample was transferred to a scintillation vial. ACS scintillation fluid (10 mL) was added and the amount of antibody-bound [^{3}H]PAF was determined with an Aloka LSC-900 liquid scintillation counter.

Extraction and chromatography of samples. Alveolar macrophages were harvested from the lungs of New Zealand White rabbits (male, 3 kg weight) according to the method of Myrvik *et al.* (7). Macrophages (5 × 10^6) were suspended in 1 mL of MEM medium (Nissui Pharmaceutical Co., Tokyo, Japan) with 20 mM Hepes buffer (pH 7.3). A trace of [^{3}H]PAF (3,000 dpm) was added to the samples for calculating recoveries. Lipids were extracted from macrophage suspensions by the method of Bligh and Dyer (8). The chloroform layer was evaporated to dryness. These samples were separated by high performance liquid chromatography (HPLC) according to the method of Hayashi *et al.* (9). Briefly, the above residue was dissolved in 60 μL of hexane/isopropanol (1:2, v/v). HPLC was performed using a Shimadzu LC-6A system (Shimadzu, Kyoto, Japan), which was connected to a UV detector. PAF was separated from other phospholipids by a 3.9 × 150-mm μ Bondasphere silica column (Waters Associates Inc., Milford, MA) eluted with isopropanol/hexane/water (110:100:20, v/v/v), and the fraction corresponding to PAF was collected. After evaporation of the PAF fraction to dryness under nitrogen, 0.5 mL of PBS containing Tween 20 (0.05%, w/v) was added. An aliquot (0.2 mL) was removed to determine percentage recovery and the remainder was used for PAF determination by RIA, as described above.

Accuracy of RIA. The accuracy of the assay was assessed by analyzing the content of PAF in (i) a standard buffer solution of PAF; and (ii) macrophage suspensions (5 × 10^6 cells) with known amounts of added PAF. These samples were extracted and chromatographed as described above prior to RIA.

Cell incubation. Macrophage suspensions preincubated at 37°C for 1 min were incubated with ionophore A23187 (1 μg/mL) for 20 min. The incubation was stopped by addition of 1 mL of ice-cold methanol, followed by extraction and chromatography of samples prior to RIA.

RESULTS

Specificity and sensitivity of RIA. Figure 1 shows the elevation of the antiserum titer after injection of the immunogens into rabbit. The titer of the antiserum increased by repeated injections of the immunogen. For the radioimmunoassay, we used the antiserum collected after the fourth injection (19 weeks). Fifty percent binding of [^{3}H]PAF was observed at an antiserum dilution of 1:200 (final concentration).

The standard curve for PAF was obtained using the antibody at 1:640 dilution, which bound 25% of the labelled PAF in the absence of standard PAF. As shown in Figure 2, increasing amounts of PAF inhibited the binding of labelled PAF to antibody in a competitive

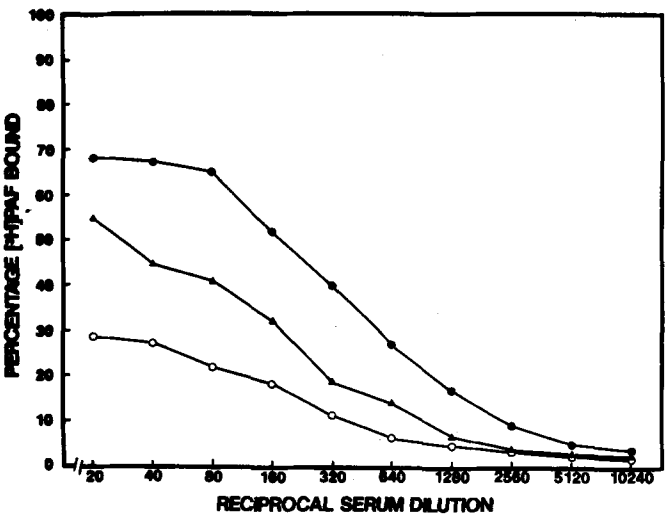


FIG. 1. Binding activity of rabbit antisera. Percentage of [^{3}H]PAF bound was measured 10 weeks (○), 13 weeks (▲) and 19 weeks (●) after the beginning of antigen injection. RIA was carried out as described in Materials and Methods.

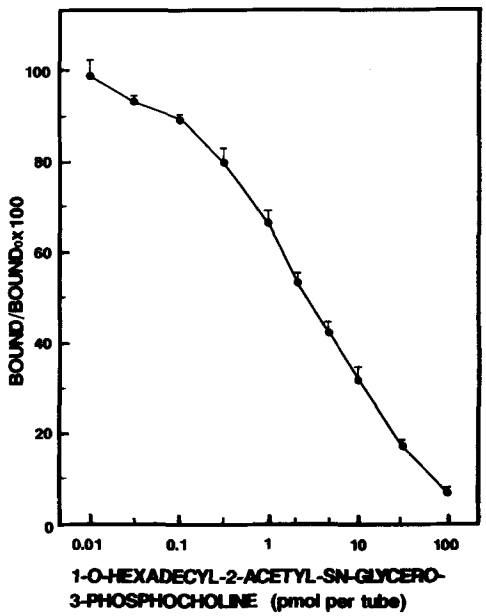


FIG. 2. Calibration curve for the quantitation of PAF. Each reaction mixture contained standard PAF (0.1 mL, 0–100 pmol), radioactive PAF (0.1 mL, 30,000 dpm, 2.03 pmol), antiserum (0.1 mL, 1:320, 0.25 mL), and 1% γ-globulin (0.05 mL). Other procedures were carried out as described in Materials and Methods. B/B_0 (%) indicates radioactivity bound to the precipitate in the presence of standard PAF over the radioactivity bound in the absence of PAF. Points represent the mean S.D. (n=3) using polyethylene glycol precipitation of antibody-bound radiolabelled PAF.

manner. The sensitivity of the standard curve, i.e., the lowest amount that could be significantly (± 2 S.D.) distinguished from zero, was 0.1 pmol (50 pg)/tube. The standard curve obtained was suitable for measurement of PAF ranging from 0.1–30 pmol. The specificity of the PAF antibody was examined in cross-reactivity studies with other phospholipids (Table 1). The antibody bound to a synthetic hapten (1-*O*-hexadecyl-2-*N,N,*-dimethylcarbamoyl-*sn*-glycero-3-phosphocholine) with higher affinity than PAF itself. Choline containing phospholipids (lysoPAF, lysoPC and PC) demonstrated little or no effect on the binding of radiolabelled PAF to antibodies. However, slight cross-reactivity (6.25%) was observed for 1-palmitoyl-2-acetyl-*sn*-glycero-3-phosphocholine. All other natural phospholipids tested (PE, SM, PI, PG and CL) showed less than 0.01% cross-reactivity. The cross-reaction of the D-isomer of PAF was only 0.047%.

Accuracy of RIA. To test the validity of the RIA, a series of known amounts of PAF (0–50 pmol/tube) were added to 1-mL aliquots of rabbit alveolar macrophage suspensions (5×10^6). The recovery of [^{3}H]PAF after Bligh and Dyer extraction followed by HPLC was 64.2%. There was a good correlation between the amount of added PAF and that measured by RIA (Table 2). A background level of 1.71 pmol PAF/mL (0.34 pmol/10^6 cells) was detected.

Determination of PAF in activated rabbit alveolar macrophages by calcium ionophore A23187. The amount of PAF produced by rabbit alveolar macrophage suspensions stimulated with ionophore A23187 was determined by RIA. Ionophore A23187 (1 μg/mL) increased the amount of PAF from a background level of 0.34 pmol/10^6 cells to 4.50 pmol/10^6 cells.

DISCUSSION

We have described here the development of a sensitive and specific RIA for PAF. The PAF antiserum showed a relatively high degree of sensitivity with a minimum detection limit for PAF of 0.1 pmol (50 pg) at a final antiserum dilution of 1:640 with 1-*O*-[^{3}H]octadecyl PAF (132 Ci/mmol, 30,000 dpm) as a tracer. If a PAF tracer with higher specific activity (e.g., ^{125}I-labelled tracer) were to be used, the detection limit would be lowered further. Cross-reactivity studies of the antiserum revealed a high specificity for PAF. Choline-containing phospholipids such as lysoPAF, lecithin or lysoPC did not cross-react with PAF antiserum (Table 1). This result is in good agreement with that obtained by the thin-layer chromatography/enzyme immunostaining method (10). Specificity studies of the antibody showed that the binding sites are against both the acetyl group at *sn*-2 and the phosphocholine moiety at *sn*-3. The PAF antiserum showed slight cross-reactivity with 1-palmitoyl-2-acetyl-*sn*-3-GPC (6.25%). This result shows that the antibody recognizes, in part, the alkyl ether bond (-CH_2-O-CH_2CH_2-) at the *sn*-1 position. The presence of a significant amount of the acyl analogue of PAF has been demonstrated in some tissues, such as bovine brain (11). Removal of the acyl analogue of PAF by hydrolysis using lipase may be required prior to RIA in a sample containing this analogue.

Recently, Baldo and his collaborators reported the production of rabbit polyclonal antibodies to PAF (12). They used a synthetic PAF analogue coupled to a protein carrier at the terminus of the alkyl chain. However, their immunogens were different from ours in two respects. First, the acetyl group at *sn*-2 was left intact. Second, the alkyl chain at *sn*-1 was very short (C_6 or C_{12}). In contrast, the immunogen we used was a non-hydrolyzable analogue of PAF containing a *N,N*-dimethylcarbamoyl group in place of the acetyl group at *sn*-2 and a hexadecyl chain at sn-1. Although the free form of PAF was rapidly degraded by serum acetylhydrolase, it is uncertain whether the acetyl group of the immunogen covalently attached to the carrier protein is hydrolyzed by serum acetylhydrolase. Serum acetylhydrolase may not have access to substrate covalently attached to a protein by steric hindrance or may not hydrolyze a substrate containing a short alkyl chain at *sn*-1, since human acetylhydrolase shows lower activity with the short alkyl chain-containing PAF derivative (13). Smal *et al.* (12) showed that a

TABLE 1

Specificity of Rabbit Polyclonal Antibody to PAF

Compound	Mass required to displace 50% bound [^{3}H]PAF (pmol)	Relative cross-reactivity[a]
1-*O*-Hexadecyl-2-acetyl-*sn*-3-GPC	2.5	100
1-*O*-Hexadecyl-2-*N,N,*-dimethylcarbamoyl-*sn*-3-GPC	1.33	188
1-*O*-Octadecyl-2-acetyl-*sn*-3-GPC	3.8	65.8
1-Palmitoyl-2-acetyl-*sn*-3-GPC	40.0	6.25
3-*O*-Hexadecyl-2-acetyl-*sn*-1-GPC (enantio-PAF)	530	0.047
1-*O*-Hexadecyl-*sn*-3-GPC (lysoPAF)	>10,000	<0.025
1-Palmitoyl-*sn*-3-GPC (lysoPC)	>10,000	<0.025
1,2-Dipalmitoyl-*sn*-3-GPC (PC)	>10,000	<0.025

[a]Relative cross-reactivity is expressed as the relative potency of each compound compared to PAF in displacing 50% of bound radioactive PAF. RIA was performed as described in Materials and Methods.

TABLE 2

Recovery of Added PAF from Macrophage Suspensions[a]

PAF added pmol/mL	Amount of PAF measured by RIA pmol/mL From buffer	From macrophage suspensions	Recovery[b] %
0	0	1.71	
5	5.22	6.37	94.9
10	10.8	12.0	102
20	18.8	22.9	105
30	28.2	29.7	93.7
50	49.7	49.5	95.7

[a]Various amounts of PAF were added to 1-mL aliquots of rabbit macrophage suspension (5×10^6). Each sample was immediately mixed with 1 mL ice-cold methanol and stored at −20°C. A trace of [^{3}H]PAF (3,000 dpm) was added to each sample for calculating recoveries. After extraction and HPLC separation, as described in Materials and Methods, an aliquot (0.2 mL) was removed for determining percentage recovery, and the remainder was analyzed for PAF content by RIA as described in Materials and Methods. Each value is the mean of two samples.

[b]Recovery was determined as: Amount of PAF measured/Amount of PAF added + background PAF × 100.

large amount of PAF, 2.5-250 ng per tube (approximately 5-500 pmol) was required for the displacement of antibody-bound labelled ligand. The detection limit of PAF was about 50 times larger than that calculated by RIA using our antiserum (0.1 pmol, Fig. 2). Possible effects of structural differences in the antigen on the sensitivity of RIA are unclear. We obtained the present antibody to PAF by injection of an immunogen, in which 1-*O*-(15′-carboxypentadecyl)-2-*N,N,*-dimethylcarbamoyl-*sn*-glycero-3-phosphocholine was covalently bound to BSA. The affinity of the antibody for the dimethylcarbamoyl derivative of PAF was higher than that for PAF. In cross-reactivity studies, the amount of the PAF dimethylcarbamoyl derivative necessary for 50% inhibition was approximately half that of PAF itself (Table 1). This result suggests that the antibody strictly recognizes the substituent at the *sn*-2 position of glycerol.

PAF is produced by a variety of inflammatory cells (basophils, neutrophils, endothelial cells, monocytes). Since the majority of newly synthesized PAF remains intracellular and is not released into the extracellular environment (14), extraction of PAF from the cells is necessary. In an RIA for PAF, however, there is a problem because of insolubility of the Bligh and Dyer extracts from samples in the assay buffer, which makes it impossible to separate antibody-bound [^{3}H]PAF from free [^{3}H]PAF. In the present study, therefore, the sample extract was subjected to HPLC for removal of insoluble materials. The fraction corresponding to the authentic PAF was soluble in the assay buffer. In the present study, addition of A23187 to rabbit alveolar macrophages increased the level of immunoreactive PAF from a background level of 0.34 pmol/10^6 cells to 4.50 pmol/10^6 cells. It has been demonstrated that alveolar macrophages produce PAF upon ionophore stimulation (15,16). Arnoux *et al.* (16) detected PAF released from human, monkey and rat alveolar macrophages by bioassay in the presence of 1-5 μg/mL ionophore A23187 (2.5 pmol of PAF from 2.5×10^5 cells). This value is of the same order as that obtained in our present experiment using rabbit alveolar macrophages. Work is in progress to determine the amount of PAF in cells and tissues in various pathological states. RIA for PAF will undoubtedly prove to be a useful tool for detailed studies on the pathological role of PAF.

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Synthesis of a PAF Immunogen and Production of PAF-Specific Antibodies[1]

Mary A. Smal[a], Brian A. Baldo[a,b,*] and John W. Redmond[c]
[a]Kolling Institute of Medical Research, Royal North Shore Hospital, St. Leonards, N.S.W. 2065, [b]Department of Medicine, University of Sydney, Sydney, N.S.W. 2006 and [c]School of Chemistry, Macquarie University, North Ryde, N.S.W. 2113, Australia

Platelet activating factor (PAF), a naturally occurring phospholipid with many potent physiological and pharmacological activities, is implicated as a mediator of many diseases. An immunoassay for PAF would greatly improve quantitation, and hence PAF-specific antibodies were required. Chemically-reactive analogs of PAF, containing an aldehyde group at the end of the 1-*O*-alkyl chain (hexyl or dodecyl), were synthesized from readily available materials. During the multi-step synthetic procedure, the aldehyde group was protected as an acetal, which was converted by mild acidic hydrolysis to the aldehyde immediately prior to protein coupling. These analogs were coupled to methylated bovine serum albumin and the resultant conjugates were injected into rabbits. Antibodies to PAF were detected using a solid phase radioimmunoassay based on Protein A-Sepharose. The dodecyl PAF conjugate proved to be the more immunogenic conjugate with more than half of the rabbits producing significant levels of antibodies (at least a 10-fold increase in radioactive uptake over pre-immune levels). Results from solid phase immunoassays employing nitrocellulose discs impregnated with PAF, lysoPAF, lecithin, lysolecithin and 2-*O*-methyl-lysoPAF indicated that the antibodies recognized only PAF. PAF-specific antibodies were isolated by affinity chromatography using a column of PAF-poly(lysine) conjugated to carboxy-activated polyacrylamide. The antibodies may be employed in a sensitive and specific immunoassay for PAF and for many other studies involving PAF.
***Lipids* 26, 1130–1135 (1991).**

Platelet activating factor (PAF) is a naturally occurring phospholipid with potent biological activities, such as the promotion of hypotension, bronchoconstriction, anaphylaxis and chemotaxis (1,2). It was originally described as a substance released from rabbit basophils during IgE-antibody mediated anaphylaxis (3) and was subsequently identified as 1-*O*-alkyl-2-acetyl-*sn*-glycero-3-phosphocholine (4). Numerous cell types, especially those involved in inflammation, have been shown to produce and release PAF under appropriate stimulation (5,6).

Because of its potent inflammatory activity, PAF has been implicated in many disease states, such as asthma (7,8), allergic diseases (9), anaphylaxis (10), endotoxic shock (11) and kidney disease (12). Elucidation of the role of PAF in disease and also in normal physiological processes is dependent upon accurately quantitating PAF in biological tissues and fluids. Currently, PAF is measured by aggregation or secretion bioassays employing rabbit platelets (13,14). Although these methods are sensitive to 10^{-10} M, they are often difficult to standardize and are unreliable because other substances can induce aggregation or inhibit the response of platelets to PAF (13).

Measurement of PAF by immunoassay would have distinct advantages, such as specificity, wide applicability and high sample through-put. It would also be independent of the ambiguous relationship between occupancy of the PAF-receptor on the platelet and functional response. To develop an immunoassay, a specific antibody to PAF was needed. In this paper we report the synthesis of a PAF-immunogen and the production of PAF-specific antibodies.

MATERIALS AND METHODS

Chemicals. Solvents were reagent grade or better and were purchased from Ajax Chemicals (Sydney, Australia). Cyclododecanone and sodium hydride were from Fluka AG (Buchs, Switzerland); C_{16}- and C_{18}PAF were from Bachem Feinchemikalien (Bubendorf, Switzerland). Biogel P-100 and nitrocellulose (NC) membrane (0.45 μm) were from Bio-Rad (Richmond, CA) and Protein A-Sepharose was from Pharmacia (Uppsala, Sweden). Complete Freund's adjuvant and the Coomassie blue protein assay reagent were from Difco Laboratories (Detroit, MI) and Pierce Chemical Company (Rockford, IL), respectively. [^{125}I]Donkey anti-rabbit F(ab')$_2$ with a specific activity of 9 μCi/μg was from Amersham (Buckinghamshire, U.K.) and 1-*O*[^{125}I]alkyl-2-acetyl-*sn*-glycero-3-phosphocholine ([^{125}I]PAF) was a generous gift from Dr. J. Czarnecki, Silenus Laboratories (Melbourne, Australia). All other chemicals and biochemicals were purchased from Sigma Chemical Company (St. Louis, MO). Cord serum (CS) solution used in immunoassays was prepared by acidifying human fetal cord serum to pH 2.5 and, after 15 min, adding phosphate buffered saline (PBS) containing 0.1% Tween 20 (7 vols).

[1]Based on a paper presented at the Third International Conference on Platelet-Activating Factor and Structurally Related Alkyl Ether Lipids, Tokyo, Japan, May 1989.

*To whom correspondence should be addressed.

Abbreviations: Ac, acetyl; BSA, bovine serum albumin; C_nPAF, PAF where the 1-*O*-alkyl chain is *n* carbons long; CS, human cord serum; DMOC$_6$PAF, 1-*O*-(6',6'-dimethoxyhexyl)-2-acetyl-*sn*-glycero-3-phosphocholine; DMOC$_{12}$PAF, 1-*O*-(12',12'-dimethoxydodecyl)-2-acetyl-*sn*-glycero-3-phosphocholine; Ig, immunoglobulin; [^{125}I]PAF, 1-*O*-[^{125}I]alkyl-2-acetyl-*sn*-glycero-3-phosphocholine; lysoPAF, 1-*O*-alkyl-*sn*-glycero-3-phosphocholine; MBSA, methylated bovine serum albumin; 2-*O*-methyl-lysoPAF, 1-*O*-octadecyl-2-*O*-methyl-*sn*-glycero-3-phosphocholine; NC, nitrocellulose; PA, polyacrylamide; PAF, platelet activating factor, 1-*O*-alkyl-2-acetyl-*sn*-glycero-3-phosphocholine; PAF-PL-PA, PAF-poly(lysine)-polyacrylamide; PBS, phosphate (50 mM) buffered saline pH 7.2; PL-PA, poly(lysine)-PA.

Synthesis of 1-O-(ω,ω-dimethoxyalkyl)-2-acetyl-sn-glycero-3-phosphocholines. *(R)*-1-(Benzyloxy)-2, 3-epoxypropane was prepared from (+)1,2-*O*-isopropylidene-*sn*-glycerol as described by Hirth and Barner (15). Choline *p*-toluenesulfonate was prepared from dimethylethanolamine and methyl *p*-toluenesulfonate, as previously reported (16). 6,6-Dimethoxy-hexan-1-ol and 12,12-dimethoxydodecan-1-ol, prepared from cyclohexanone and cyclododecanone, respectively, by the method described by Schmidt and Grafen (17), were condensed with the epoxypropane in the presence of sodium hydride; the product was acetylated, hydrogenolyzed and phosphorylated as recently described by Smal *et al.* (18) to produce 1-*O*-(6′,6′-dimethoxyhexyl)-2-acetyl-*sn*-glycero-3-phosphocholine ($DMOC_6PAF$) and 1-*O*-(12′,12′-dimethoxydodecyl)-2-acetyl-*sn*-glycero-3-phosphocholine ($DMOC_{12}PAF$) in 27% and 14% overall yields, respectively.

Deprotection and coupling to protein. The dimethoxy PAF analogs were deprotected by acidic hydrolysis to yield the free aldehydes, as described (18). Briefly, the PAF analog (130 mg) was suspended in ethyl acetate and trifluoroacetic acid/water (9:1,v/v) (170 μL) was added. The reaction was kept at room temperature until complete, and the pH was carefully adjusted to 5–6 with 1 M $NaHCO_3$. The mixture was evaporated and the residue redissolved in methanol/water (2:3, v/v). A Purpald spot test (19) indicated the presence of free aldehyde. The aldehyde-PAF analogs were coupled to either methylated bovine serum albumin (MBSA) or poly(Lys) (mol. wt 90,000) by the following method. The analogs (25–50 mg) in methanol/water were added to a methanolic solution of the protein (300 mg) and, after 0.5 hr, $NaCNBH_3$ (100 mg) was added. The pH of the solution was adjusted to 5 with 1N HCl. After 16 hr, the solution was evaporated and the residue dispersed in water. The suspension was dialyzed against water for 6 hr, and then lyophilized. The resultant material was analyzed for phosphorus (20).

Platelet aggregation. Washed rabbit platelets were prepared as described by Coeffier *et al.* (21) and platelet aggregation carried out as reported by Huo *et al.* (22). Ethanolic stock solutions of the dimethoxy-PAF analogs were diluted in PBS containing 0.25% bovine serum albumin (BSA), and these solutions (20 μL) were added to the platelet suspensions (18). Changes in optical density were monitored with a Payton dual-channel aggregometer.

Antibody production. Six-month-old New Zealand white rabbits were injected intramuscularly with either C_6PAF or $C_{12}PAF$ conjugated to MBSA (5–10 mg, 0.5-1.5 μmol PAF), emulsified with saline (0.5 mL) and complete Freund's adjuvant (0.5 mL) (18). The rabbits were boosted three weeks later with a subcutaneous injection of the same dose of conjugate in saline and were maintained on this alternating injection schedule. The rabbits were bled seven days after the boosts and the blood was allowed to clot. The serum was removed and stored at −20°C.

Determination of anti-PAF antibody titer. Serum was diluted 1 in 10 in 0.1% Tween/PBS, and 25 μL was incubated with 9 μL of swollen Protein A-Sepharose beads. After 3 hr, the beads were washed once with 0.05% Tween/PBS (3 mL), and then incubated with 40,000 cpm of [^{125}I]PAF (diluted in 0.1% Tween/PBS)(50 μL). After 16 hr, the beads were washed twice and counted. Results are expressed as a percentage of the total counts added.

Preparation of PAF-NC discs. Five-mm discs were cut from NC membrane and soaked in a solution of $C_{18}PAF$ (5 mg) in 70% ethanol (0.5 mL) plus 0.07% Tween/PBS (1.5 mL) for 16 hr. The discs were removed and blotted dry. Discs impregnated with 1-*O*-alkyl-*sn*-glycero-3-phosphocholine (lysoPAF), lecithin, lysolecithin, 1-0-octadecyl-2-*0*-methyl-*sn*-glycero-3-phosphocholine (2-*0*-methyl-lysoPAF) and 0.07% Tween alone were prepared in a similar manner, as described previously (18).

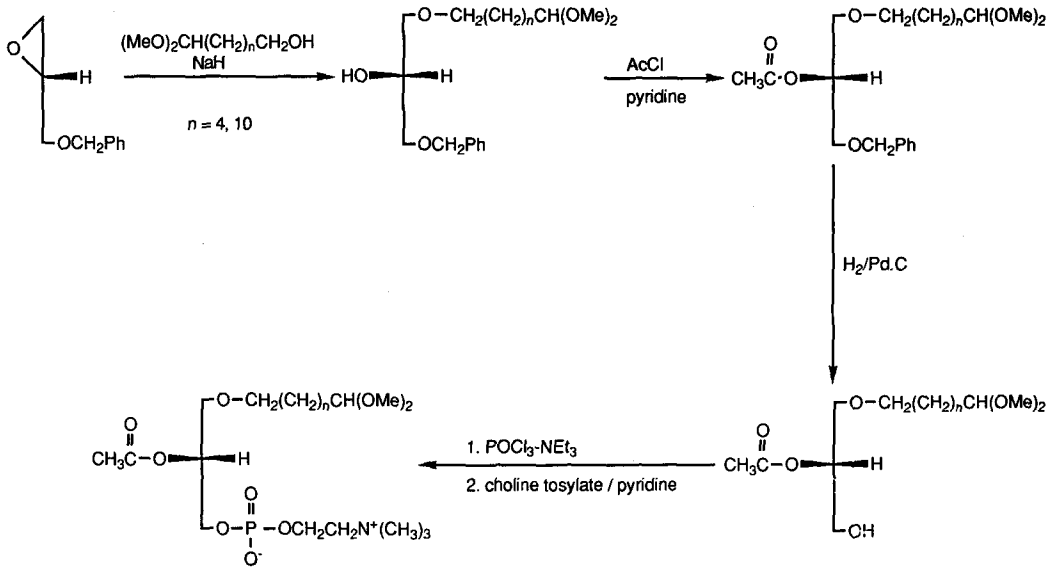
Preparation of PAF-poly(Lys)-polyacrylamide (PAF-PL-PA) and PAF-AcPL-PA. Plastic labware was used for all operations involving polyacrylamide (PA) beads. Succinylhydrazide PA was prepared from Bio-gel P100 (4 g) as previously reported (23), and $C_{12}PAF$-poly(Lys) (190 mg, approx 30 μmol PAF) was coupled to the beads in the presence of 1-ethyl-3(3-dimethylaminopropyl)carbodiimide (3.47 g) at pH 5, as described previously (18). The beads were washed extensively with water, saline, PBS and 0.1 M acetate buffer, pH 4.0. The beads (60 mL bed volume) were resuspended in water to a total volume of 120 mL. For the acetylation procedure, the bead suspension (6 mL) was mixed with PBS (5× concentrate) (6 mL) and cooled to 0°C. While gently stirring, acetic anhydride (250 μL) was added, followed by 2.5 M NaOH to maintain the pH at 6–7. The addition of acetic anhydride and NaOH was repeated, and then the beads were washed and resuspended in water to a total volume of 6 mL. For the immunoassays, the bead suspension was diluted with two parts of water.

Affinity purification of anti-PAF antibodies. The immunoglobulin (Ig) fraction of antiserum was isolated by chromatography on Protein A-Sepharose as previously reported (24), except that 0.1 M citrate (pH 3.0) and 0.1 M glycine (pH 3.0) were used for elution. The Ig fraction (4 mg protein) was loaded onto a 20-mL column of PAF-PL-PA beads equilibrated in 0.1% Tween/saline. After the non-binding material had been eluted, the elution buffer was changed to 4.5 M $MgCl_2$ in saline containing 0.1% Tween to elute the PAF-binding antibodies. The desired fractions were pooled and concentrated; protein concentrations were determined by the Bradford method (25).

Binding of anti-PAF antibodies to PAF-solid phases. Rabbit serum or purified Ig was diluted in 0.1% Tween/PBS or CS solution, respectively, and 50 μL of these solutions were incubated with a PAF-NC disc (or control disc) for 3 hr. The disc was washed three times with 0.05% Tween/PBS and then incubated with [^{125}I]donkey anti-rabbit $F(ab')_2$ (diluted in 0.005% Tween/PBS)(50 μL). After 16 hr, the disc was washed again and counted. In binding studies with PA beads, the NC disc was replaced by 50 μL of bead suspension, and the tracer was diluted in 0.1% BSA/PBS.

RESULTS

Synthesis and biological activity of protected PAF analogs. Protected PAF analogs with either a 6- or a 12-carbon chain in position 1 were prepared in moderate yields (14–27%) following the sequence of reactions



SCHEME 1. Synthesis of protected PAF analogs.

shown in Scheme 1. It was important that acidic conditions be avoided during these steps in order to preserve the acetal protecting group. The ability of these analogs to induce aggregation was tested with washed rabbit platelets (Fig. 1). Protected C_6PAF did not induce aggregation at a final concentration of 8.3 μM, whereas the protected C_{12}PAF had approximately 1% of the activity of C_{16}PAF.

Coupling of the PAF analogs to protein. Conversion of the dimethyl acetal protecting group to the free aldehyde was achieved with trifluoroacetic acid, and the PAF analog was condensed with the protein, as outlined in Scheme 2. The deprotection reaction was relatively free of by-products, and spectral analysis, as well as a Purpald test of the product, showed conversion to an aldehyde. The acetyl group was preserved during this procedure. Results of the phosphorus assay for the PAF-protein conjugates indicated a 50-70% coupling efficiency.

Antibody production. The anti-PAF antibody titer of rabbit sera was checked by the Protein A-Sepharose-based assay, and the production of antibodies over an extended time period in five rabbits is summarized in Figure 2. Of the three rabbits immunized with the C_6PAF-MBSA conjugate, only one produced significant levels of antibody. However, five out of the eight rabbits immunized with the C_{12} analog produced antibodies to PAF.

Specificity of antibodies. The specificity of antibodies was tested using NC discs to which selected analogs of PAF had been adsorbed. Both whole antiserum and the Ig fraction obtained by Protein A-Sepharose chromatography were tested (Table 1). The only significant binding occurred when PAF discs were used with antiserum or the corresponding Ig fraction. The antibodies did not react with the lysoPAF-, lecithin-, lysolecithin- or 2-*O*-methyl-lysoPAF-NC discs.

Affinity purification of anti-PAF antibodies. The PAF-poly(Lys) conjugate was immobilized onto PA beads *via* a spacer arm of succinylhydrazide. Polyacrylamide

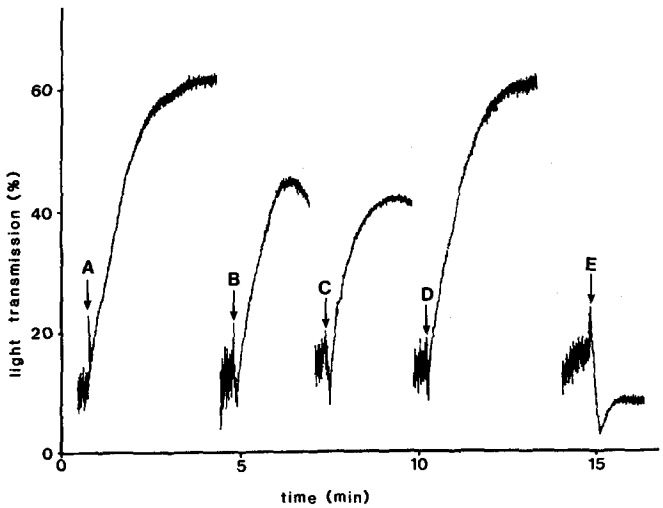
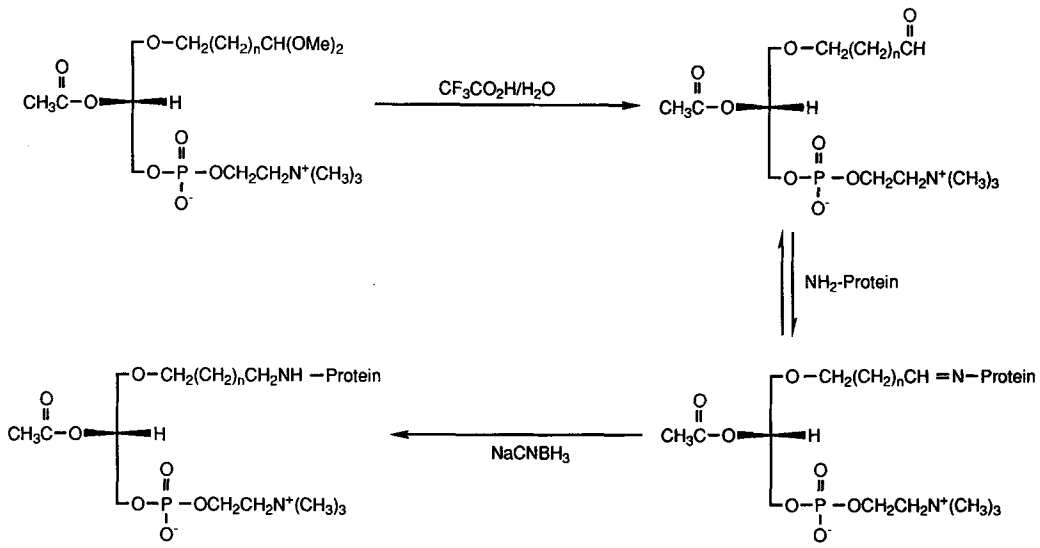


FIG. 1. Response of washed rabbit platelets to PAF and the dimethoxy PAF analogs: A, C_{16}PAF 4 nM (final concentration); B, C_{16}PAF 0.4 nM; C, DMOC_{12}PAF 30 nM; D, DMOC_{12}PAF 300 nM; and E, DMOC_6PAF 12.6 μ M.

was chosen because it gave the lowest non-specific binding of the commonly available chromatography media. Whole antisera was initially fractionated on Protein A-Sepharose to obtain the Ig fraction and remove the enzyme PAF-acetylhydrolase, which converts PAF to lysoPAF (26). When the Ig fraction was applied to a PAF-PL-PA column and the non-binding material eluted, a sharp peak representing the PAF-binding Igs was recovered with 4.5 M $MgCl_2$ (Fig. 3). Affinity purified Igs were compared with the corresponding whole Ig fraction and normal Ig for binding to various PAF solid phases (Fig. 4). By the NC assay, affinity purification did not enhance binding, but there was a slight increase in specificity, as seen by a lower binding to lecithin-NC. Affinity purifica-



SCHEME 2. Hydrolysis of protected PAF analogs and coupling to protein.

TABLE 1

Specificity of Binding of Anti-PAF Antibodies to PAF and Analogs [a]

NC disc adsorbed with:	Radioactive uptake (%) of [^{125}I]second antibody with: Normal serum [b]	Immune serum [c]	Normal Ig [d]	Immune Ig [e]
PAF	1.1	13.1	2.2	15.7
LysoPAF	4.0	3.7	1.0	1.0
Tween	1.3	2.7	—	—
Lecithin	—	—	0.5	0.9
Lysolecithin	—	—	0.9	1.1
2-*O*-Methyl-lysoPAF	—	—	0.7	1.1

[a]Diluted serum or Ig (50 μL) was incubated with a PAF or other NC disc for 3 hr. The disc was washed, incubated with [^{125}I]donkey anti-rabbit F(ab')$_2$(40,000 cpm) for 16 hr and washed again. The disc was counted to determine the bound radioactivity (% of total activity).

[b]Serum from unimmunized rabbit, diluted 1:1000 in 0.1% Tween/PBS.

[c]Serum from rabbit 7 immunized with C_6PAF-MBSA, diluted 1:1000 in 0.1% Tween/PBS.

[d]Ig fraction from normal serum, diluted to 0.23 μg Ig/mL in CS solution.

[e]Ig fraction from immune serum, diluted to 0.21 μg Ig/mL in CS solution.

tion markedly enhanced binding to PAF-PL-PA beads. However, binding to the control support, poly(lysine)-PA (PL-PA), was also significant—this binding could be abolished by acetylation of the lysine residues on the PL-PA beads without affecting the PAF-specific binding. Affinity purification was attempted with PAF-AcPL-PA, but did not yield any substantial improvement.

DISCUSSION

Since PAF has a molecular weight of only approximately 500, it is too small a molecule to elicit an immune response and must, therefore, be coupled to a high molecular weight "carrier". It was reported that antibodies to PAF were produced using an incompletely defined antigen complex of PAF, lecithin and cholesterol; however, these antibodies also recognized lysoPAF (27). In the only other report of anti-PAF antibodies, the immunogen used was a PAF analog, containing a *N,N*-dimethylcarbamate group and not an acetyl group at carbon 2, and was coupled to BSA through the end of the alkyl chain (28). These antibodies were also cross-reactive with lysoPAF.

In the present study, the acetyl group at carbon 2 was deliberately retained since it is a unique and essential feature of the PAF molecule, and PAF-specific antibodies would have to recognize this group. The most suitable site for coupling the PAF molecule to a protein is the end of the alkyl chain, and an aldehyde group was chosen as the functional group through which coupling

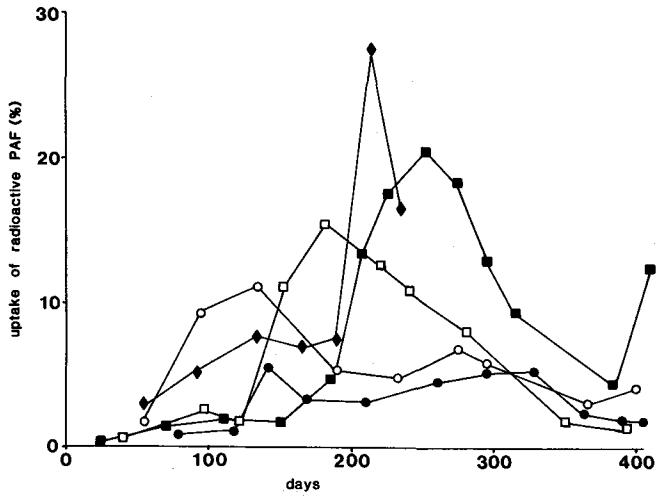


FIG. 2. Production of anti-PAF antibodies in rabbits over time. Rabbit 7 (●) was immunized with C_6PAF-MBSA, and rabbits 9 (■), 11 (○), 12(♦) and 14 (□) were immunized with C_{12}PAF-MBSA. Titer was determined by incubating diluted serum (1:10 in 0.1% Tween/PBS) with Protein A-Sepharose beads for 3 hr. The washed beads were incubated with [^{125}I]PAF for 16 hr, and then washed. The beads were counted to determine the bound radioactivity (% of total activity).

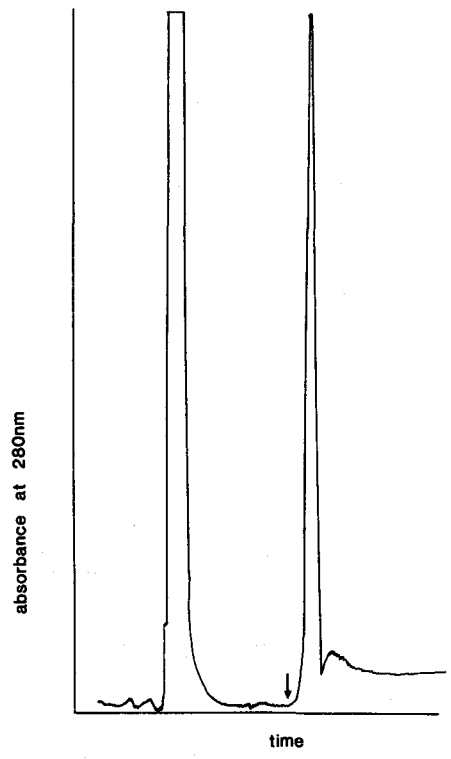


FIG. 3. Elution profile for the purification of anti-PAF antibodies by affinity chromatography. The Protein A purified Igs were applied to a PAF-PL-PA column and, after the non-binding Igs were eluted, elution of the anti-PAF antibodies was commenced with 4.5 M $MgCl_2$ (indicated by the arrow)(from ref. 18).

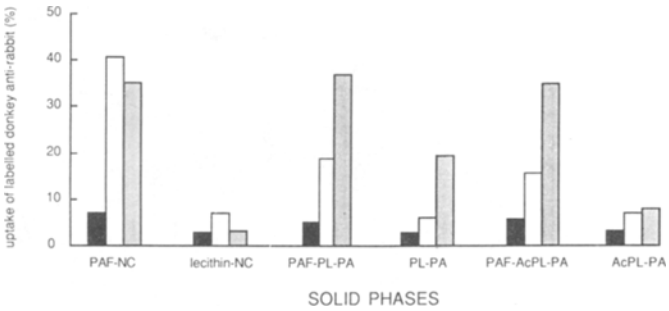


FIG. 4. The binding of Ig from unimmunized rabbits (solid bars), immune Ig from rabbit 7 (open bars) and affinity-purified immune Ig (hatched bars) to PAF- and lecithin-NC discs, and PAF-PL-PA, PL-PA, PAF-AcPL-PA and AcPL-PA beads. Binding was determined by incubating Ig (5 μg/mL diluted in CS solution; 50 μL) with either discs or bead suspension for 3 hr. These were then washed, incubated with [^{125}I] donkey anti-rabbit F(ab')$_2$(40,000 cpm) for 16 hr, washed again, and counted. The bound radioactivity is expressed as % of the total activity (data from ref. 18).

to the amino residues of the protein carrier could occur. In order to synthesize such analogs, the aldehyde group had to be protected as a dimethyl acetal during the many steps of the synthetic procedure. Two aldehyde analogs of PAF, with alkyl chain lengths of 6 and 12 carbons, were synthesized and coupled with 50–75% efficiency to MBSA. The protected C_6PAF had no activity towards platelets, but the protected C_{12}PAF had a reduced activity towards platelets as compared with C_{16}PAF. Antibodies were raised in rabbits immunized with either the C_6PAF-MBSA or C_{12}PAF-MBSA conjugates, although the latter appeared to be more immunogenic. Attempts were made to raise murine monoclonal antibodies with these antigens, but no response was observed. The NC-disc assay showed that the antibodies did not recognize lysoPAF, suggesting that the PAF immunogens were not hydrolyzed *in vivo*. The antibodies also did not recognize 2-*O*-methyl-lysoPAF nor lecithin, indicating that the antibody combining site recognizes, at least in part, a short chain acyl group at carbon 2 of the glycerol backbone. The specificity of these antibodies recently has been fully characterized (29).

The aldehyde analogs of PAF were also used to prepare a stable PAF-affinity column PAF-PL-PA for the purification of anti-PAF antibodies. A PAF-affinity column had been previously described in which PAF was bound non-covalently to albumin-Sepharose; however, under these conditions, PAF was slowly lost from the column (30). Anti-PAF antibodies, purified on the PAF-PL-PA column, showed increased binding to PAF-PL-PA beads, but no significant change in the binding to PAF-NC discs. There was also significant binding of the antibodies to poly(Lys) bound to PA beads, indicating a population of antibodies directed against lysine or PAF-lysine. This is not surprising, because the immunogen contained lysine residues, some of which were bound to PAF. Binding to lysine could be abolished by *N*-acetylation of the poly-(Lys)-PA beads.

The antibodies produced in this study are specific for PAF and the methodology outlined here has been ap-

plied to the development of a sensitive and specific radioimmunoassay for PAF (31). The antibodies have also been employed in localization studies using immunocytochemical methods (32), and have potential application in studies on the PAF receptor and various *in vitro* and *in vivo* investigations of the pharmacological, physiological and biochemical effects of PAF. The PAF-affinity column is also likely to have applications for purification of the PAF-receptor and other PAF-binding proteins.

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A Specific, Sensitive and High-Capacity Immunoassay for PAF[1]

Brian A. Baldo[a,b,*], Mary A. Smal[a] and Alistair C. McCaskill[c]

[a]Kolling Institute of Medical Research, Royal North Shore Hospital of Sydney, St. Leonards, N.S.W., 2065, [b]Department of Medicine, University of Sydney, Sydney, 2006 and [c]Silenus Laboratories, Victoria, 3122, Australia

A specific radioimmunoassay for platelet-activating factor (PAF) sensitive in the range 10–1000 pg (0.02–2 pmoles) has been developed. Detailed quantitative hapten inhibition studies showed specificity for the acetyl group at C-2 of PAF, a requirement for the ether linkage at C-1 and some tolerance for substituents on the choline nitrogen. No significant cross-reactivity was found with phosphatidylcholine and lysophosphatidylcholine or with lysoPAF.

***Lipids* 26, 1136–1139 (1991).**

The involvement and importance of platelet-activating factor (PAF) in a variety of normal and disease states make its quantitation in biological fluids an important priority. Because of its potency, detection and measurement of PAF must take place at very low concentrations. Currently, this is usually achieved by employing assays based on aggregation of human or rabbit platelets. Although these methods are sensitive, they are not usually simple or straightforward and have the drawback of the inherent variability associated with biological assays.

Application of the radioimmunoassay, which we have developed, to the measurement of PAF in some biological fluids and comparisons with results obtained by platelet aggregation has revealed that the latter method is frequently affected by the presence of inhibitors of PAF-induced aggregation. The net effect of this inhibition is that levels of PAF determined by platelet aggregation are frequently underestimated. The immunoassay is not subject to this serious drawback. The accurate determination of PAF levels in fluids from subjects with asthma, shock and allergic reactions, and a variety of other conditions, may increase our understanding of the role of PAF in these conditions.

Accurate measurement of very small amounts of PAF in normal and pathological fluids and extracts is a prerequisite for studying and understanding the role of PAF in health and disease. At present, the methods most commonly used for the quantitation of PAF rely on the interaction of PAF with platelets (1). These methods are not strictly quantitative, lack specificity, are difficult to standardize and reproduce, and do not have a high capacity. Mass spectrometric techniques (2–4) are sensitive and specific, but the cost and specialized nature of the equipment required, and the difficulty of rapidly assessing large numbers of samples side-by-side, preclude the widespread and routine use of this technique.

Introduction of a simple, specific, sensitive and high-capacity immunoassay for PAF has overcome these problems associated with the measurement of PAF, and it is now possible to routinely and accurately quantitate PAF in large numbers of samples.

MATERIALS AND METHODS

Materials. C_{16}, C_{18} and $C_{18:1}$PAF were from Bachem Feinchemikalien, Bubendorf, Switzerland. All other lipids were from Sigma Chemical Co., St. Louis, MO. [^{125}I]PAF and donkey anti-sheep immunoglobulin (Ig) fractions were supplied by Silenus Laboratories, Hawthorn, Victoria, Australia. Chloroform and methanol were supplied by Ajax Chemicals (Sydney, Australia) and polyethylene glycol MW 6000 by BDH Chemicals (Kilsyth, Victoria, Australia).

Antigen and antisera. C_{12}PAF-methylated bovine serum albumin conjugates were prepared as previously described (5) and were emulsified in Freund's complete adjuvant and injected intramuscularly into sheep (1 mg/sheep). Animals were boosted 1 mon and 4 mon later with 1-mg doses of the antigen in Freund's incomplete adjuvant before being bled eleven days after the third injection.

PAF-acetylhydrolase activity in the antiserum was destroyed by incubating the serum with an equal volume of 1 M acetic acid for 6 hr before diluting with 0.2 M phosphate buffer pH 7.2.

Radioimmunoassay (RIA). Assays were carried out in disposable polystyrene tubes (Disposable Products, Adelaide, Australia). To each tube was added 100 μL of the sample to be tested or the PAF standard solution (equal parts C_{16}PAF and C_{18}PAF in aqueous ethanol), 100 μL of diluted anti-PAF (1–8,000 in 0.05 M sodium acetate buffer pH 6 containing 0.1% Tween 20 and 0.1% sodium azide), 100 μL anti-sheep Ig (1–250 in the above buffer containing 6% glycol) and [^{125}I]PAF (40,000 cpm per tube) and tubes were incubated at room temperature for 16 hr. After the addition of 4 mL of acetate buffer, the tubes were centrifuged at 1900 × *g* for 25 min, decanted and the remaining radioactivity measured. The percent of tracer bound to the precipitate in the tubes (% B/B_0) was calculated from the formula $(B\text{-}NSB)/(B_0\text{—}NSB) \times 100$ where B_0 represents the counts from tubes containing no PAF and NSB the non-specific binding tubes which contain only the second antibody, tracer and buffer. PAF in the test samples was determined from the standard curve obtained by plotting PAF concentrations against $\%B/B_0$.

[1]Based on a paper presented at the Third International Conference on Platelet-Activating Factor and Structurally Related Alkyl Ether Lipids, Tokyo, Japan, May 1989.

*To whom correspondence should be addressed.

Abbreviations: Acetyl-C_{18}GPDME, 1-*O*-octadecyl-2-acetyl-*sn*-glycero-3-phospho-*N,N*-dimethylethanolamine; acetyl-C_{18}GPE, 1-*O*-octadecyl-2-acetyl-*sn*-glycero-3-phosphoethanolamine; acetyl-C_{16}-lysoPAF, 1-*O*-hexadecanoyl-2-acetyl-*sn*-glycero-3-phosphocholine; butyryl-C_{18}lysoPAF, 1-*O*-octadecyl-2-butyryl-*sn*-glycero-3-phosphocholine; C_{16}PAF, 1-*O*-hexadecyl-2-acetyl-*sn*-glycero-3-phosphocholine; (±)C_{16}PAF, racemic C_{16}PAF; C_{18}PAF, 1-*O*-octadecyl-2-acetyl-*sn*-glycero-3-phosphocholine; $C_{18:1}$PAF, 1-*O*-(*cis*-octadec-9′-enyl)-2-acetyl-*sn*-glycero-3-phosphocholine; DMOC_{12}PAF, 1-*O*-(12′, 12′-dimethoxydodecyl)-2-acetyl-*sn*-glycero-3-phosphocholine; Ig, immunoglobulin; [^{125}I]PAF, 1-*O*-[^{125}I]alkyl-2-acetyl-*sn*-glycero-3-phosphocholine; lysoPAF, 1-*O*-alkyl-*sn*-glycero-3-phosphocholine; 2-*O*-methyl-C_{18}lysoPAF, 1-*O*-octadecyl-2-*O*-methyl-*sn*-glycero-3-phosphocholine; PAF, platelet-activating factor; propionyl-C_{18}lysoPAF, 1-*O*-octadecyl-2-propionyl-*sn*-glycero-3-phosphocholine.

RESULTS AND DISCUSSION

Procedure, sensitivity and specificity. A diagrammatic representation of the RIA procedure is summarized in Figure 1 and a resulting typical standard curve is shown in Figure 2. Since PAF in most biological samples is a mixture of alkyl homologs with C_{16}PAF and C_{18}PAF predominating, the standard chosen at present for the RIA is an equimolar mixture of the two.

The standard curve permits accurate quantitation of PAF in the range of 10–1000 pg (0.02–2 pmol) per tube.

Quantitative hapten inhibition studies with selected PAF analogs revealed that C_{16}PAF and $C_{18:1}$PAF were better inhibitors than C_{18}PAF, 1-acyl analogs were inactive and 2-propionyl-C_{18}lysoPAF and 2-butyryl-C_{18}lysoPAF were weak inhibitors. These, and other results summarized in Table 1, led us to conclude that the best inhibitors have an acetyl group at position 2 and an ether linkage at position 1 of the glycerol backbone, whereas variations at position 3 are less critical. The latter conclusion is consistent with the finding that acetyl-C_{18} GPDME was a good inhibitor and some activity was retained by acetyl-C_{18} GPE despite the absence of the three methyls of the PAF choline group. Importantly, lysoPAF was without inhibitory activity at concentrations of 100 ng/mL and was very weakly inhibitory in the μg/mL range. No inhibition was seen with the commonly occurring lipids cholesterol, cholesteryl oleate, lysophosphatidylcholine, lysophosphatidylethanolamine, oleic acid, phosphatidylcholine, phosphatidylethanolamine, phosphatidylinositol, phosphatidylserine, sphingomyelin and triolein at concentrations up to 20 μg/mL.

Application of the RIA to the measurement of PAF in biological fluids. The RIA has so far been used to determine PAF levels in human saliva (6), sputum, serum (7) and in supernatants from embryo cultures (8) and antigen-challenged human lung (Baldo, B.A., Smal, M.A., and

TABLE 1

Inhibition of Sheep Anti-PAF Antibodies by C_{16}PAF and Some PAF Analogs[a]

Compound	Substituent at C-1, C-2, C-3	Amount (pmoles) for 50% inhibition	Reactivity relative to C_{16}PAF
	C-1		
C_{16}PAF	$-O-(CH_2)_{15}CH_3$	0.39	1.0
C_{18}PAF	$-O-(CH_2)_{17}CH_3$	0.72	1.85
$C_{18:1}$	$-O-(CH_2)_8-\underset{H}{C}=\underset{H}{C}-(CH_2)_7CH_3$	0.48	1.23
Acetyl-C_{16}lysoPAF	$-O-\overset{O}{\overset{\|}{C}}-(CH_2)_{14}CH_3$	>100	>260
	C-2		
(±) C_{16}PAF	$CH_3\overset{O}{\overset{\|}{C}}-O-$	0.84	2.15
Propionyl-C_{18}lysoPAF	$CH_3CH_2\overset{O}{\overset{\|}{C}}-O-$	1.7	4.36
Butyryl-C_{18}lysoPAF	$CH_3CH_2CH_2\overset{O}{\overset{\|}{C}}-O-$	>100	>260
2-*O*-Methyl-C_{18}lysoPAF	CH_3O-	>100	>260
	C-3		
Acetyl-C_{18}GPDME	$-O-\overset{O}{\overset{\|}{P}}(-O)-O-CH_2CH_2\overset{+}{N}HME_2$	2.3	5.9
Acetyl-C_{18}GPE	$-O-\overset{O}{\overset{\|}{P}}(-O)-O-CH_2CH_2\overset{+}{N}H_3$	21	53.8

[a]LysoPAF produced 50% inhibition at a concentration of 13,300 pmoles, a reactivity of 34,000 relative to C_{16}PAF.

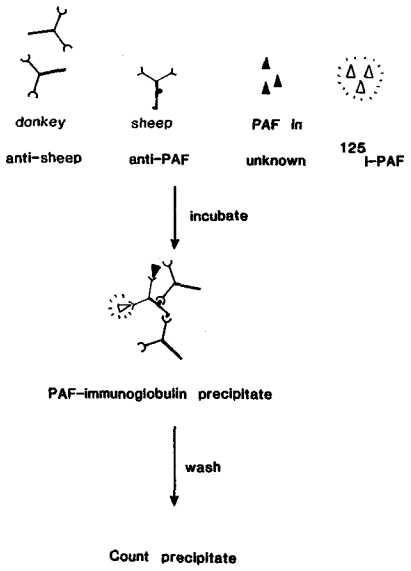


FIG. 1. Diagrammatic representation of the RIA for PAF.

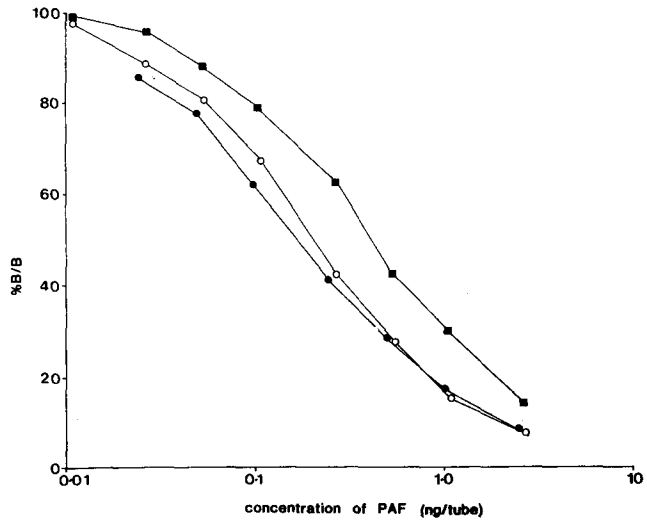


FIG. 2. Typical standard curves obtained with the RIA for PAF. (○) C_{16}PAF; (●) an equimolar mixture of C_{16}PAF and C_{18}PAF; (■) C_{18}PAF.

Cooney, S.J., unpublished). A research priority is now to establish "normal" levels of PAF in body fluids and tissues before employing the assay for the study of various disease states. As with saliva (6) and serum, it is anticipated that extraction of tissues and fluids with chloroform/methanol/water (9) will be a necessary step in preparing samples for examination by RIA.

Comparison of assays for the quantitation of PAF. A comparison of PAF levels detected in human salivas by RIA and platelet aggregation showed that figures obtained by the aggregation assay are much lower (6,10). Apart from the fact that losses are often likely to be greater during the sample preparation for platelet aggregation methods, interfering substances are clearly a problem with platelet-based assays (11–14). The presence of a substance(s) in saliva that inhibits PAF-induced platelet aggregation (14) but which does not affect the RIA highlights an important advantage of the latter method.

TABLE 2

Comparison of the Available Assays for the Routine Measurement[a] of PAF

Assay	Principle	Working range (pg/tube)	Potential problems
Radioimmunoassay	Interaction of PAF with specific antibody	10–1000	
Platelet aggregation (1)	Interaction of PAF with platelets	10–1000	—Preparation of platelet suspension —Interfering substances —Difficult to standardize —Low capacity; 1 sample at a time
Platelet degranulation (1)	Interaction of PAF with platelets and release of [^{3}H] serotonin	50–300	—As above —Labelling of platelets
Radioreceptor assay (15,16)	Binding of PAF to membrane receptor(s)	10–1000	—Preparation of membranes —Standardization of membrane batches —High non-specific binding

[a]Mass spectrometric methods are not considered because of the cost and specialized nature of the equipment required, lack of access by many medical laboratories and, in particular, the low rate of through-put of samples.

Table 2 lists the methods most commonly used to measure PAF and summarizes what we see as the main problems and disadvantages of the methods when compared to the newly developed RIA described here.

Application of the RIA should, in the near future, lead to a clearer understanding of the role of PAF in health and disease.

ACKNOWLEDGMENTS

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Quantitation by Radioimmunoassay of PAF in Human Saliva[1]

Sue J. Cooney[a], Mary A. Smal[a] and Brian A. Baldo[a,b,*]
[a]Kolling Institute of Medical Research, Royal North Shore Hospital, St. Leonards, N.S.W. 2065 and [b]Department of Medicine, University of Sydney, N.S.W. 2006, Australia

Approximately 75% of the PAF present in saliva is recovered on extraction of whole saliva (0.8 vol) with chloroform/methanol/water (2:2:1, v/v/v). PAF levels, determined by our recently developed radioimmunoassay, in saliva extracts ranged from 0.5–21 ng/mL with 59% between 2–6 ng/mL. These figures, for apparently healthy subjects, are higher than previously reported levels obtained by platelet assays. The validity of our radioimmunoassay results was checked by isolating and quantitating the PAF fraction from whole saliva. In addition, when we examined our saliva samples by platelet aggregation, low levels of PAF, comparable with the values found in the literature, were detected. Investigations revealed the presence of a substance(s) which inhibited PAF-induced platelet aggregation but which did not affect the radioimmunoassay.
Lipids 26, 1140–1143 (1991).

The biologically active phospholipid platelet activating factor (PAF) (1-*O*-alkyl-2-acetyl-*sn*-glycero-3-phosphocholine) (1) is synthesized and released by various cell types upon stimulation (2). The variety of physiological effects exhibited by PAF (3) suggests that it may be involved in a number of diseases including asthma (4), anaphylactic shock (5), gastric ulceration (6) and other allergic reactions (7).

Determination of the significance of PAF in diseased states depends, to a large degree, on the precise quantitation of PAF in biological systems. Using techniques which rely on the interaction of mediator with platelets, Cox *et al.* (8) and Janero *et al.* (9) demonstrated the presence of PAF in human saliva. However, these assays are not simple to perform and would be unsuitable for the measurement of PAF in the large numbers of samples required to determine "normal" levels and hence demonstrate deviations from normal due to any particular disease.

Recently, a specific radioimmunoassay (RIA) for PAF has been developed and extensively characterized (10,11). In this paper, we present results which validate the use of the PAF RIA for the routine measurement of PAF in saliva.

MATERIALS AND METHODS

Materials. 1-*O*-Hexadecyl-2-acetyl-*sn*-glycero-3-phosphocholine (C_{16}PAF) and 1-*O*-octadecyl-2-acetyl-*sn*-glycero-3-phosphocholine (C_{18}PAF) were purchased from Bachem Feinchemikalien (Bubendorf, Switzerland). Anti-PAF serum, donkey anti-sheep immunoglobulin (Ig) and [^{125}I]-PAF were from Silenus Laboratories Pty. Ltd. (Melbourne, Australia). [^{3}H-*alkyl*]PAF (1-*O*-[^{3}H]alkyl-2-acetyl-*sn*-glycero-3-phosphocholine, a mixture of C_{16} and C_{18} alkyl ethers, 81 Ci/mmol) was from Amersham (Buckinghamshire, U.K.) and [^{3}H-*acetyl*]PAF (1-*O*-alkyl-2-[^{3}H]acetyl-*sn*-glycero-3-phosphocholine, 10 Ci/mmol) was from DuPont NEN (Boston, MA). Polyethylene glycol MW 6000 and organic solvents were from BDH Chemicals (Kilsyth, Vic, Australia) and Ajax Chemicals (Sydney, Australia), respectively. Phospholipase A_2 attached to agarose beads was from the Sigma Chemical Company (St. Louis, MO) and silica gel 60 was purchased from E. Merck (Darmstadt, Germany). All other chemicals and reagents were from Sigma.

Saliva samples. Mixed, unstimulated human saliva samples were collected from healthy volunteers and stored at 4°C for up to 30 min. All salivas were extracted by the method of Bligh and Dyer (12) and reconstituted in the appropriate assay buffer.

PAF RIA procedure. Samples to be tested for PAF content were reconstituted in 0.05 M acetate buffer pH 6.0 containing 0.05% Tween 20 (AcT). Anti-PAF serum was diluted 1/100 in AcT buffer. The second antibody, anti-sheep Ig, was diluted 1/250 in AcT buffer containing 6% polyethylene glycol and [^{125}I]PAF was added to give 40,000 cpm per 100 μL. In the assay, each tube contained 100 μL sample to be tested, 100 μL anti-PAF serum and 100 μL anti-sheep Ig/tracer. The B_0 tubes (zero binding) contained no sample and the NSB (non-specific binding) tubes contained only anti-sheep/tracer. After the tubes were incubated for 16 hr at room temperature, 4 mL of AcT was added and the tubes centrifuged at 1900 × *g* for 25 min. Supernatants were removed and the radioactivity remaining was determined. The percent of tracer bound to the precipitate (%B/B_0) was calculated from the formula (B-NSB)/(B_0-NSB) × 100. The amount of PAF present was interpolated from a standard curve constructed using serial dilutions of a 1:1 C_{16}/C_{18}PAF stock solution (0.1 mg/mL).

Platelet aggregation. Washed rabbit platelets were prepared as previously described (13) and aliquots (50 μL) of the platelet suspension were preincubated with Tyrodes-Ca^{2+} (450 μL) (14) before stimulation. Samples to be tested were reconstituted in phosphate buffered saline (PBS) pH 7.2 containing 0.25% BSA and these solutions (20 μL) were added to the platelet suspensions. Changes in the optical transmission were monitored by a Payton dual-channel aggregometer. For quantitation of PAF a standard curve was constructed using serial dilutions of 0.1 mg/mL C_{16}/C_{18}PAF (1:1).

Phospholipase A_2 treatment. Samples were reconstituted in 10 mM Tris buffer (pH 8.0) containing 0.1% BSA. Each incubation tube contained 600 μL buffer, 100 μL reconstituted saliva sample and 100 μL phospholipase A_2 suspension or buffer. Incubations were carried out at 37°C for 4 hr with mixing to prevent settling of the enzyme

[1]Based on a paper presented at the Third International Conference on Platelet-Activating Factor and Structurally Related Alkyl Ether Lipids, Tokyo, Japan, May 1989.

*To whom correspondence should be addressed.

Abbreviations: AcT, 0.05M acetate buffer pH 6.0 with 0.05% Tween-20; B_0, binding to antibody in absence of inhibitor; BSA, bovine serum albumin; C_nPAF, PAF where the 1-*O*-alkyl chain is n carbons long; [^{3}H-*acetyl*]PAF, 1-*O*-alkyl-2-[^{3}H]acetyl-*sn*-glycero-3-phosphocholine; [^{3}H-*alkyl*]PAF, 1-*O*-[^{3}H]alkyl-2-acetyl-*sn*-glycero-3-phosphocholine; Ig, immunoglobulin; [^{125}I]PAF, 1-*O*-[^{125}I]alkyl-2-acetyl-*sn*-glycero-3-phosphocholine; lyso-PAF, 1-*O*-alkyl-*sn*-glycero-3-phosphocholine; NSB, nonspecific binding; PAF, 1-*O*-alkyl-2-acetyl-*sn*-glycero-3-phosphocholine; PBS, phosphate (50 mM) buffered saline pH 7.2; PLA_2, phospholipase A_2; RIA, radioimmunoassay.

suspension. Reactions were stopped by centrifugation to remove the phospholipase A_2 bound to the agarose beads. Supernatants were re-extracted using the Bligh-Dyer method (12) and the PAF content was determined by RIA.

Silica gel chromatography. To the total lipid extract from 5 mL of saliva, [^{3}H-*alkyl*]PAF (38,500 cpm) was added and the mixture reconstituted in 400 μL chloroform. The solution was loaded onto a silica gel column (0.5 g), which had been packed in chloroform, and the lipids were eluted sequentially with 5 mL chloroform, 5 mL chloroform/methanol (3:2, v/v), 5 mL chloroform/methanol (1:3, v/v) and 5 mL chloroform/methanol/water (2:3:1, v/v/v). Each fraction collected (1 mL) was evaporated, reconstituted in ethanol, and assayed for radioactivity and PAF content.

RESULTS AND DISCUSSION

Initially, experiments using [^{3}H-*acetyl*]PAF incubated with whole, fresh saliva showed that very little PAF was degraded to lysoPAF. This suggested that there was little PAF-acetylhydrolase activity in saliva and therefore, discrepancies in the measurement of PAF levels would not occur as a result of specific PAF hydrolysis. However, when PAF was added to whole saliva and centrifuged, the PAF was adsorbed to the precipitated sediment. In order to make accurate, quantitative PAF measurements, saliva (0.8 vol) was therefore extracted with chloroform/methanol/water (2:2:1, v/v/v) and the reconstituted lipid extracts assayed. Using [^{3}H]PAF, this method produced recoveries of greater than 75%.

RIA and PAF in saliva. The specificity of the PAF RIA has been demonstrated using a large range of naturally occurring lipids and synthetic PAF analogs (10,11). Authenticity of the PAF levels measured in saliva was confirmed by three further experiments: i) Saliva extracts were assayed at different dilutions in the presence and absence of a known amount of synthetic PAF (Table 1). Dilution of the extracts produced the desired decrease in PAF levels as determined by RIA. ii) Each fraction collected from silica gel chromatographic separation, of a pooled saliva extract spiked with [^{3}H-*alkyl*]PAF, was tested for PAF activity by platelet aggregation and RIA, the recovery of [^{3}H]PAF tracer was also determined (Table 2). Only the fractions exhibiting PAF activity (chloroform/methanol/water, 2:3:1, v/v/v, fractions) also contained [^{3}H]PAF radioactivity. When compared to the total unfractionated activity (determined by both platelet aggregation and RIA), the percentage recovery of radioactivity corresponded to the percentage recovery of PAF activity. iii) Phospholipase A_2 (PLA_2) degrades PAF to the inactive lysoPAF which is not detected by the RIA (10,11). Extracted saliva samples were incubated with PLA_2 followed by RIA to determine the levels of PAF remaining (Fig. 1). After treatment with PLA_2 all samples, including a stock solution of synthetic PAF, showed less than 1 ng/mL PAF remained. The results of these experiments and the known specificity of PLA_2 and the RIA leads to the conclusion that the PAF-like activity detected in the saliva was indeed due to PAF.

RIA vs platelet aggregation. One interesting result of this work was the observation that PAF levels determined by platelet aggregation were consistently lower than the values obtained by RIA (Table 3). PAF induces aggregation of platelets at concentrations as low as 0.1 nM (15) and results obtained by RIA showed levels of PAF in saliva within the range 4–25 nM. However, with some of these saliva extracts, stimulation of platelets did not result in aggregation. Furthermore, platelets treated with these extracts did not respond to the subsequent addition of synthetic PAF. Results of these studies with sample 3 (Table 3) are shown in Figure 2 and are compared with the response of platelets to the same amount of synthetic PAF, in the absence of saliva extract.

TABLE 1

Quantitation of PAF in Human Saliva Extract by RIA[a]

Dilution of saliva[b]	Observed PAF concentration (ng/mL)[c]	
	No added PAF	With added PAF (2.5 ng/mL)
1 in 2	1.6	4.7
1 in 4	0.83	3.3
1 in 8	0.35	3.0

[a] Saliva was extracted by the method of Bligh and Dyer (12), reconstituted to the appropriate dilution in assay buffer, and the PAF concentration determined by RIA.

[b] Relative to unextracted saliva.

[c] Average of duplicates.

TABLE 2

Separation and Recovery of Salivary PAF by Silica Gel Chromatography[a]

Fraction	Eluting solvent[b]	% Recovery of [^{3}H]PAF	% Total activity[c]	
			by RIA	by Platelet aggregation
1	A	0	ND	ND
2	A	0	ND	ND
3	A	0	ND	ND
4	A	0	ND	ND
5	A	0	ND	ND
6	B	0.1	1.6	ND
7	B	0	ND	ND
8	B	0	ND	ND
9	B	0	ND	ND
10	B	0	ND	ND
11	C	0.2	ND	ND
12	C	0.2	ND	ND
13	C	0.3	ND	ND
14	C	0.5	ND	ND
15	D	1.1	ND	ND
16	D	3.6	3.2	2.4
17	D	85.2	82.9	86.7
18	D	3.9	4.8	3.1
19	D	0	ND	ND

[a] Saliva was extracted by the Bligh and Dyer method (12), [^{3}H-*alkyl*]PAF was added and chromatographed on a silica gel column. Each fraction was assayed for radioactivity and PAF content by both RIA and platelet aggregation.

[b] Solvent A, chloroform; Solvent B, chloroform/methanol (3:2, v/v); Solvent C, chloroform/methanol (1:3, v/v); and Solvent D, chloroform/methanol/water (2:3:1, v/v/v).

[c] Relative to the PAF content (determined by either RIA or PA) of the unfractionated saliva extract. ND, below the detection limit (~1% for either method).

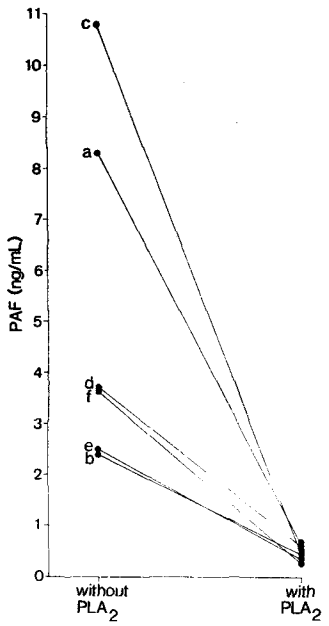


FIG. 1. The effect of phospholipase A_2 (PLA_2) treatment on PAF levels in human saliva. Saliva samples were extracted by the method of Bligh and Dyer (12). Incubations were carried out for 4 hr at 37°C in the presence or absence of both PLA_2 and synthetic PAF. After incubation, the samples were re-extracted and levels of PAF determined by RIA: a, buffer (control) + 7.2 ng/mL PAF; b, saliva 1; c, saliva 1 + 7.2 ng/mL PAF; d, saliva 2; e, saliva 3; and f, saliva 4.

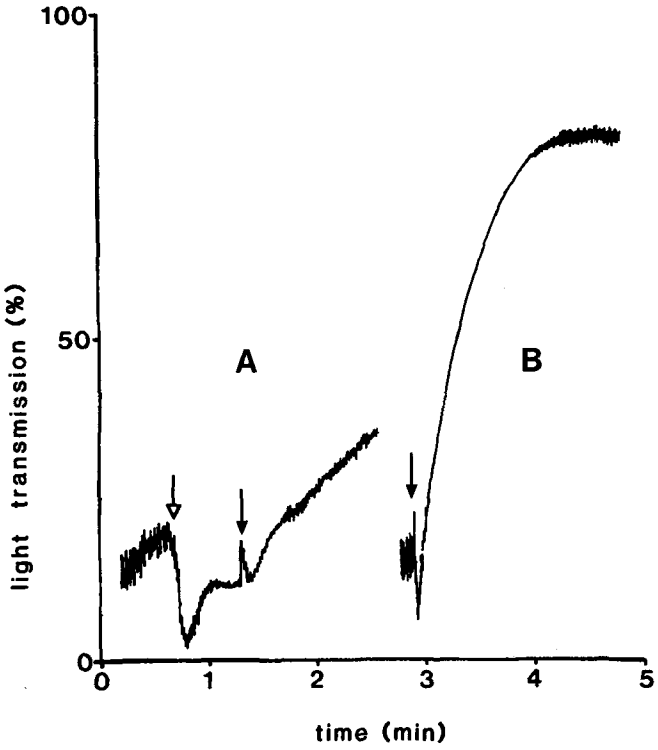


FIG. 2. A, Response of washed rabbit platelets to 20 μL lipid extract of saliva (× 10 concentrate) (open arrow) and subsequent response to 200 pg PAF (closed arrow). B, Response of platelets to 200 pg PAF alone.

TABLE 3

Comparison of RIA and Platelet Aggregation in Quantitating PAF in Saliva[a]

Sample	Observed PAF content (ng/mL saliva)	
	by RIA[b]	by Platelet aggregation[c]
1	10.6	<0.1
2	4.2	<0.1
3	3.8	<0.1
4	2.2	<0.1
5	15.6	0.22

[a]Saliva was extracted by the method of Bligh and Dyer (12), divided and reconstituted in the appropriate assay buffers.

[b]Tested as a ×2.5 concentrate relative to saliva.

[c]Tested as a ×10 concentrate relative to saliva.

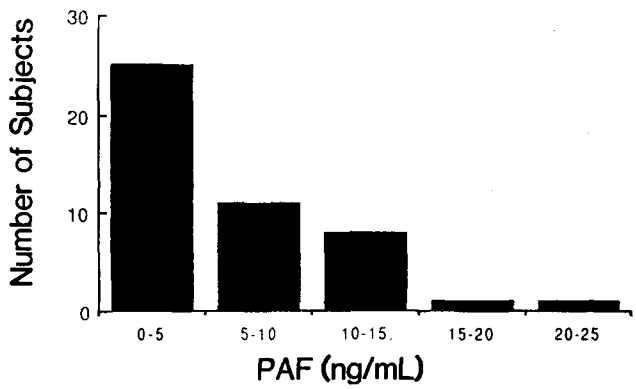


FIG. 3. Levels of PAF in human saliva. Samples of saliva were collected from 46 healthy volunteers. PAF was extracted by the method of Bligh and Dyer (12) within 15 min of sample collection. PAF levels were determined by RIA.

These observations suggest the presence in saliva of an endogenous inhibitor(s) which interferes with the determination of PAF levels using the biological assay, but does not affect the RIA. Experiments to determine the identity of these inhibitors are currently in progress (16).

PAF levels in normal human saliva. To date, only two groups have reported PAF levels in human saliva. The first study (8) employed a bioassay based on platelet degranulation and the second (9) utilized radioligand competitive binding methodology with intact canine platelets. These assay procedures may be influenced by antagonists/agonists of PAF activity in biological fluid (17). Indeed, we have shown (Fig. 2) that inhibitor(s) of platelet aggregation are present in saliva extracts and will therefore affect results using this method (16).

Having determined that the RIA is specific for PAF, is free of interfering factors and that chromatographic separation of samples is not necessary prior to RIA, the procedure was used to quantitate PAF in human saliva. Unstimulated human salivas from 46 healthy volunteers were examined (Fig. 3). The levels of PAF, not corrected for percent recovery, ranged from 0.46–21 ng/mL, with over 50% between 2–6 ng/mL, which is significantly greater than the values of 0.3–1.2 ng/mL previously reported (8,9) for apparently healthy subjects. These results are not surprising, as the latter values were obtained

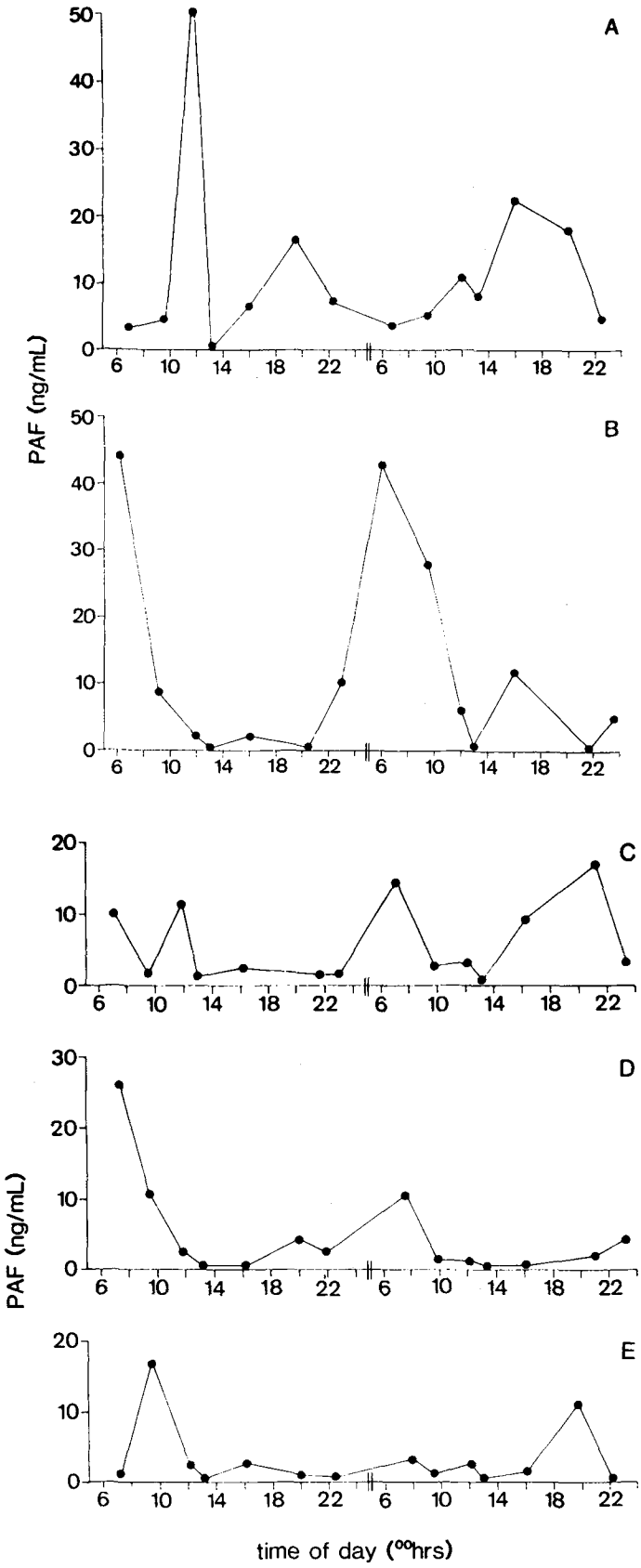


FIG. 4. Variation of PAF levels in human saliva over 48 hr. Saliva samples were collected from five volunteers at various times over a 48-hr period. Samples were stored at −20°C. After thawing samples, lipids were extracted by the Bligh and Dyer method (12). Levels of PAF were determined by RIA.

using platelet based assays. The response of platelets to endogenous PAF is diminished due to the presence of inhibitors which have now been demonstrated in rat liver (18), rat uterus (19) and, more recently, in human saliva (16). In our sample of 46, a wide range of PAF levels was observed, and therefore, the levels of PAF in the saliva from five healthy volunteers was monitored over a 48-hr period (Fig. 4). Significant variation can be seen in each individual with, in general, a peak in the morning followed by decreasing levels in the middle of the day which are lower than those at night. It is possible that these variations are due to the levels of hydration of the subject at the time of donation although further experiments are required before any firm conclusions can be reached. One clear feature of these results is the important role that standardization, not only of assay procedure but also of sample collection, will play in any future investigations on the levels of PAF in both normal and diseased states.

In summary, the RIA appears to be a suitable and reliable method for measuring PAF levels, with the added advantage of high sample capacity. Further investigations should reveal whether or not PAF levels in saliva reflect a relationship between PAF and disease.

ACKNOWLEDGMENTS

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Inhibitor(s) of Platelet-Activating Factor (PAF) in Human Saliva[1]

Mary A. Smal[a] and **Brian A. Baldo**[a,b,*]

[a]Kolling Institute of Medical Research, Royal North Shore Hospital, St. Leonards, N.S.W. 2065, and [b]Department of Medicine, University of Sydney, N.S.W. 2006, Australia

Quantitation of platelet-activating factor (PAF) in human saliva samples by radioimmunoassay indicated there was, at times, sufficient PAF present to aggregate platelets. However, in certain samples, we observed little or no aggregation, and furthermore, these samples were found to inhibit aggregation induced by PAF (200 pg). Chromatographic fractionation of pooled saliva increased the PAF activity 4-fold, and the observed inhibitory activity was found to co-migrate with the fatty acids. The inhibitory fraction was found to be active against platelet aggregation induced by arachidonic acid (3.4 nmole) as well as PAF (25 pg), but not thrombin (20 mU). These results indicate the existence of a PAF inhibitor in saliva, which may explain why potentially toxic levels of PAF can occur in the saliva of normal, healthy individuals. These findings also highlight an important advantage of the radioimmunoassay over platelet aggregation for the quantitation of PAF in, at least, some biological fluids. *Lipids 26*, 1144–1147 (1991).

Platelet-activating factor (PAF), 1-*O*-alkyl-2-acetyl-*sn*-glycero-3-phosphocholine, is a naturally occurring phospholipid with many potent biological activities (1,2). Amongst the bioactions, the capacity to induce aggregation of human and rabbit platelets at concentrations of 0.1nM is unique (3). Predictably, PAF has been implicated as a mediator of many diseased states, including asthma (4), other allergic diseases (5), septic shock (6), kidney disease (7) and gastric ulceration (8). The proposed role of PAF in these diseases and in normal physiological processes may be clarified by the quantitation of PAF in the relevant tissues or fluids.

The development of a specific and sensitive radioimmunoassay (RIA) for PAF (9) allows PAF to be accurately and routinely measured in various biological samples. Using the RIA, we quantitated PAF levels in numerous samples of mixed human saliva and found the levels to range from 0.5 to 21 ng/mL (10). These levels were higher than the previously reported data determined by bioassay (11,12) and higher than our results based on platelet aggregation measurements (10).

The most likely explanation for these observations seemed to be the presence of an inhibitor of PAF activity in the saliva extract. In this communication we report the isolation and preliminary examination of an inhibitory fraction from human saliva.

MATERIALS AND METHODS

Materials. Solvents were reagent grade or better and were obtained from Ajax Chemicals (Sydney, Australia). C_{16}PAF and C_{18}PAF were purchased from Bachem Feinchemikalien (Bubendorf, Switzerland) and an equimolar mixture of these two PAF homologs was used for all studies. Silica gel 60 for column chromatography (70–230 mesh), aluminum-backed thin-layer chromatography (TLC) plates (silica gel 60, 0.2 mm thick) and glass-backed preparative TLC plates (silica gel 60, 0.25 mm thick, 20 cm × 20 cm) were purchased from E. Merck (Darmstadt, Germany) and these were activated before use by heating to 100°C. All other chemicals and reagents were obtained from the Sigma Chemical Company (St. Louis, MO). Analytical TLC plates were developed in the standard solvents for lipid separation (13) and visualized by charring with sulfuric acid.

Extraction of saliva. Unstimulated mixed salivas (0.8 vol) were extracted with chloroform/methanol/water (2:2:1, v/v/v) by the method of Bligh and Dyer (14). The organic layer was evaporated under nitrogen, and the residue stored at −20°C. For platelet aggregation, the residue was reconstituted in phosphate-buffered saline (PBS) containing 0.25% bovine serum albumin (BSA).

Platelet aggregation. Washed rabbit platelets were prepared as previously described (15) and platelet aggregation carried out in duplicate as described by Huo *et al.* (16). PAF and arachidonic acid (AA) were diluted in 0.25% BSA/PBS and thrombin in saline. A standard dose-response curve was obtained by stimulating platelets with synthetic PAF, and this curve was used to quantitate PAF-activity in unknowns. For inhibition studies, the platelets were incubated with the saliva extracts or fractions for 1 min before addition of PAF. For the control response, platelets were pre-treated with the vehicle only before stimulation. Inhibition (%) (average) was calculated comparing the maximum height of the aggregation curve to that of the control.

Column chromatography. An extract of pooled saliva (72 mL) in chloroform (0.6 mL) was loaded onto a silica gel column (2 g) packed in chloroform. The column was eluted sequentially with 5 mL of each of the following: chloroform, chloroform/methanol (3:2, v/v), chloroform/methanol (1:3, v/v), methanol, chloroform/methanol/water (2:3:1, v/v/v) (2 lots). One-mL fractions were collected and each was analyzed by TLC. An aliquot of each fraction was evaporated, reconstituted in 0.25% BSA/PBS, and tested by platelet aggregation assay.

Preparative TLC. Extract of saliva (89 mL) in chloroform was applied to a preparative TLC plate and the plate developed in chloroform/methanol (19:1, v/v). The plate was divided into 1-cm bands, and these were scraped into glass centrifuge tubes. The silica was extracted 4 times with chloroform/methanol (2:1, v/v) (4 × 4 mL) and the combined extracts evaporated. Each

[1]Based on a paper presented at the Third International Conference on Platelet-Activating Factor and Structurally Related Alkyl Ether Lipids, Tokyo, Japan, May 1989.

*To whom correspondence should be addressed.

Abbreviations: AA, arachidonic acid; BSA, bovine serum albumin; C, cholesterol; CE, cholesteryl esters; FA, fatty acids; MG, monoglycerides; PAF, platelet-activating factor; PBS, phosphate (50mM) buffered saline pH 7.2; PC, phosphatidylcholine; PE, phosphatidylethanolamine; PL, phospholipids; RIA, radioimmunoassay; S, sphingomyelin; TG, triglycerides; TLC, thin-layer chromatography; U, unknown.

fraction was analyzed for lipids present and inhibitory activity.

RESULTS

Effect of whole saliva extract on platelet aggregation. Figure 1 shows the changes in light transmission when platelets were preincubated with several saliva extracts followed by the addition of PAF. Thirty different saliva extracts were studied; with each of the extracts, the response to PAF was diminished and with some extracts, completely abrogated. A representative selection is shown in Figure 1. Platelets aggregated when stimulated with some saliva extracts, but the response was weak (saliva B in Fig. 1). It has also been noted several times that the observed level of PAF in some saliva extracts, as determined by platelet aggregation, apparently increased as the sample was diluted.

Separation of salivary PAF activity from inhibitory activity. A sample of pooled saliva extract was fractioned by column chromatography and the fractions were analyzed for PAF and inhibitory activities (Table 1). PAF activity was observed in fractions corresponding to the more polar phospholipids (phosphatidylcholine and sphingomyelin) and this activity increased approximately 4-fold over the activity observed in the whole extract. Furthermore, PAF inhibitory activity was observed in the fractions corresponding to the neutral lipids, cholesterol and fatty acids. It is possible that there are inhibitors that co-migrate with PAF. These may be difficult to detect since the endogenous PAF would desensitize the platelets (17) and hence, it would be unclear which compounds were responsible for the observed inhibition.

A purer fraction containing the above inhibitory activity was isolated by subjecting the whole saliva extract to preparative TLC (Table 2). The only significant inhibitory activity was confined to fractions 11 and 12; analytical TLC indicated that the major component of these fractions co-migrated with the fatty acids.

Specificity of inhibitory activity. The PAF inhibitory fractions (11 and 12 from the TLC separation) were tested for their capacities to block platelet aggregation induced by AA and thrombin. The results for fraction 12 are shown in Figure 2; similar results were obtained for fraction 11.

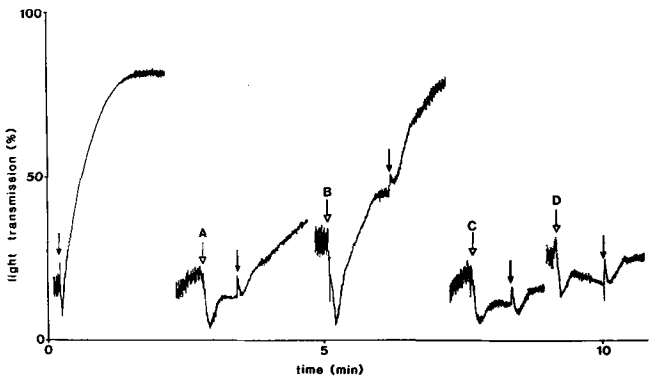


FIG. 1. The response of washed rabbit platelets (0.5 mL) following addition of lipid extracts of salivas A, B, C and D (equivalent to 0.2 mL saliva) (open arrow) and stimulation with PAF (200 pg) (closed arrow).

TABLE 1

Fractionation of Saliva by Column Chromatography[a]

Fractions	PAF concentration (ng/mL saliva)[b]	Inhibition of platelet response (%)[c]	Co-migrating lipids
1-7	0	0	CE,TG,C[d]
8-9	0	100	C,FA[e]
10-20	0	0	PE[f]
21-23	0	0	PC,S[g]
24-27	1.33 ± 0.02	not tested	PC,S
28	0	0	nil
Whole extract	0.29 ± 0.04	not tested	all

[a]The lipid extract of pooled saliva was fractionated on a silica gel column as described in Materials and Methods.

[b]Determined by the platelet aggregation assay, using at least two different sample concentrations.

[c]Platelets (0.5 mL) were incubated with an aliquot of each fraction (equivalent to 3.6 mL saliva), and followed by stimulation with PAF (50 pg).

[d]CE, cholesteryl esters; TG, triglycerides; C, cholesterol.

[e]FA, fatty acids.

[f]PE, phosphatidylethanolamine.

[g]PC, phosphatidylcholine; S, sphingomyelin.

TABLE 2

Separation of the Salivary PAF-Inhibitors by TLC[a]

Fraction	R_f[b]	Inhibition of platelet response (%)[c]	Co-migrating lipids
2	0.91	5.6	nil
3	0.86	11.3[d]	CE,TG
4	0.80	49.3[d]	CE,TG
5	0.74	42.2[d]	CE,TG
6	0.69	40.6[d]	CE,TG
7	0.63	2.8	nil
8	0.57	39.4[d]	C
9	0.51	16.9	C
10	0.46	8.5	nil
11	0.40	88.7	FA
12	0.34	69.0	FA
13	0.29	1.4	MG[e]
14	0.23	0	MG
15	0.17	0	U[f]
16	0.11	0	U
17	0.06	0	PL[g]
18	0	pro-aggregatory	PL

[a]The lipid extract of pooled saliva was separated by preparative TLC as described in Materials and Methods.

[b]On silica gel plates run in methanol/chloroform (1:19, v/v).

[c]Platelets (0.5 mL) were incubated with an aliquot of each fraction (equivalent to 2 mL saliva) and followed stimulation with PAF (100 pg).

[d]Uncorrected for the turbidity of the lipid sample; the true inhibitory potencies of these fractions were lower than observed.

[e]MG, monoglycerides.

[f]U, unknown.

[g]PL, phospholipids.

PAF- and AA- induced aggregation were completely blocked, whereas thrombin-induced aggregation was unaffected by the same dose of inhibitor. Dose-dependent inhibition was observed for PAF-induced aggregation. Studies with other aggregating agents are in progress.

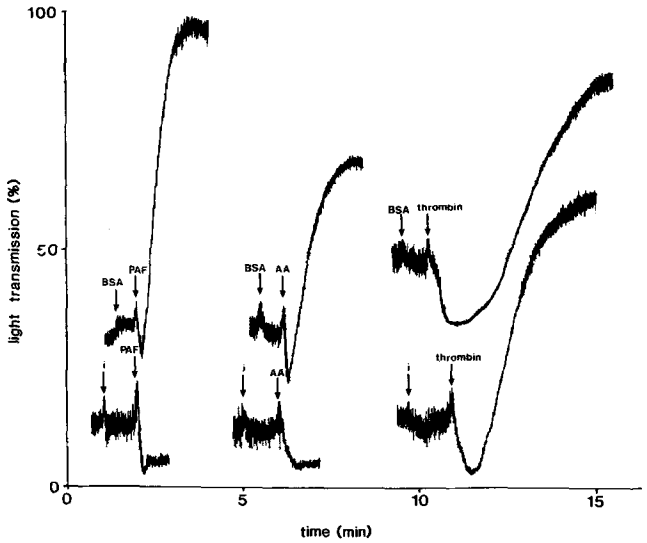


FIG. 2. The response of washed rabbit platelets (0.5 mL) following incubation with saliva fraction (i) number 12 from preparative TLC and equivalent to 2 mL saliva, followed by stimulation with either PAF (25 pg), AA (3.4 nmol) or thrombin (20 mU). The vehicle (0.25% BSA/PBS) was used for the control response.

DISCUSSION

In our previous study (10), PAF levels in whole saliva extracts were quantitated by RIA, and results indicated that there was sufficient PAF present in saliva to aggregate platelets. However, when measured by platelet aggregation, little PAF activity was found. One possible explanation for these results was that the extracts contained lipids which interfered with the functional response of platelets to the endogenous PAF.

In this study, we examined this possibility by incubating platelets with various saliva extracts before stimulation with PAF, and the diminished platelet response we observed (Fig. 1) supported this hypothesis. Upon chromatographic fractionation of whole saliva extract, the PAF activity increased 4-fold, indicating that an inhibitory factor had been removed. Indeed, inhibitory activity was observed in fractions that co-migrated with the more polar neutral lipids.

The inhibitory fraction studied here was further purified by preparative TLC, and found to co-migrate with the fatty acids. The inhibitor is suspected to be a fatty acid, and additional supportive evidence may be obtained by carrying out preparative TLC in a different solvent system. A variety of naturally-occurring fatty acids are currently being tested for PAF inhibitory activity.

The specificity of the inhibitor(s) was also investigated. AA-induced aggregation was also blocked, whereas thrombin-induced aggregation was not. This suggests that the inhibitor(s) does not act through the PAF receptor, but may affect a biochemical reaction which is common to both PAF- and AA-induced platelet aggregation. The effect of the inhibitor(s) on other cells and tissues that are sensitive to PAF has not yet been studied.

Recently, three reports have appeared in the literature on the existence of lipid-like inhibitors of PAF in mammalian tissues. Nakayama *et al.* (18) isolated two inhibitory fractions from rat uterus, and identified them to be mixtures of firstly, 1-acyl-*sn*-glycero-3-phosphocholines and 1-alkyl-*sn*-glycero-3-phosphocholines, and secondly, *N*-acyl sphing-4-enylphosphocholines. These substances were specific inhibitors of PAF-induced platelet aggregation. Masugi *et al.* (19) reported the existence of a non-specific inhibitor in renal venous blood from patients with renovascular hypertension; this inhibitor had an R_f similar to PAF and was thought to be a polar phospholipid. Miwa *et al.* (20) reported the existence of an endogenous inhibitor in rat liver that had the chromatographic properties of a non-polar lipid. This inhibitor may be similar to the salivary inhibitor(s) described here.

The existence of an endogenous inhibitor(s) in saliva extracts serves to highlight the unreliability of the platelet bioassays for quantitating PAF (3). Since the RIA is only dependent on the binding of PAF to an antibody, which is highly specific for PAF (9), the PAF levels obtained by this method are more likely to reflect the true levels. This would also explain why higher levels of PAF were observed using the RIA (10) compared to those previously reported using platelet bioassays (11,12).

The existence of a PAF inhibitor in saliva may be incidental or it may have some functional role. Since the levels of PAF in saliva appear to be sufficient to elicit biological responses, this inhibitor may serve to protect oral tissues from the inflammatory effects of PAF.

ACKNOWLEDGMENT

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REVIEW

L-659,989: A Useful Probe in the Detection of Multiple Conformational States of PAF Receptors[1]

San-Bao Hwang* and **My-Hanh Lam**
Merck Sharp & Dohme Research Laboratories, Department of Biochemical Regulation, Rahway, New Jersey 07065-0900

L-659,989 is a potent, specific and competitive platelet-activating factor (PAF) receptor antagonist. The 2,5-tritium labeled L-659,989, similar to [^{3}H]PAF, specifically binds to rabbit platelet membranes with an equilibrium dissociation constant (K_D) of 1.60 (±0.20) nM in 10 mM $MgCl_2$. However, guanosine 5′-triphosphate (GTP) and several cations affect the specific binding of [^{3}H]PAF and of [^{3}H]L-659,989 to rabbit platelet membranes in different ways. K^+, Mg^{2+}, Ca^{2+} and Mn^{2+} potentiate the specific binding of both ligands. Na^+ and Li^+ inhibit the specific [^{3}H]PAF binding, but enhance the binding of [^{3}H]L-659,989; GTP reduces the [^{3}H]PAF binding but has no effect on the binding of [^{3}H]-L-659,989. Ni^{2+} inhibits the [^{3}H]L-659-989 binding, but has no effect on the binding of [^{3}H]PAF. In the presence of 150 mM NaCl, [^{3}H]L-659,989 exhibits identical K_D and detectable binding sites (B_{max}) values as those in the presence of 10 mM $MgCl_2$, while K_D And B_{max} values of [^{3}H]PAF are dramatically reduced in the presence of 150 mM NaCl compared to those in 10 mM $MgCl_2$. These results suggest the existence of multiple conformational states of the PAF specific receptor and that PAF and L-659,989 bind differently to those states. In the presence of 150 mM NaCl and 1 mM GTP, receptors appear to exist in a single conformational state with an equilibrium dissociation constant (K_B) of 0.93 μM for PAF as derived from the Schild plot. In isolated rabbit platelets pretreated with 10 μM ETH 227, a Na^+-specific ionophore, the detectable [^{3}H]PAF binding sites drop from 260 to 100 binding sites per platelet, but the binding sites for [^{3}H]L-659,989 remain roughly the same. The Na^+ binding sites which modulate the conformation of PAF receptors are therefore protected from extracellular Na^+ until ionophore is added, and are probably located on the cytoplasmic side of the plasma membrane.
***Lipids 26,* 1148–1153 (1991).**

Platelet-activating factor (PAF) is a potent phospholipid mediator with a wide variety of biological activities, which include aggregation and degranulation of platelets and leukocytes, bronchoconstriction, vascular permeability and hypotension (1). A cell surface receptor that specifically binds PAF is generally believed to mediate cellular responses to PAF. Indeed, specific receptors for PAF have been reported in a variety of cell membranes, including those from human (2–4) and rabbit platelets (5,6), human polymorphonuclear leukocytes (PMNs) (7–9) and membranes from several types of tissues (10–12).

Working with isolated membranes, we have previously shown that a single type of PAF-specific receptor exists in human (4) and rabbit (5) platelets, human PMNs (9), human lung tissue (10), rat liver tissue (11), and rat peritoneal PMNs (9). In each case, we detected a single equilibrium dissociation constant of about 0.5 nM for [^{3}H]-C_{16}-PAF under identical ionic conditions. O'Flaherty *et al.* (8) have reported both high and low affinity binding sites for PAF in human PMNs (K_D = 0.2 and 500 nM, respectively). These could result from the existence of multiple conformational states of the receptor which could be detected under different ionic or other experimental conditions. Indeed, with the help of 2,5-tritium labeled L-659,989 (*trans*-2-(3-methoxy-5-methylsulfonyl-4-propoxyphenyl)-5-(3,4,5-trimethoxyphenyl)tetrahydrofuran, (Scheme 1), we could detect multiple conformational states of a single type of PAF receptors (13). This review summarizes these studies and extends the work with the sodium specific ionophore, ETH 227, to demonstrate that the Na^+ binding sites are probably located on the cytoplasmic side of the plasma membrane.

L-659,989 is a potent specific and competitive receptor antagonist of PAF. L-659,989 is an orally active and extremely potent, selective and competitive PAF receptor antagonist (14). It potently inhibits the [^{3}H]C_{16}-PAF (1-*O*-hexadecyl-2-*O*-acetyl-*sn*-glycero-3-phosphocholine) binding to either rabbit platelet or rabbit PMN membranes with an equilibrium constant (K_I of 1.1 nM) but is about 10 times less potent with a K_I of 14.3 nM in human platelet (Table 1), PMN, and lung membranes. This difference is consistent with the species difference in PAF receptor between human and rabbit platelets (4).

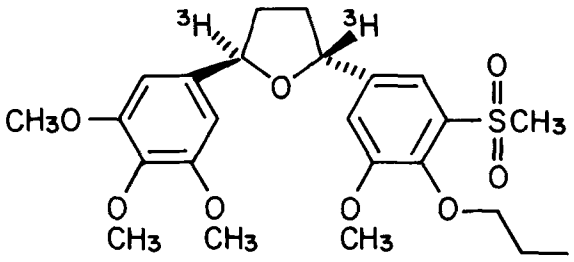
L-659,989 is one of the most potent PAF receptor antagonists reported in the literature. As shown in Table 1, it is about 10 times more potent than L-652,731 (4) and almost 100 times more potent than kadsurenone (4) in rabbit platelet membranes. L-659,989 is about 5- to 6-fold more potent than WEB2086 and about 70-fold more potent than FR72112 in human platelet membranes.

L-659,989 shares a common binding site with PAF. The specific binding of 2,5-tritium-labeled L-659,989 to rabbit platelet membranes is saturable with a single type of high affinity binding site detectable in Scatchard plots. The equilibrium dissociation constant (K_D) is 1.60 ± 0.20 nM (n = 6) in 10 mM $MgCl_2$ (13). This value is almost identical to the equilibrium inhibition constant (K_I), 1.10 ± 0.19 nM (n = 4) (Table 1) in inhibiting the

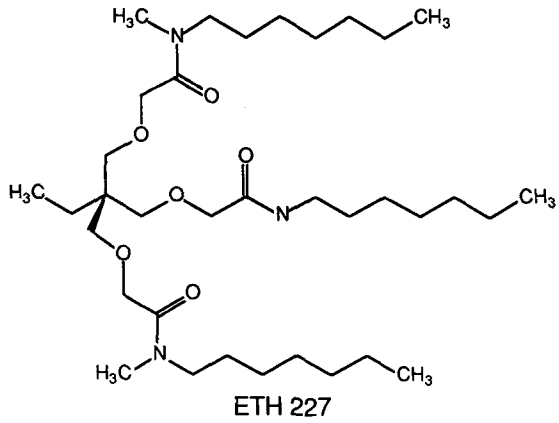
[1]Based on a paper presented at the Third International Conference on Platelet-Activating Factor and Structurally Related Alkyl Ether Lipids, Tokyo, Japan, May 1989.

*To whom correspondence should be addressed at Merck Sharp & Dohme Research Laboratories, Department of Biochemical Regulation, P.O. Box 2000 (80B7), Rahway, NJ 07065-0900.

Abbreviations: B_{max}, detectable binding sites; BSA, bovine serum albumin; DMSO, dimethylsulfoxide; ED_{50}, effective dose, 50%; GTP, guanosine 5′-triphosphate; K_B and K_D, equilibrium dissociation constants; K_I, equilibrium inhibition constant; PAF, platelet-activating factor; PMA, phorbol myristate acetate; PMN, polymorphonuclear leukocytes; R_o, state of receptor with no addition of ions; R_s, cooperativity index.



[^{3}H] L-659,989



ETH 227

SCHEME 1

TABLE 1

Equilibrium Inhibition Constants (K_I) for the Inhibition by PAF Receptor Antagonists of [^{3}H]C_{16}-PAF Binding to Rabbit and Human Platelet Membranes

Compound	K_I[a]	
	Rabbit platelet membranes	Human platelet membranes
L-659,989	1.10 (± 0.19) × 10^{-9}	1.43 (± 0.49) × 10^{-8}
L-652,731	9.83 (± 2.92) × 10^{-9}	
Kadsurenone	8.81 (± 1.4) × 10^{-8}	
WEB 2086	7.93 (± 0.50) × 10^{-8}	
FR72112		9.51 (± 1.74) × 10^{-7}

[a]Data are expressed as mean (± S.D.) of four to five experiments.

specific [^{3}H]C_{16}-PAF binding to those membranes. The specific binding of [^{3}H]L-659,989 can be fully displaced by PAF and PAF analogs and also by several selected PAF receptor antagonists of different chemical structures (13). The K_I values of these PAF analogs, PAF receptor antagonists, and PAF against [^{3}H]L-659,989 binding are about the same as their K_I values against [^{3}H]C_{16}-PAF binding. An excellent correlation ($\gamma = 0.997$, slope = 0.94) exists between the potencies of the compounds in displacing [^{3}H]C_{16}-PAF from its binding sites and in inhibiting the specific [^{3}H]-659,989 binding (13).

The specificity of [^{3}H]L-659,989 is similar to that of [^{3}H]C_{16}-PAF and [^{3}H]dihydrokadsurenone binding (13,15). Several selected pharmacologically defined drugs or agents show no significant inhibition of [^{3}H]L-659,989 binding. The competitive nature of the antagonism was demonstrated by the inhibition of [^{3}H]C_{16}-PAF binding to rabbit platelet membranes and by the inhibition of PAF-induced gel-filtered rabbit platelet aggregation (14). These results strongly indicate that L-659,989 shares with PAF a common binding site in the PAF receptor.

PAF induces a conformational change of the PAF receptor. Although L-659,989 is a competitive inhibitor of [^{3}H]PAF binding (see above), the reverse is not true. L-659,989 alters the apparent dissociation constant (K_D') of specific [^{3}H]C_{16}-PAF binding to rabbit platelet membranes, but not the number of detectable binding sites (B_{max}) as shown by Scatchard plots (Fig. 1A). However, C_{16}-PAF alters not only the K_D' of [^{3}H]L-659,989 binding to the receptors in rabbit platelet membranes but also the B_{max} (Fig. 1B). In the presence of 30 nM C_{16}-PAF, the B_{max} drops from 3.84 ± 0.08 pmol/mg protein (n = 3) to 2.05 ± 0.23 pmol/mg protein (n = 3, mean ± S.D.), nearly 50% lower than that in the absence of C_{16}-PAF (13). Since L-659,989 is a competitive PAF receptor antagonist, the most likely explanation is that PAF but not the antagonist induces a conformational change in the PAF receptor from a high-affinity state for L-659,989 to a low-affinity state. This change could also account for the difference in the detectable receptor number between [^{3}H]C_{16}-PAF and [^{3}H]L-659,989. The B_{max} for [^{3}H]L-659,989 (4.27 ± 0.34 pmol/mg protein, n = 6) is about 25% higher than the B_{max} of [^{3}H]C_{16}-PAF binding sites (3.19 ± 0.39 pmol/mg protein, n = 3) assayed in the same membranes under identical ionic conditions (13). Therefore, PAF could also convert the PAF receptor from a high-affinity state to a low-affinity state for binding of PAF itself. However, we cannot rule out the possibility that the detection of the lower B_{max} value for [^{3}H]PAF is due to the difference in the dissociation rate constants between the two ligands.

Modulation by ions and GTP of the binding of [^{3}H]C_{16}-PAF and [^{3}H]L-659,989 to rabbit platelet membranes. Na^+ and Li^+ inhibit, but K^+, Mn^{2+}, Ca^{2+}, and Mg^{2+} potentiate the specific [^{3}H]C_{16}-PAF binding to rabbit (16) and human (9) platelet membranes. The Na^+ inhibitory effect is apparently due to an increase in the equilibrium dissociation constant of the PAF binding, but not in the number of receptor sites. However, the increase in the specific [^{3}H]C_{16}-PAF binding by divalent cations is mainly due to an increase in the total number of the detectable binding sites (16). On the other hand, the specific [^{3}H]L-659,989 binding to rabbit platelet membranes is potentiated not only by K^+, Mg^{2+}, Ca^{2+} and Mn^{2+} but also equally by Na^+ and Li^+ (13). A detailed Scatchard analysis of the specific [^{3}H]L-659,989 binding shows that the potentiation effects of Na^+ and Mg^{2+} are apparently due to the increase in B_{max} with a minimal alteration in the K_D. In the presence of either 150 mM NaCl or 10 mM $MgCl_2$, the PAF receptors show identical affinity and similar detectable binding sites for L-659,989. Ni^{2+} inhibits the specific [^{3}H]L-659,989 binding, even though it shows no effect on the specific [^{3}H]C_{16}-PAF binding to rabbit platelet membranes (17). GTP specifically inhibits the [^{3}H]PAF binding (16) but

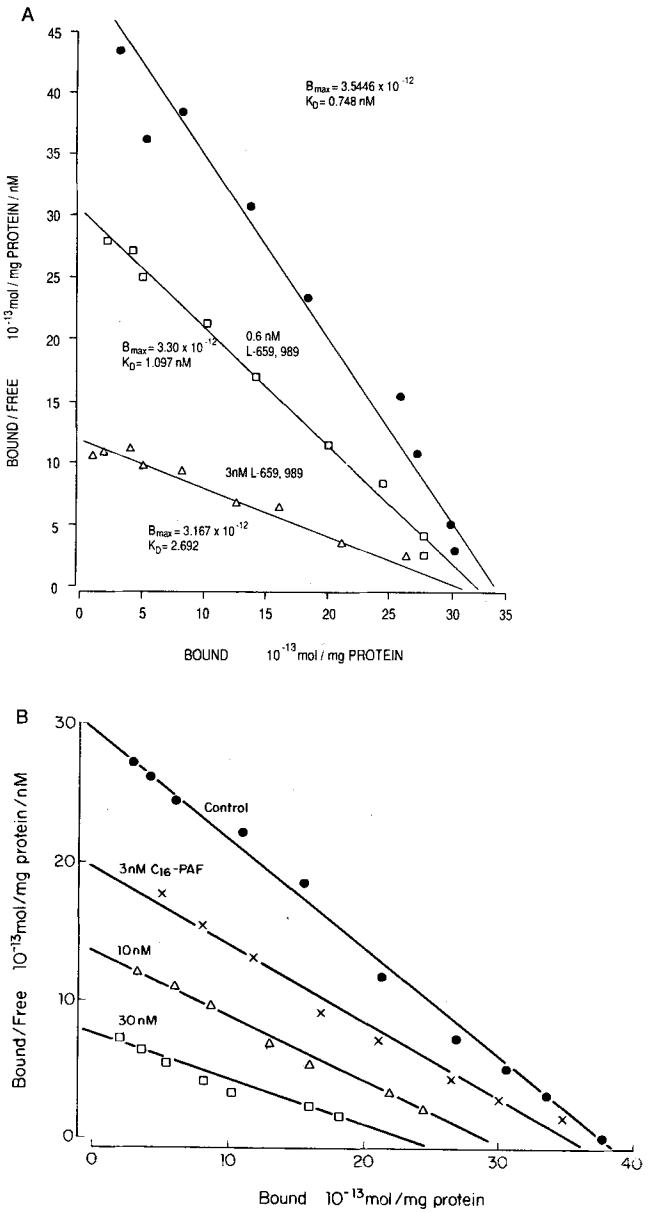


FIG. 1A: Scatchard plots of [^{3}H]C_{16}-PAF binding to rabbit platelet membranes without (●) and with L-659,989 at a concentration of 0.6 nM (□) and 3 nM (△). The assay medium was 10 mM $MgCl_2$, 10 mM Tris and 0.25% BSA at pH 7.0. Lines were plotted by linear regression. The data points are the mean of duplicate determinations. B: Scatchard plots of the specific [^{3}H]L-659,989 binding to rabbit platelet membranes in the absence (●) and in the presence of unlabeled PAF at 3 nM (X), 10 nM (△) and 30 nM (□) (13).

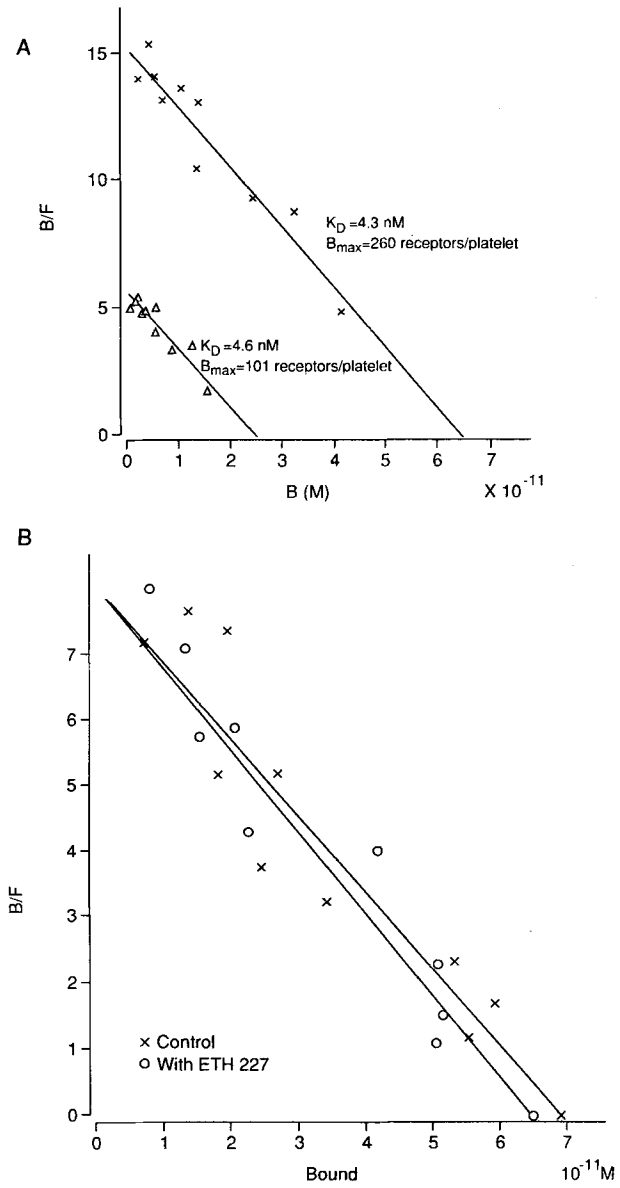


FIG. 2. Scatchard plots of the binding of [^{3}H]C_{18}-PAF (2A) and [^{3}H]L-659,989 (2B) to gel-filtered rabbit platelets with or without 10 μM ETH 227. Platelets were prepared as previously described (5) and incubated (3 × 10^8 platelets/mL) at 37°C for 2 min either with or without 20 μM ETH 227. ETH 227 (4 mM) was dissolved in dimethylsulfoxide (DMSO). DMSO (5 μL) was added as control. Cells (250 μL) incubated either with or without ETH 227 in column buffer were added to Microwell tube strips (Skatron, Inc., Stirling, VA) which contained 250 μL of 0.1 to 6 nM [^{3}HC_{18}-PAF (Fig. 2A) or [^{3}H]L-659,989 (Fig. 2B) such that the final cell number was 1.5 × 10^8 platelets per mL and the concentration of ETH 227 was 0 or 10 μM. Nonspecific binding was measured in the presence of 1,000-fold excess of unlabeled C_{18}-PAF or L-659,989. After 2 hr incubation at 0°C, bound and unbound radioligands were separated through filtermat with a 12 well cell harvester (Skatron, Inc.) and radioactivity was counted.

shows no effect on the binding of [^{3}H]L-659,989 (13). Differences in the modulation by ions and GTP of the binding of [^{3}H]C_{16}-PAF and [^{3}H]L-659,989 to rabbit platelet membranes seem to be clear. Such a clear change in the binding of agonist and antagonist caused by ionic conditions strongly suggests that the variation in the maximal number of binding sites under different ionic conditions results from a direct receptor effect rather than from altered membrane structure and that these ions and GTP could modulate the conformation of the PAF receptor. Thus, multiple conformational states could exist in a possible single type of PAF receptor.

The Na^+ binding site is located at the cytoplasmic side of plasma membranes. As discussed above, Na^+ and Li^+ inhibit, but K^+ and divalent cations such as Mn^{2+}, Ca^{2+} and Mg^{2+} potentiate the specific [^{3}H]PAF binding (16). In the presence of 10 mM $MgCl_2$, the detectable

TABLE 2

Specific Binding of [^{3}H]C_{18}-PAF of [^{3}H]L-659,989 to Intact Gel-Filtered Rabbit Platelets Without and With Na^+-Ionophore Treatment

Ligand	ETH 227	K_D (nM)	B_{max} (receptors/platelet)
[^{3}H]C_{18}-PAF	0	3.62 (± 1.23)	259 ± 7 (n = 3)
[^{3}H]C_{18}-PAF	10 μM	3.14 (± 1.62)	110 ± 9 (n = 3)
[^{3}H]L-659-989	0	1.75 (± 0.79)	267 ± 16 (n = 4)
[^{3}H]L-659,989	10 μM	1.44 (± 0.35)	251 ± 9 (n = 4)

receptor number of [^{3}H]PAF in rabbit platelet membranes is about twice of that found in the presence of 150 mM NaCl (16). The decrease in the detectable receptor number in the presence of Na^+ is apparently due to the presence of the reduced affinity of the receptor for [^{3}H]PAF since binding with a K_D value in the μM range cannot be detected in Scatchard plots of experiments with the radioligand in the nanomolar concentration range (13). On the other hand, the specific binding of [^{3}H]L-659,989 is potentiated not only by K^+, Mg^{2+}, Ca^{2+} and Mn^{2+}, but also equally potentiated by Na^+ and Li^+. Therefore, in intact gel-filtered rabbit platelets, if the Na^+ binding sites which modulate the specific binding of [^{3}H]PAF are located in the cytoplasmic side of the plasma membranes, we suspect that induction of Na^+ inward movement will decrease the detectable binding sites of [^{3}H]PAF but not those of [^{3}H]L-659,989. Indeed, Na^+ influx induced by a Na^+ specific ionophore, ETH 227 [*N,N',N''*-triheptyl-*N,N',N''*-trimethyl-4,4',4''-prophylidyne-*tris*(3-oxabutyramide)] (Scheme 1) (10 μM), decreases the [^{3}H]PAF binding sites by half with no significant change of [^{3}H]L-659,989 binding sites as demonstrated by the experiments described below.

The tritium-labeled C_{18}-PAF (1-*O*-[9',10'-^{3}H]octadecyl-2-*O*-acetyl-*sn*-glycero-3-phosphocholine) specifically binds to isolated rabbit platelet membranes with K_D of 1.99 ± 0.09 nM (mean ± S.D., n = 3) in an assay medium of 10 mM $MgCl_2$ 10 mM Tris, and 0.25% bovine serum albumin (BSA) at pH 7.0. However, it binds to gel-filtered rabbit platelets with a K_D of 3.62 (±1.23) nM (mean ± S.D., n = 3) in a sodium-containing buffer (126 mM NaCl, 14.5 mM Tris, 0.1% glucose, 5.4 mM KCl, 10 mM $MgCl_2$, 0.05 mM $CaCl_2$ and 0.25% BSA at pH 7.6). A sodium specific ionophore, ETH 227, (18,19) was used to determine whether a Na^+-sensitive site might be shielded from extracellular Na^+ in the intact platelets. The specific binding of [^{3}H]C_{18}-PAF to intact rabbit platelets in the presence of 10 μM ETH 227 was reduced to about half of the binding in the control experiments. Scatchard plots indicated that the reduction in the specific binding of [^{3}H]C_{18}-PAF is due to a decrease in the B_{max} without a significant alteration in the K_D. The detectable PAF binding sites decreased from 260 to 110 receptors per platelet in the presence of 10 μM ETH 227 (Fig. 2A and Table 2). In contrast, ETH 227 showed no significant effect on either the K_D or B_{max} of [^{3}H]-L-659,989 binding (Fig. 2B and Table 2). A single class of binding sites was observed for [^{3}H]L-659,989 binding to intact rabbit platelets with a K_D value close to 1.5 nM, which is the K_D value also found in the isolated rabbit platelet membranes (13). The B_{max} of [^{3}H]L-659,989 either with or without ETH 227 treatment was similar to the B_{max} value for [^{3}H]C_{18}-PAF in the control experiments. These results indicate that in platelets the Na^+ binding sites which modulate the affinity of PAF for specific PAF receptors are inaccessible to extracellular Na^+ and are probably located in the cytoplasmic side of the plasma membranes. Similar conclusions have been reached in the α_2-adrenergic receptor system of human platelets (20). Our data do not distinguish whether the Na^+ binding site is on the PAF receptor itself or on a regulatory protein. Also, since the receptors which cannot be detected by [^{3}H]C_{18}-PAF in the ETH 227-treated rabbit platelets are still detectable by [^{3}H]L-659,989, the reduction in [^{3}H]PAF binding is not due to the receptors being either embedded into the membrane or translocated into the inner membrane surface, but is due to the interaction of receptors with the ionophore-carried sodium such that the receptors' affinity is reduced. This lower-affinity binding cannot be detected in conventional Scatchard plots with radioligand in the nM range concentration. The identity of B_{max} for [^{3}H]C_{18}-PAF and [^{3}H]L-659,989 in the untreated rabbit platelets support the conclusion that L-659,989 is a specific PAF receptor antagonist and that both L-659,989 and PAF bind to the same receptor and share a common binding site in the receptor.

Multiple conformational states of a single type of PAF receptor. As discussed above, the K_D of [^{3}H]L-659,989 binding to rabbit platelet membranes is the same in 10 mM $MgCl_2$ and in 150 mM NaCl and is not altered by GTP. On the other hand, Na^+ and GTP do greatly affect the binding of [^{3}H]L-659,989 binding. In the presence of 10 mM $MgCl_2$, the effective dose, 50% (ED_{50}) of C_{16}-PAF to inhibit the specific [H]L-659,989 binding is 1.4 nM, whereas in the presence of 150 mM NaCl, the ED_{50} value is shifted to about 1 μM, almost 1,000-fold higher than that in 10 mM $MgCl_2$ (Fig. 3). GTP at 1 mM also significantly shifts the C_{16}-PAF competition curves to the right, from an ED_{50} value of 1.4 nM to 10 nM in the presence of 10 mM $MgCl_2$ and from 1 μM to 2 μM in the presence of 150 mM NaCl. The competition curves of C_{16}-PAF in the presence of either 10 mM $MgCl_2$ or 10 mM $MgCl_2$ and 1 mM GTP belong to a "shallow" competition curve, with a cooperativity index, R_s, value >81, indicating that multiple orders of binding sites of C_{16}-PAF are occurring in the competition binding (see Ref. 21 for the definition of R_s value). Two straight lines with slope equal to −1 and −0.5 in the indirect Hill plots better fit the data of C_{16}-PAF in the presence of either 10 mM $MgCl_2$ or 10 mM $MgCl_2$ and 1 mM GTP (13). Indeed, the competitive binding data under the assay conditions of 10 mM $MgCl_2$, 10 mM $MgCl_2$ and 1 mM GTP,

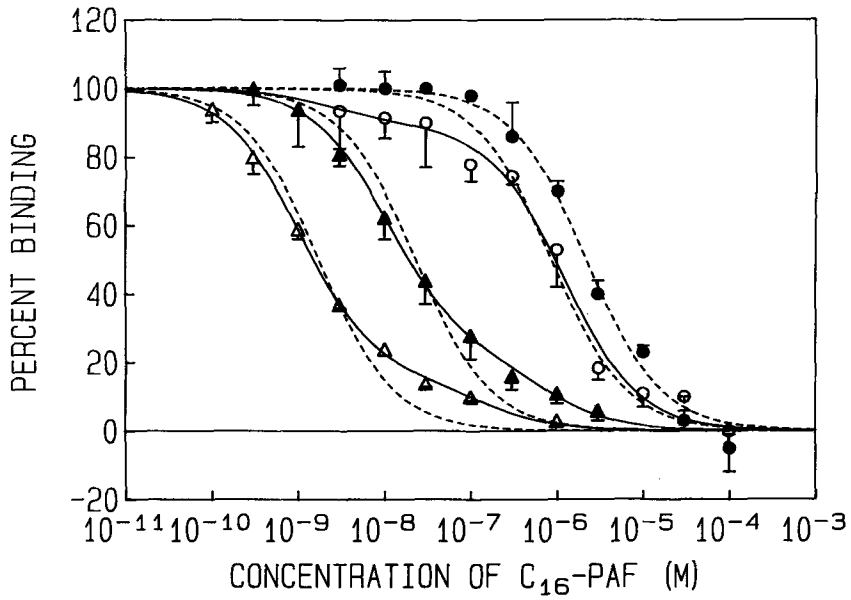


FIG. 3. Inhibition of [^{3}H]L-659,989 binding to rabbit platelet membranes by C_{16}-PAF. Membrane protein (100 μg) and 1 nM [^{3}H]L-659,989 were used in each tube in the assay medium containing 10 mM Tris and 0.25% BSA at pH 7.0 with 10 mM $MgCl_2$ (△), 10 mM $MgCl_2$ and 1 mM GTP (▲), 150 mM NaCl (○), and 150 mM NaCl and 1 mM GTP (●). Data points and error bars are mean and standard deviation of two or three experiments (13). Solid lines were plotted by fitting the competitive binding data with two components by a nonlinear regression computer software, GraphPAD, version 2.0 (GraphPAD Software, San Diego, CA). Dashed lines were plotted by fitting competitive binding data with one site by the same computer software.

or 150 mM NaCl can be better fitted with two sites (solid lines in Fig. 3) than with one site (dashed lines in Fig. 3). However, in the presence of 150 mM NaCl and 1 mM GTP, the competitive binding data can be best fitted with one component (Fig. 3). A normal steepness competition with a pseudo-Hill coefficient equal to −1 was observed in the indirect-Hill plot (13). A simple competitive antagonism of C_{16}-PAF on the specific binding of [^{3}H]-L-659,989 is therefore expected under this experimental condition. In fact, in the Scatchard plots of the saturation binding isotherms of [^{3}H]L-659,989 in the presence of 150 mM NaCl and 1 mM GTP, C_{16}-PAF alters the apparent dissociation constant (K_D') of [^{3}H]L-659,989 to its receptor but not the intercept at the x-axis or the maximal number of receptor sites (B_{max}). The equilibrium dissociation constant (K_B) of the unlabeled C_{16}-PAF could thus be determined indirectly from the Schild plot and is found to be 0.93 μM in the presence of 150 mM NaCl and 1 mM GTP (13), which is more than 1,000-fold higher than the K_D value (0.53 nM) obtained directly from the C_{16}-PAF binding isotherms in 10 mM $MgCl_2$ (16).

We conclude that PAF receptors exist in several distinct conformational forms with different affinity either to PAF or to a PAF receptor antagonist, L-659,989. Figure 4 shows a schematic representation. R_o is the state of the receptor with no addition of ions which shows low affinity for both [^{3}H]PAF and [^{3}H]L-659,989. Mg^{2+} and possibly other divalent cations, such as Ca^{2+} and Mg^{2+}, convert the receptor from R_o to $R_{Mg^{2+}}$ state, possibly through the interaction between PAF receptor and guanine nucleotide-binding protein, which is unrelated to the calmodulin system (17). $R_{Mg^{2+}}$ is the state with a high affinity for both PAF and L-659,989 (K_D = 0.53 nM and 1.60 nM, respectively). GTP in the presence of Mg^{2+} converts $R_{Mg^{2+}}$ into $R_{Mg^{2+}\cdot GTP}$, which has a lower affinity for PAF but not for L-659,989. Na^+ and

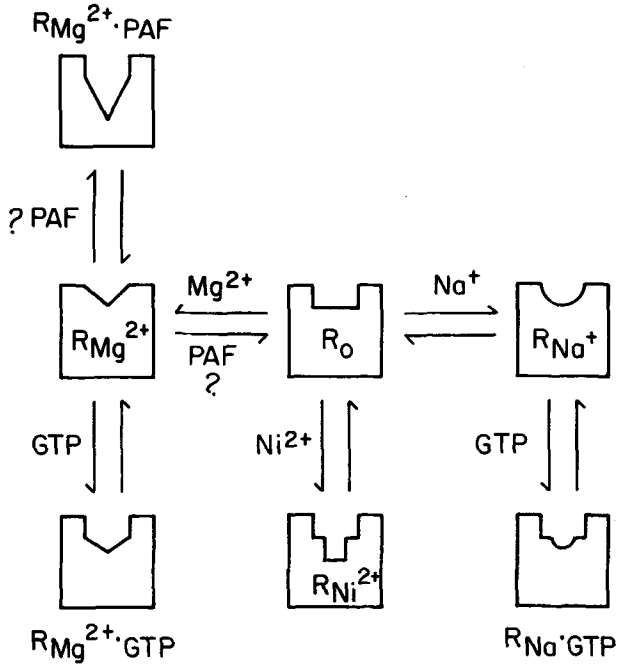


FIG. 4. Possible conformational states of PAF receptors in rabbit platelet membranes (13).

possibly Li^+ bind to the cytoplasmic side of plasma membrane and convert R_o into R_{Na^+}, which shows low affinity for PAF (K_D in the submicromolar range) but high affinity for L-659,989 (K_D = 1.5 nM). GTP can similarly transform R_{Na^+} into $R_{Na^+ \cdot GTP}$, possibly the lowest affinity state for PAF (K_D = 0.93 μM). L-659,989 shows similar affinity for receptor states in the forms of $R_{Mg^{2+}}$, $R_{Mg^{2+} \cdot GTP}$, R_{Na^+}, and $R_{Na^+ \cdot GTP}$ but shows low affinity for R_o. PAF could induce a conformational change of the receptor, possibly through the interaction between the receptor and guanine nucleotide-binding protein, resulting in a transition from $R_{Mg^{2+}}$ to either R_o or $R_{Mg^{2+} \cdot PAF}$, another conformation state of the receptor, which shows low affinity for L-659,989. Ni^{2+} could possibly induce another conformation of the receptor $R_{Ni^{2+}}$.

O'Flaherty *et al.* (22) recently reported that phorbol myristate acetate (PMA) reduced the availability of high affinity receptors for PAF in PMNs. Also, depletion of high affinity receptors for PAF paralleled inhibition of PAF-induced increase in cytosolic calcium. Losses in high affinity receptors may therefore be responsible for reducing the calcium mobilization response (12), and multiple conformational states of receptors may regulate the function of PAF.

CONCLUSIONS

L-659,989 is a potent, specific and competitive receptor antagonist. With the combined use of [^{3}H]PAF and [^{3}H]-L-659,989, we have demonstrated that PAF itself can induce a conformational change of its receptor and that the PAF receptor can exist in multiple conformational states which could possibly regulate and mediate the function of PAF.

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REVIEW

Chemical and Biochemical Characterization of Lignan Analogs as Novel PAF Receptor Antagonists[1]

T.Y. Shen
Department of Chemistry, University of Virginia, Charlottesville, Virginia 22901

Various derivatives and isosteres of neolignans of the 2,5-diaryl tetrahydrofuran type have been synthesized as antagonists of platelet-activating factor (PAF). A detailed analysis of their structure-activity relationship (SAR) has revealed a clear preference for an asymmetrical molecular configuration with a high degree of stereo and chiral specificity associated with greater potency. The *trans*-2S,5S enantiomers are generally 10–200 times more potent *in vitro* than their corresponding *cis* or *trans*-2R,5R isomers. A similar stereochemical preference is indicated by the recently reported PAF antagonist MK-287 which has undergone clinical evaluation. An azido derivative L-662,025 has been characterized as a photolabile irreversible antagonist of PAF for the investigation of solubilized and partially purified PAF binding proteins from cell membranes. The biological justification for concomitant inhibition of both PAF receptor and 5-lipoxygenase in inflammation is well recognized. The feasibility of developing such dual-functional agents has been demonstrated by a group of dithiolane analogs of neolignans and several derivatives of futoenone.
***Lipids* 26, 1154–1156 (1991).**

With therapeutic applications of platelet-activating factor (PAF) antagonists closer to reality, increasing attention is being given to further improvement of the antagonists' potency, pharmacodynamics and, possibly, tissue selectivity. Following the initial discovery of CV-3988 and kadsurenone, and the subsequent identification of L-652,731, ginkgolide B (BN 52021) and 48,740 R.P. as moderately active, specific receptor antagonists (1,2), a wide variety of chemical structures have been found to possess similar properties, several of which are active *in vitro* at nM concentrations and are undergoing clinical evaluations (3). In conjunction with a better understanding of the cellular biology of PAF (for reviews, see refs. 4,5), classification of PAF receptors and antagonists into subtypes may now be possible according to specific criteria, such as the relative receptor affinity for PAF and antagonists, the influence of metal ions on ligand binding to the receptor, and the differential effects of various inhibitors of the signal transduction mechanism on receptor function (6,7). Irreversible PAF antagonists have now been developed as biochemical probes for the characterization of PAF receptors. Based on the structure-activity relationship of PAF antagonists of the lignan type, some progress has also been made toward the development of dual-functional inhibitors of both PAF and 5-lipoxygenase (5-LO) as potentially more effective anti-inflammatory and anti-allergic agents.

STRUCTURE-ACTIVITY RELATIONSHIP OF LIGNAN ANALOGS

The structure-activity relationship (SAR) of receptor agonists and specific antagonists may reflect the topography of the receptor binding domain. In a detailed SAR analysis of the lignan-type PAF antagonists, we have previously noted that there is a high degree of stereospecificity in receptor blockade. For both kadsurenone (8) and 2,5-diaryl-tetrahydrofuran analogs (9), the *trans* isomers are generally more potent than the corresponding *cis* isomers. Similar correlations have since been extended to other isosteres of lignans with terahydrothiophene, cyclopentane, dioxolane and dithiolane ring systems (Fig. 1).

The asymmetrical configuration of the more potent antagonists, which is presumably complementary to a high affinity receptor binding site, is clearly indicated by two lignan derivatives, L-659,989 (10) in Figure 2 and its β-hydroxyethylsulfonyl analog L-680,573 (MK-287) (11), which are 10 times more potent than the symmetrical prototype L-652,731. The chirality of the molecule is also important. In a comparison of several pairs of optical isomers, which were obtained either by resolution of the racemic mixture on a chirapack column in high-performance liquid chromatography (HPLC) or through stereospecific synthesis using newly developed chiral reducing reagents (12,13), the 2S,5S-enantiomers are more active than their mirror images, *i.e.*, the 2R,5R-enantiomers. The potency ratio of S,S- over R,R-enantiomer is higher for antagonists with greater potency. For example, the K_i value of the (−) 2S,5S-isomer of

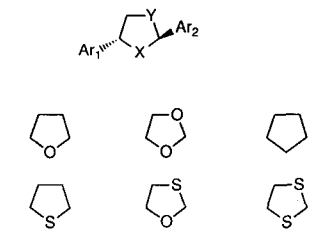


FIG. 1. Isosteres of 3,5-diaryl-tetrahydrofuran lignans possessing PAF antagonistic activities. The X and Y in the center ring may be oxygen (tetrahydrofuran, dioxolane), carbon (cyclopentane) and sulfur (tetrahydrothiophene, oxathiolane, dithiolane) as shown from left to right. Ar_1 and Ar_2 are substituted phenyl groups.

[1]Based on a paper presented at the Third International Conference on Platelet-Activating Factor and Structurally Related Alkyl Ether Lipids, Tokyo, Japan, May 1989.

Abbreviations: HPLC, high-performance liquid chromatography; LO, lipoxygenase; PAF, platelet-activating factor, 1-*O*-alkyl-2-acetyl-*sn*-glycero-3-phosphocholine; SAR, structure-activity relationship.

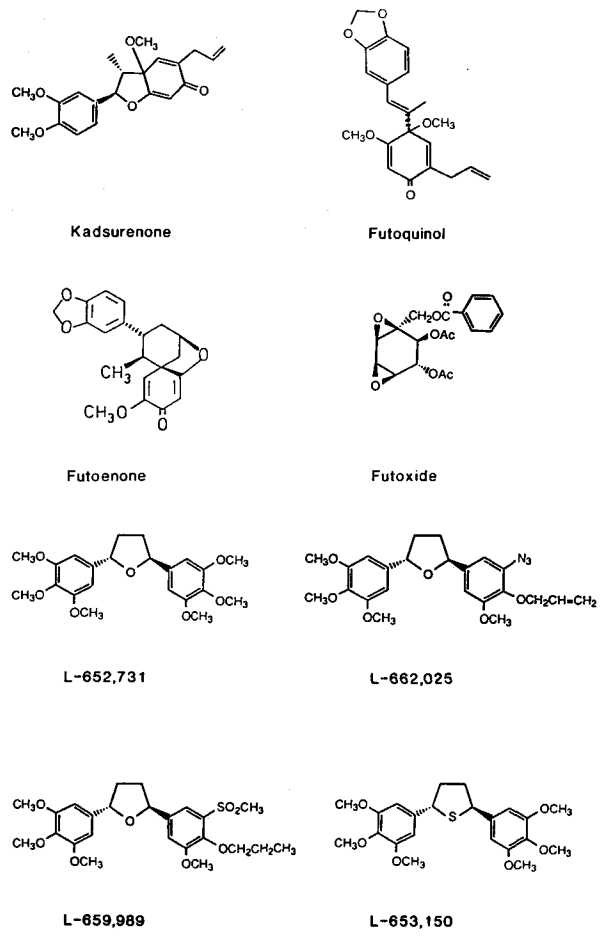


FIG. 2. Chemical formulae of neolignans and synthetic analogs which are PAF antagonists.

L-659,989 (1 nM) is 20–30 times lower than that of the (+) 2R,5R-isomer (14). The geometry of the binding domain near the tetrahydrofuran ring oxygen was clarified by a recent report (15) on a series of highly potent cyclopentane analogs of L-659-989 (Fig. 3). The IC_{50} value of two derivatives with R being α-allyloxy and α-propargyloxy analogs in the human platelet aggregation assay are in the range of 4 nM, nearly 10 times more potent than L-659,989. Based on X-ray crystallographic studies, the compound with an α-propargyloxy substituent in the center of the cyclopentane ring is 15 times more active than its β-isomer. The analog with a smaller α-methoxy substituent is less active, but it is still more potent than L-659,989 which has the ether group in the ring. It appears that at this position there is a deep hydrophobic trough that is asymmetrically oriented and thus can discriminate against the β-isomer.

Obviously, for this series of lignan-type PAF antagonists, especially for those with greater affinity for the receptor, the hypothetical "receptor contour" is highly stereospecific and significantly different from the symmetrical "ear-muffs" model described recently (16), which seems to accommodate some general features of kadsurenone, BN 52021 and other less active ginkgolides better.

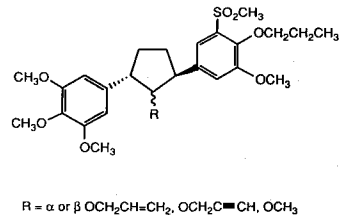


FIG. 3. Potent cyclopentane analogs of neolignans.

SOLUBILIZATION AND PARTIAL PURIFICATION OF PAF BINDING PROTEINS

In an attempt to elucidate the structural features of the binding domain of the PAF receptor more definitely, we have solubilized the PAF binding proteins of human platelet and bovine lung with 1% w/v digitonin and significantly increased their specific binding to [^{3}H]PAF through partial purification by ion exchange and affinity chromatography. Similar solubilization and purification conditions have been reported (17,18). Further chemical and biochemical characterization of purified binding proteins may help to clarify the subtype diversity of PAF receptors and antagonists.

As a research probe of PAF receptor and a specific marker in the identification of PAF binding proteins, an azido analog (L-662,025) of the antagonist L-659,989 was recently characterized as a photo-activatable, irreversible PAF antagonist (19). Upon irradiation at 260–320 nm for 15 min, L-662,025 selectively and irreversibly inactivates the PAF receptor on human platelets or in solubilized membrane preparations from bovine lung. As the PAF receptor from the guinea pig lung was cloned and expressed most recently (20), it would be interesting to identify the binding domains of PAF and its antagonists, and shed further light on the molecular interactions involved.

PAF ANTAGONISTS WITH DUAL ACTIONS

The synergism and potentiation of PAF with other inflammatory mediators are well recognized. To achieve greater therapeutic efficacy in clinical disorders such as asthma, which is mediated by both PAF as well as leukotrienes, there are ample biological justification and experimental data to support the use of a mixture of two agents (21), or preferably a single compound, which can inhibit the synthesis or function of PAF and leukotrienes. The search for such dual-function inhibitors of two inflammatory mediators is reminiscent of the recent study of dual inhibitors of both cyclooxygenase and 5-lipoxygenase as new antiarthritic agents.

We have previously shown that it is feasible to achieve both PAF antagonism and 5-lipoxygenase inhibition with one chemical structure. The tetrahydrothiophene analog L-653,150 (Fig. 2) was an early example (1). Recently, we have found that the 1,3-dithiolane analog of L-653,150 possesses even greater potency in blocking PAF-induced

aggregation of human platelets and the biosynthesis of LTB_4 by calcium ionophore-stimulated human monocytes at micromolar concentrations (22). In addition, three other constituents of the Chinese herbal plant *piper futokadsura*, futoxide, futoenone and futoquinol (Fig. 2) have recently been found to be effective PAF antagonists. The latter two are also useful as intermediates in the synthesis of dual PAF and 5-lipoxygenase inhibitors (23).

In summary, some progress has been made in the synthesis of lignan analogs as potent PAF antagonists, photoactivatable receptor probe, and dual functional inhibitors. Encouraged by the recent structure elucidation of a PAF receptor protein (20), further chemical and biological studies may facilitate the discovery of second generation PAF antagonists with greater potency, dual actions and possibly tissue selectivity.

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REVIEW

Thieno-triazolo-1,4-diazepines as Antagonists of Platelet-Activating Factor: Present Status[1]

Jorge Casals-Stenzel*
Boehringer Ingelheim KG, 6507 Ingelheim am Rhein, Germany

The platelet-activating factor, 1-*O*-alkyl-2-acetyl-*sn*-glycero-3-phosphocholine, (PAF) antagonistic activity of thienotriazolodiazepines has recently been described. The lead compound in this series was brotizolam, which also exhibits sedative and hypnotic effects. By combination of brotizolam with the benzodiazepine receptor antagonist RO 15-1788, PAF antagonistic and central nervous system (CNS) activities could be segregated. Systematic structure variation has led to potent and selective PAF antagonists without CNS effects. WEB 2086 and its analogues WEB 2170 and STY 2108 are representative examples of this structural type and have shown a high potency and selectivity in PAF-induced and PAF-dependent *in vitro* tests and in experimental models. Studies in healthy volunteers have demonstrated potent pharmacological activity and good safety and tolerance of oral, intravenous or inhaled WEB 2086 in man. These agents should therefore prove useful for the further elucidation of the pathophysiological role of PAF and provide an opportunity for therapeutic applications in diseases in which the involvement of PAF has been implicated.
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Platelet-activating factor (PAF, 1-*O*-alkyl-2-acetyl-*sn*-glycero-3-phosphocholine) was first described by Benveniste *et al.* in 1972 (1). In the following decade, a variety of physiological and pharmacological responses *in vitro* and *in vivo* have been attributed to PAF (2,3). However, the pathophysiological role of PAF remained poorly understood. Thus, a search for specific PAF antagonists became essential for the further elucidation of the functional significance of PAF.

Basically three types of compounds of different origin and structure have been shown to act as PAF antagonists: (i) PAF analogues (*e.g.* CV-3988); (ii) natural products (*e.g.* kadsurenone, and BN 52 021); and (iii) other synthetic compounds with a chemical structure different from that of PAF (*e.g.* the thieno-triazolo-1,4-diazepines, such as brotizolam and WEB 2086) (4).

The thieno-triazolo-1,4-diazepines were already known as powerful sedative and hypnotic agents for some 15 years (5). In 1983, the PAF antagonistic activity of this type of substances, benzo- as well as thieno-triazolo-1,4-diazepines, was first observed in the laboratories of Boehringer Ingelheim (Ingelheim, Germany). Using the PAF-induced human platelet aggregation assay and by screening about 200 carefully selected compounds with different structures and pharmacological properties, it was possible to select certain substances, such as calcium antagonists and benzodiazepines, which showed PAF antagonism (6–8). Among the benzodiazepines, the most powerful compounds were those containing an annelated triazolo ring (9). Triazolam and alprazolam, two *benzo*-triazolo-1,4-diazepines, and particularly brotizolam, a *thieno*-triazolo-1,4-diazepine, which was synthetized by Weber *et al.* (10) at Boehringer Ingelheim some years earlier, showed pronounced and specific PAF antagonistic activities in these first *in vitro* tests (9). These observations were the key to the development of the thieno-triazolo-1,4-diazepines as PAF antagonists. At approximately the same time, Kornecki *et al.* (11) described the triazolobenzodiazepines which also acted as antagonists to PAF.

A major problem with the diazepine derivatives with specific PAF antagonistic activity *in vitro* was their potent sedative and hypnotic activity. In the first experiments that were carried out to demonstrate anti-PAF activity *in vivo*, the animals died due to apnoea induced by the relatively high triazolam or brotizolam doses needed in addition to urethane anaesthesia (9). The question arose whether the effect on the central nervous system (CNS) and the PAF antagonistic activity of these compounds would be associated with the same structural features of the compounds. Clarification of this question was the first step toward the development and the successful application of the thieno-triazolo-1,4-diazepines as PAF antagonists.

DISSOCIATION OF CNS AND ANTI-PAF ACTIVITY

Central and peripheral type benzodiazepine (BDZ) receptors, as well as PAF binding sites in human and animal tissues, were already known before the anti-PAF activity of BDZ was described (12–14). The possibility that the PAF antagonistic effects of the *thieno*- and *benzo*-triazolo-1,4-diazepines would be mediated by a central or peripheral type BDZ receptor was examined using the specific BDZ receptor ligands RO 15-1788 (central antagonist) and RO 5-4864 (peripheral ligand) in combination with brotizolam and triazolam. It was expected that the BDZ antagonist would counteract the typical BDZ and CNS activities of these drugs.

In vitro, RO 15-1788 inhibited the platelet aggregatory activity of PAF at relatively high concentrations. When RO 15-1788 at a concentration of 10–1000 μM was combined with brotizolam (1 μM) or triazolam (20 μM), the PAF antagonistic activity of either compound was not only not inhibited, but was in fact increased (15). These findings were confirmed by *in vivo* experiments in guinea pigs. The PAF antagonistic activity of brotizolam (10 mg/kg p.o.) and triazolam (200 mg/kg p.o.) was not affected but actually (numerically) enhanced when either compound was used in combination with RO 15-1788 (20–100

[1]Based on a paper presented at the Third International Conference on Platelet-Activating Factor and Structurally Related Alkyl Ether Lipids, Tokyo, Japan, May 1989.

*To whom correspondence should be addressed at Starnbergerstrasse 17, 8000 München 71, Germany.

Abbreviations: ADP, adenosine diphosphate; BDZ, benzodiazepine; CNS, central nervous system; ConA, concanavalin A; FMLP, *N*-formyl-L-methionyl-L-leucyl-L-phenylalanine; IC_{50}, inhibitory concentration 50%; LD_{50}, lethal dose 50%; LTB_4, leukotriene B_4; PAF, platelet-activating factor, 1-*O*-alkyl-2-acetyl-*sn*-glycero-3-phosphocholine.

or 400 mg/kg p.o., respectively) in a ratio of 1:2 or 1:10 (agonist/antagonist). By comparison, the BDZ antagonist, RO 15-1788, clearly blocked the motor depressant and hypnotic effect of these triazolodiazepines in guinea pigs. RO 15-1788 itself, given at high doses (100 mg/kg p.o. or 10 mg/kg i.v.), did not prevent the lethal effect of PAF.

The relationship between the peripheral-type BDZ receptors, RO 5-4864, and the activity of PAF on platelets was also studied *in vitro*. RO 5-4864 had virtually no effect on PAF-induced human platelet aggregation and did not inhibit the PAF antagonistic activity of brotizolam (15). Consequently, these studies showed that the PAF antagonistic activity of triazolo-diazepines in general, and thieno-triazolo-1,4-diazepines in particular, is independent of their activity on the central or peripheral BDZ binding sites. This clear segregation of PAF-antagonistic activity and CNS activity allowed the development of new thieno-triazolo-1,4-diazepine derivatives with potent PAF antagonistic properties.

SYNTHESIS OF NEW COMPOUNDS: WEB 2086

Brotizolam (1) (inhibitory concentration 50% (IC_{50}) 0.54 μM) and its alkyl analog etizolam (2) (IC_{50} 0.5 μM) (16) served as lead compounds for the search and synthesis of PAF antagonists without CNS effects (Fig. 1). After systematic structural variations, a series of new compounds were found which proved to be highly potent PAF antagonists (Table 1).

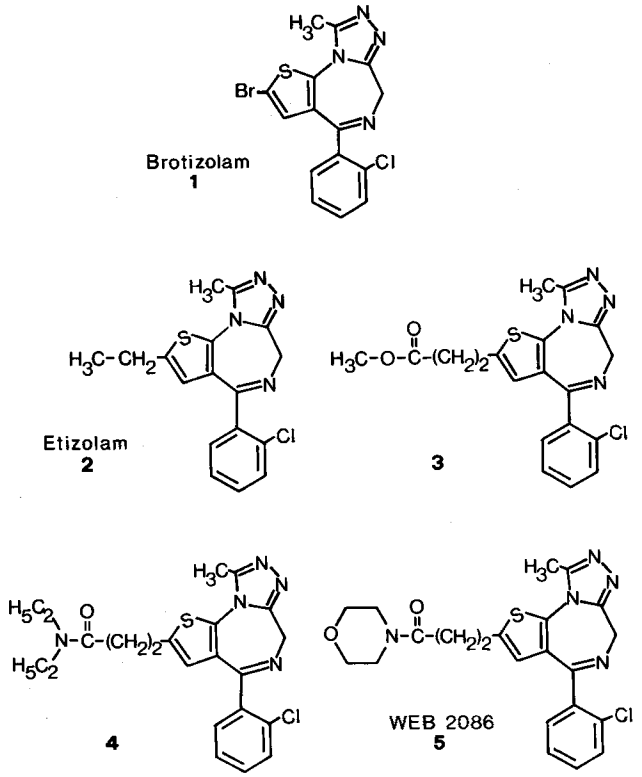


FIG. 1. Brotizolam (1) and etizolam (2) served as lead compounds for the synthesis of WEB 2086 (5). Compounds 3 and 4 are conceptual steps between 1 and 5.

TABLE 1

Relative Potencies of PAF Antagonists of Different Sources in Comparison to Thieno-triazolo-1,4-diazepines[a]

PAF antagonist	IC_{50} (μM)
Thieno-triazolo-1,4-diazepines	
Brotizolam	0.54
WEB 2086	0.17
WEB 2170	0.32
WEB 2347	0.30
Benzo-triazolo-1,4-diazepines	
Triazolam	7.6
Alprazolam	13.7
PAF derivatives	
CV-3988	46.2
RO 19-3704	0.83
Natural products	
Ginkgolide B	0.82
Kadsurenone	6.7
Other synthetic compounds	
L 652,731	1.3
48 740 RP	48.7

[a]Inhibition of PAF (5×10^{-8}M)-induced human platelet aggregation.

The replacement of the ethyl side chain at position-2 of etizolam (2) by an alkoxycarbonyl or ester function led to compound (3) which had encouraging anti-PAF activity *in vitro* (IC_{50} 0.21 μM). Unfortunately, compound (3) was completely ineffective *in vivo*. This was explained by hydrolitic cleavage of the ester side chain *in vivo*. A corresponding analog with a free propionic acid side chain at position-2 was inactive in the *in vitro* test.

The ester function was replaced by a carboxamide alkyl function which resulted in the water soluble derivative (4) (IC_{50} 0.72 μM). Because of its high selectivity and potency *in vitro* and *in vivo*, it became the first candidate for further pharmacological characterization. The side chain at position-2 not only increased water solubility of the compound but was also responsible for its lack of CNS side effects. Several potent amides were synthesized and characterized pharmacologically (17). Among them, WEB 2086 (5) became the final candidate for drug development (18). The introduction of this compound in PAF research set new standards in this field (19), and stimulated broad interest in the synthesis of closely related structures.

At Boehringer Ingelheim, a second series of very active compounds was obtained by ring closure (6) between the side chain at position-2 and the thiophene ring. The resulting new tetracyclic compounds (Sty 2108 (7), WEB 2170 (8) and WEB 2347 (9)) show lower BDZ receptor affinity than the tricyclic thieno-triazolo-1,4-diazepines (Fig. 2) (20,21). These tetracyclic compounds possess a chiral center in the new ring and were prepared in racemic form. In WEB 2170, the chiral carboxamide side chain has a high degree of stereospecificity: the S (−) optical enantiomer (K_i 14 nM) proved much more active than the R (+) isomer (K_i 660 nM) (22).

Other substituents at position-2 have recently been introduced. An aralkyl moiety (isobutyl-phenyl-ethyl, Y-24180) (10) also improved PAF activity and reduced CNS effects (23). In addition, side chains consisting of aryl or bi- and tricyclic heterocyclic moieties bound to the

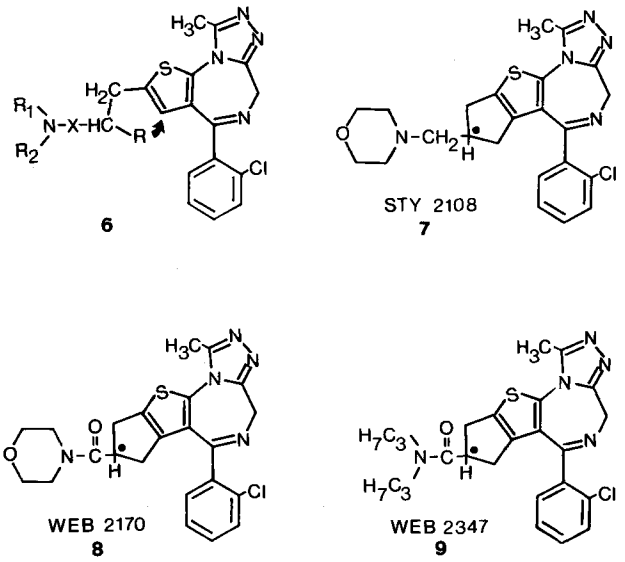


FIG. 2. Tetracyclic thieno-triazolo-1,4-diazepines: Structure 6 represents schematically the ring closure between the side chain and the thiophene ring. Compounds 7 to 9 are new tetracyclic PAF antagonists.

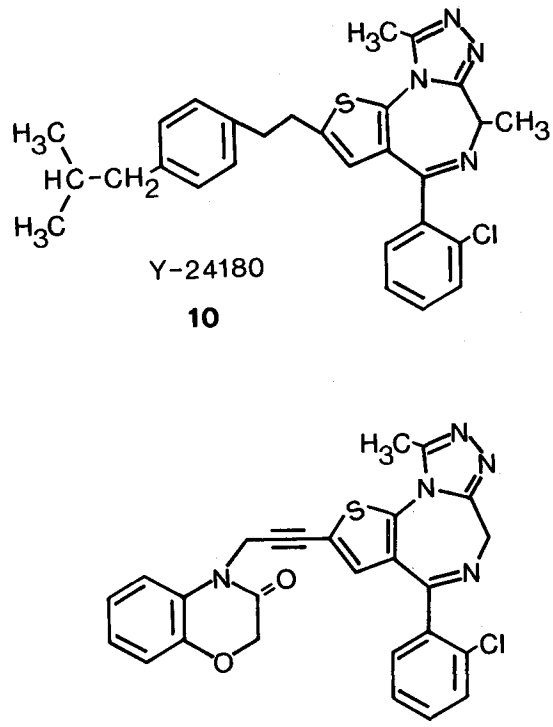


FIG. 3. Other thieno-triazolo-1,4-diazepines with different substituents at position-2.

2-position of the thiophene ring by a propynyl bridge (11) led to powerful PAF-antagonists (24) (Fig. 3).

Other compounds of this family are likely to emerge in the future.

STRUCTURE ACTIVITY RELATIONSHIP

Some important and basic findings in regard to the structure-activity relationship of thieno-triazolo-1,4-diazepines as PAF antagonists are summarized in Figure 4. The abbreviated scheme describes a simplified situation, but it focuses on the possibility of producing potent PAF antagonists without CNS effects.

It is apparent that changes at the 1,4-diazepine and chlorophenyl moiety are very poorly tolerated in respect to the PAF antagonistic effect. For example the substitution of the chlorine in triazolam (IC_{50} 7.6 μM) by a hydrogen (alprazolam, IC_{50} 13.7 μM) reduces anti-PAF activity (9). By contrast, the introduction of a methyl group in position-6 at the diazepine ring can increase anti-PAF potency. But in general, major changes in these structures will reduce or completely abolish the activity.

The 1,2,4-triazolo or an imidazole ring is essential for anti-PAF activity, *i.e.* for affinity to the PAF-receptor. Diazepines without such rings have little or no effect against PAF (8,9,21). Moreover, the substituent at 9-position (R_1 in Fig. 4) drastically influences the affinity to the PAF receptor, and in consequence the PAF antagonistic activity depends on the bulkiness of the substituent (25). Small substituents such as methyl, bromo, ethyl and cyclo-propyl groups appear optimal; the hydrogen analogues possess a lower affinity in comparison to the methyl-substituted compounds (19,21).

The thiophene ring itself is not essential for anti-PAF activity, but it improves it. This feature is well illustrated by comparing triazolam with brotizolam. Compounds with a benzene ring at this position are up to 20 times less active than the thieno derivatives (9).

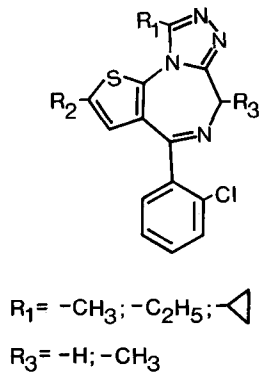


FIG. 4. Structure/activity relationship of thieno-triazolo-1,4-diazepines as PAF antagonists: R_1 is essential for affinity to the PAF receptor but not to the BDZ receptor. Optimal substituents are shown above. R_2 is important for dissociation of CNS and anti-PAF activity, for the increase of anti-PAF potency and water solubility. Changes at R_3 generally cause loss of activity. Optimal substituents are shown in the figure.

The substituent at position-2 of the thiophene ring (R_2 in Fig. 4) is essential for several reasons. It is responsible for the dissociation of PAF antagonistic from CNS activity which is achieved by a complete lack of, or by a low affinity to, the central type BDZ receptor (17,26), causing a corresponding decrease or abolition of the CNS side effects. Furthermore, the water solubility of the thieno-triazolo-1,4-diazepine molecule can be improved, and its lipophilicity can be reduced resulting in low partition coefficients (log D for brotizolam 2.44, WEB 2086 0.8 and

WEB 2170 1.0). Reduced lipophilicity is important to minimize penetration of this type of compounds through the blood-brain barrier. The search for the optimal and most potent substituent at position-2 of the thiophene ring continues. It was evident from the beginning that in principle various types of substitution at the 2-position of the thiophene ring would result in potent PAF antagonists with more or less dissociated CNS effects (17,26). Although there are numerous possibilities for the synthesis of PAF antagonists with a thieno-triazolo-1,4-diazepine structure, one would not expect that upcoming new compounds will add superior insights to the basic findings described (17,21).

SELECTIVITY FOR THE PAF RECEPTOR

Thieno-triazolo-1,4-diazepines generally show a high affinity for the central type BDZ receptor. By introduction of bulky substituents in position-2 of the thiophene ring, it is possible to separate PAF-receptor binding from BDZ-receptor binding in ratios up to 1:5000, *e.g.* in the tetracyclic compound WEB 2347 (20). In general the tetracyclic compounds have lower BDZ receptor affinity than the tricyclic thieno-triazolo-1,4-diazepines. A simple elongation of the side chain further increases the affinity for this receptor (17,26).

It has been shown that the PAF antagonistic activity of WEB 2086 is specific and competitive (27). In PAF-induced human platelet or neutrophil aggregation experiments *in vitro*, the thieno-triazolo-1,4-diazepines (brotizolam, WEB 2086, etc.) and the benzo derivatives (triazolam, alprazolam) had little or no effect on the cellular responses to other aggregating agents, such as ADP, arachidonic acid, epinephrine, and collagen in the platelet aggregation experiments and Concanavalin A (Con A), *N*-formyl-L-methionyl-L-leucyl-L-phenylalanine (FMLP), and leukotriene B_4 (LTB_4), in the neutrophil aggregation tests.

The competitive character of this antagonism has been shown in platelet aggregation and binding studies. In PAF-induced human platelet aggregation *in vitro*, concentration/response curves of WEB 2086 were shifted to the right in a parallel manner by increasing concentrations of PAF (28,29). In binding studies performed on human and rabbit platelets, specific binding and competitive displacement of WEB 2086 from the PAF receptor was shown. WEB 2086 inhibits [^{3}H]PAF binding at the PAF receptor while [^{3}H]WEB 2086 is displaced by PAF and WEB 2086 (27,28,30).

The specific interaction of WEB 2086 with the PAF receptor has also been shown in other cell types (eosinophils, endothelial cells, lung, etc.) (31–33).

PHARMACOLOGY IN ANIMALS AND MAN

The pharmacological properties of the thieno-triazolo-1,4-diazepines in respect to their PAF antagonism have been described in detail (9,18,34–36). The *in vitro* and *in vivo* pharmacologies of the PAF antagonists known so far, regardless of their source or structure, are very similar, particularly in experiments where exogenous PAF is used as inducer.

Nevertheless, there are three features which distinguish these compounds from other antagonists. First, the potency of this group is generally very high; *in vitro*, the concentrations needed are in the nM range (Table 1). *In vivo* the very low doses reach the μg range per kg body weight (18,19). Secondly, these compounds have a long duration of action with biological half-lives ($t_{1/2}$) of up to 15 hr depending on the route of administration (19). Thirdly, the thieno-triazolo-1,4-diazepines are active orally, intravenously and after inhalation (9,18,19). In guinea pigs, the oral dose-range is about 10 times higher than the intravenous one. The bioavailability of WEB 2086 is very good, independently of the route of administration. Bronchoconstriction induced by intravenous PAF in guinea pigs can be blocked by inhaled WEB 2086 and *vice versa* (18). Oral WEB 2086 antagonizes both intravenously administered and inhaled PAF.

Thieno-triazolo-1,4-diazepines are essentially non-toxic. The lethal dose 50% (LD_{50}) values of WEB 2086 in mice were very high (4600 mg/kg p.o. and 540 mg/kg i.v.) (18). In chronic toxicity studies, WEB 2086 did not cause any problems which would argue against clinical trials with this compound. In man, some double-blind and placebo controlled tolerance studies have been performed with WEB 2086 (37–39). Healthy volunteers received WEB 2086 either orally (1.25–400 mg), intravenously (0.5–50 mg) or by inhalation (0.5 and 1 mg) in several doses. During the following 24 hr, inhibition of PAF-induced platelet aggregation *ex vivo* was recorded as an index of pharmacological action. The PAF-antagonistic activity *ex vivo* was dose-dependent and maximal at doses of 20 mg p.o., 10 mg i.v., and 1 mg inhaled. No side effects, particularly on the central nervous system, were observed, thus confirming the dissociation of anti-PAF and CNS effects in man. Therefore, in man as well, WEB 2086 shows a high potency, good tolerance and is safety independent of the route of administration.

CONCLUSION

The synthesis and production of thieno-triazolo-1,4-diazepines with potent anti-PAF activity, but free from CNS side effects, can now be carried out without difficulty. PAF antagonists of this family, such as the well known WEB 2086 or the newer compounds WEB 2170 and WEB 2374, are very potent, highly specific, long acting, non-toxic and, as known to date, well-tolerated substances in animal and man. Hence, potential drugs with almost ideal features are available. Results from animal experiments suggest that PAF antagonists have a beneficial effect in diseases such as endotoxic shock and anaphylaxis (34,35).

For biochemical and pharmacological purposes there appears to be no need for further compounds of this type. Hence, all efforts should be concentrated on well-conducted clinical studies which can prove the utility of these compounds in man.

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REVIEW

PAF Receptor Structure: A Hypothesis[1]

J.-J. Godfroid[a,*], G. Dive[b], J. Lamotte-Brasseur[b], J.-P. Batt[a] and F. Heymans[a]

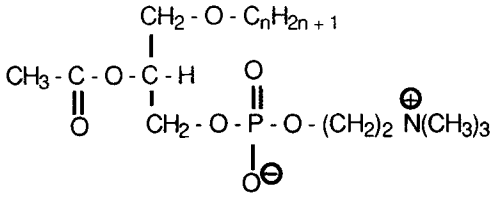
[a]Laboratoire de Pharmacochimie Moléculaire, Université Paris 7, Paris, France and [b]Centre d'Ingéniérie des Protéines, Institut de Chimie, Liège-Sart-Tilman, Belgium

Different hypotheses of the structure of platelet-activating factor (PAF) receptor based on structure-activity relationships of agonists and antagonists are reviewed. For an agonistic effect, strong hydrophobic interactions and an ether function are required in position-1 of the glycerol backbone; chain length limitations and steric hindrance demand a small group in position-2. The unusual structural properties of non-PAF-like antagonists required 3-D electrostatic potential calculations. This method applied to seven potent antagonists suggests a strong "Cache-oreilles" (ear-muff) effect, *i.e.*, two strong electronegative wells (isocontour at −10 Kcal/mole) are located at 180° to each other and at a relatively constant distance. Initial consideration of the "Cache-oreilles" effect implied the structure of a bipolarized cylinder of 10–12 Å diameter for the receptor. However, very recent results on studies with agonists and antagonists structurally similar to PAF suggest that the receptor may in fact be a multi-polarized cylinder.
***Lipids 26*, 1162–1166 (1991).**

The diverse structural properties of platelet-activating factor (PAF) antagonists (reviewed in refs. 1–4) and the existence of specific high affinity binding sites for the mediator (reviewed in ref. 5) invite a comparison of the common electronic features of non-PAF-like molecules. In this review we will examine the structural requirements for potent agonistic activity (2,6) and the 3-D electrostatic potential maps of non-PAF-like high potency antagonists selected for their diversity in chemical structure (7). We will also present a simplified model of the PAF receptor.

PAF RECEPTOR AGONISTS

A wide variety of PAF analogs have been synthesized to establish the structural requirements for potent PAF-like activity (reviewed in refs. 2, 3, 6 and 8) and to distinguish antagonist from agonistic activities. The existence of specific binding sites is consistent with the enantiospecificity of platelet stimulation. Synthetic PAF in its natural R configuration (n = 16 and 18) is 1000 times more potent than its S enantiomer (9–12).



The effects of modifying the PAF structure at the three positions of the glycerol backbone have been investigated. The hydrophobicity of the ether chain in position-1 is one of the key factors that permits interaction of PAF with its receptor (13; reviewed in ref. 6). Godfroid and coworkers (13,14; reviewed in ref. 8) have demonstrated by quantitative structure activity relationship (QSAR) calculations that regression analyses performed with data for 15 PAF analogs are significant with respect to a parabolic correlation between lipophilicity of the chain and platelet stimulation. This result is characteristic of a hydrophobic interaction between the agonist and the PAF receptor. Maximum activity corresponds to the lipophilicity of a C_{16}–C_{18} linear chain. The interaction energy is estimated to be 11–13 Kcal/mole. The ether function in position-1 is an absolute requirement for platelet stimulating potency (13), and changes in the steric effect around the glycerol skeleton decrease or nullify agonistic activity (reviewed in ref. 8).

In position-2, steric hindrance limits chain length to small groups (reviewed in refs. 2, 3, 6 and 8), such as *N*-methylcarbamate, propionate or ethoxy. For example, the butanoyl analog shows only residual aggregating activity amounting to 10% of that of PAF (11; reviewed in ref. 8).

In position-3, replacement of the phosphate group by a phosphonate, carboxylate or ether group decreases or abolishes platelet stimulation (reviewed in ref. 1–4, 6, 8 and 15). Agonistic activity rapidly decreases with the number of methylenes inserted between the phosphate group and the ammonium function, while antagonistic activity appears and becomes maximal with six methylenes inserted (16 and 17; reviewed in ref. 8). The latter features are observed in some (18), but not in all (19), series of PAF analogs. Replacement of trimethylammonium of PAF by triethylammonium, methylpiperidinium, methylpyrrolidinium or other bulky or hydrophobic groups enhances the stimulatory effect on platelets above that of PAF itself (20–22; reviewed in refs. 6 and 8).

3-D ELECTROSTATIC MAPS OF SELECTED PAF ANTAGONISTS: THE "CACHE-OREILLES" EFFECT

Godfroid and Braquet (6) and Braquet and Godfroid (2) initially presented a model of interaction at the binding site which suggested a probable role of the oxygen doublets of the ether group of PAF (agonist) as a site of protonation *vs.* those present in antagonists, such as kadsurenone, gingkolides and neolignan derivatives, which

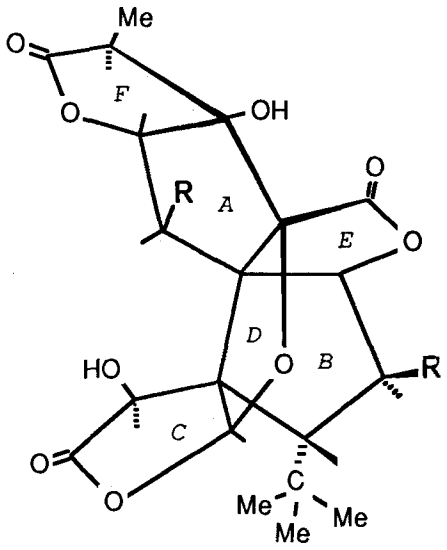
[1]Based on a paper presented at the Third International Conference on Platelet-Activating Factor and Structurally Related Alkyl Ether Lipids, Tokyo, Japan, May 1989.

*To whom correspondence should be addressed at Laboratoire de Pharmacochimie Moléculaire, Université Paris 7, 2, Place Jussieu, 75251 Paris Cedex 05, France.

Abbreviations: 3-D, three-dimensional (electrostatic potential map); PAF, platelet-activating factor; QSAR, quantitative structure activity relationship.

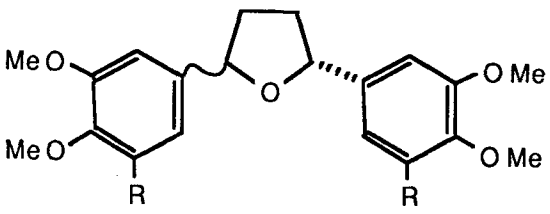
possess a tetrahydrofuran ring. These oxygens were postulated to function as key anchor points for the interaction with the receptor. The structural diversity of synthetic and natural PAF antagonists, including examples without oxygenated rings, which have since appeared in the literature (reviewed in refs. 3 and 4), required a new approach to account for more subtle structural similarities.

The 3-D electrostatic potential map is the best "fingerprint" of a molecule, and takes into account volume, conformation and electronic properties. In a recent paper (7), the 3-D electrostatic potential maps of non-PAF-like high potency antagonists selected for their diversity in chemical structure and for their structural difference from PAF (absence of fatty chain and charged moieties) were calculated. These are the gingkolides A, B and C (*i.e.*, compounds 1, 2, and 3, which are BN 52020, 52021 and 52022, respectively),



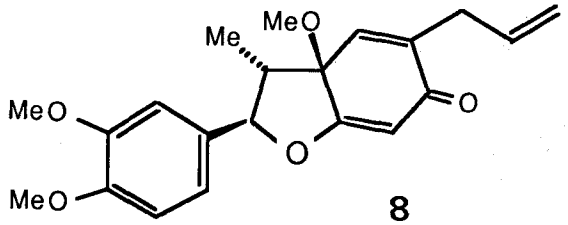
1 : R = R' = H BN 52020
2 : R = OH ; R' = H BN 52021
3 : R = R' = OH BN 52022

the synthetic furanoid neolignan L-652,731 (compound 5),

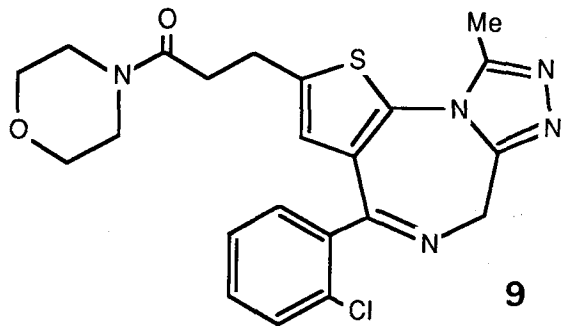


4 : R = OMe; *trans*
5 : R = OMe; *cis*
6 : R = H; *trans*
7 : R = H; *cis*

the neolignan kadsurenone 8,



and the triazolothienobenzodiazepin WEB 2086 (compound 9).



The magnitude of the contour level significantly determines the shape of the electrostatic potential cloud. Three-dimensional maps were contoured at −10 Kcal/mole, as the electrostatic clouds were defined best at this level. The negative isocontours revealed the existence of the so-called "Cache-oreilles" ("ear-muffs"), which comprise two electronegative wells located at 180° to each other.

In the gingkolides, the lactone rings are the functional groups generating the negative wells. For instance, in BN 52021 (compound 3), two distinct areas appear on the left (Fig. 1) and a third well is generated on the right by the

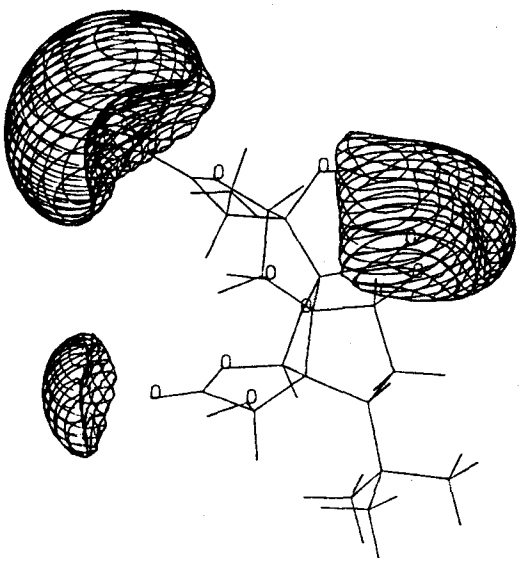


FIG. 1. 3-D electrostatic map (isocontours drawn at −10 Kcal/mole) calculated for BN 52021 (2). For details, see ref. 7.

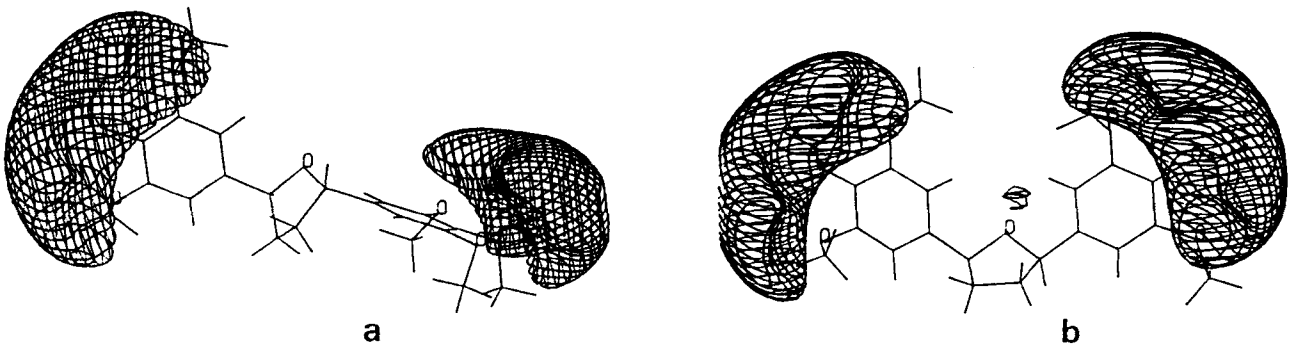


FIG. 2. 3-D electrostatic maps calculated for the 2,5-diaryltetrahydrofurans; a) *trans*-2,5-*bis*-(3,4,5-trimethoxyphenyl)tetrahydrofuran 4, L-652,731; b) the *cis*-isomer of 4 (5).

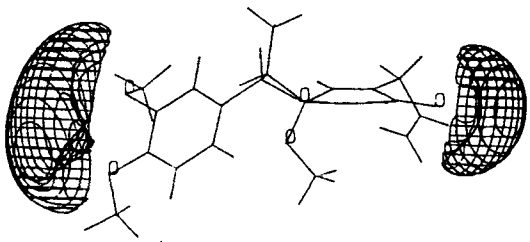


FIG. 3. 3-D electrostatic map calculated for kadsurenone 8.

other lactone group. The *tert*-butyl group may function as the hydrophobic binding site. For the three gingkolides 1, 2 and 3, no significant negative potential is generated around the oxygen of the innermost tetrahydrofuran ring, a feature which is inconsistent with the earlier hypothesis.

The latter observation was also made for *trans*-2,5-*bis*-(3′,4′,5′-trimethoxyphenyl)tetrahydrofuran 4, for which the "cache-oreilles" are generated by the trimethoxy groups. This compound does not bear a hydrophobic moiety (Fig. 2). When the distance between the atoms generating the negative clouds was shortened in *cis*-isomer 5, a negative capsule occurs around the oxygen of the tetrahydrofuran ring. The binding of this isomer to rabbit platelets was 1000 times less pronounced than that of the *trans* isomer 4. The negative capsule was also observed with *cis*- and *trans*-2,5-*bis*-(3′,4′-dimethoxyphenyl)tetrahydrofuran (6,7), and both were less potent than L 652,731 (4).

In extended conformations, the more the substituents become negatively charged, the less significant is the electronic contribution of the bearing skeleton. Dispersion of negative charges decreases biological activity, which is inconsistent with the earlier hypothesis which emphasized the likely importance of oxygen doublets.

Kadsurenone also represents a "cache-oreilles" system that is generated by the dimethoxy substituents and by the carbonyl oxygen of the quinone ring (Fig. 3). Once again, no weak negative cloud was observed around the tetrahydrofuran oxygen. The alkyl group may be a moderately hydrophobic tail.

In WEB 2086, the negative wells are generated by the triazolo ring and the carbonyl oxygen of the amide group bearing the morpholino ring. The orthochlorophenyl group may serve as hydrophobic binding site (Fig. 4).

Suggested requirements for potent agonistic activity include a site at position-1 for strong hydrophobic interaction with the receptor and conformational restrictions imposed by the acetyl group in position-2 of the glycerol backbone. In a recent publication (23), after examining the electronic and conformational properties of 14 PAF analogs (agonists and antagonists), we concluded that the folded conformation of the substituent in position-2 induces agonistic activity. In contrast, an extended conformation in position-2 induces antagonistic activity. 3-D electrostatic potential maps of the PAF analogs show a strong electronegative effect generated by the phosphate and acetate groups. The positive contour mainly is located close to the zwitterionic system and N-substitution enhances the volume of the positive cloud. Consequently, we re-examined the activity of several PAF analogs. For example, the 2-butanoyl PAF derivative is described in the literature as only a very weak agonist. We therefore concluded that this derivative exists in an extended conformation. We found that the 2-butanoyl PAF analog inhibited rabbit platelet aggregation at 10^{-7} M (23).

The results in regard to the selected non-PAF-like antagonists suggest a major electronic effect which induces interaction with subcomponents of the PAF receptor. The distances between atoms generating the "cache-oreilles", and the maximum distances between the envelopes of both isocontours (22–27 Å) are remarkably constant, with the gingkolides showing the shortest distances. Except for the synthetic neolignan L 652,731 (4), all antagonists have a moderately hydrophobic tail.

CONCLUSION

A first step towards constructing a mirror image of the PAF receptor of platelets is to compare common electronic features of molecules belonging to the heterogeneous series. Dive *et al.* (7) concluded that as a first approximation the features resemble interaction with a polarized cylinder of 10–12 Å diameter (Fig. 4).

Except for the tetrahydrofuran derivatives, well-defined hydrophobic sites may interact with a third site located inside the receptor. The hydrophobic tail may better position the electronegative clouds and does not make a

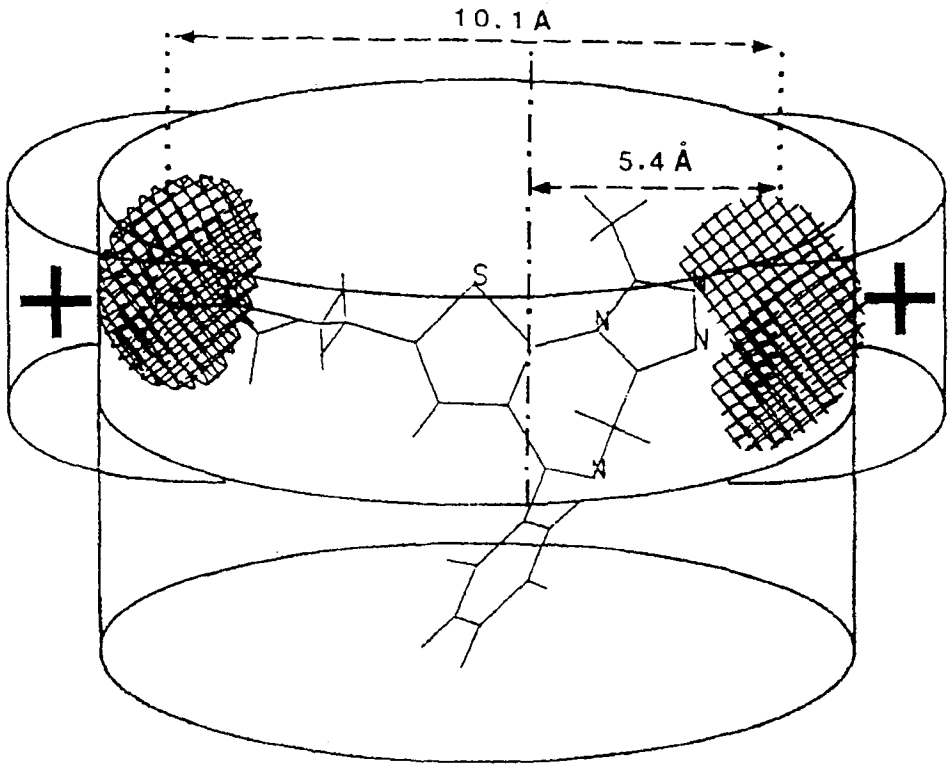


FIG. 4. 3-D electrostatic map calculated for WEB 2086 included in the simplified model of the "polarized cylinder" (see text); (+) represents the positive counterpart at this site of the receptor. Note the hydrophobic pocket inside the receptor. For details, see ref. 7.

"cache-oreilles" effect mandatory, as is observed with the tetrahydrofuran compounds.

The significant information that is provided by the negative electrostatic potential isocontours raises questions about molecules bearing only one strong electronegative cloud together with a weak negative well and a moderately hydrophobic tail. An extensive theoretical study of PAF and its analogs has been published (23) which updates the simple original model. The "cylinder," it appears, is probably multipolar, alternatively electronegative and electropositive, with at least two hydrophobic zones (24).

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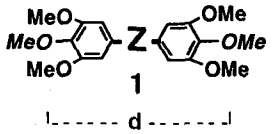
PAF Receptor And "Cache-Oreilles" Effect. Simple PAF Antagonists[1]

J. Lamotte-Brasseur[a], F. Heymans[b], G. Dive[a], A. Lamouri[b], J.-P. Batt[b], C. Redeuilh[b], D. Hosford[c], P. Braquet[c] and J.-J. Godfroid[b,*]

[a]Centre d'Ingéniérie des Protéines, Institut de Chimie, Liège-Sart-Tilman, Belgium, [b]Laboratoire de Pharmacochimie Moléculaire, Université Paris 7, Paris, France and [c]Institut Henri Beaufour, Le Plessis Robinson, France

Nine simple and structurally flexible PAF antagonists were synthesized and their inhibitory effects on PAF induced platelet aggregation were measured. Compounds with PAF antagonistic activity exhibited a negative electrostatic potential generated by two trimethoxyphenyl groups (isocontour at −10 Kcal/mole) at various distances between the negative clouds. The optimal distance between the atoms generating the "cache-oreilles" system for exhibiting potent PAF antagonistic activity is estimated to be 11–13 Å. In the flexible molecules studied, the dispersion of the electronic distribution is not necessarily favorable for anti-PAF activity. The data support the simple bipolarized model for the PAF receptor that has been proposed by the authors.
***Lipids 26*, 1167–1171 (1991).**

In our attempt to test the "Cache-oreilles" model for the platelet-activating factor (PAF) receptor (1) based on 3-D electrostatic potential maps of several PAF antagonists, a series of simple compounds of the general formula 1 were synthesized. The two 3,4,5-trimethoxyphenyl groups that generate the negative wells were linked by a flexible straight or branched chain Z.



1

We have previously suggested (1) that the structure of the PAF receptor appears to be cylindrical, having a diameter of 10–12 Å (for a review, see ref. 2). In the present study, two factors in molecular design have been taken into consideration—the distance between the atoms generating the "Cache-oreilles" system, and the general location of these negative clouds within the molecular skeleton of 1. These two characteristics seem critical when we want to test whether our simple model of the PAF receptor is correct.

In the present study we have attempted: (i) to estimate the ideal distance (d) between the atoms that generate the negative wells that is required to attain maximum antagonistic activity; and (ii) to assess the effect of the dispersion of the negative electrostatic potential (isocontour at −10 Kcal/mole) on antagonistic activity. The studies were carried out on flexible molecules with moderate antagonistic activity as a first step towards studies on more rigid and complex structures.

MATERIALS AND METHODS

Chemical synthesis. All compounds of the general formula 1 were prepared in one or two synthetic steps following standard methods. The syntheses are outlined in Scheme 1 (R = 3,4,5-trimethoxyphenyl).

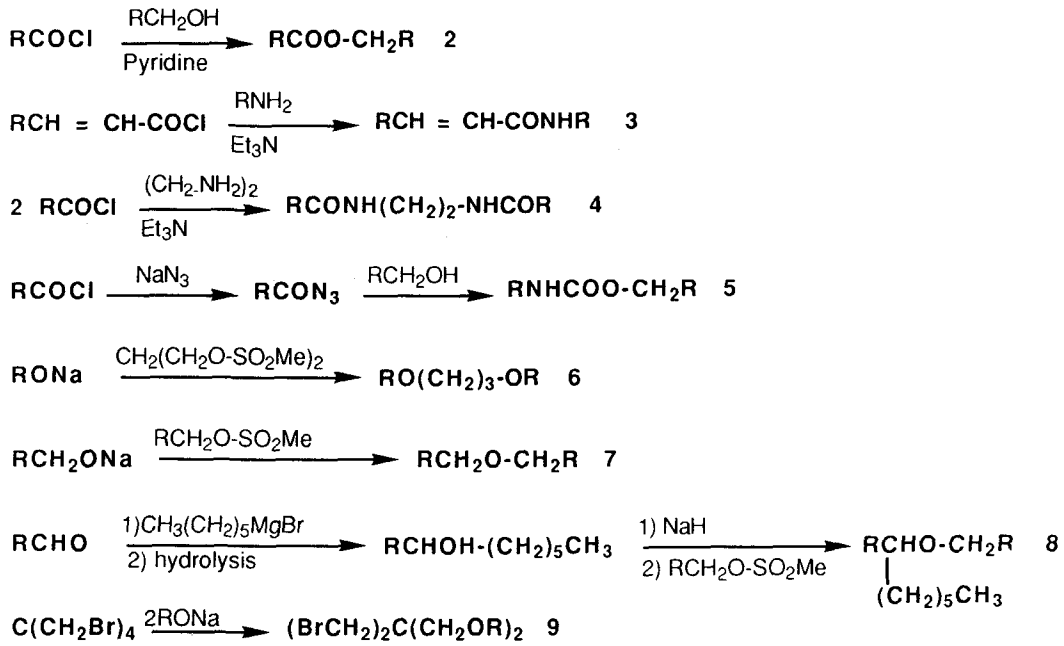
The 3′,4′,5′-trimethoxybenzyl 3,4,5-trimethoxybenzoate 2 was obtained by reaction of 3,4,5-trimethoxybenzylalcohol with an adequate amount of the acyl chloride. Treatment of 3,4,5-trimethoxyaniline with the corresponding cinnamoyl chloride led to *N*-(3′,4′,5′-trimethoxyphenyl) 3,4,5-trimethoxycinnamamide 3. *N,N′*-Di-3,4,5-trimethoxybenzoylethylenediamine 4 was prepared starting from ethylenediamine and the corresponding acyl chloride. The action of 3,4,5-trimethoxybenzoyl azide prepared following the method of Munch-Peterson (3) on 3,4,5-trimethoxybenzyl alcohol in boiling benzene afforded the *N*-(3′,4′,5′-trimethoxyphenyl)carbamoyloxymethyl 3,4,5-trimethoxybenzene 5. Ethers were synthesized by reacting sodium alcoholates with methanesulfonates. The reaction between sodium 3,4,5-trimethoxyphenoxide and 1,3-dimethylsulfonyloxypropane yielded the 1,3-di-3′,4′,5′-trimethoxyphenoxypropane 6, while sodium 3,4,5-trimethoxybenzyloxide with 3,4,5-trimethoxybenzyl methanesulfonate formed the di-(3,4,5-trimethoxybenzyl) ether 7. When 3,4,5-trimethoxybenzyl methanesulfonate was reacted with the sodium salt of 1-(3′,4′,5′-trimethoxyphenyl)hexan-1-ol prepared by Grignard reaction, it yielded the 1-(3,4,5-trimethoxyphenyl) 1-(3′,4′,5′-trimethoxybenzyloxy) hexane 8. Pentaerythrityl tetrabromide was used to prepare the 1,3-di-(3′,4′,5′-trimethoxyphenoxy) 2,2-dibromomethyl propane 9 by reaction with sodium 3,4,5-trimethoxyphenoxide and to obtain the 1,3-di-(3′,4′,5′-trimethoxyphenoxy) 2-*o*-chlorophenoxymethyl-2-bromomethyl propane 10 by successive reaction with sodium *o*-chlorophenoxide and sodium 3,4,5-trimethoxyphenoxide.

Materials. Solvents were all reagent grade. The purity of each product was checked by thin-layer chromatography (TLC) on silica gel plates (Merck, Darmstadt, Germany; Kieselgel 60 F_{254}, thickness 0.2 mm). Column chromatography was carried out on silica gel (Merck, particle size 0.063–0.200 mm) without any special treatment. Melting points were determined on a digital melting point apparatus (Electrothermal) using capillary tubes. The structures of all compounds were confirmed by infrared (IR) (SP_3-200, Pye-Unicam Ltd., Cambridge, England) and ^1H nuclear magnetic resonance (NMR) spectroscopy

[1]Based on a paper presented at the Third International Conference on Platelet-Activating Factor and Structurally Related Alkyl Ether Lipids, Tokyo, Japan, May 1989.

*To whom correspondence should be addressed at Laboratoire de Pharmacochimie Moléculaire, Université Paris 7, 2, Place Jussieu, 75251 Paris Cedex 05, France.

Abbreviations: CNDO, complete neglect of differential overlap; 3-D, three dimensional (electrostatic potential map); DMF, dimethylformamide; HMDS, hexamethyldisiloxane; IC_{50}, 50% inhibition; IR, infrared spectroscopy; MM2, molecular mechanics version 2; MMP2, molecular mechanics π systems version 2; MNDO, modified neglect of differential overlap; NMR, nuclear magnetic resonance; PAF, platelet activating factor, 1-*O*-alkyl-2-acetyl-*sn*-glycero-3-phosphocholine; PRP, platelet-rich plasma; TLC, thin-layer chromatography.



SCHEME 1

(WP 80 Bruker, Wissembourg, France) in $CDCl_3$ with hexamethyldisiloxane (HMDS) as internal standard. Elemental analyses were consistent with the proposed structures.

3',4',5'-Trimethoxybenzyl 3,4,5-trimethoxybenzoate (2). A solution of 2.3 g (10 mmoles) of 3,4,5-trimethoxybenzoyl chloride in benzene (20 mL) was added dropwise to a stirred solution of 1.98 g (10 mmoles) of 3,4,5-trimethoxybenzyl alcohol in dry benzene (20 mL) and pyridine (5 mL) at room temperature. The mixture was stirred for 1 hr and then evaporated *in vacuo*, leading to a residue which was diluted with H_2O and extracted with $CHCl_3$. The organic layer was washed twice with H_2O and dried ($MgSO_4$); the solvent was evaporated. The crude compound 2 was then chromatographed on a silica gel column using diethyl ether/petroleum hydrocarbon (50:50, v/v) as eluent to give 1.6 g (40%) of pure 2 as white crystals; mp 113°C. IR (film in nujol): 1705 (C=O), 1590 (aromatic C=C), 1130 (C-O) cm^{-1}. ^1H NMR δ: 7.42 (*s*, 2H, benzyl aromatic H), 6.77 (*s*, 2H, benzoyl aromatic H), 5.35 (*s*, 2H, CH_2), 3.92 (*s*, 18H, CH_3O).

N-(3',4',5'-Trimethoxyphenyl) 3,4,5-trimethoxycinnamamide (3). A solution of 2.6 g (10 mmoles) of 3,4,5-trimethoxycinnamoyl chloride in 10 mL of benzene was added dropwise to a stirred solution of 1.83 g (10 mmoles) of 3,4,5-trimethoxyaniline in dry benzene (20 mL) and triethylamine (5 mL) at room temperature. After stirring for one more hour, the solvent was evaporated, and the residue, after extraction as described for 2 above, was chromatographed on a silica gel column using $CHCl_3$/MeOH (99:1, v/v) as eluent. Compound 3 was recovered (3.1 g, 77%) as yellow crystals; mp 162°C. IR (film in nujol): 3270 (N-H), 1650 (C=O), 1600 (C=C) and 1580 (aromatic C=C) cm^{-1}. ^1H NMR δ: 8.40 (*s*, 1H, NH), 7.67 (*d*, J = 15 Hz, 1H, Ar-CH=), 7.02 (*s*, 2H, anilide aromatic H), 6.72 (*s*, 2H, aromatic H), 6.61 (*d*, J = 15 Hz, 1H, =CH-CO), 3.80 (*s*, 18H, CH_3O).

N,N'-Di-3,4,5-trimethoxybenzoylethylenediamine (4). A solution of 7.65 g (33.2 mmoles) of 3,4,5-trimethoxybenzoyl chloride in benzene (20 mL) was added dropwise to a stirred solution of 1 g (16.6 mmoles) of ethylenediamine in benzene (20 mL) and triethylamine (5 mL) at room temperature. After stirring for one more hour, the white precipitate was filtered and washed with H_2O. The precipitate was subsequently washed with a 5% $NaHCO_3$ solution, then with MeOH, and finally dried *in vacuo*. The very insoluble compound 4 (7.4 g, yield 100%) was obtained as white crystals; mp 255°C. IR (film in nujol): 3310 (N-H), 1645 (C=O), 1585 (aromatic C=C) cm^{-1}. ^1H NMR δ: 7.25 (*s*, 2H, NH), 7.05 (*s*, 4H, aromatic H), 3.87 (*s*, 18H, CH_3O), 3.70 (*t*, 4H, CH_2).

N-(3',4',5'-Trimethoxyphenyl)carbamoyloxymethyl 3,4,5-trimethoxybenzene (5). A solution of 23 g (100 mmoles) of 3,4,5-trimethoxybenzoyl chloride in 30 mL of acetone was added dropwise to a stirred solution of 7.8 g (120 mmoles) of NaN_3 in water (50 mL) maintained at 20-25°C. After stirring for 30 min, the white precipitate of 3,4,5-trimethoxybenzoyl azide was filtered, washed with H_2O, and dried *in vacuo* (100% yield) for use in the next step without further purification. 3,4,5-Trimethoxybenzyl alcohol (1 g, 5 mmoles) and the azide (1.15 g, 6 mmoles) were then refluxed in dry benzene (15 mL) for 2 hr. After cooling, the solvent was evaporated, and the residue was chromatographed on a silica gel column using $CHCl_3$/MeOH (99:1, v/v) as eluent to recover 2 g (98%) of 5 as white crystals; mp 108°C. IR (film in nujol): 3300 (N-H), 1730 (C=O), 1600 (aromatic C=C) cm^{-1}. ^1H NMR δ: 6.87 (*s*, 1H, NH), 6.67 (*s*, 2H, anilide aromatic H), 6.60 (*s*, 2H, benzyl aromatic H), 5.10 (*s*, 2H, CH_2), 3.82 (*s*, 18H, CH_3O).

1,3-Di-(3',4',5'-trimethoxyphenoxy)propane (6). One g (25 mmoles) of NaH (60% dispersion in mineral oil) was added carefully in small portions to a stirred solution of 3 g (16.3 mmoles) of 3,4,5-trimethoxyphenol in 30 mL of dry dimethylformamide (DMF). The suspension was heated (50°C, oil bath) for 30 min, cooled, and added dropwise to a solution of freshly prepared 1,3-dimethylsulfonyloxypropane (1.89 g, 8.15 moles). The mixture was stirred overnight at room temperature, and excess NaH was destroyed by adding 5 mL of MeOH dropwise while cooling. After evaporation of DMF, the residue was taken up in water and extracted with $CHCl_3$. The organic layer was washed with water to pH 7, dried ($MgSO_4$), and evaporated. Silica gel column chromatography using diethyl ether/petroleum hydrocarbon (50:50, v/v) as eluent yielded 2.4 g (72%) of **6** as white crystals; mp 107°C. IR (film in nujol): 1590 (aromatic C=C), 1130 (C-O) cm^{-1}. ^1H NMR δ: 6.13 (*s*, 4H, aromatic H), 4.06 (*t*, 4H, CH_2O), 3.75 (*s*, 18H, CH_3O), 2.15 (*quintet*, 2H, CH_2).

Di-(3,4,5-trimethoxybenzyl) ether (7). 3,4,5-Trimethoxybenzyl methanesulfonate was freshly prepared from 3,4,5-trimethoxybenzyl alcohol and methanesulfonylchloride in dry benzene in the presence of Et_3N in 74% yield. An aliquot of the methanesulfonate (2.9 g, 10.5 mmoles) was added to a stirred suspension of sodium 3,4,5-trimethoxybenzyloxide (1.9 g, 8.7 mmoles) prepared as described above in 50 mL of dry DMF. The mixture was heated (80°C) and stirred for 2 hr, then cooled and excess NaH was destroyed as above. After extraction and purification as described for **6**, compound **7** was recovered (2.5 g, 76%) as white crystals; mp 88°C. IR (film in nujol): 1595 (aromatic C=C), 1130 (C-O) cm^{-1}. ^1H NMR δ: 6.61 (*s*, 4H, aromatic H), 4.50 (*s*, 4H, CH_2), 3.86 (*s*, 18H, CH_3O).

1-(3',4',5'-Trimethoxybenzyloxy) 1-(3,4,5-trimethoxyphenyl) hexane (8). n-Hexyl bromide (24.7 g, 150 mmoles) in 100 mL of diethyl ether was added dropwise to a stirred suspension of Mg turnings in 10 mL of dry diethyl ether (3.6 g, 150 mmoles) at 0°C (ice bath). The mixture was stirred at room temperature for 1 hr. A solution of 19.6 g (100 mmoles) of 3,4,5-trimethoxybenzaldehyde in 70 mL of dry benzene was then added dropwise, while the solution was cooled (0°C) and stirred. After two more hours of stirring at room temperature, the mixture was cooled again (0°C) and hydrolyzed by dropwise addition of 10 mL of acetic acid in 100 mL of water. After stirring for 30 min, the solution was filtered. The organic layer was washed with water until pH 7, dried ($MgSO_4$), and evaporated to give 29 g of crude 1-(3',4',5'-trimethoxyphenyl)hexan-1-ol as a liquid which was characterized spectroscopically and used without purification in the next step. An aliquot (2.8 g, 10 mmoles) of the alcohol was diluted with dry DMF (30 mL), and NaH (0.6 g, 15 mmoles) was added in small portions. After stirring for 1 hr at 60°C, a solution of 3.31 g (12 mmoles) of 3,4,5-trimethoxybenzyl methanesulfonate in 10 mL of DMF was added, the mixture was heated at 80°C and stirred for 2 hr. After cooling, excess NaH was destroyed and compound **8** was obtained as a syrup, following the same extraction and purification procedures as for **6**, in 65% yield. IR (film in nujol): 3000 (aromatic C-H), 2940, 2860 (C-H), 1595 (aromatic C=C), 1130 (C-O) cm^{-1}. ^1H NMR δ: 6.52 (*s*, 4H, aromatic H), 4.55-4.07 (*m*, 3H, CH-O-CH_2), 3.80 (*s*, 18H, CH_3O), 1.65 (*m*, 2H, CH_2-C-O), 1.25 (*s*, 8H, $(CH_2)_4$), 0.81 (*t*, 3H, CH_3).

1,3-Di-(3',4',5'-trimethoxyphenoxy) 2,2-di-bromomethyl propane (9). A suspension of sodium 3,4,5-trimethoxyphenoxide prepared as described above from 5.71 g (31 mmoles) of 3,4,5-trimethoxyphenol and 2 g (50 mmoles) of NaH in 100 mL of DMF was added dropwise to a stirred solution of 12 g (31 mmoles) of pentaerythrityl tetrabromide in 60 mL of DMF. The mixture was heated (80°C) and stirred for 2 hr. After the same treatment as described for **6**, column chromatography using first diethyl ether/petroleum hydrocarbon (50:50, v/v), then $CHCl_3$/MeOH (99:1, v/v), eluted successively 2.72 g of monosubstituted derivative and 3.19 g 34.6%) of compound **9** as white crystals; mp 152°C. IR (film in nujol): 1600 (aromatic C=C), 1130 (C-O) cm^{-1}. ^1H NMR δ: 6.11 (*s*, 4H, aromatic H), 4.02 (*s*, 4H, CH_2O), 3.77 (*s*, 18H, CH_3O), 3.72 (*s*, 4H, CH_2Br).

1,3-Di-(3',4',5'-trimethoxyphenoxy) 2-o-chlorophenoxymethyl-2-bromomethyl propane (10). 1-*o*-Chlorophenoxy-3-bromo-2,2-dibromomethyl propane was obtained from pentaerythrityl tetrabromide and sodium *o*-chlorophenoxide following the procedure described above. Its reaction with two equivalents of sodium 3,4,5-trimethoxyphenoxide under conditions as for **9** yielded compound **10** (50%) as white crystals; mp 140°C. IR: 1600 (aromatic C=C), 1130 (C-O) cm^{-1}. ^1H NMR δ: 7.42-6.76 (*m*, 4H, *o*-chlorophenyl H), 6.17 (*s*, 4H, aromatic H), 4.26 (*s*, 5H, CH_2 and *o*-chlorophenyl O-CH), 3.91 (*s*, 1H, *o*-chlorophenyl O-CH), 3.78 (*s*, 18H, CH_3O), 3.75 (*s*, 2H, CH_2Br).

Geometry calculations. Molecular geometrics were obtained by the energy minimization method of the MM2 program (Molecular Mechanics, 1987, Force Field; Quantum Chemistry Program Exchange, Dept. of Chemistry, Indiana University, Bloomington, IN) (4). The quality of the generated conformations was evaluated by the semi-empirical MNDO (modified neglect of differential overlap) method applied to the full optimization of compound **5**.

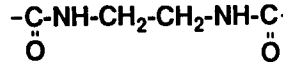
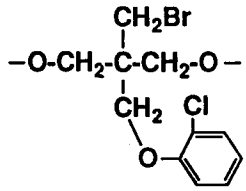
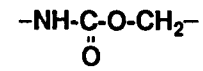
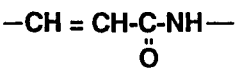
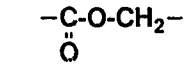
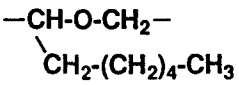
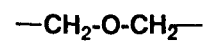
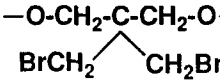
Electrostatic potential calculations. The electrostatic potential was calculated within a complete CNDO (complete neglect of differential orbitals) scheme (5,6), excluding the off-diagonal matrix elements. The nuclear attraction integrals were approximated by substracting the repulsion integrals between S orbitals (7). A regular 3-D grid was calculated around each molecule. The dimensions of the box were defined as the dimensions of the molecules increased by 5 Å in each direction. From these data sets, contouring algorithm (Jorgensen W.I., program PS177, Purdue University, West Lafayette QCPE 340) was applied joining the points at the level of −10 Kcal/mole.

Electrostatic potentials could not be calculated for compounds **9** and **10** as no nuclear attraction integral calculation was available for the bromide atom. The choice of CNDO electrostatic potential calculation at the MNDO geometry was guided by the consistency and the quality of the results derived by this method (8). The electrostatic repulsion between the trimethoxyphenyls, within the same negative environment, generates an extended conformation of the skeleton which was confirmed by the MNDO conformational analysis.

The MM2 minimizations were conducted on a Gould NP1 at the Paris 6 computer center (ccr-p6) and on an Apple MacIntosh using the Chem-3D-Plus program. The

TABLE 1

Biological Activities and Distances (d) Between the Atoms Generating the "Cache-Oreilles" for *bis*-3,4,5-Trimethoxyphenyl Compounds 1

Compound	-Z-	Inhibition[a] of platelet aggregation IC_{50} (μM)	d[d] (Å)
4		9% at 100[a]	16.5
10		12% at 100[a]	14.9
5		24% at 100[a]	13.8
3		32% at 100[a]	12.7
2		100	12.0
6		100	14.3
8		24	11.5
7		20	10.9
9		10	13.2

[a]When an IC_{50} value could not be obtained, % inhibition at 100 μM was recorded.
[b]See text for details.

other computer calculations were performed in the Centre d'Ingéniérie des Protéines (Institut de Chimie, Université de Liège) with a FPS164 processor linked to a VAX 11/780. The program PS177 was interfaced with the DI3000 library implemented on a Data General MV7800 (1).

Platelet aggregation assay. Inhibition of platelet aggregation was measured using platelet-rich plasma (PRP) of New Zealand rabbits following the method of Cazenave *et al.* (9). Blood samples were collected from the auricular artery into citrate buffer (3.8%; pH 7.4) and PRP was obtained by centrifugation for 15 min at 1200 rpm. The antagonists were solubilized in DMSO at concentrations from 10^{-2} to 10^{-7} M and added to the incubated and stirred PRP for 1 min before PAF (2.5 nM) challenge. Platelet aggregation induced by PAF in the presence of the antagonist was monitored by continuous recording of light transmission in a dual-channel recorder (Cronolog Coultronics Apparatus) and was compared to a control aggregation induced by PAF alone. The drug concentration required to produce 50% inhibition (IC_{50}) was calculated from dose-response curves.

RESULTS AND DISCUSSION

The compounds we synthesized were tested for their inhibitory effect (IC_{50}) on PAF induced rabbit platelet aggregation and classified from least to most active (Table 1). For several of the compounds we observed that the electrostatic repulsion between the trimethoxyphenyls generates an extended conformation of the skeleton (Fig. 1). In addition, these molecules exhibit large negative electrostatic potential clouds (at −10 Kcal/mole) which are located as a "Cache-oreilles" system (compounds 6–8) at various distances (d) between the respective parathoxy substituents. Conversely, compounds 2, 4 and 5 generate

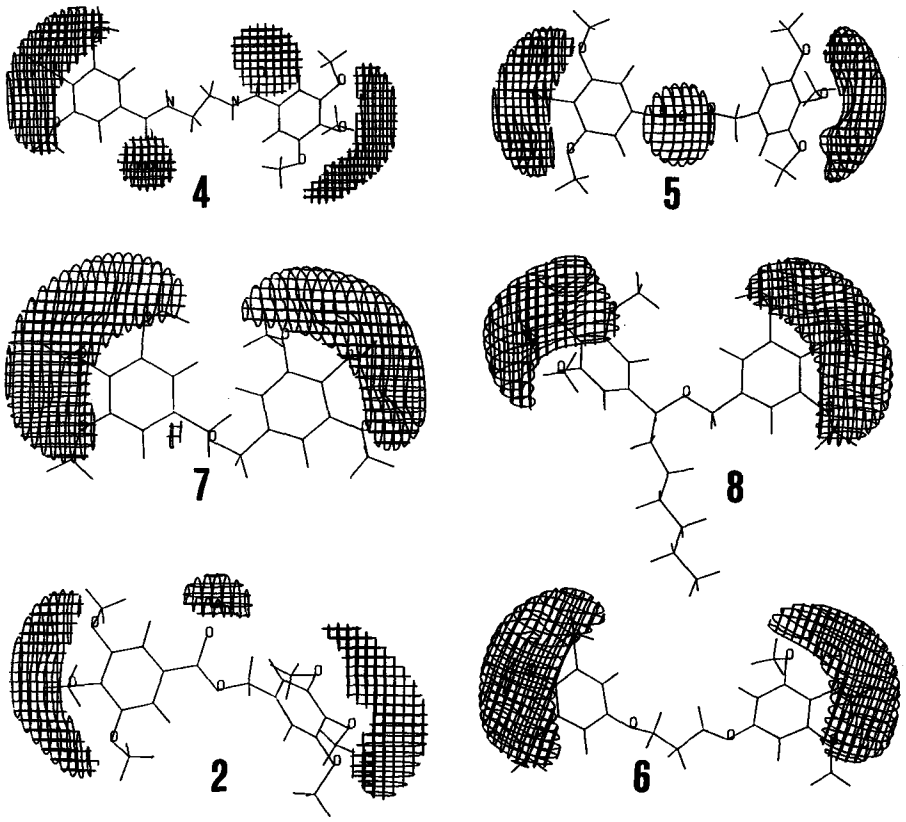


FIG. 1. 3-D electrostatic map calculated for selected compounds.

secondary negative wells around the ester, amide and carbamate groups. Compound **9** exhibits the highest antagonistic activity (d = 13 Å). In comparison with compound **6**, *bis*-bromomethylene substitution enhances the inhibition of PAF induced platelet aggregation; the distance of d = 13 Å is consistent with our PAF receptor hypothesis. Compounds **7** and **8** represent ideal "Cache-oreilles" structures consistent with our model and they also exhibit considerable antagonistic activity; however, the hydrophobic tail in compound **9** does not increase the antagonistic activity of this compound. The activity of the other compounds is weaker. This is probably due to the greater distance (d) in the case of compound **4** (16.5 Å) and dispersion of the electronic distribution in compounds **2**, **4** and **5**.

In conclusion, the flexibility of the molecules selected makes it difficult to assess whether the extended conformation is always the arrangement preferred by the receptor. However, the strong electrostatic repulsion of the trimethoxy groups may be sufficient to assume that the extended conformation is the likely one to interact at the receptor level. Several of these flexible molecules synthesized for the present study exhibit interesting PAF antagonistic activities and their molecular conformation is in accordance with our simple PAF receptor model. In a follow-up study, we have recently synthesized several semi-rigid compounds with a distance (d) of 11–13 Å between the atoms that generate the clouds of negative potential. These compounds exhibit more potent PAF antagonistic activity (10), adding further support to the "Cache-oreilles" hypothesis for the conformation of the PAF receptor. PAF antagonists, such as the ginkgolides, which possess a shorter distance (d, 7–8 Å), (10) can also be accommodated within the hypothesis if the structure of the receptor is considered a multi-polarized cylinder, rather than a bi-polarized structure.

ACKNOWLEDGMENT

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Discovery and Preliminary Pharmacology of Sch 37370, a Dual Antagonist of PAF and Histamine[1]

M. Motasim Billah[a], Robert W. Egan[a], Ashit K. Ganguly[b], Michael J. Green[b,*], William Kreutner[a], John J. Piwinski[b], Marvin I. Siegel[a], Frank J. Villani[b] and Jesse K. Wong[b]

Departments of [b]Chemistry and [a]Allergy and Inflammation Biology Schering-Plough Research, Schering-Plough Corporation, Bloomfield, New Jersey 07003

From a series of amide analogs of the histamine H_1 antagonist, azatadine, a potent, orally active, dual platelet-activating factor (PAF) and histamine antagonist, Sch 37370, namely 1-acetyl-4-(8-chloro-5,6-dihydro-11*H*-benzo-[5,6]cyclohepta[1,2-b]pyridin-11-ylidine)piperidine, was discovered. Sch 37370 selectively inhibits PAF-induced aggregation of human platelets *in vitro* (IC_{50} = 0.6 μM), and *in vivo* inhibits PAF- and histamine-induced bronchospasm in guinea pigs with ED_{50} values of 6.0 and 2.4 mg/kg p.o., respectively. Sch 37370 is expected to be more efficacious than single mediator antagonists in allergic diseases, such as asthma.

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Allergic diseases such as asthma are thought to be due to immediate hypersensitivity reactions mediated by IgE (1,2). In this scenario, the interaction of antigen with IgE-primed mast cells causes the release of preformed mediators, *e.g.* histamine, and turns on the synthesis of others, *e.g.* leukotrienes, platelet-activating factor (PAF), and thromboxane A_2. These mediators elicit the physiological effects that characterize the disease. These include smooth muscle contraction, influx of inflammatory cells and edema (3). The importance of the contribution that any one of these mediators makes to clinical asthma is largely unknown. H_1 histamine antagonists have not been very effective in treating asthma (4), although one of the very potent compounds, ketotifen, is reported to have antiallergic activity in man (5). The newer non-sedating antihistamines such as terfenadine and cetirizine are also being tested clinically in asthma (6). Selective antagonists of other mediators, leukotriene D_4, PAF and thromboxane A_2, have been developed (7-9) and are also currently being evaluated in clinical trials in asthma.

The approach in our laboratories has been to search for compounds that inhibit more than one of these mediators (10), with the expectation that they would be clinically more effective than single mediator antagonists. Here we report the discovery of the first dual antagonist of PAF and histamine, Sch 37370.

The physiological effects of histamine (11,12) and PAF (13) are mainly smooth muscle contraction (bronchospasm) and edema formation. However, for histamine, these are of rapid onset and short duration, whereas, for PAF, they are of slow onset and long duration. Therefore, inhibition of both mediators should be complementary. Furthermore, histamine causes the release of PAF from cultured endothelial cells (14) and the H_1 antihistamines, ketotifen and terfenadine, largely block the cutaneous response to PAF in man (15,16). Lastly, we have a great deal of experience testing histamine antagonists clinically. With a dual PAF/histamine antagonist we can define the safety profile and bioavailability using antihistamine activity, and then investigate the therapeutic consequences of additional PAF blockade.

MATERIALS AND METHODS

Sch 37370 and the analogs reported here were synthesized as described (17). Other compounds that were used in this work were obtained as follows: PAF (Calbiochem; Los Angeles, CA); azatadine, loratadine, L-652,731, CV-3988 (Schering-Plough Research, Bloomfield, NJ); WEB 2086 (Boehringer Ingelheim; Elmsford, NY); ketotifen fumarate (Sandoz; East Hanover, NJ); terfenadine (Merrell-Dow, Cincinnati, OH); astemizole (Janssen, Beerse, Belgium); alprazolam (Upjohn, Kalamazoo, MI).

Platelet aggregation assay. Human blood (50 mL) was collected from healthy male donors in an anticoagulant solution (5 mL) containing sodium citrate (3.8%) and dextrose (2%). Blood was centrifuged at 110 × *g* for 15 min to obtain platelet-rich plasma. The aggregation assays were performed using a dual-channel aggregometer (Model 440, Chrono-Log Corp., Haverton, PA). Solutions of the test compounds or vehicle were added to the platelet-rich plasma, and, after incubation for 2 min, 10-15 μL aliquots of the PAF solution were added to achieve a final concentration of 1-5 × 10^{-8}M. In different experiments the aggregatory response was kept within a set limit by varying the concentration of PAF. Incubations were continued until the increase in light transmission reached a maximum (usually 2 min). Values for inhibition were calculated by comparing the rate of aggregation obtained in the absence and in the presence of the compound. For each experiment, alprazolam, a benzodiazepine with anti-PAF activity, was used as a positive control.

Spasmogen-induced bronchospasm in guinea pigs. Male Hartley guinea pigs (450-550 g) were obtained from Charles River Breeding Labs. (Wilmington, MA). The animals were fasted overnight and were anesthetized the following day with 0.9 mL/kg of dialurethane i.p. (containing 0.1 g/mL diallylbarbituric acid, 0.4 g/mL ethylurea and 0.4 g/mL urethane). The left jugular vein was cannulated for the administration of the compounds. The trachea was cannulated and the animals were ventilated by a rodent respirator at 55 strokes/min with a stroke volume of 4 mL. A side arm to the tracheal cannula was connected to a pressure transducer to obtain a continuous measure of inflation pressure. Bronchoconstriction was measured as the percent increase in inflation pressure that peaked within 5 min after the challenge with spasmogen. The animals

[1]Based on papers presented at the Third International Conference on Platelet-Activating Factor and Structurally Related Alkyl Ether Lipids, Tokyo, Japan, May 1989.

*To whom correspondence should be addressed.

Abbreviations: BSA, bovine serum albumin; PAF, platelet-activating factor.

were challenged intraveneously with either PAF (0.4 μg/kg in isotonic saline containing 0.25% bovine serum albumin —BSA), histamine (10 μg/kg) or methacholine (10 μg/kg).

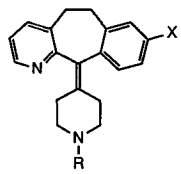
RESULTS AND DISCUSSION

Sch 37370 **6** is structurally related to two clinically used H_1 antihistamines, azatadine **1** (18) and loratadine **2** (19) (Fig. 1). However, these potent antihistamines have no significant PAF antagonist activity. The main structural difference between these compounds and Sch 37370 **6** is the group attached to the piperidine nitrogen atom. In Sch 37370, this group is an acetamide. We have attached a great variety of groups to the piperidine nitrogen and found that only compounds that contain an amide or, to a lesser extent, a thioamide group have good PAF antagonist activity. Some of these compounds are listed in Figure 1 with their *in vitro* biological activity listed in Table 1. Thus, the acetamide **6** is much more potent than the thioacetamide **5**, which in turn is more potent than the methylamine **3** and the methylsulfonamide **4**. The size of the alkyl moiety of the amide derivative is important for high PAF antagonist potency, and we found the methyl group, as in Sch 37370, to be optimal. We also investigated the effect on *in vitro* PAF antagonist potency of changing the C-8 substituent in this series (Table 1). The fluoro **8** and bromo **9** analogs are both equipotent to Sch 37370 **6**, which is somewhat more potent than the unsubstituted compound **7**. However, *in vivo*, the chloro derivative, Sch 37370, had the most desirable profile.

In PAF-induced platelet aggregation assays, Sch 37370 caused a concentration-dependent shift of the PAF dose response curve to the right, indicating competitive inhibition (20). When compared to other PAF antagonists (13,21) in this assay, Sch 37370 was approximately equipotent to L-652,731, more potent than CV-3988 and alprazolam, and less potent than WEB-2086 (Table 2).

In vivo, Sch 37370 was active against PAF- and histamine-induced responses in guinea pigs. It caused dose-dependent inhibitions of both PAF- and histamine-induced bronchospasms when given intravenously or orally (Table 3). After i.v. administration, Sch 37370, with an ED_{50} of 0.7 mg/kg against PAF, was equipotent to L-652,731 and more potent than alprazolam, while WEB 2086 was more potent than Sch 37370. WEB 2086 also showed greater oral potency than Sch 37370, which had an ED_{50} of 6 mg/kg, while L-652,731 and alprazolam were inactive at 30 mg/kg. Several standard antihistamines showed little or no inhibition of PAF-induced bronchospasms except at doses that greatly exceeded their antihistamine dose. Sch 37370 also inhibited histamine-induced bronchospasms with ED_{50} values of 0.6 mg/kg i.v. and 2.4 mg/kg p.o. The specificity for the PAF and histamine receptors was indicated by the much weaker inhibition of the methacholine-induced bronchospasm (20).

In summary, Sch 37370 is a dual antagonist of PAF and histamine *in vitro* and *in vivo*. It is active in anti-PAF and antihistamine assays both upon intravenous and oral administration. While it is neither the most potent PAF antagonist nor the most potent antihistamine, it is the first agent that embodies the unique combination of anti-PAF and antihistamine activities in the same molecule. This combination of activities warrants future testing of Sch 37370 in man.



1	R = CH_3	X = H	(Azatadine)
2	R = $CO_2C_2H_5$	X = Cl	(Loratadine)
3	R = CH_3	X = Cl	
4	R = SO_2CH_3	X = Cl	
5	R = $CSCH_3$	X = Cl	
6	R = $COCH_3$	X = Cl	(Sch 37370)
7	R = $COCH_3$	X = H	
8	R = $COCH_3$	X = F	
9	R = $COCH_3$	X = Br	

FIG. 1. Structures of compounds.

TABLE 1

***In Vitro* PAF Antagonist Activity of Sch 37370 and Analogs[a]**

Compound	Inhibition of PAF-induced platelet aggregation IC_{50} (μM)[b]
1	>50
2	>50
3	⩾50[c]
4	>50[c]
5	29 ± 10
6	0.61 ± 0.05[d]
7	1.4 ± 0.3
8	0.67 ± 0.03
9	0.81 ± 0.09

[a]Values are a measure of the concentration of drug required to cause a 50% inhibition of PAF-induced platelet aggregation of human platelet-rich plasma when challenged with PAF. In different experiments the aggregatory response was kept to within a set limit by varying the concentration of PAF between 10–50 nM.

[b]Unless otherwise noted, the values represent the mean of two independent experiments with the associated errors representing the range from the mean if appropriate.

[c]Value was determined from three independent experiments.

[d]Value is the mean ± the standard error of the mean for 11 independent experiments.

TABLE 2

***In Vitro* Potency of Sch 37370 and Selected Anti-PAF and Antihistamine Standards Against PAF-Induced Platelet Aggregation[a]**

Compound	Primary pharmacological activity	Inhibition of PAF-induced platelet aggregation IC_{50}(μM)[b]
Sch 37370	—	0.61 ± 0.05
WEB 2086	anti-PAF	0.04 ± 0.005
L-652,731	anti-PAF	1.5 ± 0.5
Alprazolam	anti-PAF	9.3 ± 1.0
CV-3988	anti-PAF	11 ± 4
Azatadine	antihistamine	>50[c]
Diphenhydramine	antihistamine	>50[c]

[a]Values are a measure of the concentration of drug required to cause a 50% inhibition of PAF-induced platelet aggregation of human platelet-rich plasma when challenged with PAF. In different experiments the aggregatory response was kept to within a set limit by varying the concentration of PAF between 10–50 nM.

[b]Unless otherwise noted the values represent the mean of four to eleven independent experiments with the associated errors representing the standard error of the mean.

[c]Value represents the mean of two independent experiments.

TABLE 3

***In Vivo* Anti-PAF and Antihistamine Potency of Sch 37370 and Selected Standards in Guinea Pigs[a]**

Compound	ED_{50} (mg/kg) against bronchospasm due to[b]			
	PAF		Histamine	
	i.v.	p.o.	i.v.	p.o.
Sch 37370	0.71	6.0	0.57	2.4
WEB 2086	0.04	0.8	NT[c]	>8.0
L-652,731	0.51	>30	NT	NT
Alprazolam	2.9	>30	>4.0	NT
Terfenadine	NT	25	NT	1.8
Ketotifen	NT	>10	0.005	0.006

[a]Compounds administered either 10 min (i.v.) or 2 hr (p.o.) before i.v. challenge with 0.4 μg/kg of PAF or 10 μg/kg of histamine.

[b]ED_{50} values were obtained by linear regression analysis of dose-response curves (constructed using at least three doses for each compound with three to six animals per dose) of each compound.

[c]Not tested.

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PAF Inhibitory Activity of Diketopiperazines: Structure-Activity Relationships[1]

Norihiko Shimazaki, Ichiro Shima, Masanori Okamoto, Keizo Yoshida, Keiji Hemmi* and Masashi Hashimoto

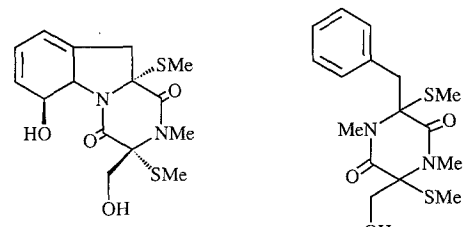
Exploratory Research Laboratories, Fujisawa Pharmaceutical Co., Ltd., Tsukuba, Ibaraki, Japan

FR900452, a natural product isolated from the culture broth of *Streptomyces phaeofaciens* No. 7739, was found to inhibit PAF-induced rabbit platelet aggregation with an IC_{50} of 3.7×10^{-7}M. FR900452, 1-methyl-3-[1-[5-methylthiomethyl-6-oxo-3-(2-oxo-3-cyclopenten-1-ylidene)-2-piperazinyl]ethyl]-2-indoline, has an oxocyclopentylidene group incorporated as a vinylogous amide in a diketopiperazine skeleton. This unique structure led us to synthesize diketopiperazine derivatives, 3-arylalkyl-6-substituted-piperazine-2,5-diones. Their observed PAF inhibitory activity suggest that the D-D configuration of diketopiperazine is an important factor for anti-PAF activity and that the hydrophobic aromatic portion may play a specific role in the binding of the diketopiperazine to the PAF receptor.

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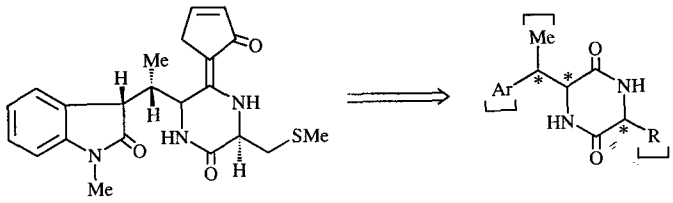
Platelet-activating factor (PAF), an endogenous phospholipid, has been suggested to act as a potent mediator in pathological processes such as anaphylaxis and inflammation (1-3). Our interest in the pathophysiological function of PAF led to the search for PAF inhibitors of microbial origin. For this purpose, a wide range of fermentation broths were tested for their inhibitory effect against PAF-induced rabbit platelet aggregation. Two natural products, designated FR49175 and FR106969 (Scheme 1), were isolated from the culture filtrate of *Penicillium terlikowskii* No. 5348 and *Penicillium citrinum* No. 2973, respectively (4-6). Both compounds have a diketopiperazine skeleton and a *bis*(methylthio) function as common structural units.

Recently, a new compound, designated FR900452, was isolated from the culture broth of *Streptomyces phaeofaciens* No. 7739 and identified as 1-methyl-3-[1-[5-methylthiomethyl-6-oxo-3-(2-oxo-3-cyclopenten-1-ylidene)-2-piperazinyl]ethyl]-2-indolinone (Scheme 2) (7,8). The compound showed high inhibitory potency against PAF-induced rabbit platelet aggregation with an IC_{50} of 3.7×10^{-7} M. The structure of this compound is unique as the oxocyclopentylidene group is incorporated as a vinylogous amide into the diketopiperazine skeleton. Moreover, structurally simpler analogues with diketopiperazine skeleton were prepared to examine whether the vinylogous diketopiperazine moiety contributes to the ability of the molecule to bind to the PAF receptor site.



FR49175 FR106969

SCHEME 1



FR900452

SCHEME 2

In the synthesis of the derivatives, we focused on: i) the stereochemistry of the diketopiperazine; ii) the modification of the oxindole moiety; iii) the modification of the *S*-methylcysteine part; and iv) the effect of the β-methyl group (Scheme 2).

In the present study, the relationship between the structure of diketopiperazine derivatives and the ability of the derivatives to inhibit PAF was examined. Our results suggest that the stereochemistry of the diketopiperazine moiety is an important factor in the inhibition of PAF activity and that the hydrophobic aromatic portion plays an important role in the binding of the diketopiperazine to the PAF receptor.

MATERIALS AND METHODS

Synthesis. Diketopiperazine derivatives were synthesized by peptide coupling using the dicyclohexylcarbodiimide method, followed by removal of the *N*-protecting group with hydrogen chloride in ethyl acetate and subsequent cyclization in alcoholic ammonia (9). Derivatives prepared from *dl-erythro* and *dl-threo-N*-Boc-1, β-dimethyltryptophans and *S*-methylcysteine have already been reported (10). Optically active (αR, βR)-1,β-dimethyltryptophans were prepared by reaction of aziridine-2-carboxylate and *N*-alkylindoles in the presence of boron trifluoride etherate (11). Purities and structures of the diketopiperazines were confirmed by thin-layer

[1]Based on a paper presented at the Third International Conference on Platelet-Activating Factor and Structurally Related Ether Lipids Tokyo, Japan, May 1989.

*To whom correspondence should be addressed at Exploratory Research Laboratories, Fujisawa Pharmaceutical Co., Ltd., 5-2-3 Tokodai, Tsukuba, Ibaraki 300-26, Japan.

Abbreviations: IC_{50}, concentration required to inhibit the platelet aggregation response by 50%; PAF, platelet-activating factor, 1-*O*-alkyl-2-acetyl-*sn*-glycero-3-phosphocholine; PRP, platelet-rich plasma.

chromatography, nuclear magnetic resonance spectroscopy, and elemental analysis.

Inhibition of platelet aggregation. Platelet aggregation was measured using a Sienco (Morrison, CO) dual-sample aggregometor (DP-247E). Blood was collected in 3.8% sodium citrate (1 mL for 9 mL of blood) from the carotide arteries of male Japanese white rabbits (2.5–3 kg body weight). Blood was then centrifuged at 150 × *g* for 10 min at 10°C to prepare platelet-rich plasma (PRP). The remaining blood was further centrifuged at 1000 × *g* to obtain platelet-poor plasma for adjusting the concentration of platelets to 4.0×10^5 platelets/mL. PRP (0.3 mL) was incubated with drugs or vehicle for 2 min before PAF (1×10^{-8} M) was added. Activity of inhibition was expressed as IC_{50} value, *i.e.*, the concentration required to inhibit the platelet aggregation response by 50%.

RESULTS

Stereochemistry of the diketopiperazine. The four stereoisomers with L-L, D-L, L-D, and D-D configuration were prepared starting from *N*-Boc-1-methyl-L- and D-tryptophan and *S*-methyl-L- and D-cysteine ethyl ester to examine which stereochemistry of the diketopiperazine is preferable to express optimal inhibitory activity. The inhibitory activities of the compounds are listed in Table 1. Of the four stereoisomers, compound 1 with D-D configuration was the most effective inhibitor of PAF; the other diastereoisomers were less potent or essentially inactive. The derivative 5 prepared from D-tryptophan and D-methionine also showed potent inhibitory activity.

Modification of the oxindole moiety. The inhibitory activities of the derivatives modified at the oxindole moiety are given in Table 2. The naphthyl derivative 8 was more potent than the phenyl derivatives 6 and 7. The 1-methylindole derivative 5 was more potent than the indole derivative 10. The activity of the naphthyl derivative 8 was almost the same as that of the 1-methylindole derivative 5. The pyridyl derivative 9 showed very weak activity.

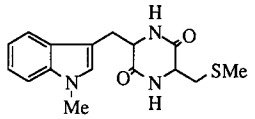
Modification of the S-methylcysteine moiety. The inhibitory activities of the derivatives modified at the *S*-methylcysteine moiety are listed in Table 3. The most potent analogue was the n-butyl derivative 11. The long-chain analogue 12 showed only weak activity. The benzyl derivative 13 was as potent as the methylthioethyl derivative 5. The α-picolyl derivative 16 showed more potent activity than the β-picolyl 17 and γ-picolyl derivative 18. The hydrophilic derivatives 19 and 20 exhibited only weak activities, and the corresponding ester derivatives 14 and 15 showed relatively high potencies.

Effect of the β-methyl group. The inhibitory activities of the four stereoisomers which have a β-methyl group in the tryptophan residue are shown in Table 4. Compound 21 with (7R)-β-methyl and D-D diketopiperazine configuration was the most active. The inhibitory activity of the (7S)-methyl counterpart 22 was about five-fold less than that of compound 21. Compounds 23 and 24 with L-D diketopiperazine configuration were essentially inactive.

Effect of substituents on the indole moiety of tryptophan. Compound 16 was modified to examine the effect of substituents on the indole moiety. The inhibitory activities of these derivatives are shown in Table 5. The inhibitory activities of the *N*-ethyl (26) and the *N*-propyl (27) derivatives were approximately three-fold higher than that of the *N*-methyl derivative 25. Compounds 30 and 31, with the hydrophobic chloro and methyl substituents at the 5-position of the indole, respectively, showed about a three-fold increase in potency compared to the inhibitory

TABLE 1

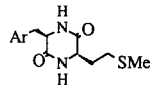
Inhibitory Activity of PAF-Induced Rabbit Platelet Aggregation



Compound	N-Me-tryptophan	S-Me-cysteine	IC_{50} (μM)
1	L(S)	L	250
2	D(R)	L	36
3	L(S)	D	140
4	D(R)	D	7.6
5	D(R)	D-methionine	2.3

TABLE 2

Inhibitory Activity of PAF-Induced Rabbit Platelet Aggregation



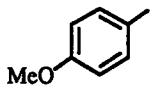
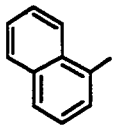
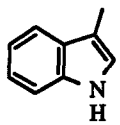
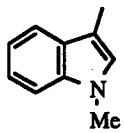
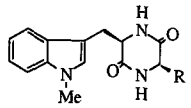
Compound	Ar	IC_{50} (μM)
6		43
7		89
8		1.5
9		660
10		43
5		2.3

TABLE 3

Inhibitory Activity of PAF-Induced Rabbit Platelet Aggregation



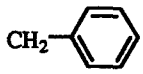
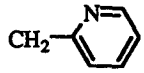
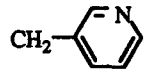
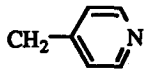
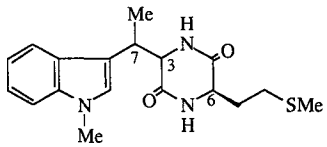
Compound	R	IC_{50} (μM)
4	CH_2SCH_3	7.6
5	$CH_2CH_2SCH_3$	2.3
11	$CH_2CH_2CH_2CH_3$	1.9
12	$(CH_2)_{11}CH_3$	25
13		2.5
14	CH_2CH_2COOEt	15
15	CH_2OCOCH_3	29
16		5.2
17		140
18		86
19	CH_2CH_2COOH	150
20	CH_2OH	150

TABLE 4

Inhibitory Activity of PAF-Induced Rabbit Platelet Aggregation

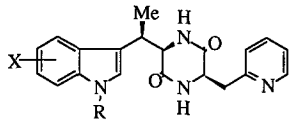


Compound	Configuration of C-7	C-3	C-6	IC_{50} (μM)
21	R	R(D)	R(D)	0.70
22	S	R(D)	R(D)	3.6
23	S	S(L)	R(D)	>230.0
24	R	S(L)	R(D)	>230.0

activity of compound 25, whereas the methoxy 28 and fluoro derivatives 29 showed almost the same activity as compound 25. Compound 32, a regioisomer of compound 31, showed less than one-tenth of the activity of 31. Compound 33 showed the most potent activity among the compounds in this series.

TABLE 5

Inhibitory Activity of PAF-Induced Rabbit Platelet Aggregation



Compound	X	R	IC_{50} (μM)
25	H	Me	0.44
26	H	Et	0.16
27	H	n-Pr	0.15
28	5-OMe	Me	0.51
29	5-F	Me	0.47
30	5-Cl	Me	0.15
31	5-Me	Me	0.21
32	6-Me	Me	2.9
33	5-Cl	Et	0.036

DISCUSSION

Four stereoisomers of diketopiperazines from *N*-methyltryptophan and *S*-methylcysteine were synthesized to determine which diketopiperazine stereochemistry was most desirable for inhibitory activity (Table 1). The most active compound had D-D configuration and the compounds prepared from L-N-methyltryptophan showed only weak activities. Thus the D-D configuration is necessary for potent inhibitory activity in this diketopiperazine series. Next, the effect of modification of the oxindole moiety on inhibitory activity was examined (Table 2). The hydrophobic α-naphthyl and *N*-methylindole derivatives showed high inhibitory activity, whereas the hydrophilic pyridyl derivative showed very weak activity. These results suggest that the hydrophobic aromatic moiety is important in the inhibition of PAF. Hydrophobic, moderately hydrophobic, and hydrophilic derivatives were synthesized to examine the effect of the modification of the *N*-methylcysteine part (Table 3). The hydrophobic derivatives 5, 11, and 13 showed high potency. The hydrophilic derivatives 19 and 20 showed only weak activities, whereas the corresponding esters 14 and 15, which were more hydrophobic, showed moderate potency. These results suggest that a hydrophobic side chain is preferable in inhibiting PAF activity. The long chain derivative 12, which is the most hydrophobic compound prepared in this study, showed relatively weak activity, probably due to steric hindrance. The difference in activities between the three picolyl isomers suggests that there may be an ionic and/or hydrogen bonding interaction in addition to a hydrophobic interaction between the inhibitors and the receptor. The effect of the β-methyl group in the tryptophan residue was examined to see whether the methyl group is necessary for inhibitory activity (Table 4). The inhibitory activity of the derivative with (7R)-methyl and D-D diketopiperazine configuration was about three times higher than that of the corresponding desmethyl derivative. The activity of the (7S)-methyl counterpart was almost the same as that of the desmethyl derivative. These results suggest that the configuration of the β-methyl group affects the inhibitory activities of diketopiperazines.

Finally, the effect of substituents on the indole ring were examined (Table 5). Compounds having the hydrophobic group showed potent inhibitory activities. This result is similar to that observed when the oxindole part was modified. Compound **32**, a regioisomer of compound **31**, showed less than one-tenth of the activity of compound **31**. This suggests that a steric limitation may exist at the receptor site which interacts with the aromatic portion of diketopiperazines.

In summary, our results suggest the stereochemistry of the diketopiperazine, *i.e.*, the D-D configuration, is an important factor in the inhibition of PAF. In addition, the hydrophobic aromatic portion and hydrophobic side chain play important roles in the binding of the diketopiperazine to the PAF receptor.

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Pharmacological Properties of YM461, a New Orally Active Platelet-Activating Factor Antagonist[1]

Toshimitsu Yamada, Munetoshi Saito, Toshiyasu Mase, Hiromu Hara, Hitoshi Nagaoka, Kiyoshi Murase and Kenichi Tomioka*

Medicinal Research Laboratories, Yamanouchi Pharmaceutical Co., Ltd., Tsukuba, Ibaraki 305, Japan

The antagonistic effect of YM461 [1-(3-phenylpropyl)-4-[2-(3-pyridyl)thiazolidin-4-ylcarbonyl]piperazine fumarate] against platelet-activating factor (PAF) was examined in several *in vitro* and *in vivo* systems. We found that YM461 inhibited [^{3}H]PAF binding to rabbit platelet membranes with a pK_i value of 8.90. YM461 inhibited PAF induced rabbit and human platelet aggregation with pA_2 values of 7.52 and 7.29, respectively; the slopes of the Schild plots were 1.07 and 1.01, respectively. However, YM461 at 10^{-4}M did not affect rabbit and human platelet aggregation induced by ADP, collagen, arachidonic acid or epinephrine. YM461 inhibited PAF induced death in mice with an ED_{50} (50% effective dose) value of 0.35 mg/kg *p.o.* YM461 at doses above 0.3 mg/kg *i.v.* inhibited PAF induced hypotension in rats. YM461 showed a dose-dependent inhibition of PAF induced hemoconcentration in rats with ED_{50} values of 0.15 and 0.21 mg/kg *p.o.*, respectively, at 0.5 and 1 hr after oral administration. The anti-PAF effect of YM461 persisted more than 6 hr after 3 mg/kg *p.o.* in rats. YM461 inhibited the bronchoconstriction induced by PAF with an ED_{50} value of 1.2 mg/kg *p.o.* in anesthetized guinea pigs. Furthermore, the compound at doses above 3 mg/kg *p.o.* significantly inhibited antigen-induced anaphylactic asthma in conscious guinea pigs pretreated with mepyramine and propranolol. These results indicate that YM461 is a selective, potent and orally active PAF antagonist.
***Lipids* 26, 1179–1183 (1991).**

Platelet-activating factor (PAF) is a phospholipid (1,2) that is synthesized by various cells including neutrophils (3), eosinophils (4), basophils (1), macrophages (5), platelets (6) and endothelial cells (7). PAF exerts various biological effects such as platelet aggregation (8), neutrophil degranulation (9), cardiac and renal dysfunction (10), hypotension (11), bronchoconstriction (12) and enhancement of vascular permeability (13). Because of its various potent activities, PAF has been implicated to possibly play an important role in asthma, shock, glomerulonephritis and other diseases. Therefore, inhibitors of the formation and the action of PAF are thought to be of therapeutic value in the treatment of these diseases. In fact, beneficial effects of PAF antagonists in several animal models and in human diseases have been reported. For example, WEB 2086, a thienotriazolodiazepine derivative, inhibits antigen-induced bronchoconstriction in guinea pigs (14). BN52063, an extract of the Ginkgo biloba, improves symptoms in asthmatic patients (15). CV-3988, a structural analogue of PAF, inhibits endotoxin-induced shock in rats (16). L-652,731, a trimethoxyphenyl tetrahydrofuran derivative, protects the rat from renal injury in nephrotoxic glomerulonephritis (17).

We have synthesized several thiazolidin derivatives, tested their anti-PAF effects, and found several orally active PAF antagonists. In the present study, we describe the pharmacological properties of 1-(3-phenylpropyl)-4-[2-(3-pyridyl)thiazolidin-4-ylcarbonyl]piperazine fumarate (YM461, Fig. 1), one of the orally active PAF antagonists, in several *in vitro* and *in vivo* test systems.

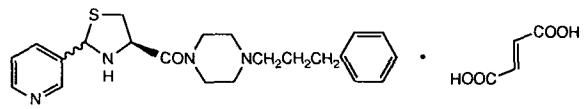


FIG. 1. Chemical structure of YM461. YM461 is a mixture of *cis* and *trans* isomers; the *cis/trans* ratio reaches equilibrium (*cis/trans*, 3:2) in aqueous solution within 2 min.

MATERIALS AND METHODS

Chemicals. Tritium-labeled PAF ([^{3}H]PAF) was purchased from Amersham (Buckinghamshire, England). PAF was obtained from Funakoshi Pharmaceuticals (Tokyo, Japan) and kept at −80°C in a stock solution of saline containing 0.25% bovine serum albumin (BSA, Sigma, St. Louis, MO). Dilutions were made with 0.25% or 0.1% (for platelet aggregation) BSA-saline on a daily basis as needed. Mepyramine, DL-propranolol hydrochloride and arachidonic acid were purchased from Sigma, ADP from Boehringer Mannheim (Mannheim, Germany), collagen from Niko Bioscience (Tokyo, Japan), and epinephrine from Daiichi (Tokyo, Japan). YM461 and WEB 2086 were synthesized in our labolatories.

Animals. Male, Japanese, white strain rabbits were obtained from the Clean experimental animal center (Saitama, Japan). Male ICR mice and Wistar rats were obtained from Japan SLC (Hamamatsu, Japan). Male Hartley guinea pigs were from Hitachi Animal Medical Research Institute (Ibaraki, Japan).

[^{3}H]PAF binding to rabbit platelet membranes. [^{3}H]-PAF binding to rabbit platelet membranes was determined by the method of Hwang *et al.* (18). Briefly, platelets from male, Japanese, white strain rabbits, weighing about 3 kg, were separated by centrifugation and the platelet membranes were obtained by repeated freezing and thawing. The incubation mixture contained 1.5 nM [^{3}H]PAF (222 dpm/fmol), 0.25% BSA in the presence or absence of unlabeled PAF or test compound, and rabbit platelet membrane protein (75 μg) in 0.5 mL of incubation buffer containing 5 mM $MgCl_2$, 2 mM EDTA and 10 mM Tris-HCl (pH 7.0). Incubations were performed at 0°C for

[1] Based on a paper presented at the Third International Conference on Platelet-Activating Factor and Structurally Related Alkyl Ether Lipids, Tokyo, Japan, May 1989.

*To whom correspondence should be addressed at Medicinal Research Laboratories, Yamanouchi Pharmaceutical Co., Ltd., 21 Miyukigaoka, Tsukuba, Ibaraki 305, Japan.

Abbreviations: BSA, bovine serum albumin; EC_{50}, 50% effective concentration; ED_{50}, 50% effective dose; IC_{50}, 50% inhibitory concentration; PAF, platelet-activating factor, 1-*O*-alkyl-2-acetyl-*sn*-glycero-3-phosphocholine; PPP, platelet-poor plasma; PRP, platelet-rich plasma.

120 min and the mixtures were rapidly filtered. The radioactivity on the filter was counted in a Packard liquid scintillation counter linked to an HP-85 computer (Hewlett Packard, San Diego, CA). The radioligand competition activity (K_i) of each compound was calculated from the equation: $K_i = IC_{50}/(1 + [[^3H]PAF]/K_d)$ where IC_{50} is the concentration required to inhibit specific binding by 50%, and K_d is the equilibrium dissociation constant for [^{3}H]PAF binding to specific binding sites on the rabbit platelet membrane. The pK_i values were calculated from the negative logarithm of the K_i values.

Platelet aggregation. Rabbit and human blood were collected in plastic syringes with 1 volume of 3.8% sodium citrate. The blood was centrifuged at 270 × *g* for 10 min. The upper platelet-rich plasma (PRP) was removed carefully. The pellet was further centrifuged and the supernatant was used as platelet-poor plasma (PPP). The platelet concentrations were adjusted with PPP to 5×10^8 cells/mL for rabbit and 3×10^8 cells/mL for human. Platelet aggregation was measured by the method of Born (19) using a Hema Tracer IV (Niko Bioscience, Tokyo, Japan). Test compounds at various concentrations were added to PRP 2 min prior to the aggregant.

PAF-induced death in mice. Six-week-old male ICR mice were injected with 75 μg/kg PAF through the tail vein, and the mortality of animals was estimated at 24 hr after PAF injection. Test compounds were administered orally 1 hr prior to PAF.

PAF-induced hypotension. Wistar rats weighing 295 to 330 g were anesthetized with sodium pentobarbital (50 mg/kg *i.p.*). Blood pressure was monitored from the right carotid artery. The test compound was injected into the right femoral vein 5 min prior to bolus injection of PAF (0.5 μg/kg *i.v.*).

PAF-induced hemoconcentration. Wistar rats weighing 250 to 315 g were anesthetized with sodium pentobarbital (50 mg/kg *i.p.*) and cannulated in the right carotid artery for blood sampling. Hemoconcentration was determined by measuring the changes of hematocrit between before and 10 min after *i.v.* injection of 1.65 μg/kg PAF. The test compound was administered orally 0.5, 1, 2, 3, 4 or 6 hr before injection of PAF.

PAF-induced bronchoconstriction. Airway resistance was determined according to the method of Konzett and Rössler (20). Guinea pigs, weighing 500 to 690 g, were anesthetized with 1.2 g/kg *i.p.* urethane, and intratracheal cannula and catheter were inserted into the jugular vein for drug administration. Animals were then ventilated with a respirator (Shinano, Tokyo, Japan) at 60 strokes/min with a volume of 1 mL/100 g body weight/cycle; gallamine (1 mg/kg *i.v.*) was injected to suppress spontaneous breathing. Intrapulmonary pressure was measured with a bronchospasm transducer (Ugo Basile, Comerio, Italy) connected to AD-611G (Nihon Kohden, Tokyo, Japan) and bronchoconstriction was expressed as percentage of maximal response obtained by clamping the trachea. The test compound was administered orally 1 hr prior to bolus injection of 50 ng/kg PAF.

Anaphylactic asthma in conscious guinea pigs. Hartley guinea pigs, weighing 370 to 430 g, were passively sensitized by intravenous injection of 1 mL/kg of rabbit anti-BSA serum (passive hemagglutination titer, 20480) (21, 22). Twenty-four hr after sensitization, the animals were pretreated with mepyramine (2 mg/kg *i.v.*) and propranolol (0.3 mg/kg *i.v.*) 5 min prior to antigen challenge. The animals were placed in an 11-L chamber connected to a glass nebulizer (KG-20, Kinoshita, Tokyo, Japan), and 1% solution of BSA was sprayed into the chamber for 30 sec. The animals were exposed to the antigen aerosol for 2 min and observed for 20 min after antigen challenge. The time from the start of inhalation to the onset of coughing and the mortality of animals were recorded. The test compound was administered orally 1 hr before antigen challenge.

Statistics. The IC_{50}, EC_{50} and ED_{50} values were calculated by the Probit method (23). The pA_2 values for the antagonists were obtained from Schild plots of the log of the molar concentration of the antagonist against the log of (concentration ratio −1) as described by Arunlakshana and Schild (24). Statistical significance of the data was estimated using Dunnett's multiple comparison test or Student's *t*-test.

RESULTS

[^{3}H]PAF binding to rabbit platelet membranes. K_d and B_{max} for [^{3}H]PAF binding to rabbit platelet membranes were 1.8 nM and 0.53 nmole/mg protein, respectively. YM461, PAF and WEB 2086 inhibited [^{3}H]PAF binding to rabbit platelet membranes in a dose-dependent manner, and their pK_i values were 8.90 (8.80–9.00), 8.48 (8.36–8.62) and 7.74 (7.64–7.82), respectively (Fig. 2).

Platelet aggregation in rabbit and human platelet-rich plasma. PAF produced a dose-dependent aggregation of rabbit platelets with EC_{50} value of 1.7×10^{-9} M (Fig. 3). YM461 shifted the PAF dose-response curve to the right in a parallel manner without reduction of the maximal response (Fig. 3). The pA_2 value for YM461 was 7.52 and the slope of the regression line of the Schild plot was 1.07. WEB 2086 also showed a dose-dependent inhibition of rabbit platelet aggregation with a pA_2 value of 7.31 (data not shown). This pA_2 value was similar to that reported by O'Donnell and Barnet (25).

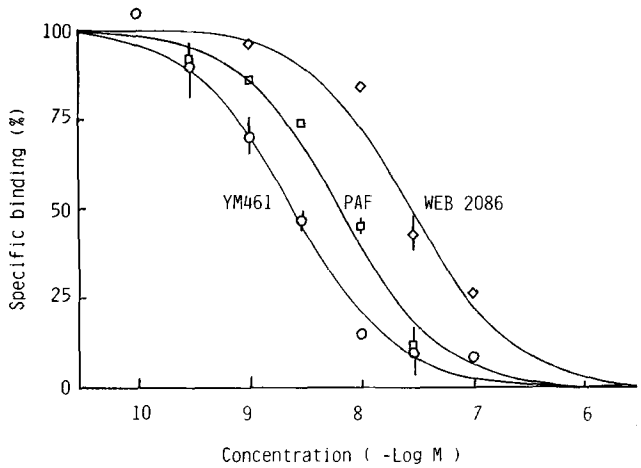


FIG. 2. Effects of YM461, PAF and WEB 2086 on [^{3}H]PAF binding to rabbit platelet membranes. Various concentrations of test compounds, 75 μg membrane protein and 1.5 nM [^{3}H]PAF were added to the medium containing 5 mM $MgCl_2$, 2 mM EDTA and 0.25% BSA and 10 mM Tris-HCl (pH 7.0). Each point represents the mean ± SEM of six experiments.

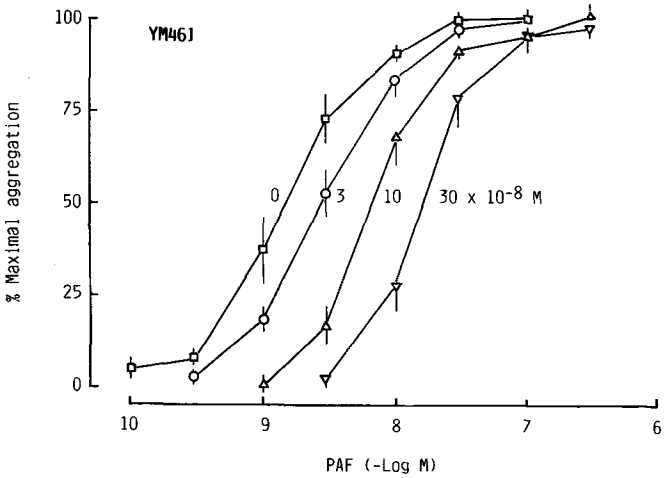


FIG. 3. Inhibition of PAF induced rabbit platelet aggregation by YM461. The % aggregation is relative to the maximal response (100%) obtained with 10^{-7} M PAF in corresponding control (0 M) platelets. Each point represents the mean ± SEM of seven experiments.

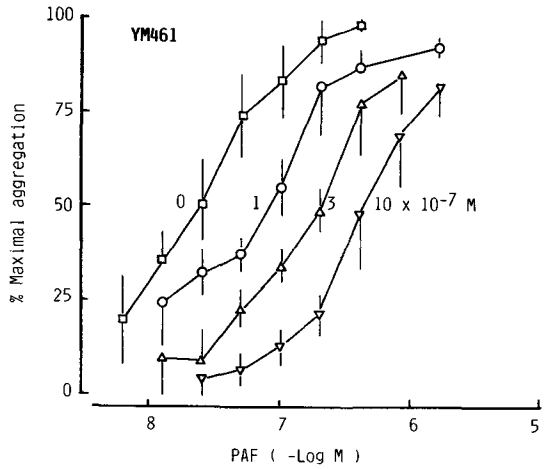


FIG. 4. Inhibition of PAF induced human platelet aggregation by YM461. The % aggregation is relative to the maximal response (100%) obtained with 4×10^{-7} M PAF in corresponding control (0 M) platelets. Each point represents the mean ± SEM of four experiments.

The effect of YM461 on PAF induced human platelet aggregation is shown in Figure 4. PAF induced dose-dependent aggregation of human platelets with an EC_{50} value of 2.8×10^{-8} M. As with rabbit platelets, YM461 showed a parallel shift of the PAF dose-response curve to the right without altering the maximal response. The pA_2 value for YM461 and its slope were 7.29 and 1.01, respectively.

As shown above, YM461 dose-dependently inhibited PAF induced rabbit and human platelet aggregation. However, there is a possibility that YM461 exerts non-specific inhibitory effect on platelets. Therefore, we determined next the effect of YM461 on rabbit and human platelet aggregation induced by PAF, ADP, collagen, arachidonic acid or epinephrine. As shown in Table 1, YM461 inhibited PAF induced rabbit and human platelet aggregation with IC_{50} values of 7.1×10^{-8} and 2.2×10^{-7} M, respectively. However, YM461 at 10^{-4}M did not show any significant inhibitory effect on platelet aggregation induced by ADP, collagen, arachidonic acid or epineprine.

TABLE 1

Effect of YM461 on Platelet Aggregation Induced by PAF, ADP, Collagen, Arachidonic Acid or Epinephrine in Rabbit and Human Platelet Rich Plasma[a]

Agonist	Concentration of agonist (μm)	IC_{50} (μM) Rabbit	Human
PAF	0.01,[b] 0.1∼0.4[c]	0.22	0.071
ADP	3	> 100	> 100
Collagen	10[d]	> 100	> 100
Arachidonic acid	100	> 100	NT[e]
Epinephrine	1∼5	—	> 100

[a]Concentrations of platelets were adjusted to 5×10^8 and 3×10^8 cells/mL, respectively, for rabbit and human. IC_{50} values were calculated by the Probit method (23) (N=3∼9).
[b]Rabbit. [c]Human. [d]μg/mL. [e]NT, not tested.

TABLE 2

Protective Effects of YM461 and WEB 2086 on PAF Induced Death in Mice

Drug[a]	Dose (mg/kg *p.o.*)	Mortality (death/tested)	Inhibition (%)	ED_{50} (mg/kg *p.o.*)
Control	—	10/10	—	
YM461	0.03	10/10	0	
	0.1	8/11	27	0.35
	0.3	7/10	30	(0.17-0.73)[b]
	1.0	2/10	80	
	3.0	0/10	100	
Control	—	9/10	—	
WEB 2086	0.1	8/10	11	0.57
	0.3	6/10	33	(0.33-0.98)[b]
	1.0	4/10	56	
	3.0	0/10	100	

[a]Drugs were administered orally 1 hr prior to PAF (75 μg/kg *i.v.*).
[b]95% Confidence limit of ED_{50} value calculated by Probit method (23).

Effect on PAF induced death in mice. As shown in Table 2, the mortality in control mice was 90 to 100% at 24 hr after PAF injection. YM461 and WEB 2086 dose-dependently inhibited PAF induced death in mice; the 50% protective dose of these compounds were 0.35 and 0.57 mg/kg *p.o.*, respectively.

Effect on PAF induced hypotension in anesthetized rats. Bolus injection of PAF produced a marked hypotension and reached a peak effect (36% decrease in blood pressure) at 2 min after injection. YM461 dose-dependently inhibited PAF induced hypotension; complete inhibition was observed at 0.3 mg/kg YM461 (Fig. 5).

PAF induced hemoconcentration in rats. In 35 control rats, the hematocrit value increased by 43 ± 1% at 10 min after PAF injection. YM461 dose-dependently inhibited PAF induced hemoconcentration (Fig. 6). When the anti-PAF effect of YM461 was plotted as a function of time, the peak effect was observed between 0.5 and 1 hr after oral administration, and ED_{50} values were 0.15 and

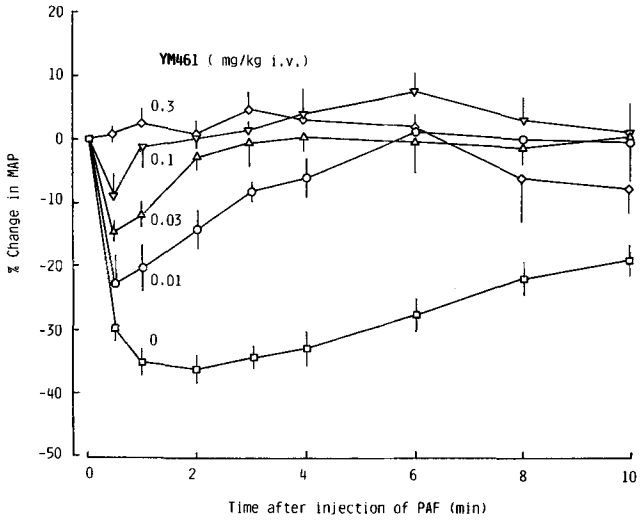


FIG. 5. Effect of YM461 on PAF induced hypotension in anesthetized rats. YM461 was administered intravenously 5 min prior to bolus injection of 0.5 μg/kg PAF. Each point represents the mean ± SEM of 4 to 16 rats.

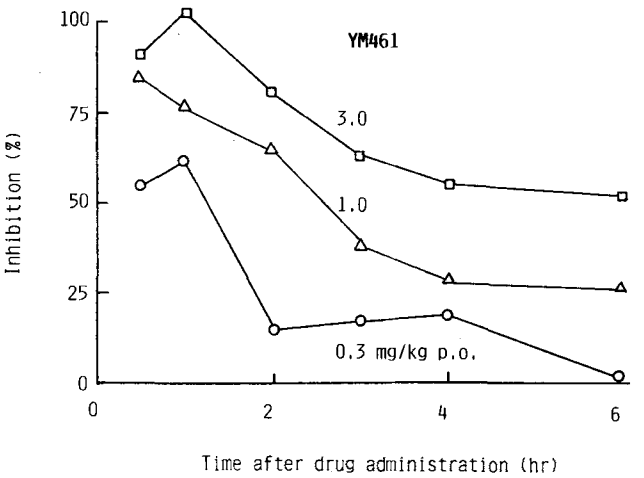


FIG. 6. Inhibitory effect of YM461 on PAF induced hemoconcentration in rats. YM461 was administered orally 0.5 to 6 hr before PAF (1.65 μg/kg *i.v.*). Each point represents the mean ± SEM of 3 to 13 rats.

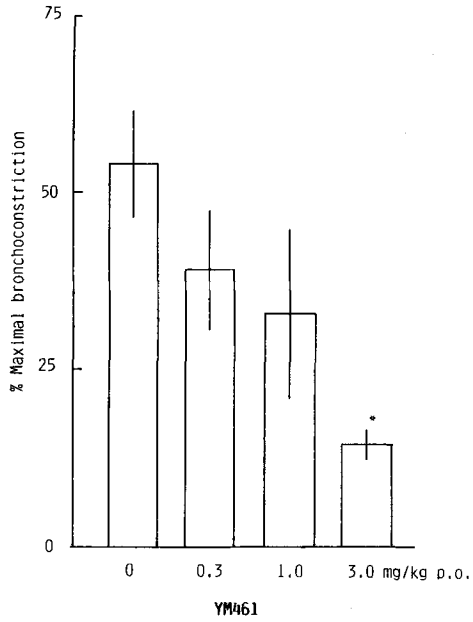


FIG. 7. Effect of YM461 on PAF induced bronchoconstriction in anesthetized guinea pigs. PAF (50 ng/kg) was injected in the jugular vein. YM461 was administered orally 1 hr before PAF. Bronchoconstriction is expressed as a percentage of the maximal response obtained by clamping the trachea. The data represent the mean ± SEM of 4 to 12 guinea pigs. *Significantly different from the value of control group at $p<0.05$ by Dunnett's multiple comparison test.

TABLE 3

Effects of YM461 and WEB 2086 on Antigen-Induced Anaphylactic Asthma in Guinea Pigs[a]

Compound[b]	Dose (mg/kg *p.o.*)	N	Time to onset of cough[c] (s)	Mortality
Control	—	8	415 ± 46	1/8
YM461	1	8	516 ± 49	2/8
Control	—	8	369 ± 42	4/8
YM461	3	8	540 ± 40[d]	0/8
Control	—	8	361 ± 39	4/8
YM461	10	8	479 ± 37[d]	1/8
Control	—	8	454 ± 57	4/8
WEB 2086	1	8	532 ± 29	3/8
Control	—	8	417 ± 49	2/8
WEB 2086	3	8	484 ± 43	0/8
Control	—	8	433 ± 53	3/8
WEB 2086	10	8	562 ± 26[d]	1/8

[a] Guinea pigs were passively sensitized with rabbit anti-BSA serum one day before antigen challenge, and pretreated with mepyramine (2 mg/kg *i.v.*) and propranolol (0.3 mg/kg *i.v.*) 5 min prior to antigen challenge.

[b] Test compounds were administered orally 1 hr prior to inhalation of 1% BSA solution.

[c] When animals did not cough over 10 min after challenge, time was calculated as 600 sec.

[d] Significantly different from the value of control group at $p<0.05$ by Student's *t*-test.

0.21 mg/kg *p.o.*, respectively. The anti-PAF effect of YM461 at 0.1 and 0.3 mg/kg *p.o.* returned to near control levels at 3 hr, but at 1 and 3 mg/kg *p.o.* the compound showed 25 and 58% inhibition, respectively, 6 hr after treatment.

PAF induced bronchoconstriction in guinea pigs. As shown in Figure 7, bolus injection of PAF produced 54% bronchoconstriction of maximal response in control group. YM461 at 0.3, 1 and 3 mg/kg *p.o.* dose-dependently inhibited PAF induced bronchoconstriction; the ED_{50} value was 1.2 mg/kg *p.o.*

Antigen-induced anaphylactic asthma in conscious guinea pigs. The guinea pigs in the control group exhibited cough within an average of 361 to 454 sec following inhalation of antigen; 1/8 to 1/2 of the animals died within 20 min. YM461 at 3 and 10 mg/kg *p.o.* significantly prolonged the time till onset of cough and tended to reduce the mortality of animals. WEB2086 at 10 mg/kg also prolonged the time interval by onset of cough (Table 3).

DISCUSSION

YM461 was shown to possess the activity profile of a PAF antagonist in several *in vitro* and *in vivo* test systems. YM461, PAF and WEB 2086 inhibited [^{3}H]PAF binding to rabbit platelet membranes with pK_i values of 8.90, 8.48 and 7.74, respectively (Fig. 4). The affinity of YM461 to PAF receptors on platelet membranes was 2.6, and 14 times higher than those of PAF and WEB 2086. Furthermore, YM461 inhibited PAF induced rabbit and human platelet aggregation with pA_2 values of 7.52 (Fig. 3) and 7.29 (Fig. 4), respectively. Competitive antagonism of YM461 against PAF induced rabbit and human platelet aggregation was confirmed by the slope of unity of the Schild plot. YM461 at 10^{-4} M did not show any inhibitory effect on rabbit and human platelet aggregation induced by ADP, collagen, arachidonic acid or epinephrine (Table 1). These results indicate that YM461 is a selective and competitive PAF antagonist.

Next, we examined the anti-PAF effect of YM461 using five *in vivo* models. Intravenous injection of PAF into ICR mice produced a lethal shock which resembled anaphylactic action (26). Several mediators, especially lipoxygenase products, may contribute to this reaction, because different types of drugs, including steroids, β-adrenoceptor agonists, lipoxygenase inhibitors and PAF antagonists, protect mice from PAF induced death (27).

YM461 dose-dependently protected the mice from PAF induced death with ED_{50} value of 0.35 mg/kg *p.o.* (Table 2). YM461 is not a steroid and has no β-agonistic action because the compound inhibits anaphylactic asthma in the presence of propranolol (Table 3). Furthermore, YM461 does not act as a leukotriene antagonist or a 5-lipoxygenase inhibitor, because the compound at 10^{-5} or 10^{-4} M inhibits neither LTD_4 (10^{-8} M) induced contraction of guinea pig ileum nor 5-lipoxygenase from mastocytoma P-815 cells (unpublished data). These results indicate that YM461 inhibits PAF induced death in mice *via* its anti-PAF effect.

Intravenous PAF injection induced marked hypotension and hemoconcentration in rats. YM461 at doses above 0.01 mg/kg *i.v.* and 0.1 mg/kg *p.o.* dose-dependently inhibited PAF induced hypotension and hemoconcentration. The compound at 3 mg/kg *p.o.* inhibited hemoconcentration by 58% even at 6 hr after oral administration, indicating YM461 has a relatively long duration of action. PAF induced reactions in rats are brought about through platelet-independent mechanisms because rat platelets are insensitive to PAF (28). Accordingly, YM461 can block PAF actions both in platelet-dependent and platelet-independent systems.

YM461 at doses of 0.3 to 3.0 mg/kg *p.o.* dose-dependently inhibited PAF induced bronchoconstriction in guinea pigs (Fig. 7). YM461 at doses above 3 mg/kg *p.o.* also inhibited antigen-induced anaphylactic asthma in passively sensitized guinea pigs (Table 3). Furthermore, YM461 inhibited antigen-induced late airway responses and associated airway hyperresponsiveness in allergic sheep (29). These results indicate that oral YM461 is able to inhibit the actions induced not only by exogenously administered PAF but also by endogenously liberated PAF.

In summary, our results indicate that YM461 is a selective, potent and orally active PAF antagonist. YM461 may therefore be useful in the treatment of pathological conditions associated with excessive PAF production.

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REVIEW

Bioactions of 5-Hydroxyicosatetraenoate and its Interaction with Platelet-Activating Factor[1]

Adriano G. Rossi* and **Joseph T. O'Flaherty**
Department of Medicine, Section of Infectious Diseases, Wake Forest University Medical Center, Winston-Salem, NC 27103

In a variety of stimulated cells, platelet-activating factor (PAF) and numerous arachidonate derivatives are co-products that form as a consequence of receptor-mediated phospholipid mobilization. These lipid co-products produce a plethora of biological effects in a wide variety of cell systems. Furthermore, they often have a fascinating, although less widely appreciated, interaction. 5-HETE, at submicromolar concentrations, exerts relatively few direct bioactions. It does, however, potently (16–160 nM) raise cytosolic free calcium $[Ca^{2+}]_i$ and augment PAF-induced responses in human polymorphonuclear neutrophils (PMN) by as much as 100- to 1000-fold. 5-HETE acts on PMN by a structurally specific, stereospecific and pertussis toxin-inhibitable mechanism. In addition, PMN exposed to 5-HETE exhibit homologous but not heterologous desensitization. These findings suggest that 5-HETE, like PAF, may bind to its own specific plasmalemmal receptors to exert its unique set of bioactions. However, further investigation is required to demonstrate any putative 5-HETE receptors. Other potential mechanisms of 5-HETE-induced bioactions together with the possible effects of 5-HETE on PAF transduction mechanisms are also discussed.
Lipids 26, 1184–1188 (1991).

Many stimuli (S) elicit function by inducing target cells to convert their resident phospholipids into a series of signalling molecules (Fig. 1) (1,2). They generally achieve this by binding with specific plasmalemmal receptors (R) that operate through guanine nucleotide-binding proteins (G-proteins) to activate phospholipase(s) C (PLC). The activated phospholipase(s) cleaves phosphatidylinositol 4,5-bisphosphate (PIP_2) into two critical secondary messengers—inositol 1,4,5-triphosphate (IP_3) and diacylglycerol (DAG). IP_3 mobilizes storage calcium to elevate cytosolic free Ca^{2+}($[Ca^{2+}]_i$), whereas DAG activates protein kinase C (PKC) by a Ca^{2+}-enhanced interaction (3–9). Concurrently with these events, the stimuli also cause target cells to attack their phospholipids by a second route involving a calcium-dependent phospholipase(s) A_2 (PLA_2). The enzymes principally release

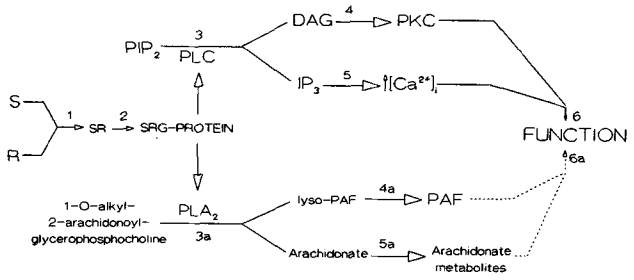


FIG. 1. Simplified schematic representation of stimulus-response coupling. See text for details.

arachidonate from the *sn*-2 position of phospholipids. When the substrate phospholipid is 1-*O*-alkyl-2-acyl-glycerophosphocholine, 1-*O*-alkyl-2-lyso-glycerophosphocholine is formed. Acetyltransferase converts this lyso intermediate into platelet-activating factor (PAF) (10–12). Finally, cyclooxygenase and various lipoxygenases oxygenate the arachidonate released by the phospholipase(s)-mediated cleavage reaction. Products of these oxygenations include prostaglandins (PGs), thromboxanes (TXs), hydroxyicosatetraenoates (HETEs), leukotrienes (LTs), and lipoxins (LXs) (13–20). Many of the above phospholipid derived products interact to influence cellular responsiveness. This review article focuses on some of these intriguing interactions.

PAF BIOSYNTHESIS, METABOLISM, AND MECHANISMS OF ACTION

As already indicated, cells commonly form PAF during stimulation by deacylating their resident 1-*O*-alkyl-2-acyl-glycerophosphocholine and then acetylating the lyso intermediate (10–12). In addition to the deacylation/acetylation pathways, cells form PAF by a *de novo* pathway involving the formation of 1-alkyl-2-acetyl-glycerophosphates and ultimate conversion of 1-alkyl-2-acetyl glycerol to PAF by a cholinephosphotransferase (12).

Cells also rapidly and quantitatively inactivate PAF. The principal pathway of cellular PAF metabolism uses an acetylhydrolase to convert PAF to its bioinactive derivative, 1-*O*-alkyl-2-lyso-glycerophosphocholine. The product is then rapidly acylated (commonly with arachidonate) by acyltransferase. 1-*O*-alkyl-2-acyl-glycerophosphocholine is stored but available for reconversion to PAF by the deacylation/acetylation pathway during cell stimulation (10–12). Minor pathways of PAF degradation (e.g., cleavage at *sn*-3) have also been reported (21–24).

PAF possesses a broad range of bioactions upon diverse cell types. Many, if not all, the bioactions of PAF proceed through a classical transduction mechanism (Fig. 1). That is, PAF binds with specific plasma membrane receptors

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*To whom correspondence should be addressed.

Abbreviations: $[Ca^{2+}]_i$, cytosolic free calcium ions; DAG, diacylglycerol; G-proteins, guanine nucleotide-binding proteins; HETE, hydroxyicosatetraenoate; HPETE, hydroperoxyicosatetraenoate; IP_3, inositol 1,4,5-triphosphate; LT, leukotriene; PAF, platelet-activating factor; PG, prostaglandin; PIP_2, phosphatidylinositol 4,5-bisphosphate; PKC, protein kinase C; PL, phospholipase; PMN, polymorphonuclear neutrophils; R, receptor; S, stimulus; TX, thromboxane.

that couple to G-proteins resulting in the activation of phospholipases C and A_2. Hence, PAF-challenged cells form DAG and IP_3, mobilize storage Ca^{2+} and PKC, and synthesize various arachidonate metabolites (11,12, 25–34). Three unique aspects of the PAF transduction cascade merit emphasis. First, G-proteins linked to PAF receptors may have somewhat different sensitivities to pertussis and cholera toxins than G-proteins linked with other receptor elements (35–39). Second, PAF receptors may couple with plasmalemmal calcium channels (36,37,40). Third, PAF, in utilizing the standard transduction cascade, stimulates its own synthesis (41,42). It should also be noted that PAF stimulates 5-HETE synthesis in target cells capable of forming this arachidonate metabolite (33,34).

5-HETE BIOSYNTHESIS, METABOLISM, AND STRUCTURAL CONSIDERATIONS

As indicated earlier, stimulated cells release arachidonate from phospholipids. Depending on the arachidonate pools utilized and the active enzymatic machinery available within a particular cell type, liberated arachidonate is converted to a variety of bioactive compounds. In leukocytes and mast cells, for example, arachidonate is predominantly attacked by the calcium- and ATP-dependent 5-lipoxygenase. This enzyme attaches molecular oxygen to carbon 5 of the fatty acid. The product may abstract a proton to form 5-hydroperoxyicosatetraenoate (HPETE), which is rapidly reduced to 5-HETE, or, alternatively, form the 5,6-epoxide, LTA_4. This unstable epoxide is metabolized enzymatically to the dihydroxy derivative, LTB_4, or converted non-enzymatically into isomers of LTB_4 (13–18,43,44). LTB_4, but not its isomers, is an extremely potent leukocyte (45,46) and smooth muscle (46–49) stimulus. It operates by binding to specific plasmalemmal receptors (50–53). While LTB_4 is a 5,12-diHETE, and therefore contains a 5-hydroxy residue, it should be noted that LTB_4 differs from 5-HETE in double bound positions and geometry. LTB_4 is a 5(S),12(R)-dihydroxy-6-*cis*, 8-*trans*,10-*trans*,14-*cis*-eicosatetraenoate, whereas 5-HETE is a 5(S)-hydroxy-6-*trans*-8-*cis*,11-*cis*, 13-*cis*-eicosatetraenoate. The two compounds have remarkably different spacial configurations and thus may not be structural analogs with similar bioactions. On the other hand, two other endogenously formed diHETEs, 5,15-diHETE and 5,20-diHETE, are 5(S),15(R)-dihydroxy-6-*trans*, 8-*cis*,11-*cis*,13-*trans*- and 5(S),20-dihydroxy-6-*trans*, 8-*cis*,11-*cis*,13-*cis*-eicosatetraenoates. These compounds more closely resemble 5-HETE and therefore might have biological analogies to 5-HETE.

After producing 5-HETE, cells may release the product extracellularly or metabolize it further by one of two alternate routes. First, many cells incorporate 5-HETE into glycerolipids, principally triglycerides but also various species of phospholipids. This pathway proceeds rapidly but is readily saturated (54–57). The second pathway slowly converts 5-HETE to its C-20 hydroxy derivative, 5,20-diHETE. This C-20 hydroxylation may be catalyzed by the same C-20 hydroxylase that converts LTB_4 to 20-OH-LTB_4 (57,58). Note that 5,20-diHETE and 20-OH-LTB_4 are 10–100-fold less potent than their respective parent compounds (57,59–62). The potency of certain PGs also appears to be reduced by C-20 hydroxylation (63). Thus, C-20 hydroxylation generally results in the inactivation of diverse bioactive arachidonate metabolites.

BIOACTIONS OF 5-HETE

Agonist activity. In many assays, 5-HETE by itself exerts relatively weak, but nevertheless definite, bioactions (17). For example, at concentrations ≥ 1 μM, it activates leukocytes (64–73), contracts smooth muscle-containing tissues [e.g., guinea pig lung strip and human isolated bronchial muscle (74)] and releases insulin from isolated rat pancreatic cells (75), luteinizing hormone from cultured rat pituitary cells (76), mucus from human airways (77), amylase from isolated guinea pig pancreatic acini (78), and intestinal secretions from rabbit colonic mucosa (79). In most of the above studies, various other mono-HETEs were shown to be comparably inactive. Given the concentration of 5-HETE required, the physiological relevancy of these bioactions remains uncertain. On the other hand, 5-HETE exhibits one of its most potent effects in raising $[Ca^{2+}]_i$—it mobilizes calcium in human PMN at ≥ 16 nM (80–83).

5-HETE interaction with PAF. In addition to its direct actions, 5-HETE has strikingly prominent influences on certain PAF-induced responses. For example, it increases the potency of PAF in stimulating PMN degranulation and oxidative metabolism by as much as 1000-fold (84,85). Under the same assay conditions, 5-HETE by itself fails to induce function. Thus, 5-HETE potentiates PAF bioactions. 5-HETE also potentiates the PMN stimulating actions of DAG and, to a much lesser extent, LTB_4 (86). However, it has no influence on functional responses to chemotactic peptides or ionophoretic stimuli. Thus, the potentiating actions of 5-HETE are stimulus-selective and, intriguingly, restricted to those stimuli that are generated in activated cells. Other arachidonate metabolites have 5-HETE-like bioactions. For example, 5-HPETE is as potent as 5-HETE at inducing many of the above responses. In addition, 5,15-diHETE and 5,20-diHETE also potentiate the PMN degranulating actions of PAF: these compounds are ~30 and ~100-fold, respectively, less potent than 5-HETE. Various other analogs, such as 8-HETE, 11-HETE, 12-HETE, 8,15-diHETE, and LTB_4 do not promote the actions of PAF (84–88).

More recent studies reveal that 5-HETE increases the bioactions of PAF at a very fundamental level—in PMN, it potentiates the ability of PAF to activate PKC (89–92) and mobilize phospholipids (42,93,94). These activities have mechanistic implications which are discussed below.

5-HETE interaction with other stimuli. 5-HETE potentiates antigen-stimulated histamine release from human basophilic leukocytes (95,96), IgE-stimulated histamine release from rat mast cells (97), FMLP-stimulated arachidonate release from dimethylsulfoxide differentiated HL60 granulocytes (98), LTD_4-induced contractions of isolated guinea pig trachea (99) and histamine-induced contractions of isolated human bronchial muscle (74). Unfortunately, the mechanisms and consequences of these effects have not been reported.

MECHANISMS OF ACTION

It seems quite unlikely that any single mechanism will successfully explain the many diverse bioactions of 5-HETE. We suggest that 5-HETE has a multiplicity of effects, any one of which may be pertinent to a particular

response, stimulus, or assay condition. The proposed mechanisms of 5-HETE's bioactions fall into five areas.

Membrane perturbation. As noted above, PMN incorporate 5-HETE into cellular triglycerides and phospholipids (54–57). Stenson and Parker (54) have suggested that this alters membrane physiochemistry to promote function. This mechanism, however, fails to explain the agonist-selectiveness of 5-HETE. The arachidonate metabolite does not promote degranulation per se, but rather enhances the degranulation and other bioactions of a restricted few types of stimuli.

Antimetabolic action. PMN convert 5-HETE and LTB_4 to C-20 oxidized derivatives (57–61). It is likely that this oxidation is achieved by a common C-20 hydrolase. Thus 5-HETE might compete for the hydrolase to prolong the metabolic half-life and bioavailability of LTB_4. This effect could potentiate the stimulatory actions of the leukotriene. Indeed, we have observed that 5-HETE approximately doubles the half-life of LTB_4 in PMN under the same conditions in which it increases LTB_4 potency (unpublished observations). However, we find that 5-HETE does not inhibit PAF metabolism. The antimetabolic hypothesis, then, is unlikely to explain the actions of 5-HETE on PAF, the actions of 5-HETE on direct PKC activators (e.g., DAG and phorbol myristate acetate) or the ability of 5-HETE to stimulate responses by itself. However, if a significant part of the response induced by PAF or PKC activators is mediated by LTB_4, the antimetabolic hypothesis may have broader application to the potentiating actions of 5-HETE. Further investigation is required to clarify this possibility.

5-HETE receptors. The bioactions of 5-HETE resemble those of other receptor-mediated agonists in several respects. First, 5-HETE mobilizes Ca^{2+} and potentiates PAF at nanomolar concentrations. Therefore, these effects might require the amplification produced by receptors. In contrast, other actions of 5-HETE (e.g., potentiation of LTB_4) occur only in the near micromolar range—it is not certain that these findings are of more than a pharmacological interest. Second, 5-HETE bioactions are structurally-specific, as well as stereospecific. Thus, the unnatural enantiomer of 5-HETE, 5(R)-HETE is 20 to 100-fold less potent than the natural 5-HETE in raising PMN $[Ca^{2+}]_i$ and potentiating PAF. Since this unnatural 5-HETE preparation likely contained 1–3% natural 5-HETE enantiomer, the potency difference may be even more pronounced (83). In any event, these structural effects support the receptor hypothesis. Third, in PMN, 5-HETE rapidly desensitizes to itself in assays of Ca^{2+} mobilization (82) and PAF potentiation (84,88). Furthermore, PMN desensitized to 5-HETE raise $[Ca^{2+}]_i$ almost normally in response to LTB_4 or PAF (82). This stimulus-selective desensitization seems best explained as resulting from receptor down-regulation. Finally, the bioactions of 5-HETE are blocked by agents that interfere with G-protein/receptor interactions like pertussis and cholera toxins (100).

We have attempted to define PMN receptors for 5-HETE using [^{3}H]-labeled analogs. Unfortunately, the cells (as well as their isolated membrane preparations) quantitatively metabolized [^{3}H] 5-HETE as well as [^{3}H] 5-HETE methyl ester even at 4°C. This has interferred with our ability to assay these receptors. Nevertheless, the four findings listed above support further efforts to demonstrate 5-HETE receptors. Studies have demonstrated 15-HETE receptors in selected cell types (101,102).

Ca^{2+} mobilization. As discussed previously, 5-HETE potently mobilizes storage Ca^{2+} in PMN. Such mobilization raises $[Ca^{2+}]_i$ and appears responsible for increasing the PKC-activating potency of DAG as well as other direct PKC activators (81,103). It cannot explain other potentiating effects of 5-HETE. For example, 5-HETE does not enhance LTB_4-induced PKC mobilization (81). Furthermore, although 5-HETE potentiates PAF-induced PKC mobilization, studies discussed below suggest that this effect may result from a more fundamental interaction between PAF and 5-HETE.

Effects of 5-HETE on other receptors. 5-HETE exhibits little or no ability to cross-desensitize PMN to LTB_4 and PAF, or to interfere directly with the binding of these two latter ligands to their receptor sites (82). Nevertheless, 5-HETE may have a less direct actions on PAF receptors. This possibility is revealed by analyzing how PAF activates PMN. As indicated by the diagram, PAF operates in at least six discrete steps: step 1, PAF-receptor (R) binding; step 2, formation of a ternary complex with G-protein; step 3, activation of phospholipase C to cleave PIP_2; step 4, DAG mediated activation of PKC; and step 5, release of Ca^{2+} by IP_3. Concurrently, PAF also triggers a parallel pathway involving *sn*-2-cleavage of 1-*O*-alkyl-2-arachidonoyl-glycerophosphocholine (step 3a) and further metabolism of the cleavage products (step 4a and 5a). Following these steps, a complex set of poorly understood events (here termed steps 6 and 6a) produce function. Initial studies on the effects of 5-HETE in potentiating PAF-induced function indicated a site of action at or before step 6. More recent studies, however, show that 5-HETE enhances PAF-induced PKC mobilization (step 4) (81), Ca^{2+} transients (step 5) (unpublished observations) and PAF synthesis (step 4a) (42). Thus, 5-HETE appears to influence the actions of PAF at a very early stage, i.e., possibly at the PAF receptor or the receptor linked G-proteins. It therefore seems likely that 5-HETE will have a fundamental effect on the PAF transduction mechanism: the arachidonate metabolite may up-regulate PAF receptors and/or enhance these receptors' interactions with G-proteins.

CONCLUSION

5-HETE, one of many phospholipid derived products of activated cells, exerts unique bioactions and potently enhances specific agonist-induced responses. Its effects are structurally specific, stereospecific, and pertussis toxin sensitive. Furthermore, cells exposed to 5-HETE are desensitized to a second 5-HETE challenge but not to other receptor-mediated agonists. These observations suggest that 5-HETE operates through a specific receptor mechanism. However, the presence of putative 5-HETE receptors has not been demonstrated and therefore awaits documentation. In any event, the influences of 5-HETE on the bioactions of other agonists seem to reflect various mechanisms. Some of these may be "5-HETE-receptor" independent. Thus, 5-HETE may promote the bioactions of LTB_4 as well as LTB_4-dependent stimuli by directly interferring with LTB_4 metabolic inactivation. On the other hand, 5-HETE may potentiate direct PKC

activators by raising $[C_a^{2+}]_i$ through a receptor-dependent route. Finally, 5-HETE/PAF interactions could reflect 5-HETE-induced up-regulation of PAF receptors. The many other effects of 5-HETE on diverse agonists in other systems have yet to be explored. These studies will not only help decipher novel mechanisms involved in stimulus-response coupling, but also give insight into the role of multiple mediators in various biological processes.

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Platelet-Activating Factor (PAF) Receptor Antagonists Inhibit Arachidonic Acid Induced Platelet Aggregation in Rabbit Whole Blood[1]

Alaina Jean Ammit and Chris O'Neill*
Human Reproduction Unit, Royal North Shore Hospital of Sydney, St. Leonards, 2065, N.S.W., Australia

The potency of several platelet-activating factor (PAF) receptor antagonists was measured by observing their inhibitory effects against PAF induced platelet aggregation. Their selectivity was assessed by monitoring their effect on platelet aggregation induced by arachidonic acid (AA) and adenosine diphosphate (ADP). The antagonists inhibited platelet aggregation induced at the PAF EC_{50} (0.023 μM) with the following rank order of potency: WEB 2086> WEB 2170> SRI 64-412> SRI 63-675> BN 52021> kadsurenone> SRI 63-441> alprazolam. While the antagonists had no inhibitory effect at the EC_{50} for ADP (10 μM), they did inhibit platelet aggregation induced at the EC_{50} for AA (55 μM). However, there was considerable variability in the slope of the inhibitory response and the relative potency of each antagonist against PAF induced platelet aggregation as compared to AA induced platelet aggregation. The antagonist IC_{50} (μM) against PAF and AA were as follows, with those that showed significantly different ($p < 0.01$) slopes indicated by an asterisk: SRI 63-441* (3.8, 15.1); SRI 63-675 (1.4, 36.2); SRI 64-412 (0.5, 10.5); BN 52021* (2.4, 58.9); kadsurenone* (2.8, 28.3); alprazolam* (10, 25); WEB 2086 (0.055, 0.220), and WEB 2170 (0.107, 0.534). Therefore, in rabbit whole blood the antagonists were potent, although not completely selective, inhibitors of PAF induced platelet aggregation. These results suggest that the mode of action of PAF and AA induced platelet aggregation may share some common features. However, since the slope of the inhibitory response against PAF and AA for some antagonists differed, mechanistic differences in their action appear to exist.

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Platelet-activating factor (PAF) induces platelet aggregation *via* receptor-mediated mechanisms. Using a rapid *in vitro* bioassay for the quantification of platelet aggregation (1), our aim was to measure the potency and selectivity of several PAF receptor antagonists in rabbit whole blood. The antagonists examined were either synthesized compounds based on the PAF structure, such as SRI 63-441, SRI 63-675 and SRI 64-412; natural products, such as the terpene, BN 52021 and the neolignan, kadsurenone; or pharmacological agents such as the triazolobenzodiazepine alprazolam and the thienotriazolobenzodiazepines, WEB 2086 and WEB 2170. Antagonist potency was measured by observing their inhibitory effects at PAF EC_{50} (*i.e.*, the effective concentration required to induce 50% platelet aggregation), and was expressed as the IC_{50} (*i.e.*, the lowest concentration required to totally inhibit the EC_{50}). Antagonist selectivity was assessed by monitoring their effect on the platelet aggregation induced at the EC_{50} for arachidonic acid (AA) and adenosine diphosphate (ADP).

MATERIALS AND METHODS

Whole blood (0.8 volumes) was collected, using 20G teflon catheters (Surflo, Terumo, Tokyo, Japan), from the marginal ear vein of male New Zealand White rabbits into 0.2 volumes of citrate 3.2% (w/v) (trisodium salt-dihydrate, Sigma Chemicals Co., St. Louis, MO). The citrated rabbit whole blood was added, in 50 μL aliquots, to each of the eight wells of a teflon micromixing device, followed by 25 μL of antagonist/vehicle. After a 30-sec temperature equilibration period, 25 μL of the agonist EC_{50}/vehicle was added. A 10 μL sample was taken immediately and after 15 min incubation at 37°C for counting of the single, non-aggregated platelets (3–24 μm^3) using the Baker Instruments Series 810 Platelet Analyzer (Baker Instruments, Allentown, PA). The results were expressed as the platelet aggregation index, *i.e.*, $1\text{-}P_{15}/P_0$, where P_{15} and P_0 are the platelet count at 15 and 0 min, respectively. Therefore, an index of 0 signifies no platelet aggregation and, conversely, 1 is total platelet aggregation. The platelet count for male New Zealand White rabbits was approximately $250 \times 10^3/\mu L$, and there was minimal variation between experimental animals. The bioassay was performed immediately after blood collection.

PAF receptor antagonists examined were: SRI 63-441 (*cis*-(±)-1-[2-[hydroxy[[tetrahydro-5-[(octadecylaminocarbonyl)oxy]methyl]furan-2-yl]methoxy-phosphinyloxy]-ethyl]quinolinium hydroxide, inner salt; Sandoz Research Institute, East Hanover, NJ) dissolved in calcium/magnesium-free phosphate buffered saline (PBS) (140 mM NaCl, 2.6 mM KCl, 8.1 mM Na_2HPO_4, 1.5 mM KH_2PO_4; Sigma); SRI 63-675 (*cis*-(±)-1-[2-[hydroxy[[tetrahydro-2, 5-dimethyl-5-[(octadecylaminocarbonyl)oxy]methyl]furan-2-yl]methoxy-phosphinyloxy]ethyl]quinolinium hydroxide, inner salt; Sandoz) dissolved in PBS; SRI 64-412 (5-[4'-(3,4,5-trimethoxy phenylethyl)phenyl]-2,3-dihydroimidazo[2,1-a]isoquinoline hydrochloride; Sandoz) dissolved in 2% (v/v) acetic acid (AR grade; BDH Chemicals, Melbourne, Vic., Australia) in distilled water; BN 52021 (9H-1,7a-(epoxymethano)-1H,6a-H-cyclopenta-[c]-furo[2,3-b]furo[3',2':3,4]cyclopenta[1,2-d]furan-5,9,12-(4H)-trione,3-*tert*-butylhexahydro-4,7b-11-hydroxy-8-methyl; IHB-IPSEN, Le Plessis Robinson, France) dissolved in methanol (AR grade; BDH); kadsurenone (2-(3,4-dimethoxyphenyl)-2,3-dihydro-3a-methoxy-3-methyl-5-(allyl)-6-2H-oxobenzofuran; Merck, Sharp and Dohme Research Laboratories, Rahway, NJ) dissolved in ethanol (AR grade; BDH); alprazolam(8-chloro-1-methyl-6-phenyl-4H-2-tri-

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*To whom correspondence should be addressed.

Abbreviations: AA, arachidonic acid; ADP, adenosine diphosphate; EC_{50}, activator concentration required to induce 50% platelet aggregation; IC_{50}, antagonist concentration required to inhibit platelet aggregation by the EC_{50}; PAF, platelet-activating factor; PBS, phosphate buffered saline.

azolo[4,3-a][1,4]benzodiazepine; Upjohn, Kalamazoo, MI) dissolved in ethanol; WEB 2086 (3-[4-(2-chlorophenyl)-9-methyl-6H-thieno[3,2-f][1,2,4]triazolo-[4,3-a][1,4]diazepin-2-yl]-1-(4-morpholinyl)-1-propanone; Boehringer Ingelheim KG, Ingelheim am Rhein, Germany) dissolved in PBS; and WEB 2170 (6-(2-chloro-phenyl)-8,9-dihydro-1-methyl-8-(4-morpholinyl-carbonyl)-4H,7H-cyclopenta[4,5]-thieno[3,2-f][1,2,4]triazolo-[4,3-a][1,4]diazepine; Boehringer Ingelheim) dissolved in PBS. All dilutions were in PBS.

Activators used were: PAF (1-*O*-alkyl-2-acetyl-*sn*-glycero-3-phosphocholine; Sigma) dissolved in chloroform (AR grade; BDH), solvent was evaporated with N_2 and the sample was resuspended and diluted in PBS containing 0.25% (w/v) bovine serum albumin (CSL, Melbourne, Vic., Australia); AA (from porcine liver; Sigma) dissolved in ethanol, solvent was evaporated with N_2 and the sample was resuspended in 10 mM Na_2CO_3 (Sigma) and diluted in 0.9% (w/v) NaCl (Sigma); and ADP (sodium salt, grade I, from equine muscle; Sigma) dissolved and diluted in 0.9% NaCl. The concentrations of activators used were the EC_{50} as determined in a previous study (1).

Statistical analysis was performed by estimating regression models for the linear section of the inhibition dose-response curve (after correcting for differences in antagonist concentration), and by examining equality of slopes by analysis of covariance.

RESULTS

PAF receptor antagonists inhibited platelet aggregation induced by PAF at the EC_{50} (0.023 μm) in rabbit whole blood with the following rank order of potency: WEB 2086> WEB 2170> SRI 64-412> SRI 63-675> BN 52021> kadsurenone> SRI 63-441> alprazolam.

While the antagonists had no inhibitory effect at the EC_{50} for ADP (10 μM), they did inhibit platelet aggregation induced at the EC_{50} for AA (55 μM). The IC_{50} of the antagonist, the slope of the inhibitory response, and the relative potency of each antagonist against PAF and AA induced platelet aggregation are given in Table 1. Half of the antagonists examined showed significantly different ($p < 0.01$) slopes for the inhibition of PAF induced platelet aggregation as compared to AA induced platelet aggregation. Also, the relative potency of each PAF receptor antagonist ranged widely, *i.e.*, from 2.5 to 25.8. Based on these differences, the antagonists can be categorized into four groups: i) Those with parallel slopes and small differences in relative potency, *i.e.*, WEB 2086 and WEB 2170; ii) those with different slopes and small differences in relative potency, *i.e.*, SRI 63-441 and alprazolam; iii) those with parallel slopes and large differences in relative potency, *i.e.*, SRI 63-675 and SRI 64-412; and iv) those with different slopes and large differences in relative potency, *i.e.*, BN 52021 and kadsurenone. The inhibitory responses for antagonists that are representative of each group are shown in Figure 1 (a-d).

TABLE 1

Effect of PAF Receptor Antagonists on PAF Induced and Arachidonic Acid Induced Platelet Aggregation in Rabbit Whole Blood[a]

Antagonist	PAF		AA		Relative potency
	IC_{50} (μM)	Slope	IC_{50} (μM)	Slope	
SRI 63-441[b]	3.8	−0.10	15.1	−0.02	4.0
SRI 63-675	1.4	−0.28	36.2	−0.28	25.8
SRI 64-412	0.5	−0.79	10.5	−0.78	21.0
BN 52021[b]	2.4	−0.18	58.9	−0.06	24.5
Kadsurenone[b]	2.8	−0.15	28.3	−0.04	10.1
Alprazolam[b]	10.0	−0.03	25.0	−0.09	2.5
WEB 2086	0.055	−6.15	0.220	−6.12	4.0
WEB 2170	0.107	−3.64	0.534	−2.96	5.0

[a]The IC_{50} (μM), the slope of the inhibitory response and the relative potency of each PAF receptor antagonist against platelet aggregation induced at the EC_{50} for PAF (0.023 μM) and AA (55 μM) after 15 min incubation were determined with citrated rabbit whole blood.

[b]Significantly different slopes at $p < 0.01$.

To investigate whether the antagonists affected any of the whole blood cellular constituents or were acting as partial agonists, high concentrations of antagonists alone were incubated with rabbit whole blood and examined for blood smear abnormalities or platelet aggregation by phase contrast microscopy. WEB 2086 and WEB 2170 had no effect on the morphology of any of the whole blood cells when tested up to 55 μM, while SRI 63-441 and SRI 63-675 caused platelet and erythrocyte lysis at 150 μM and 362 μM, respectively. None of these antagonists acted as partial agonists. There was difficulty, however, in performing the same examinations with antagonists dissolved in non-aqueous solvents, since the solvent alone can cause non-specific effects. The solvent for SRI 64-412 was 2% acetic acid in distilled water. At a concentration of 25000 ppm in citrated rabbit whole blood this solvent caused platelet and erythrocyte lysis (unpublished data). The solvent for BN 52021 was methanol, while kadsurenone and alprazolam were dissolved in ethanol. Methanol and ethanol at 100000 and 25000 ppm, respectively, completely blocked the ability of platelets to respond to an activating signal, since they non-specifically inhibited platelet aggregation induced by ADP, AA and PAF (unpublished data). Therefore, due to the properties of the solvent, the highest antagonist concentrations that could be tested were 21, 118, 28 and 25 μM for SRI 64-412, BN 52021, kadsurenone and alprazolam, respectively. At these concentrations the antagonists did not affect the morphology of the whole blood cellular constituents or induce platelet aggregation. Therefore, we can exclude the possibility that the results obtained in this study were due to the PAF-receptor antagonists acting as partial agonists or causing haemolysis of platelets or erythrocytes.

DISCUSSION

In rabbit whole blood, PAF receptor antagonists were potent inhibitors of PAF induced platelet aggregation. The slope of the inhibition response was correlated with antagonist potency, and reflects the differing affinities of the antagonists for the platelet PAF receptor (2). Comparative studies on the potency of PAF receptor antagonists are rare, and this is the first which performs the examination on whole blood. Since various experimental conditions can affect PAF-induced platelet aggregation, antagonist potency obtained from separate experiments cannot be strictly compared. Therefore, it is difficult to relate our

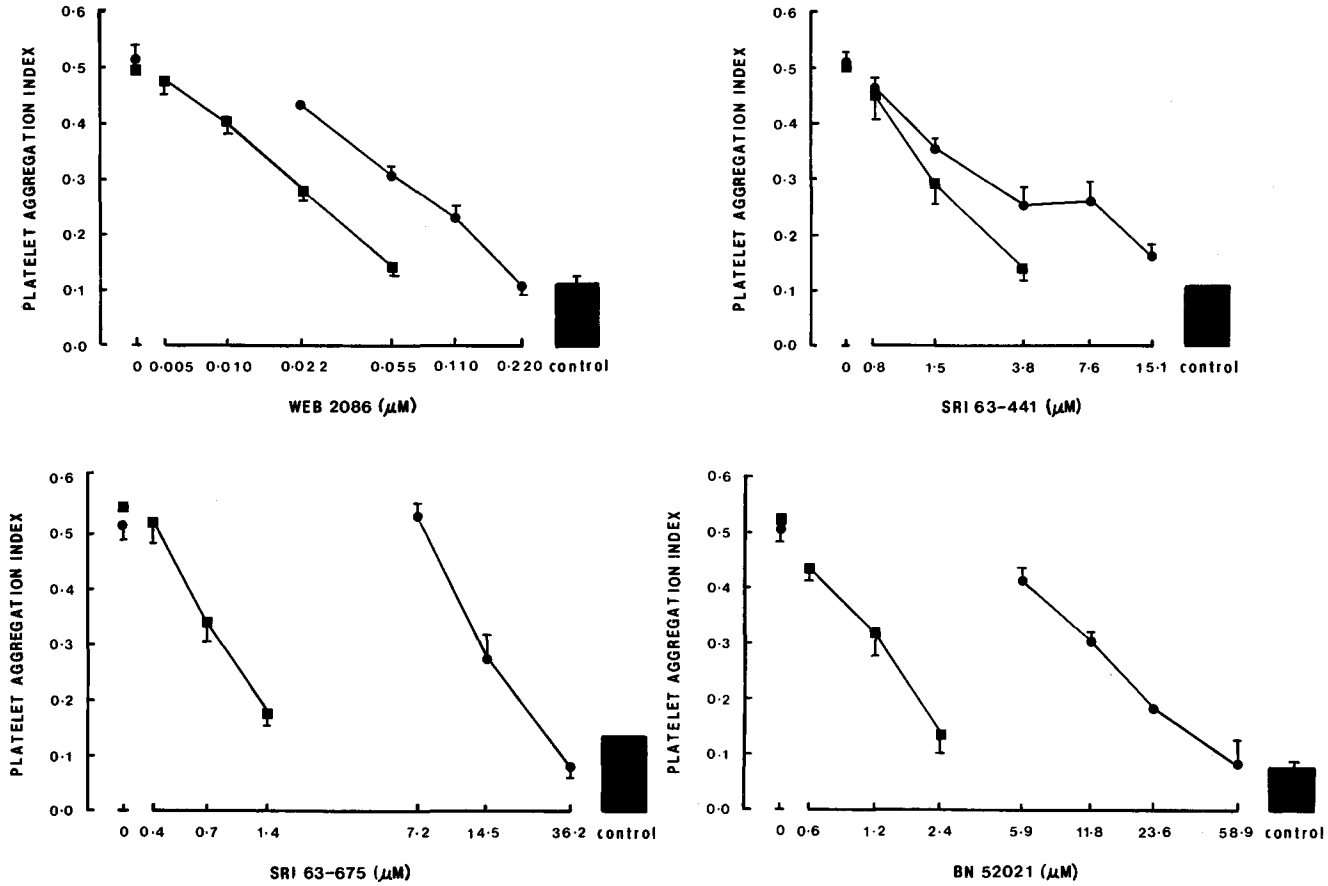


FIG. 1. Effect of increasing concentrations of PAF receptor antagonists on platelet aggregation induced at the EC_{50} for PAF (0.023 μM; ■) and AA (55 μM; ●), after 15 min incubation with citrated rabbit whole blood. Each point represents the mean ± SEM of ⩾ 3 replicates. The effect of vehicle alone is represented by the histogram (control), which is the mean ± SEM of ⩾ 11 replicates.

results to published values. However, some of the antagonists used in this study were examined in a comparative study with human platelet-rich plasma using turbidometric aggregometry (3). These authors obtained a similar rank order of potency, WEB 2086> BN 52021> kadsurenone> alprazolam.

The PAF receptor antagonists were not completely selective for PAF since, at concentrations that were 2.5- to 25.8-fold higher than required for the inhibition of PAF, they also inhibited platelet aggregation induced by AA. For four of the eight PAF receptor antagonists studied, *i.e.*, SRI 63-675, SRI 64-412, BN 52021 and kadsurenone, the antagonist concentration required to inhibit AA induced platelet aggregation were 10-fold higher than those required for PAF. Although this may be considered reasonably selective by pharmacological standards, these results are in contrast to previous reports where PAF receptor antagonists were shown to have either no effect on AA induced platelet aggregation (4,5), or only minimal effects requiring concentrations that were 200- to 1100-fold higher than those required for inhibition of PAF (6,7). These studies were performed using isolated human platelets.

There are a number of possible explanations for the inhibition of AA induced platelet aggregation by PAF receptor antagonists, although we are not able to define the actual mechanism from this study. The effect could be due to either inhibition of the (membrane-associated) enzymes responsible for the conversion of AA to its activator products, prostaglandin G_2, prostaglandin H_2 and thromboxane A_2; or *via* direct competitive inhibition of the platelet receptor for these AA metabolites. Currently there is no literature supporting these suggestions. A more likely explanation, given the mixed population of cells in whole blood and the increasing evidence supporting the involvement of AA in PAF induced platelet aggregation (8,9), is that PAF may be involved in AA induced platelet aggregation. This could be either autocrine (*i.e.*, AA induces platelets to produce PAF) or paracrine (*i.e.*, generation of PAF by other cells in the whole blood, *e.g.*, neutrophils). Although there was a general trend for the slope of the inhibitory response against AA induced platelet aggregation to be similar to that against PAF in magnitude, half of the antagonists showed significant differences. This, together with the wide range of relative potency observed, suggests that the inhibitory effect of the antagonists on AA induced platelet aggregation is not solely *via* a direct competitive inhibition of the platelet PAF receptor. Therefore, the PAF receptor antagonists may be demonstrating intrinsic pharmacological selectivity for the PAF receptor, as previously demonstrated in defined systems which examined isolated cell types, but

the results are a reflection of the mixed population of cells in whole blood and the poorly understood complex interplay understood complex interplay between PAF and AA metabolites.

Therefore, in an *in vitro* bioassay which uses whole blood to closely mimic the *in vivo* situation, PAF receptor antagonists were potent, although not completely selective inhibitors of PAF induced platelet aggregation. To clarify the nature of the inhibition of AA induced platelet aggregation by PAF antagonists, and to allow a better comparison with previous data, it would be of interest to perform these experiments with rabbit platelets prepared as platelet-rich plasma and/or washed platelets, and to examine human platelet responses as well. Our results would appear to be relevant to *in vivo* studies using PAF receptor antagonists and indicate that whole blood seems more appropriate for the *in vitro* assessment of antagonist selectivity.

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The authors would like to thank Michael P. Jones for his assistance with the statistical analysis, and the following companies for their generous gifts of PAF receptor antagonists: Sandoz Research Institute; IHB-IPSEN; Merck, Sharp and Dohme Research Laboratories; Upjohn; and Boehringer Ingelheim.

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Biological Response of Guinea Pig Peritoneal Macrophages to Platelet-Activating Factor[1]

Hidetoshi Hayashi[a,2], Ichiro Kudo[a], Shoshichi Nojima[b] and Keizo Inoue[a,*]

[a]Faculty of Pharmaceutical Sciences, The University of Tokyo, Hongo, Bunkyo-ku, Tokyo 113, and [b]Faculty of Pharmaceutical Sciences, Teikyo University, Sagamikomachi, Tsukui-gun, Kanagawa 199-01, Japan

We investigated the effects of platelet-activating factor (PAF) on guinea pig peritoneal macrophages. Specific and high-affinity binding sites for PAF were detected on guinea pig peritoneal macrophages. Scatchard analysis of PAF binding revealed high affinity binding sites (7.9×10^4/cell) with a dissociation constant of 2.3×10^{-10} M. When treated with 10^{-9}–10^{-5} M PAF, guinea pig peritoneal macrophages released hydrogen peroxide into the medium in a time-dependent manner. The release reaction upon stimulation with 10^{-5} M PAF reached a plateau within 30 min and the extent of release was twice as high as that when stimulated by *N*-formyl-L-methionyl-leucyl-L-phenylalanine (fMLP; 2 μM)-treated cells. Neither lysoPAF nor the PAF enantiomer was effective. PAF-induced H_2O_2 release was inhibited specifically by PAF antagonists, suggesting that PAF activated macrophages through binding to specific sites. Lysosomal enzyme (*N*-acetyl-β-D-glucosaminidase) was released from guinea pig peritoneal macrophages upon treatment with 10^{-5} M PAF for 60 min. Guinea pig peritoneal macrophages were treated with PAF for 8 hr and the conditioned medium was examined for cytokines. The medium exhibited cytocidal activity against mouse fibroblast L929 cells [tumor necrosis factor (TNF) activity], and this activity was comparable to that detected after treatment of cells with the bacterial lipopolysaccharide (LPS). Furthermore, the same conditioned medium also showed colony-stimulating factor (CSF) activity. Generation of these cytokines was stereospecific. Our findings suggest that PAF is a unique macrophage activator that potentiates both respiratory burst/lysosomal enzyme release (early-phase response) and monokine production/glucose consumption (late-phase response).

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Since its discovery, platelet-activating factor (PAF) has been widely viewed as an inflammatory mediator of various physiological and pathological reactions. In addition, recent evidence suggests that PAF is a potent modulator of cellular immune response (1). Cells of the macrophage/monocyte lineage produce PAF upon stimulation. It has also been shown that macrophages or related cells respond to PAF when activated under certain conditions. We have previously reported that PAF stimulated glucose consumption of guinea pig peritoneal macrophages through its binding to specific sites on the cells (2). Hartung (3) showed that PAF stimulated oxidative burst in macrophages. Production of monokines such as tumor necrosis factor (TNF) or interleukin-1 (IL-1) by human monocytes in response to PAF has also been observed (4,5). Recently, specific binding sites for PAF on human peripheral monocytes were detected (6).

In the present study, we examined the effect of PAF on macrophages. The possible role of PAF in the regulation of macrophage function under physiological and pathological conditions will be discussed.

MATERIALS AND METHODS

Chemicals. Fetal bovine serum (FBS) and RPMI1640 medium were obtained from Hy Clone Laboratories (Logan, UT) and MA Bioproducts (Walkerville, MD), respectively. Eagle's minimum essential medium (EMEM) and Hank's balanced salt solution (HBSS) were obtained from Nissui Pharmaceutical Co. Ltd. (Tokyo, Japan). LPS (lipopolysaccharide W, *E. coli* 0127:B8) and Freund's complete adjuvant were obtained from Difco Laboratories (Detroit, MI). 5-Hydroxy-6,8,11,14-eicosatetraenoic acid (5-HETE) and leukotriene B_4 (LTB_4) were purchased from Cayman Chemical Co. (Ann Arbor, MI). Other chemicals were obtained from Sigma Chemical Co. (St. Louis, MO).

PAF, PAF agonists and PAF antagonists. PAF, racemic 1-*O*-octadecyl-2-*O*-(*N*,*N*-dimethylcarbamoyl)-glycero-3-phosphocholine (dimethylcarbamoyl-OGPC), racemic 1-*O*-octadecyl-2-acetamido-2-deoxyglycero-3-phosphocholine (acetamido-OGPC), 1-*O*-ocdadecyl-2-*O*-methyl-*sn*-glycero-3-phosphocholine [methyl-OGPC(*sn*-3)], 3-*O*-octadecyl-2-*O*-methyl-*sn*-glycero-1-phosphocholine [methyl-OGPC(*sn*-1)] (2) and the PAF antagonists, CV6209 (7) and NT176, were provided by Takeda Chemical Industries (Osaka, Japan). Respective structures of PAF and its analogs are listed in Scheme 1. FR900452 (8) was kindly provided by Fujisawa Pharmaceutical Co. (Osaka, Japan). [^{3}H]Octadecyl PAF (80 Ci/mmol) was obtained from Amersham (Buckinghamshire, U.K.).

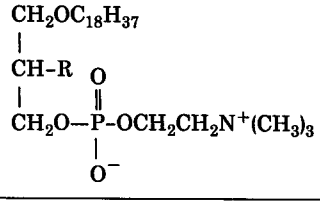
Animals and macrophage preparation. Female Hartley guinea pigs (body weight 300–500 g) and fe-

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[2]Present address: Faculty of Pharmaceutical Sciences, Nagoya City University, 3-1 Tanabe-dori, Mizuho-ku, Nagoya, 467, Japan.

*To whom correspondence should be addressed at The University of Tokyo, 7-3-1 Hongo, Bunkyo-ku, Tokyo 113, Japan.

Abbreviations: BSA, bovine serum albumin; CSF, colony-stimulating factor; EMEM, Eagle's minimum essential medium; FBS, fetal bovine serum; fMLP, *N*-formyl-methionyl-leucyl-phenylalanine; HBSS, Hank's balanced salt solution; 5-HETE, 5-hydroxy-6,8,11,14 eicosatetraenoic acid; IL-1, interleukin-1; LPS, lipopolysaccharide; LTB_4, leukotriene B_4; lysoPAF, lyso platelet-activating factor, 1-*O*-alkyl-*sn*-glycero-3-phosphocholine; NDGA, nordihydroguaiaretic acid; OGPC, octadecyl-glycerophosphocholine; PAF, platelet-activating factor, 1-*O*-alkyl-2-acetyl-*sn*-glycero-3-phosphocholine; PEC, peritoneal exudate cell(s); PMA, phorbol myristate acetate; TMB-8, 8-(*N*,*N*-diethylamino)octyl-3,4,5-trimethoxybenzoate; TNF, tumor necrosis factor; W-7, *N*-(6-aminohexyl)-5-chloro-1-naphthalene sulfonamide.



Compound	-R
PAF	$-OCOCH_3$
Dimethylcarbamoyl-OGPC	$-OCON(CH_3)_2$
Acetamido-OGPC	$-NHCOCH_3$
Methyl-OGPC (*sn*-3)	$-OCH_3$
Methyl-OGPC (*sn*-1)	$-OCH_3$
LysoPAF	-OH

SCHEME 1

male Japanese white rabbits (body weight 1.5–2.5 kg) were obtained from Japan Biosupply (Tokyo, Japan). Specific pathogen-free female ICR mice (7–10-wk-old) were purchased from Japan SLC, Inc. (Hamamatsu, Japan). Guinea pig peritoneal exudate cells (PEC) were prepared as described previously (2). Murine PEC were prepared by induction with casein, as described previously (9,10). Rabbit alveolar macrophages were prepared from pulmonary lavage fluid of rabbits injected intravenously with 1 mL Freund's complete adjuvant 3 wk before the animals were killed.

Measurement of H_2O_2 release from macrophages. Release of H_2O_2 from macrophages was measured by a modification of the procedure described by Cohen *et al.* (9). PEC (2.5×10^6 cells/mL) suspended in RPMI 1640/10% FBS were cultured in wells of 24-well multiplates (25820, Corning, NY) at 37°C for 150 min in an atmosphere of 5% CO_2 in air. After culture, the plates were rinsed twice with warm HBSS and then incubated in 1.0 mL HBSS (without phenol red) containing 10 μM scopoletin, 1 mM NaN_3 and 5 U/mL horseradish peroxidase. After 60 min of incubation, the cells were quickly chilled on ice and centrifuged at 500 $\times g$ for 5 min. Then 0.2-mL aliquots of the cell culture were transferred to wells of 96-well multiplates (Imuron 2, flat bottom), and the fluorescence at 450 nm upon activation by 365-nm light was measured using a microplate reader (MTP-32, Corona, Ibaraki, Japan). The difference in fluorescence between preparations incubated with and without peroxidase was converted to the concentration of H_2O_2 released from the cells.

Lysosomal enzyme secretion. The activity of secreted *N*-acetyl-β-D-glucosaminidase was determined by the method of Homma *et al.* (11). Guinea pig peritoneal macrophage monolayers (3×10^5 cells/mL) prepared in the wells of 96-well multiplates (flat bottom) were preincubated in 1.0 mL of HBSS (without phenol red) containing 5 μg/mL cytochalasin B for 10 min at 37°C, and cultured with PAF and its analogs (10^{-5} M) for 60 min at 37°C in an atmosphere of 5% CO_2 in air. The cells were quickly chilled on ice and centrifuged at 500 $\times g$ for 10 min at 37°C. Fifty microliters of supernatant was incubated with 4 mM *p*-nitrophenyl-*N*-acetyl-β-D-glucosaminide in 0.05 M sodium citrate buffer (pH 4.5) in a total volume of 500 μL for 30 min at 37°C. The reaction was terminated by the addition of 0.4 M glycine/NaOH buffer (pH 10.5). The nitrophenol released was measured at 410 nm. Total enzyme activity within macrophages (100% activity) was determined by addition of 0.1% Triton X-100.

Preparation of culture medium of guinea pig peritoneal macrophages and TNF assay. PEC at 5×10^6 cells per 2 mL were placed in one well of a 6-well culture plate (25810, Corning) and incubated for 150 min at 37°C in a 5% CO_2 incubator. Non-adherent cells were removed by washing with warmed HBSS 3 times. EMEM/10% FBS-containing stimulants were then added. After a predetermined incubation period, culture supernatants were harvested and centrifuged at 500 $\times$ *g* for 5 min. Cell-free supernatants were dialyzed against RPMI 1640 medium containing 0.1% bovine serum albumin (BSA). The supernatants were then filtered with a disposable syringe filter (21053 FCA, Corning). The activity of TNF was determined by the L929 fibroblast cell lytic assay according to Tamatani *et al.* (12). Fifty μL of an appropriate dilution of culture medium and 50 μL of a L929 cell suspension (2.5×10^4 cells) in the presence of actinomycin D (1 μg/mL) were added to flat-bottomed, 96-well tissue culture plates. After 18-hr culture, the plates were washed and the degree of cell lysis was determined by staining the plates with 0.1% crystal violet in methanol/water (1:4, v/v). The dye-stained cells were solubilized with 0.1 mL of 1% SDS and absorbance at 550 nm was measured by a microplate reader (MTP-32, Corona). One unit of TNF activity was defined as the reciprocal of the sample dilution that lysed 50% of the test cells.

Quantitation of PAF binding. [^{3}H]PAF binding to macrophages was measured by a modification of the procedure decribed previously (13). Guinea pig peritoneal macrophages were prepared by the method of Kumagai *et al.* (14). [^{3}H]PAF solution (50 μL) with or without a 1000-fold molar excess of unlabeled PAF was added to 200 μL of macrophage suspension (2.5×10^6 cells per mL) in Ca^{2+}, Mg^{2+}-free HBSS with 0.1% BSA (binding buffer) in an Eppendorf tube (1.5 mL). After incubation at 24°C for 10 min, 200 μL of the cell suspension was filtered through a glass fiber filter (GF/C, Whatman, Maidstone, England) using a vacuum apparatus (SM16547, Sartorius, Goettingen, Germany) attached to an aspirator. Each filter was rapidly washed four times with 5 mL of ice-cold binding buffer and dried. Radioactivity bound to filter was then measured.

RESULTS

Specific binding of PAF to macrophages. Guinea pig peritoneal macrophages were examined for specific binding sites for PAF. At 24°C, PAF binding reached plateau saturation within 10 min, and minimal PAF metabolism was observed at the time when binding saturation was achieved (data not shown). The total amount of radioactive PAF bound to the cells increased when more radioactive PAF was added to the cell suspension. Non-specific binding, the binding of radioactive PAF in the presence of a 1000-fold molar excess of non-labeled PAF was not saturable, whereas specific binding (total binding minus non-specific binding) was saturable (Fig. 1A). Two dis-

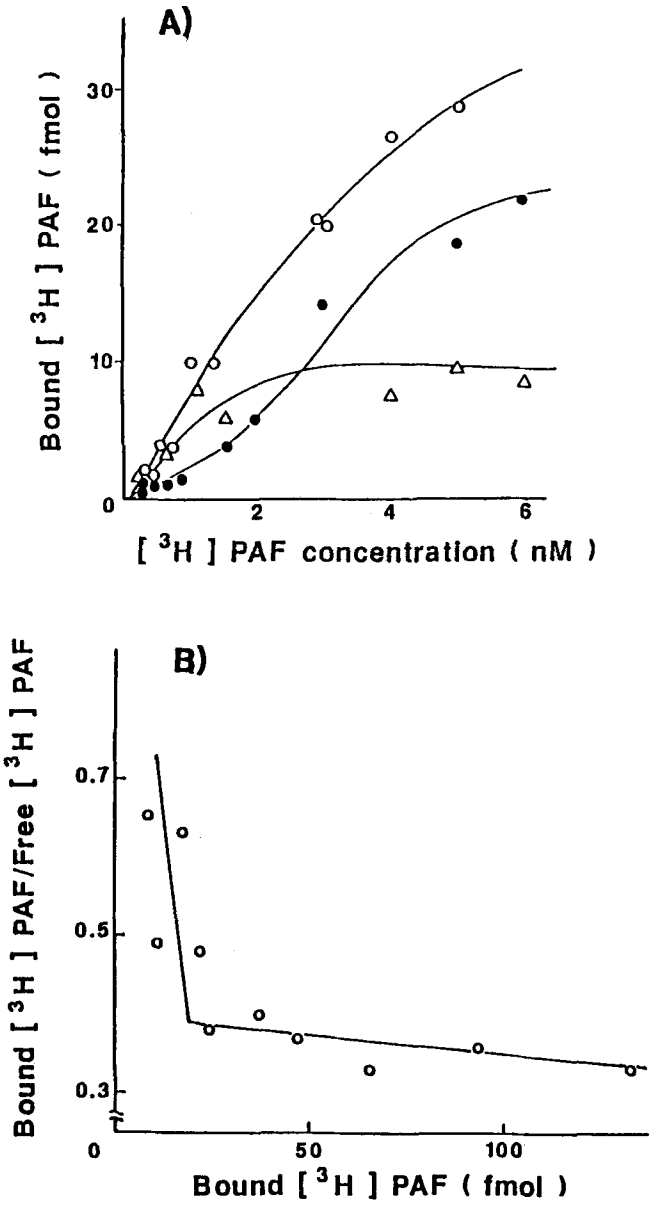


FIG. 1. (A) Binding of PAF to guinea pig peritoneal macrophages. Experiments were performed as described in Materials and Methods. Total binding, ○; non-specific binding, ●; specific binding, △. Each point is the mean of triplicate determinations; standard deviation is less than 10% of the count. (B) Scatchard plot of [^{3}H]PAF binding data.

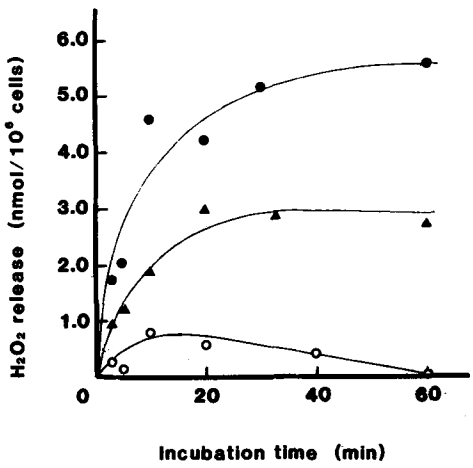


FIG. 2. Kinetics of H_2O_2 release from guinea pig peritoneal macrophages. Macrophages were treated with 10^{-6} M PAF or 2×10^{-6} M fMLP in HBSS containing 10 μM scopoletin, 1 mM NaN_3, and 5 U/mL horseradish peroxidase at the indicated times. Generation of H_2O_2 was determined as described in Materials and Methods. PAF, ●; fMLP, ○; control, △.

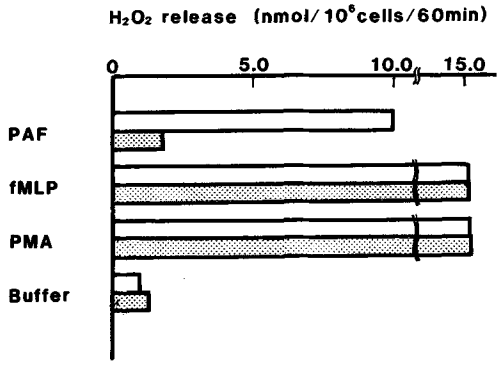


FIG. 3. Desensitization of H_2O_2 release from guinea pig peritoneal macrophages by PAF. Macrophages were incubated with (grey bars) or without (open bars) 10^{-8} M PAF for 10 min, washed, and then stimulated with PAF (10^{-5} M), fMLP (2×10^{-6} M) or PMA (6×10^{-7} M) for 60 min.

tinct binding sites for PAF were tentatively determined by Scatchard plot analysis. One of the sites exhibited high affinity of Kd = 2.3×10^{-10} M and a capacity of 7.9×10^4 binding sites per macrophage (mean, n = 3). The other site, with a substantially lower affinity and greater binding capacity, probably corresponds to nonreceptor-related uptake of PAF into the membrane.

Generation of H_2O_2 from macrophages by PAF. Generation of active oxygen was induced in guinea pig peritoneal macrophages upon incubation with 10^{-5} M of PAF. The H_2O_2 generation reached a plateau within 30 min (Fig. 2). The chemotactic peptide, fMLP (2 μM), also released H_2O_2 from the macrophages with the same time course as did PAF. PAF and dimethylcarbamoyl-OGPC were the most active in releasing H_2O_2, followed by acetamido-OGPC (Table 1), whereas no appreciable release was observed with methyl-OGPC (*sn*-3), the PAF enantiomer, or lysoPAF. The effects of a series of PAF agonists on macrophages roughly parallelled their relative potencies in inducing platelet activation, as determined by serotonin release from washed guinea pig platelets, except for methyl-OGPC (Table 1). These findings indicated that PAF induced the release of H_2O_2 through a recognition mechanism similar to that of platelets.

We performed desensitization experiments to clarify whether H_2O_2 release by PAF was induced through a specific recognition mechanism. Macrophages were preincubated in the presence of PAF at a low dose (10^{-8} M) with 5 mM ethyleneglycol *bis*(β-aminoethyl-ether)*N*, *N*, *N'*, *N'*-tetraacetic acid (EGTA) for 10 min at 37°C.

TABLE 1

Effects of PAF and Its Agonists on Guinea Pig Macrophages and Platelets[a]

	H_2O_2 release from macrophages (ED_{50}; M)	Serotonin release from platelets (ED_{50}; M)
PAF	5.0×10^{-9}	2.9×10^{-10}
Dimethylcarbamoyl-OGPC	5.8×10^{-9}	5.5×10^{-10}
Acetamido-OGPC	1.6×10^{-7}	3.4×10^{-8}
Methyl-OGPC (*sn*-3)	$> 10^{-6}$	1.4×10^{-6}

[a]H_2O_2 release from guinea pig peritoneal macrophages was measured as described in Figure 2. [^{14}C]Serotonin release from guinea pig platelets was measured as described previously (2). Effective doses of PAF or its agonists required for 50% maximum activation were determined.

TABLE 2

Effects of PAF Antagonists on H_2O_2 Release from PAF-Induced Guinea Pig Peritoneal Macrophages[a]

		H_2O_2 release nmol/10^6 cells/60 min (% of control)		
Stimulants		NT176	FR900452	HBSS
PAF	10^{-6} M	5.17 (69.4)	4.82 (67.5)	7.45 (100)
	10^{-8} M	1.52 (38.6)	1.88 (47.7)	3.94 (100)
PMA	6×10^{-7} M	15.24 (99.7)	15.26 (99.8)	15.29 (100)
Control		1.88 (119.0)	1.74 (110.1)	1.58 (100)

[a]Macrophages were pretreated with PAF antagonists, NT176 (10^{-6} M) or FR900452 (10^{-6} M), for 10 min before stimulation.

TABLE 3

H_2O_2 Release from Macrophages of Various Animal Species[a]

		H_2O_2 release (nmol/10^6 cells/60 min)		
Stimulants		Guinea pig peritoneal macrophages	Rabbit alveolar macrophages	Mouse peritoneal macrophages
PAF	10^{-6} M	6.43	7.02	1.25
	10^{-8} M	6.41	n.d.	1.95
PMA	6×10^{-7} M	8.36	7.22	5.26
Control		1.03	5.65	2.02

[a]H_2O_2 release from guinea pig peritoneal macrophages, rabbit alveolar macrophages and caseinate-induced murine peritoneal macrophages were determined as described in Figure 2; n.d., not determined.

Under these conditions, no appreciable induction of H_2O_2 was detected. After washing the cells, H_2O_2 release by 10^{-5} M PAF was suppressed almost to the basal state (88% suppression) (Fig. 3). This was also observed after addition of low amounts of the PAF agonist, dimethylcarbamoyl-OGPC (data not shown). By comparison, fMLP or phorbol myristate acetate (PMA) induced H_2O_2 release from the PAF-desensitized macrophages in the same manner as non-treated macrophages. These results suggested that desensitization by PAF was stimulus-dependent. Moreover, the PAF antagonists, NT176 and FR900452, specifically inhibited the generation of H_2O_2 by PAF, but they did not inhibit the reaction induced by PMA (0.6 μM) (Table 2).

Species differences in H_2O_2 release from macrophages. Table 3 shows the release of H_2O_2 from macrophages of various species. Treatment of rabbit alveolar macrophages with PAF or its agonist, dimethylcarbamoyl-OGPC, caused a similar amount of H_2O_2 release as PMA. Unlike guinea pig and rabbit macrophages, casein-induced or resident murine peritoneal macrophages failed to demonstrate enhanced H_2O_2 release in response to either PAF or dimethylcarbamoyl-OGPC, whereas PMA (0.6 μM) stimulated the release of H_2O_2 from the murine macrophages. One possible explanation for the non-responsiveness to PAF may be a lack of specific binding of PAF to cells; we therefore carried out PAF binding assays on rabbit and mouse macrophages. Rabbit alveolar macrophages carried saturable specific binding sites for PAF (Kd = 9.4×10^{-10} M, 8.1×10^4 sites/cell). By contrast, we couldn't detect the specific binding of PAF to murine peritoneal macrophages

TABLE 4

Effect of EGTA on H_2O_2 Release from Guinea Pig Peritoneal Macrophages[a]

Stimulants		H_2O_2 release nmol/10^6 cells/60 min (% of control)	
		Control	EGTA 5 mM
PAF	10^{-5} M	7.06 (100)	3.05 (43.2)
	10^{-8} M	4.40 (100)	0.68 (15.5)
fMLP	2×10^{-6} M	8.41 (100)	0.13 (1.5)
Control		0.07	0

[a]5 mM EGTA was added 10 min prior to PAF or fMLP treatment.

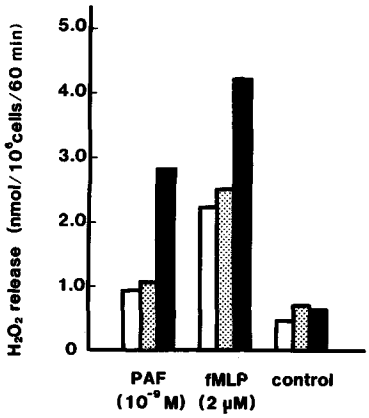


FIG. 4. Effect of 5-HETE on H_2O_2 release from macrophages treated with PAF. Macrophages were incubated with PAF (10^{-9} M) or fMLP (2 μM) in the absence (open bars) or presence of 5-HETE (50 nM, grey bars; 500 nM, solid bars).

in this study (data not shown). Casein-induced rat peritoneal macrophages had no appreciable, specific binding sites for PAF (data not shown).

Mode of action of PAF in H_2O_2 release. The presence of EGTA in the medium suppressed the PAF-induced generation of H_2O_2 by macrophages (Table 4). An antagonist of calmodulin [*N*-(6-aminohexyl)5-chloro-1-naphthalene sulfonamide; W-7] at 10^{-4} M and an intracellular Ca^{2+} chelator [8-(*N*,*N*-diethylamino)octyl-3,4,5-trimethoxybenzoate; TMB-8] at 10^{-3} M inhibited the PAF-induced generation of H_2O_2 appreciably (49% and 74%, respectively). PAF-induced H_2O_2 release was effectively inhibited by nordihydroguaiaretic acid (NDGA); addition of 1, 3, 10, or 30 μM NDGA to the incubation mixture suppressed the H_2O_2 generation to 81, 44, 16, or 3.5% of the control, respectively. This observation strongly suggested that the response was mediated by some lipoxygenase product(s). A low dose (10^{-9} M) of PAF induced no appreciable release from the cells. In the presence of 500 nM 5-HETE, however, stimulation of H_2O_2 release by PAF was observed (Fig. 4). 5-HETE alone failed to induce any H_2O_2 release at this concentration, suggesting that 5-HETE stimulates the PAF-induced reaction synergistically. Another lipoxygenase product, LTB_4, had no effect up to a concentration of 1 mM.

TABLE 5

Effects of PAF or Its Analogs on *N*-acetyl-β-D-glucosaminidase Secretion from Guinea Pig Peritoneal Macrophages[a]

PAF analogs	Control (%)
PAF	24.6 ± 1.3
Dimethylcarbamoyl-OGPC	26.4 ± 1.3
Acetamido-OGPC	29.1 ± 3.3
Methyl-OGPC (*sn*-3)	19.4 ± 4.6
LysoPAF	8.4 ± 2.1
Enantio-PAF (*sn*-1)	4.9 ± 2.3

[a]Experiments were performed as described in Materials and Methods. Results are expressed as percentage of total activity within macrophages (cells lysed with 0.1% TritonX-100).

TABLE 6

TNF Activity Release from Guinea Pig Peritoneal Macrophages[a]

	Time (hr)		
	1.5	8	19
		(units/mL)	
Experiment 1			
PAF (10^{-5} M)	15.4	22.2	2.9
LPS (1 μg/mL)	4.7	39.5	4.8
Control	< 1.0	< 1.0	< 1.0
Experiment 2			
Methy-OGPC (*sn*-1) (10^{-5} M)		< 1.0	
Methy-OGPC (*sn*-3) (10^{-5} M)		11.4	
Control		< 1.0	
Experiment 3			
PAF (10^{-5} M)		54.6	
(10^{-6} M)		18.3	
Dimethylcarbamoyl-OGPC (10^{-7} M)		81.4	
(10^{-8} M)		25.5	
Control		11.2	

[a]Macrophages were treated with stimulants for the indicated times, and the culture supernatants were collected. The TNF activity in culture supernatants was measured as described in Materials and Methods.

TABLE 7

CSF Activity Released from Guinea Pig Peritoneal Macrophages[a]

Stimulants		Number of colonies (colonies/plate)
PAF	(10^{-5} M)	258
Methyl-OGPC (*sn*-3)	(10^{-5} M)	271
Methyl-OGPC (*sn*-1)	(10^{-5} M)	31
LPS	(1.0 μg/mL)	178
Control		1

[a]Conditioned medium of guinea pig peritoneal macrophages treated with 10^{-5} M PAF, methyl-OGPC or 1 μg/mL LPS for 6 hr was examined for ability to induce colonies of guinea pig bone marrow cells in the semisolid medium. After 7 days of culture, the numbers of colonies formed were counted.

Indomethacin, a cyclooxygenase inhibitor, had no effect or only a slightly stimulatory effect on H_2O_2 release (data not shown).

Release of lysosomal enzyme. The macrophages were incubated in the presence of cytochalasin B, together with PAF or its analogs, for 60 min. Release of *N*-acetyl-β-D-glucosaminidase was induced by PAF or its agonists (dimethylcarbamoyl- and acetamido-OGPC) (Table 5). No appreciable release of lactate dehydrogenase, a marker enzyme of cytosol, was observed under the same experimental conditions. Both lysoPAF and an enantiomer of PAF failed to produce any appreciable release of lysosomal enzyme. These findings indicate that PAF induces the release of lysosomal enzymes through a specific mechanism.

Release of monokines. When guinea pig peritoneal macrophages were treated with 10^{-5} M PAF, cytotoxic activity against murine fibroblast L929 cells was detected in the culture medium, suggesting that macrophages in the presence of PAF produce TNF activity and release it into the medium. This activity reached a maximum 8 hr after the addition of PAF (Table 6). LPS also induced the release of TNF activity from cells. Significant activity was detected in the medium of PAF-treated cells after 1.5 hr of incubation, whereas no appreciable activity was observed after 1.5 hr of incubation with LPS.

One of the potent PAF agonists, dimethylcarbamoyl-OGPC, which is resistant to metabolic inactivation, possessed a marked ability to release TNF activity (Table 6). LysoPAF, a major metabolite of PAF, showed no activity even at 10^{-5} M (data not shown). Another PAF agonist, methyl-OGPC, with an *sn*-3 configuration, induced TNF production, whereas its enantiomer (the *sn*-1 isomer) possessed no activity at all (Table 6). Pretreatment of macrophages with a specific PAF antagonist, CV6209 (10^{-6} M), abolished the stimulatory effect of PAF on release of activity (from 43 units/mL to 10 units/mL).

We detected colony-stimulating factor (CSF) activity in the culture medium of macrophages stimulated by PAF or methyl-OGPC (*sn*-3) (Table 7).

DISCUSSION

We demonstrated specific binding sites for PAF on guinea pig peritoneal macrophages. In the H_2O_2 release experiment, preincubation of the cells with a sub-stimulatory dose of PAF (10^{-8} M) caused desensitization against subsequent PAF stimulation. In addition, a PAF-specific antagonist inhibited PAF-induced H_2O_2 release, suggesting that this reaction was mediated through the binding of PAF to the specific binding site.

Since both EGTA and TMB-8 inhibited the stimulatory effect of PAF on H_2O_2 release, extracellular Ca^{2+} uptake and intracellular Ca^{2+} mobilization in the macrophages may be a necessary part of the process leading to macrophage activation. This idea is further supported by the experiment showing PAF-induced uptake of $^{45}CaCl_2$ by macrophages (data not shown). Moreover, Maudsley and Morris (15) and Ward and Westwick (16) had previously shown a rapid but transient increase in the intracellular free Ca^{2+} concentration in a human monocyte/macrophage-like cell line, U937.

Macrophage activation by PAF was inhibited by the lipoxygenase inhibitor, nordihydroguaiaretic acid (NDGA), but not by the cyclooxygenase inhibitor, indomethacin. One of the lipoxygenase products, 5-HETE, activated macrophages with PAF synergistically. These findings suggest that, similar to the activation of neutrophils (17,18), the activation of macrophages by PAF may be mediated through the products of the lipoxygenase pathway.

PAF-induced generation of H_2O_2 was observed not only with guinea pig macrophages but also with rabbit alveolar macrophages. We detected specific binding sites for PAF on rabbit alveolar macrophages; however, no appreciable reactivity was observed with mouse peritoneal macrophages under the same conditions. In preliminary experiments, we also found that rat peritoneal macrophages failed to respond to PAF. No specific binding of PAF was observed either with rat or mouse macrophages, indicating that peripheral macrophages in these animals have few receptors for PAF, and do not respond to it. These findings are in agreement with the fact that no specific PAF receptor binding has been found on the rat alveolar macrophage membrane (19). No effects were elicited on the platelets of either rat or mouse, although platelets from human, rabbit and guinea pig were highly sensitive to PAF (20). Platelets from rat or mouse that are insensitive to PAF also lack specific binding (21, 22). Prepic *et al.* (23) reported the presence of specific receptors for PAF on purified membranes derived from murine peritoneal macrophages. They showed that PAF induced the generation of breakdown products of polyphosphoinositides and elevated the intracellular level of Ca^{2+}. We have no explanation for this discrepancy at present.

We showed that PAF induced the production of TNF activity by guinea pig peritoneal macrophages. This reaction was stereospecific and inhibited by a PAF-specific antagonist, suggesting that it was also mediated through a specific mechanism. In our preliminary experiment, antibody to recombinant murine TNF neutralized the cytotoxic activity against L929 cells, indicating that this factor may be guinea pig TNF. Since TNF, IL-1 and γ-interferon (INF-γ) are known to stimulate PAF synthesis in macrophages (24), activation of monocyte/macrophage lineage cells by PAF may be further stimulated through this positive feedback mechanism. Valone *et al.* (5) reported that PAF enhanced the cytotoxicity of human monocytes for WEHI 164 cells mediated by TNF production. However, we were unable to detect any TNF production by murine macrophages treated with PAF or its agonists (data not shown).

Trinchieri *et al.* (25) showed that TNF induced the monocytic differentiation of a human myeloid leukemic cell line into macrophage-like cells. Highly purified guinea pig TNF also induced differentiation of a mouse myeloid leukemic cell line, M1, into macrophage-like cells in the presence of IL-1 (12). We recently reported that PAF affected the proliferation of one or more classes of guinea pig bone marrow cells through release of soluble factor(s) (26) and induced their candidacidal activity (13). These results indicate that the CSF activity observed in the present study and the effect of PAF on bone marrow cells observed previously is mediated by this TNF-like factor.

Macrophage activation can be classified into two types: (i) the activation detected when cells are incubated with reagents for a rather short period (5 min

to several hr); and (ii) the activation that is detected only when cells are incubated for a longer period (several hours). A complement degradation product (C5a), PMA, fMLP, opsonized zymosan or Ca^{2+} ionophore (A23187) induces early responses in macrophages (release of lysosomal enzymes and respiratory burst), which are detectable within a short incubation period. Lipopolysaccharides (LPS), γ-interferon (INF-γ), muramyldipeptide (MDP), CSF or A23187 induces late responses in macrophages (TNF release, stimulation of glucose consumption, expression of microbicidal and tumoricidal activity), which are detected after a long incubation time. However, none of these factors can induce both early and late responses in macrophages except for ionophore A23187.

PAF is a unique macrophage activator present in nature, which acts both as a short-term and a long-term activator of macrophages. The long-term activation requires a much higher amount of PAF than the short-term activation. PAF may manifest its activity on macrophages *in vivo* under conditions where it is produced continuously by the macrophages themselves or by some other cells such as neutrophils or endothelial cells, located near the target macrophages. The majority of newly synthesized PAF appears to be retained within these PAF-producing cells and not released (27). We previously reported that PAF incorporated into liposomes increased glucose consumption by macrophages (long-term culture; 2–3 days) about 400-times more effectively than did PAF in its free form (28). In addition to the increased resistance to metabolic inactivation of PAF incorporated in liposomes, the marked activity of liposomal PAF on macrophages may be explained by a more efficient mechanism of PAF transfer.

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Production of Platelet-Activating Factor by Human Normodense and Hypodense Eosinophils[1]

Ayako Ojima-Uchiyama[a], Yasuo Masuzawa[a], Takayuki Sugiura[a,*], Keizo Waku[a], Takeshi Fukuda[b,2] and Sohei Makino[b,2]

[a]Faculty of Pharmaceutical Sciences, Teikyo University, Kanagawa 199-01, Japan and [b]Dokkyo University, School of Medicine, Tochigi 321-02, Japan

Normodense eosinophils and neutrophils from normal donors produced considerable amounts of platelet-activating factor (PAF) when stimulated with ionophore A23187. PAF produced by eosinophils appeared to be degraded more rapidly than PAF formed by neutrophils, suggesting a higher activity of PAF-degrading enzyme in eosinophils. Substantial proportions of PAF newly formed by both eosinophils and neutrophils were shown to be cell-associated. By comparison, hypodense eosinophils obtained from a patient with idiopathic hypereosinophilic syndrome produced an extremely large amount of PAF and released much of it into the incubation medium. The accelerated formation of PAF in hypodense eosinophils may be related to various cardiovascular complications associated with hypereosinophilic syndrome.
Lipids 26, 1200–1203 (1991).

It is generally accepted that eosinophils play an important role in various allergic reactions. For instance, the role of eosinophils in active bronchial asthma and in parasitic infections have been documented in recent years (1–5). The granular proteins of eosinophils have been found at the sites of epithelial damage and denudation in asthma (6), and in skin tissues of patients with atopic dermatitis (7,8). These toxic proteins have been assumed to induce various pathological changes in airways and in skin.

Eosinophils are also known to produce potent lipid mediators such as platelet-activating factor (PAF) (9) and leukotriene C_4 (LTC_4) (10–12) upon exposure to various stimuli. These lipid mediators have also been suggested to participate in the pathogenesis of various allergic reactions. In particular PAF is currently receiving much attention, because it has been reported that the administration of PAF results in changes that mimic the pathology of asthma (13–15) and of anaphylactic shock (16). Thus, in order to better understand allergic processes, it is important to investigate in detail the production of PAF by various inflammatory cells, including eosinophils, as well as the effects of PAF on eosinophil function.

In a previous communication, we reported that an unusually large amount of the stored PAF precursor, alkylacylglycerophosphocholine (alkylacyl-GPC), is present in human eosinophils (75.5% in choline glycerophospholipids) (17). In this communication, we compare the production of PAF in A23187-stimulated human eosinophils with that in stimulated human neutrophils. We also describe the production of PAF by hypodense and normodense eosinophils from a patient with idiopathic hypereosinophilic syndrome (HES), because the two types of eosinophils are known to be considerably different from each other in several biological properties (18–25). We found that a substantially higher amount of PAF was produced in hypodense eosinophils than was in normodense eosinophils.

MATERIALS AND METHODS

Chemicals. All chemicals were of reagent grade. 1-*O*-Hexadecyl-2-acetyl-*sn*-glycero-3-phosphocholine (16:0-PAF) was purchased from Bachem (Bubendorf, Switzerland). 1-*O*-[^{3}H]Octadecyl-2-acetyl-*sn*-glycero-3-phosphocholine (92 Ci/mmol, [^{3}H]PAF) was obtained from Amersham (Amersham, United Kingdom). A23187 was from Calbiochem (La Jolla, CA). CV3988, a PAF-antagonist, was a generous gift from Dr. Tsushima, Takeda Chemical Industries (Osaka, Japan). Pre-coated silica gel plates (layer thickness 0.25 mm) were from Merck (Darmstadt, Germany).

Cells. Human eosinophils and neutrophils were separated from peripheral blood of normal healthy donors and a patient with idiopathic hypereosinophilic syndrome (HES), using a Percoll discontinuous density gradient (26) as described in a previous report (17). Eosinophils having a density lower than 1.082 are defined as "hypodense"; others as "normodense" (27). The peak density of "hypodense" eosinophils was 1.075–1.077, that of "normodense" was 1.085–1.090. More than 70% of the leukocytes of the HES patient were eosinophils, and most of them were "hypodense". The purity of the eosinophils from a HES patient was usually >95%. The purity of eosinophils from normal donors was usually 85–90% and that of neutrophils was 85–95%.

PAF production by eosinophils and neutrophils. Eosinophils or neutrophils (2.5×10^6 cells) in 0.5 mL of 5 mM Hepes-Hanks balanced salt solution (pH 7.4) containing 0.25% bovine serum albumin (BSA) were preincubated at 37°C. Fifteen minutes later, cells were stimulated by the addition of A23187 (final concentration, 1 μg/mL). After a further incubation (0–30 min), the reaction was stopped by adding chloroform/methanol (1:2, v/v), and [^{3}H]PAF (approximately 8000 dpm) was added as internal standard to monitor the recovery of PAF. Total lipids were extracted by the method of Bligh and Dyer (28). In some experiments, after incubation, cells were immediately spun down (2500 rpm for 5 min) at 4°C, and the lipids from the cells and from the supernatant were extracted as described above.

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*To whom correspondence should be addressed at Faculty of Pharmaceutical Sciences, Teikyo University, Sagamiko, Kanagawa 199-01, Japan.

[2]Mailing address: Dokkyo University, School of Medicine, Mibu, Tochigi 321-02, Japan.

Abbreviations: BSA, bovine serum albumin; CGP, choline glycerophospholipids; EGP, ethanolamine glycerophospholipids; GLC, gas-liquid chromatography; GPC, *sn*-glycero-3-phosphocholine; Hepes, *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid; HES, hypereosinophilic syndrome; LTC_4, leukotriene C_4; PAF, platelet-activating factor; TLC, thin-layer chromatography.

Estimation of PAF production. Individual phospholipid classes were separated by thin-layer chromatography (TLC) using chloroform/methanol/water (65:35:6, v/v/v) as developing solvent. The area corresponding to authentic PAF was scraped off and extracted by the method of Bligh and Dyer (28). The chloroform layer was removed and evaporated to dryness. The residue was dissolved in chloroform/methanol (1:2, v/v), and an aliquot of the solution was placed into a scintillation vial to determine the extraction recovery of PAF. Another aliquot of solution was evaporated to dryness, and the residue was resuspended in Hepes-buffered Tyrode's solution (pH 7.4) containing 0.25% BSA. The amount of PAF was measured by estimating the aggregating activity toward washed rabbit platelets (29), using 16:0-PAF as a standard. The aggregation of rabbit platelets by the PAF fraction was completely inhibited by pretreatment of the cells with 10 μM CV3988.

Phospholipid and fatty acid analyses. Phospholipids and fatty acids were analyzed as described previously (17). Briefly, lipids were extracted by the method of Bligh and Dyer (28) and fractionated by TLC. Choline and ethanolamine glycerophospholipids (CGP and EGP) were extracted separately, and then fractionated into the alkenylacyl, alkylacyl and diacyl subclasses. Their fatty acids were analyzed as methyl esters by gas-liquid chromatography (GLC). The methyl esters were separated on a capillary column coated with SP-2330, using helium as the carrier gas. The amounts of the subclasses were estimated from their fatty acid contents, using methyl heptadecanoate as an internal standard.

RESULTS AND DISCUSSION

Production of PAF by neutrophils and eosinophils from a normal donor. The biosynthesis and release of PAF by neutrophils have been studied by several investigators (for a review, see ref. 30). However, no precise data comparing the production of PAF by neutrophils and by eosinophils are available so far.

Figure 1 shows the time course of PAF production by human neutrophils and eosinophils stimulated with A23187. The production of PAF by neutrophils reached a maximum at 5–15 min, and gradually decreased thereafter. Throughout the incubation, large proportions of newly formed PAF were found to be cell-associated. About 98% (2 min) and 90% (30 min) of PAF were retained in cells. These results are in good agreement with previous observations of others (31–33). The profiles of PAF production by eosinophils are considerably different from those of neutrophil-produced PAF. The amounts of total PAF reached a peak within 2 min after stimulation and then rapidly declined during prolonged incubation. Most of the PAF generated by eosinophils was shown to be cell-associated. However, the percentage of retained PAF was lower at 30 min (78%) than at 2 min (97%). These observations may be due to the rapid degradation of intracellular PAF in this type of cell. Lee *et al.* (34) had already demonstrated that the activity of PAF degrading enzyme, acetylhydrolase, was about four times higher in human eosinophils than in neutrophils.

Production of PAF by hypodense and normodense eosinophils. Recent studies with purified eosinophils from individuals with eosinophilia have demonstrated that

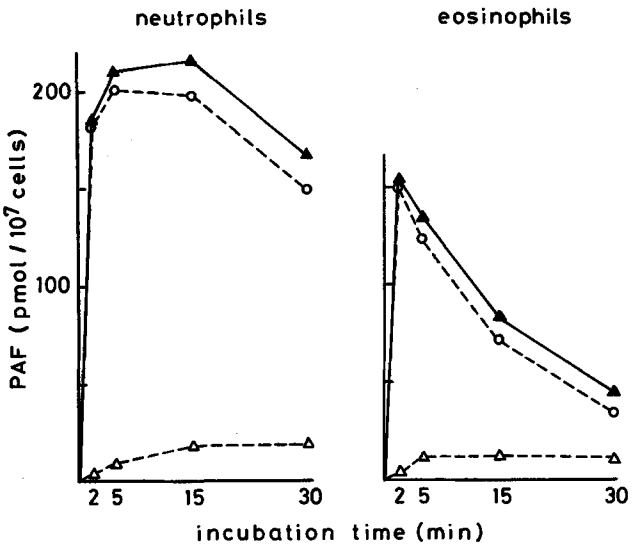


FIG. 1. PAF production by neutrophils (left) and eosinophils (right) from a normal donor as a function of time. After incubation, the cells were spun down. The amount of PAF in the external medium (△) and cell-associated PAF (○) were measured, and the sum of them is represented by closed triangles (▲). The number of the axis of ordinate in the right side of the figure is the same as that in the left side. The data represent means of two or three determinations.

TABLE 1

PAF Production by Hypodense and Normodense Eosinophils from a Patient with Hypereosinophilic Syndrome[a]

	PAF (pmol/10^7 cells/15 min)		
	Exp. 1	Exp. 2	Exp. 3
Hypodense	753.8 ± 80.6[b]	501.1 ± 24.7[d]	660.5 ± 143.2[d]
Normodense	213.9 ± 31.2[c]	252.6 ± 44.4[d]	193.0 ± 48.8[d]

[a]Eosinophils (2.5 × 10^6 cells/0.5 mL) from a patient with idiopathic hypereosinophilic syndrome were stimulated by A23187 (1 μg/mL) for 15 min. The total lipids were extracted. PAF was separated by TLC and assayed by measuring the aggregating activity toward washed rabbit platelets. The experiments were repeated with different preparations of eosinophils (Experiments 1–3). The data represent means ± S.D. from (b) seven, (c) eight and (d) three determinations. Exp., experiment.

eosinophils consist of cells with varying densities and with different biological activities (18–25,27). Eosinophils from blood of normal donors, used in the experiment of Figure 1, are usually normodense (d = 1.085–1.09). By contrast, eosinophils from bronchoalveolar lavages of subjects with asthma or blood of patients with hypereosinophilic syndrome (HES) are known to be mainly hypodense (d = 1.075–1.077) (18,20,22,27). These observations prompted us to investigate the production of PAF in two populations of eosinophils obtained from a patient with HES. Table 1 shows the amounts of PAF produced by hypodense and normodense eosinophils, both of which had been stimulated with A23187 for 15 min. The striking observation was that hypodense eosinophils produced 2–3

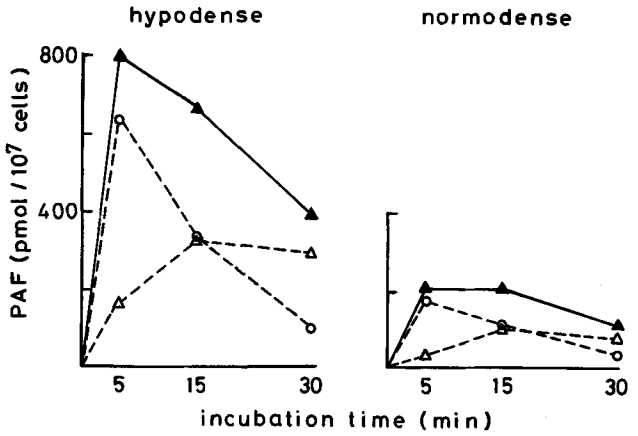


FIG. 2. PAF production by hypodense (left) and normodense (right) eosinophils from a patient with hypereosinophilic syndrome (HES) as a function of time. PAF produced by both types of cells was estimated as described in the legend to Figure 1. Symbols are the same as in Figure 1. The number of the axis of ordinate in the right side of the figure is the same as that in the left side. The data represent means of two or three determinations.

times more PAF than did normodense eosinophils. The amount of PAF synthesized by hypodense eosinophils was considerably higher than that synthesized by neutrophils from normal subjects (151.4 ± 40.9 pmole/10^7 cells/15 min, mean ± S.D., n = 6). Thus it appears that among human blood cells hypodense eosinophils are one of the most effective in generating PAF (30).

Figure 2 shows the time course of PAF production by hypodense and normodense eosinophils. The amounts of total PAF produced reached a maximum within 5 min after stimulation with A23187 and then decreased with time in both hypodense and normodense eosinophils. A similar decrease was observed for normodense eosinophils from normal donors (Fig. 1). However, the relative proportions of the released PAF were higher in both hypodense (49% at 15 min) and normodense (47% at 15 min) eosinophils than in normodense eosinophils obtained from normal subjects (23 ± 5% at 15 min, mean ± S.D., n = 4). The data suggest that, in HES patient, considerable amounts of PAF can be released into biological fluids if eosinophils are activated with appropriate stimuli. Small vessel and large vessel thrombotic occlusions are characteristic features of HES (35). Furthermore, exclusive cardiac ventricular intracavity thrombi can develop in some patients with HES (36). PAF released from hypodense eosinophils may therefore play a role in thromboembolic complications. It is furthermore possible that hypodense eosinophil-derived PAF is involved in the recruitment of eosinophils at tissue sites close to allergic and inflammatory lesions, as PAF is known to be a potent chemotactic agent to eosinophils (37). The question whether PAF actually mediates the symptoms of HES still needs to be answered.

The mechanism of accelerated PAF synthesis in hypodense eosinophils is presently not known. The levels of alkylacyl-GPC, a "storage form" of PAF precursor, were found to be relatively similar in hypodense and normodense eosinophils (64.2% and 75.6%, respectively). In addition, the fatty acid profiles of alkylacyl-GPC in hypodense and normodense eosinophils are quite similar (data not shown). Regulation of the activities of the enzymes involved in PAF biosynthesis, or the supply of the immediate PAF precursors (lysoPAF and acetyl-CoA) may, therefore, be quite different in these two types of cells. Alternatively, differences in the intracellular processing of PAF may affect the amounts of PAF produced.

Earlier studies had already demonstrated that a number of biological properties seem to differ between these two types of eosinophils. Hypodense eosinophils contain significantly smaller amounts of major basic proteins than normodense eosinophils (18). Furthermore, hypodense eosinophils have enhanced antibody-mediated cytotoxicity (19,20), increased consumption of oxygen and glucose (20–22) and enhanced expression of IgE-low affinity receptors (23) as compared to normodense eosinophils. Increased LTC_4 formation has also been demonstrated in hypodense eosinophils (24), although divergent results were reported in regard to the mode of LTC_4 formation (25). Further and more detailed studies on the synthesis and release of PAF, as well as LTC_4 by both hypodense and normodense eosinophils should contribute to a better understanding of the pathogenesis of various diseases in which eosinophils have been implicated.

ACKNOWLEDGMENT

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Immunoregulatory Functions of Paf-Acether. VI. Dual Effect on Human B Cell Proliferation[1]

Corinne Leprince[a], Eric Vivier[b], Dominique Treton[a], Pierre Galanaud[a], Jacques Benveniste[b], Yolande Richard[a] and Yolene Thomas[b,*]

[a]Laboratoire d'Immunopathologie et d'Immunologie Virale, Institut National de la Santé et de la Recherche Médicale, U131, and [b]Laboratoire d'Immunopharmacologie de l'Allergie et de l'Inflammation, Institut National de la Santé et de la Récherche Medicale, U200, Universite Paris-Sud, 92140 Clamart, France

The role of paf-acether (paf), a phospholipid cytokine, in the modulation of human B cell function was investigated. Paf, from 1×10^{-5} M to 10^{-6} M, decreased B cell proliferation induced by both phorbol myristate acetate (PMA) and anti-IgM antibodies (anti-IgM Ab). By contrast, 1×10^{-7} M to 1×10^{-9} M paf enhanced PMA triggered, but not anti-IgM triggered B cell proliferation. B cell proliferation was modulated between 24 and 72 hr of culture indicating that the effect of paf did not merely reflect a shift in proliferation kinetics. Interestingly, paf also enhanced the spontaneous proliferation of a Burkitt lymphoma-derived B cell line, Raji, which suggests that paf can directly act on B cells. The modulatory effect of paf on peripheral blood B cells was independent of PMA concentration, yet the effect on Raji cells was dependent upon cell density. The data suggest that paf is a potent modulator of B cell function, and may be involved in the control of humoral immune response.
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At initiation of humoral immune response, mature B lymphocytes recognize specific antigen through interaction with their surface immunoglobulins (Ig), a signal which stimulates transition from the G0 to the G1 phase of the cell cycle (1). Once in G1, activated B cells can progress to the S/M phase and proliferate before final maturation to antibody-secreting cells. This differentiation process is dependent on regulatory signals generated by either cellular interactions or soluble factors (2). For instance, B cell proliferation has been shown to be controlled by proteic mediators such as interleukin 2 (IL-2), IL-4, and/or B cell growth factors (2,3). In some situations, as in those found in Epstein-Barr virus (EBV) infected B cell lines, homogenous populations of "activated-cycling" B cells are generated (4). Transmembrane signalling related to surface Ig cross-linking relies at least in part on protein kinase C (PKC) activation *via* membrane phosphoinositide breakdown, generation of diacylglycerol, and mobilization of intracytoplasmic Ca^{2+}. These biochemical events can be triggered *in vitro* by anti-IgM Ab (1,5–8). Alternatively, intracytoplasmic PKC can be directly stimulated by phorbol esters such as phorbol myristate acetate (PMA) (7). Although both reagents, anti-IgM Ab and PMA, are well defined as polyclonal B cell activators inducing the G1 entry of resting B cells, they have also been shown to act as B cell mitogens (9,10).

Paf-acether (1-*O*-alkyl-2-acetyl-*sn*-glycero-3-phosphocholine, paf) is a phospholipid originally characterized as platelet-activating factor released by IgE-sensitized basophils upon antigenic stimulation (11). Paf is involved in a wide variety of proinflammatory events *in vitro* and *in vivo*, and exerts an immunoregulatory function (12,13). For instance, in addition to its capacity to downregulate CD2 and CD3 expression (14), paf inhibits T cell activation induced by phytohemagglutinin (PHA) (15,16) or anti-CD3 mAb, and potentiates activation induced by anti-CD2 mAb (17). Moreover, paf upregulates IL-1, and tumor necrosis factor (TNF) α production by human monocytes, suggesting that paf may be involved in the control of monocyte/T cell interactions (18,19).

Although previous studies have demonstrated the release of paf-like material by long term B cell lines or leukemic B cells stimulated with Ca^{2+} ionophore (20,21), little is known about the interactions between paf and B lymphocytes. In the present study, we found that paf exerts a dual effect on peripheral blood B cell proliferation induced by PMA but inhibits only the effect induced by anti-IgM Ab. Further, addition of paf to the B cell line, Raji, derived from an EBV infected Burkitt lymphoma resulted in a substantial increase in DNA synthesis indicating that paf may directly act on B cells. The data suggest that paf interferes with the processes leading to B cell activation.

MATERIALS AND METHODS

Media and reagents. The final medium used for B cell cultures consisted of Iscove's modified medium (Gibco, Grand Island, NY) supplemented with 50 μg/mL human transferrin, 5 μg/mL bovin insulin, 2 μg/mL each of oleic, linoleic and palmitic acids (Sigma, St. Louis, MO), 1% L-glutamin, 1% antibiotics (Gibco), 0.25% human serum albumin (HSA, Bio Merieux, Lyon, France) (22) and 2% heat inactivated fetal calf serum (FCS, Gibco). Synthetic C_{18}paf (1-*O*-octadecyl-2-acetyl-*sn*-glycero-3-phosphocholine; Bachem, Bubendorf, Switzerland) was dissolved in ethanol (60% v/v) at a concentration of 10 mM. The solution was diluted in 0.9% NaCl containing 0.25% HSA at a concentration of 100 μM and stored at −20°C. In all further dilutions, the concentration of 0.25% HSA was maintained. The effect of NaCl/HSA (0.25%) used as vehicle was tested at the same final dilution as used in the paf-treated cultures.

B cells and B cell line. Mononuclear cells were isolated from blood of healthy volunteers using Ficoll-Hypaque (Pharmacia, Uppsala, Sweden) density gradient centrifugation. Monocytes and macrophages were removed

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*To whom correspondence should be addressed at Laboratoire d'Immunopharmacologie de l'Allergie et de l'Inflammation, Institut National de la Santé et de la Recherche Médicale, U200, Université Paris-Sud, 32 rue des Carnets, Clamart, France.

Abbreviations: anti-IgM Ab, anti-IgM antibodies; EBV, Epstein-Barr virus; FCS, fetal calf serum; HSA, human serum albumin; IL, interleukin; paf, paf-acether, 1-*O*-alkyl-2-acetyl-*sn*-glycero-3-phosphocholine; PHA, phytohemagglutinin A; PKC, protein kinase C; PMA, phorbol myristate acetate; TNF, tumor necrosis factor.

by L-leucine methyl ester (Sigma) treatment (23). Monocyte-depleted mononuclear cells were incubated with sheep red blood cells treated with 2-aminoethyl-isothiouronium bromide hydrobromide (Sigma). The non-rosetting cell fractions that were recovered after two cycles of incubation were highly enriched B cell preparations containing 83 ± 3% $CD19^+$, 3 ± 1% $CD2^+$ and 6 ± 1% $CD11b^+$ cells.

The Burkitt lymphoma B cell line, Raji, was kindly provided by Dr. T. Tursz (CNRS UA 1156, Villejuif, France). The cell line was cultured in RPMI 1640 medium supplemented with 1% glutamine, 1% antibiotics, 1 mM sodium pyruvate and 10% heat inactivated fetal calf serum (Gibco), and was readjusted every 3 days to a density of 3×10^5 cells/mL. Before the assay, cells were subcultured for 24 hr and harvested in the log growth phase.

Proliferation assays. All culture experiments were carried out in triplicate in microtest tissue culture dishes (Falcon, Oxnard, CA) kept in a humidified atmosphere containing 7% CO_2 in air at 37°C. Peripheral blood B cells (1×10^5/well) were cultured for different periods of time (24 to 72 hr) in 200 μL final medium in the presence of either paf (1×10^{-5} to 10^{-11} M) or vehicle. Stimuli included PMA (Sigma; 1 to 10 ng/mL) or goat anti-IgM Ab coupled to sepharose beads (Biorad, Richmond, CA; 7 μg/mL).

Raji cells were cultured (1 to 20 × 10^3/well) in 200 μL final medium without addition of fetal calf serum, for 24 to 72 hr in the presence of either paf (1×10^{-5} to 10^{-11} M) or vehicle. During the last 12 hr of culture, peripheral blood B cells or Raji cells were pulsed with 0.5 μCi/well (18.5 KBq/well) of [^{3}H]thymidine (CEA, Saclay, France) and then harvested using a skatron cell apparatus. Incorporation of [^{3}H]thymidine was measured by liquid scintillation counting. Results are expressed as mean counts per min (cpm) ± SEM of triplicate samples rounded to the nearest hundred. Cell counts and viability tests (by trypan blue exclusion) were performed on all cell cultures at the time of the assay.

Statistical analysis. Mean of results obtained from at least three independent experiments were compared using the paired Student t-test.

RESULTS

The effect of paf on mitogen-induced B cell proliferation was investigated. Highly purified peripheral blood B cells (1×10^5/well) were stimulated with PMA or anti-IgM Ab in the presence of different concentrations of paf or vehicle. [^{3}H]Thymidine incorporation was assessed after 72 hr of culture. As shown in a representative experiment (Fig. 1), stimulation of human B lymphocytes with anti-IgM Ab or PMA was sufficient to trigger B cell proliferation. When B cells were stimulated with anti-IgM Ab (7 μg/mL), 1×10^{-5} and 1×10^{-6} M paf inhibited the proliferative response; 1×10^{-5} M paf decreased cell proliferation by 44.9 ± 6.4% (mean ± SEM, n = 22). By contrast, when B cells were stimulated with PMA, a biphasic effect was observed. Paf, at 1×10^{-5} and 1×10^{-6} M inhibited PMA-induced B cell proliferation, whereas 1×10^{-7} to 1×10^{-9} M paf potentiated it. Upregulation (paf 1×10^{-8} M) and downregulation (paf 1×10^{-5} M) were 34.5 ± 9.5% and 39.5 ± 6.2% (mean ± SEM, n = 22), respectively. Although the magnitude of the modulation

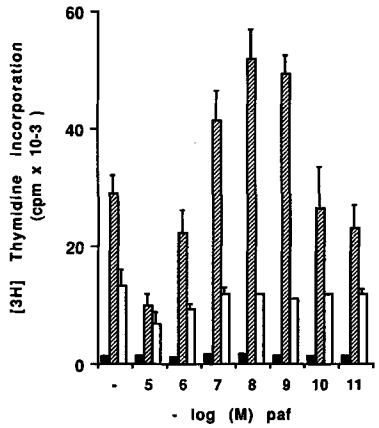


FIG. 1. Effect of paf on mitogen-induced B cell proliferation. B cells (1×10^5/well) were cultured with medium (solid bar), PMA at 5 ng/mL (hatched bar) or anti-IgM Ab (open bar) in the presence of paf (1×10^{-5} to 10^{-11} M) or vehicle (v). After 72 hr of culture, B cell proliferation was assessed by determining [^{3}H]thymidine incorporation. Results of one representative experiment are shown and expressed as mean cpm × 10^{-3} ± SEM of triplicate cultures. When error bar is absent, SEM is within the symbol.

was quite variable among different individuals tested, the effects of paf were statistically significant ($p<0.001$). In the absence of stimuli, paf alone did not modulate the background B cell proliferative response. Furthermore, no cytotoxic effect of paf was observed at all concentrations tested, as judged by cell counts and trypan blue exclusion. The results suggest that paf up- or downregulates B cell proliferation induced by PMA, but only inhibits proliferation induced by anti-IgM Ab.

In the following experiments, we focused on the dual effect of paf (at 1×10^{-5} and 10^{-8} M) on PMA-induced B cell proliferative response. B cells were incubated with varying concentrations of PMA in the presence of paf or vehicle for 72 hr to investigate whether the changes induced by paf were dependent upon the level of PMA-induced B cell proliferative response. As shown in Table 1, 1×10^{-5} and 1×10^{-8} M paf, respectively, down- and upregulated [^{3}H]thymidine incorporation at all PMA concentration tested. The data suggest that both effects of paf are not dependent on PMA concentration and occur at suboptimal as well as optimal cell stimulation. In the next set of experiments, the kinetics of PMA-induced B cell proliferation in the presence of paf (1×10^{-5} and 10^{-8} M) were investigated. As depicted in Figure 2, paf-induced downregulation and upregulation of B cell proliferation were detectable as early as 24 hr after initiation of the culture, increased by 48 and 72 hr, and plateaued thereafter (96 to 120 hr, data not shown). Upregulation (1×10^{-8} M paf) was 30.1 ± 4.2% (n = 5) and 34.5 ± 9.5% (n = 22) at 48 and 72 hr, respectively. Downregulation (1×10^{-5} M paf) was 37.2 ± 6.6% (n = 5) and 39.5 ± 6.2% (n = 22) at 48 and 72 hr, respectively. These results indicate that paf does not merely modify the kinetics of PMA-induced B cell proliferation.

Highly enriched B cell populations were not totally devoid of residual T cells and/or monocytes, and the possibility remained that the primary targets of paf were not B cells, but rather T cells or monocytes. Therefore, the

TABLE 1

Modulation by Paf of PMA-Induced B Cell Proliferation[a,b]

PMA (ng/mL)	Vehicle	Paf 1×10^{-5} M	Paf 1×10^{-8} M
0	1.8 ± 0.5	1.3 ± 0.3	2.0 ± 0.5
	1.3 ± 0.5	1.1 ± 0.1	1.7 ± 0.4
1	3.1 ± 0.4	1.4 ± 0.2	3.7 ± 0.3
	2.8 ± 0.1	NT[c]	6.7 ± 0.4
2	21.6 ± 2.0	12.1 ± 1.9	34.8 ± 1.8
	3.9 ± 3.0	2.8 ± 0.1	7.6 ± 1.0
5	30.0 ± 2.7	19.8 ± 2.0	49.4 ± 3.5
	8.1 ± 1.0	6.8 ± 0.5	13.8 ± 2.0
10	40.0 ± 5.0	28.0 ± 2.5	50.6 ± 3.0
	15.2 ± 1.2	8.5 ± 0.9	27.1 ± 2.7

[a] B cells (1×10^5/well) were incubated with different concentrations of PMA in the presence of paf or vehicle. After 72 hr of culture, B cell proliferation was assessed by determining [^{3}H]thymidine incorporation. Results are expressed as mean (cpm $\times 10^{-3}$) ± SEM of triplicate cultures.

[b] Upper line at each PMA concentration refers to experiment 1, lower line refers to experiment 2.

[c] Not tested.

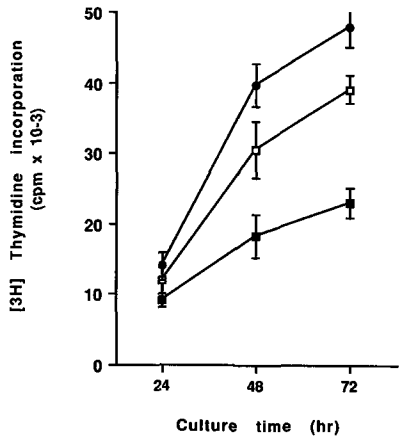


FIG. 2. Kinetics of paf effect on PMA-induced B cell proliferation. B cells (1×10^5/well) were stimulated with PMA (5 ng/mL) in the presence of 1×10^{-5} M paf (black square), 1×10^{-8} M paf (black circle), or vehicle (open square). B cell proliferation after 24, 48 or 72 hr of culture was assessed by determining [^{3}H]thymidine incorporation. Results of one representative experiment are shown and expressed as mean $\times 10^{-3}$ ± 1 SEM of triplicate cultures.

effect of paf on the proliferation of a homogeneous population of autoproliferating cells, the Burkitt lymphoma-derived B cell line Raji was investigated. Raji cells (10^4 cells/well) were grown in a chemically defined, serum-free medium in the presence of various concentrations of paf or vehicle. Within 72 hr after addition of 1×10^{-6} M paf, spontaneous proliferation of Raji cells increased by 167 ± 33% (mean ± SEM, n = 8, $p<0.005$). Paf from 1×10^{-7} to 1×10^{-11} M did not affect the proliferation (data not shown). As expected, addition of higher concentrations of paf (1×10^{-5} M) produced cytotoxic effects because in serum-free medium, paf is not hydrolyzed by serum acetyl hydrolase (24).

As the autonomous growth of Raji cells depends on cell density, the effect of paf at various cell concentrations was tested. As shown in Figure 3, the potentiating effect of paf at 1×10^{-6} M was observed at all cell concentrations that can sustain autonomous growth. This latter result suggests that paf may act as a cofactor in the spontaneous proliferation of Raji cells. The two-log difference in the upregulating concentration of paf between peripheral blood and Raji B cells, could be the difference between normal and malignant cell types, the latter being able to take up and/or to metabolize paf more efficiently. Kinetic analysis of paf-induced modulation of Raji cell proliferation revealed that the increase in [^{3}H]thymidine incorporation by Raji cells in the presence of 1×10^{-6} M paf occurred at 24 as well as at 48 and 72 hr after initiation of the culture (Table 2). The latter data suggest that paf does not delay the Raji cell cycle but facilitates Raji cell growth at each time point of the culture. Furthermore, upregulation of DNA synthesis was directly related to cell proliferation as determined by the increase in cell counts at 24, 48 and 72 hr of culture (data not shown). The results

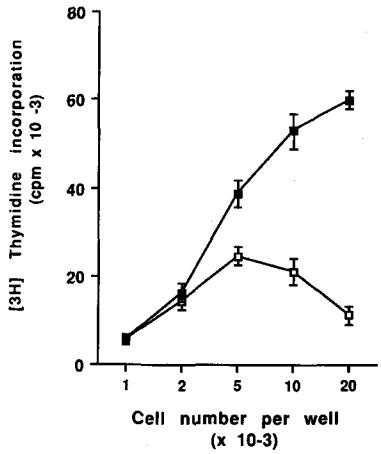


FIG. 3. Paf-induced modulation of Raji cell proliferation. Raji cells were cultured from 1×10^3 to 2×10^4 cells/well in the presence of 1×10^{-6} M paf (black square) or vehicle (open square). After 72 hr of culture, B cell proliferation was assessed by determining [^{3}H]thymidine incorporation. Results of one representative experiment out of 8 are shown and expressed as mean cpm $\times 10^{-3}$ ± 1 SEM of triplicate cultures.

TABLE 2

Kinetics of Paf Effect on Spontaneous Raji Cell Proliferation[a,b]

Length of culture (hr)	Vehicle	Paf 1×10^{-6} M
24	26.4 ± 2.2	70.7 ± 5.0
48	47.8 ± 3.1	97.3 ± 2.1
72	40.4 ± 2.7	95.3 ± 6.4

[a] Raji cells (1×10^4/well) were cultured for the times indicated in the presence of paf (1×10^{-6} M) or vehicle.

[b] Raji cell proliferation was assessed by determining [^{3}H]thymidine incorporation. Results are expressed as mean cpm $\times 10^{-3}$ ± SEM of triplicate cultures. Results of one representative experiment out of three are shown.

obtained on pure B cell populations would indicate that B lymphocytes are potential targets for the immunoregulatory action of paf, and specifically in their proliferative stage.

DISCUSSION

In this study, we have shown that paf, a naturally occurring phospholipid, has potent *in vitro* effects on B cell function. In particular, paf could exert dual biological activities depending on its concentration and on the B cell stimulus. At 1×10^{-5} and 1×10^{-6} M, paf downregulated PMA induced as well as anti-IgM induced B cell proliferation, whereas from 1×10^{-7} to 1×10^{-9} M paf upregulated proliferation induced by PMA, irrespective of the magnitude of the proliferative response. The magnitude of the paf effect was quite variable between different individuals tested and did not reflect differences in the activation and differentiation status of the responsive B cells. No inherent differences in the ability of B cells from either peripheral blood or tonsils to respond to paf were found (data not shown), although the latter can be considered as potentially *in vivo* activated (25). Thus, the variability of the paf effects may be due to differences in contaminating T cells and monocytes generating soluble factors that in turn regulate B cell function (26,27). In this regard, paf has been shown to increase monocyte production of IL-1 or TNF-α (18,19). Irrespective of which of these interpretations is correct, the fact that paf can act directly on B cells is clear because 1×10^{-6} M paf upregulated the spontaneous proliferation of the Burkitt lymphoma B cell line, Raji.

The inhibitory effect of paf was not merely due to a shift in proliferation kinetics, nor to cytotoxicity. It could be argued that at 1×10^{-5} M paf, cells could be affected by this phospholipid concentration even if no lytic effect was apparent. However, lysophosphatidylcholine, a phospholipid about as lytic to cell membranes as paf, did not affect B cell proliferation (Vivier, E., unpublished observation). Paf at 1×10^{-5} M may act during the early steps of B cell activation, limiting the pool of cells entering into the G1 phase of the cell cycle. Alternatively, paf may suppress the proliferation of activated B cells, *i.e.* their progression in the cell cycle. We favor the latter interpretation since paf at 1×10^{-5} M was also shown to downregulate high affinity IL-2 receptors on T cells, a mechanism which may explain the inhibition of mitogen-induced T cell proliferation (15,28). By analogy, a similar concentration of phospholipid may diminish the density of functional growth factor binding sites on B cell membranes. In this regard, a preliminary report seems to indicate that paf inhibits activated B cell proliferative response to IL-2 and to a low molecular weight B cell growth factor (29).

It is intriguing that paf-induced upregulation of peripheral blood B cell proliferation was dependent upon the stimuli used, since no potentiation of anti-IgM proliferative response was observed. First, it is possible that paf from 1×10^{-7} to 1×10^{-9} M synergizes with biochemical events triggered by PMA and not by anti-IgM Ab. The quantitative difference between both reagents and their capacity to stimulate, either directly or indirectly, intracytoplasmic PKC may be important (1). Further, PKC activation, either by paf alone or in synergy with PMA, has been previously reported in human neutrophils (30-32). Second, paf may differentially regulate the proliferation of distinct functional B cell subsets responsive to PMA or anti-IgM Ab stimulation. Both hypothesis are not mutually exclusive.

Finally, the precise mechanisms whereby paf could directly enhance the proliferation of human peripheral blood B cells or Raji cells have not been delineated. One possibility is that paf enhances B cell production of factors such as IL-1, TNF α and IL-6 which are known to modulate B cell function (33-37). Alternatively, since paf seems to enhance CD23 expression on B cells (38), its enhancing activity could be due to an increased production of soluble CD23 which in turn acts as a B cell growth factor (39). This seems unlikely since preliminary results suggest that paf also modulates proliferation of Rjcl3, a mutant cell line from Raji that displayed a HLA-DR$^-$ CD23$^-$ cell surface phenotype (data not shown).

According to our data, paf modulates human B cell function producing a singular or dual effect, *i.e.* down- and upregulating B cell proliferation, depending on the first signal applied. The mechanism underlying such a dual effect may involve interactions with high and low affinity paf binding sites coupled to different signaling pathways. Two classes of paf receptors have been previously described in human neutrophils (40). Further studies on B cells using the cDNA probe to the paf receptor will clarify this issue (41). Finally, paf may be an important immunoregulatory factor regulating human B cell function either directly or indirectly through its functional interactions with T cell or monocyte derived cytokines.

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Intracellular Ca^{2+} Concentration and H_2O_2 Production in Mouse Peritoneal Macrophages Are Stimulated by Platelet Activating Factor[1]

Masayuki Sasaki, Kazutaka Maeyama* and Takehiko Watanabe
Department of Pharmacology I, Tohoku University School of Medicine, Sendai 980, Miyagi, Japan

The intracellular calcium concentration ($[Ca^{2+}]i$) in mouse peritoneal macrophages cultured on a coverglass was measured in the superfusion system using fura-2 as a fluorescent calcium probe and platelet activating factor (PAF) as a stimulant. In the presence of extracellular Ca^{2+}, 10^{-7} M PAF sharply increased $[Ca^{2+}]i$ from a basal level of 90 nM to 340 nM. Thereafter, the $[Ca^{2+}]i$ level gradually decreased in two phases, in an initial rapid phase and a subsequent slow phase of decrease. The calcium response was dependent on the PAF concentration. In the absence of extracellular Ca^{2+}, a single sharp peak was observed, suggesting two different modes of Ca^{2+} movement, one from intracellular stores and the other from the extracellular medium. A simple, sensitive fluorometric assay system was developed for measuring H_2O_2 in the superfusate of macrophages after stimulation by use of immobilized peroxidase and 3-(*p*-hydroxyphenyl)propionic acid as a fluorogenic substrate. With this system, as little as 2 pmol of H_2O_2 could be measured. PAF (1 μM) increased H_2O_2 production in peritoneal macrophages in the presence of extracellular Ca^{2+}, but H_2O_2 production was not observed in the absence of extracellular Ca^{2+}.

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Mouse macrophages bear various kinds of surface receptors specific for various ligands, such as immunoglobulins and chemical mediators, and are stimulated by the binding of these ligands. The stimulations are transmitted by a second messenger system such as cyclic adenosinemonophosphate (AMP), inositol phosphates and calcium resulting in various cell responses, e.g., phagocytosis, respiratory burst and secretion of lysosomal enzymes (1–3). Platelet activating factor (PAF), 1-*O*-alkyl-2-acetyl-*sn*-glycero-3-phosphocholine, is a phospholipid mediator released from a variety of inflammatory cells, including macrophages (4). PAF induces various physiological and pathological responses, such as platelet aggregation, degranulation of neutrophils, hypotension and bronchoconstriction (5,6). It is also a potent stimulant of macrophages and causes release of prostanoids (7,8), glucose consumption in guinea pig macrophages (9), production of active oxygen species and aggregation in human monocytes (10,11). Meanwhile, $[Ca^{2+}]i$ has been measured in the murine macrophage cell line J774 and mouse peritoneal macrophages stimulated with immunocomplexes (12). Recently, a new fluorescent calcium probe, fura-2-, which is generally thought to be superior to quin2, has been widely used for measurement for intracellular concentration of Ca^{2+} ($[Ca^{2+}]i$) in cell suspensions, monolayered cells and single cells (13–15). We developed a new system for measurement of $[Ca^{2+}]i$ in which cell monolayers on coverslips are superfused and $[Ca^{2+}]i$ is calculated with a microcomputer from the ratio of fura-2 fluorescences excited at two wavelengths (16). Using this system, we previously measured $[Ca^{2+}]i$ in J774.2 cells and suggested that there are two sources of $[Ca^{2+}]i$, the intracellular sources and Ca^{2+} derived from the medium by influx across the plasma membrane (16). Unfortunately, J774.2 cells showed no secondary cell response to a calcium signal, such as H_2O_2 production and release of lysosomal enzymes, and we could not study the coupling of the increase in $[Ca^{2+}]i$ and the cellular response. In the present work, using mouse peritoneal macrophages, we confirmed the above hypothesis of two locations for mobilization of Ca^{2+}, and also measured H_2O_2 production. For H_2O_2 measurement we developed a new fluorometric system using immobilized peroxidase, which condenses 3-(*p*-hydroxyphenyl)propionic acid (HPPA) with H_2O_2 (17).

MATERIALS AND METHODS

Cell preparation. Mouse macrophages were obtained from the peritoneal cavity of male ddY mice (25–30 g, Shizuoka Laboratory Animal Center, Shizuoka, Japan) after i.p. injection of 0.5 mL liquid paraffin. Ten days later, peritoneal exudate cells were collected in Hanks' balanced salt solution (HBSS) containing 10 U/mL heparin. The cells were washed twice with HBSS and layered on a coverslip (0.9 × 2.2 cm) in RPMI 1640 medium containing 15% fetal calf serum and antibiotics (100 U/mL penicillin, 100 μg/mL streptomycin and 0.25 μg/mL Fungizone), and cultured at 37°C in an atmosphere of 5% CO_2 and 95% air.

Measurement of $[Ca^{2+}]i$ in peritoneal macrophages. The intracellular concentration of Ca^{2+}, $[Ca^{2+}]i$, was measured by the method described by Sasaki *et al.* (16). Details of the method will be reported elsewhere. Briefly, macrophages on a coverslip were loaded with 5 μM fura-2 acetoxymethylester (fura-2/AM) for 30 min at 37°C in (PIPES) buffer (PIPES, 25 mM; NaCl, 119 mM; KCl, 5 mM; glucose, 5.6 mM; $MgCl_2$, 0.4 mM; $CaCl_2$, 1.0 mM; and NaOH, 40 mM; pH 7.2). After 30-min incubation to allow hydrolysis of fura-2/AM to

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*To whom correspondence should be addressed at Dept. of Pharmacology 1, Tohoku University School of Medicine, 2-1 Seiryo-machi, Aoba-ku, Sendai 980, Miyagi, Japan.

AMP, adenosine monophosphate; $[Ca^{2+}]i$, intracellular concentration of Ca^{2+}, EGTA, ethyleneglycol *bis*-β(aminoethyl ether)*N,N,N',N'*-tetraacetic acid; fMLP, formyl Met-Leu-Phe; fura-2/AM, fura-2 acetoxymethylester; HBSS, Hanks' balanced salt solution; HPPA, 3-(*p*-hydroxyphenyl)propionic acid, PAF, platelet activating factor, 1-*O*-alkyl-2-acetyl-*sn*-glycero-3-phosphocholine; PIPES, piperazine-*N,N'-bis*(2-ethane sulfonic acid).

fura-2 by esterase in the cells, the coverslip was inserted into a quartz cuvette (1 cm × 4 cm, 0.2 cm width) with a tube at its bottom. The cuvette was placed diagonally to the light path in a holder of a fluoromonitor (Fi-4000, Hitachi, Tokyo, Japan), and capped with silicon rubber with an outlet tube. The cells were superfused upwards with PIPES buffer containing stimuli such as PAF and formyl Met-Leu-Phe (fMLP) using a peristaltic pump (ATTO, Tokyo, Japan) and a flow rate of 1 mL/min. Fractions were collected every minute for measurement of H_2O_2 as described below. The cuvette and medium were kept at 37°C. The fluorescences of fura-2 were monitored with a Hitachi fluoromonitor (F-4000) at an emission wavelength of 505 nm with excitations at 340 nm and 380 nm, which were changed automatically. $[Ca^{2+}]i$ was calculated with a microcomputer from the ratio of the fluorescence intensities obtained with excitations at 340 and 380 nm by the methods described by Grynkiewicz *et al.* (13) and Scanlon *et al.* (18).

Measurement of H_2O_2 in the superfusates of peritoneal macrophages stimulated by PAF. H_2O_2 was measured by the method described by Zaitsu and Ohkura (17) in combination with immobilized peroxidase using a flow system. As shown in Figure 1, a sample was injected via an injector in 25 mM PIPES buffer, pH 7.2, delivered by Pump 1 into a column of Affi-Prep 10 with fixed peroxidase. Pump 2 delivered a reagent, HPPA, and an fluorescence enhancer, 1 M NaOH-0.05 M boric acid, and the resulting fluorescence was measured by a fluoromonitor (Hitachi F-1100) with excitation and emission wavelengths of 340 nm and 404 nm, respectively. H_2O_2 levels in fractions were measured by this method. Alternatively, the superfusion system was directly connected to the immobilized peroxidase so that the relationship between calcium response and H_2O_2 production were directly compared.

Chemicals. Fura-2/AM and HPPA were purchased from Dojindo Laboratories (Kumamoto, Japan). PAF (1-*O*-octadecyl-2-acetyl-*sn*-glycero-3-phosphocholine) was obtained from Bachem Feinchemilkalien AG (Bubendorf, Switzerland) and ionomycin was from Calbiochem Corp. (La Jolla, CA). Formyl-methionyl-leucyl-phenylalanine (fMLP) and peroxidase (horseradish) were from Sigma Chemical Co. (St. Louis, MO) and RPMI1640 powder was from Gibco Laboratories (Grand Island, NY). Fetal calf serum, penicillin, streptomycin and Fungison were from Whittaker M.A. Bioproducts (Wakersville, MD). Affi-Prep 10 was purchased from Bio-Rad Laboratories (Richmond, CA). Other chemicals were analytical grade reagents from Wako Pure Chemical Industries (Osaka, Japan).

RESULTS

Measurement of $[Ca^{2+}]i$ in mouse peritoneal macrophages stimulated by PAF or fMLP. $[Ca^{2+}]i$ in peritoneal macrophages was measured using fura-2 as a fluorescent probe. The two thin lines in Figure 2 show typical fluorescences at 505 nm excited at 340 and 380 nm in mouse macrophages loaded with fura-2 and stimulated by PAF. After addition of 10^{-7} M PAF, the two fluorescences changed inversely—the fluorescence excited at 340 nm (upper line) increased with a corresponding decrease of the fluorescence excited at 380 nm (lower line). For calibration of Ca^{2+} concentrations in the course of each experiment, the fluorescence intensities at the maximal and zero Ca^{2+} levels and the spontaneous fluorescence were obtained by addition of 1 μM ionomycin, 1 mM ethyleneglycol *bis*-β(aminoethyl ether) *N,N,N',N'*-tetraacetic acid (EGTA) and 5 mM $MnCl_2$, respectively. Using these parameters, $[Ca^{2+}]i$ was calculated as described by Sasaki *et al.* (16). As shown by the bold line in Figure 2, after stimulation with 10^{-7} M PAF in medium containing 1 mM Ca^{2+}, $[Ca^{2+}]i$ increased rapidly, reaching a peak (340

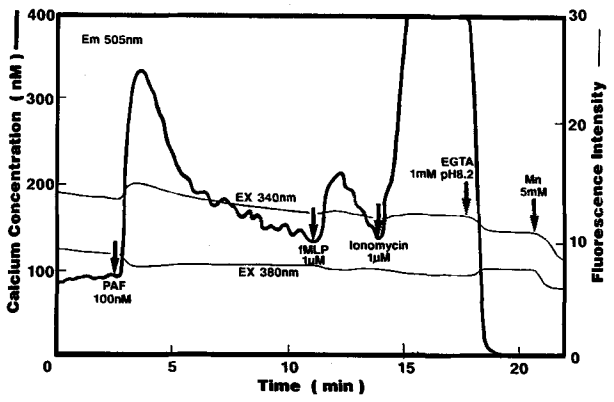


FIG. 1. Flow diagram of H_2O_2 analysis. The sample is injected via the injector in 25 mM PIPES, pH 7.2 (in A), delivered by Pump 1 to a column of Affi-Prep 10 with fixed peroxidase. Pump 2 delivers HPPA (in B) and an alkaline enhancer, 1 M NaOH-0.05 M boric acid (in C), and the resulting fluorescence is measured in a fluoromonitor at excitation and emission wavelengths of 340 nm and 404 nm, respectively.

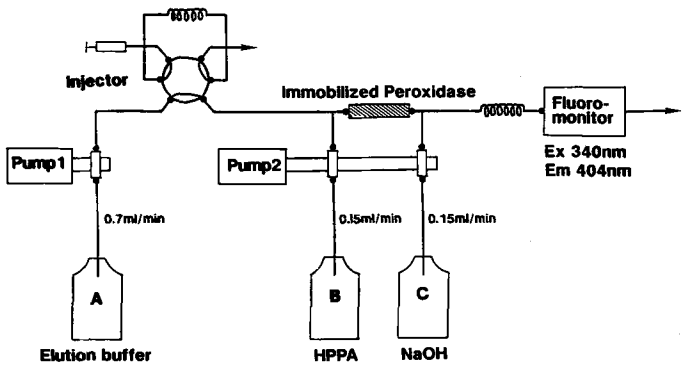


FIG. 2. Increase in $[Ca^{2+}]i$ in peritoneal macrophages stimulated by PAF or fMLP. Peritoneal macrophages were stimulated with 100 nM PAF or 1 μM fMLP, and $[Ca^{2+}]i$ was measured with fura-2 as a fluorescent probe as described in Materials and Methods. After the experiments, for calibration of Ca^{2+} concentrations, 1 μM ionomycin, 1 mM EGTA and 5 mM $MnCl_2$ were added to obtain the values for maximum and minimum fluorescences and autofluorescence, respectively.

nM) at 1 min, and then decreased, first rapidly, and from 3 min more slowly, to the basal level (90 nM). A chemotactic peptide, fMLP, increased $[Ca^{2+}]i$ (220 nM) even after the calcium response was induced by PAF. The dose dependence of the increase of $[Ca^{2+}]i$ by PAF is shown in Figure 3. The response was observed at a PAF concentration as low as 10^{-9} M and reached a plateau at 10^{-7} M PAF—in this concentration range of PAF, the calcium response increased dose-dependently. PAF is labile and metabolized rapidly during incubation with cells. Since macrophages were superfused with PAF, the dose response curve as shown in Figure 3 is quite similar to that obtained by stimulation with liposome-incorporated PAF (19).

Upon removal of extracellular Ca^{2+} with 0.2 mM EGTA, 10^{-7} M PAF caused a sharp increase in $[Ca^{2+}]i$, followed by a rapid decrease to a level lower than the base after 2 min (Fig. 4b). Addition of 1 mM Ca^{2+} resulted in rapid increase of $[Ca^{2+}]i$ to more than 500 nM (Fig. 4b). For comparison, the calcium signal in the presence of 1 mM Ca^{2+} in the medium is shown in Figure 4a.

Measurement of H_2O_2 production in peritoneal macrophages stimulated by PAF. As described in Materials and Methods, we developed a new method for measurement of H_2O_2 by combining immobilized peroxidase with the fluorometric assay described by Zaitsu and Ohkura (17). Figure 1 shows the flow diagram of the method. A sample containing H_2O_2 is delivered to a column of Affi-Prep 10 with fixed horseradish peroxidase, which oxidizes HPPA giving rise to a fluorophore. The fluorescence is then measured on line with a fluoromonitor. Figure 5 shows typical measurements, demonstrating that 1 pmol H_2O_2 gives a two-fold higher peak than buffer. The linear standard curve with 1–20 pmol of H_2O_2 is shown in the Figure 5 insert. Upon addition of catalase to the samples, no peaks were seen (data not shown).

Using the new method for assay of H_2O_2 described here, we measured H_2O_2 production in the superfusate of peritoneal macrophages stimulated by PAF. Immediately after addition of 1 μM PAF to the medium, the rate of H_2O_2 production (Fig. 6, closed circles) increased sharply and reached a maximum within 5 min. H_2O_2 production then remained at this level while PAF was present. Upon removal of PAF from the medium, the rate of H_2O_2 production began to decrease. Total H_2O_2 production reached 2 nmol/10^6 cells/10 min; this was calculated by summation of the production rates and is in good agreement with values reported by others (20,21). The effect of calcium-free superfusion medium

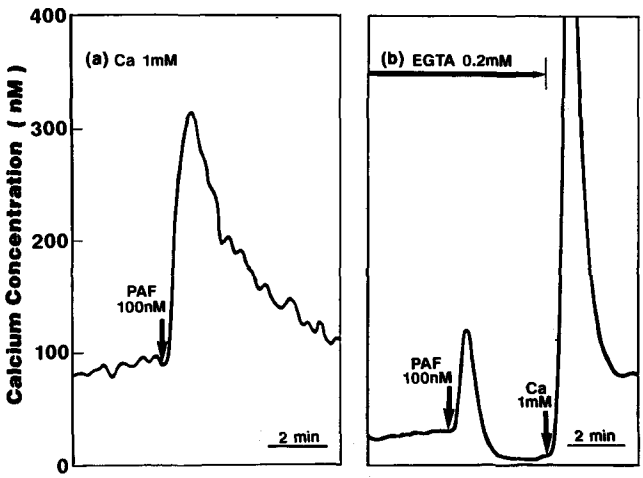


FIG. 4. Effect of extracellular calcium on increase in $[Ca^{2+}]i$ in peritoneal macrophages stimulated by PAF. The calcium signal in macrophages stimulated by 10^{-7} M PAF was measured in the presence of 1 mM Ca^{2+} (a) and in the absence of Ca^{2+} (b) in the extracellular medium. In (b), 1 mM Ca^{2+} was added to the superfusion medium at the time points indicated by arrows.

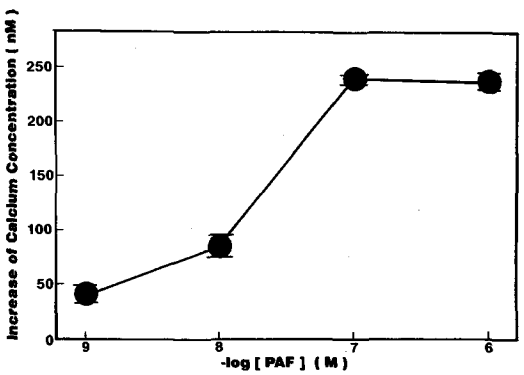


FIG. 3. Dose response curve of increase in $[Ca^{2+}]i$ by PAF. $[Ca^{2+}]i$ in peritoneal macrophages stimulated by PAF at various concentrations was measured as described in Materials and Methods. Values are means ± S.E.M. of three determinations.

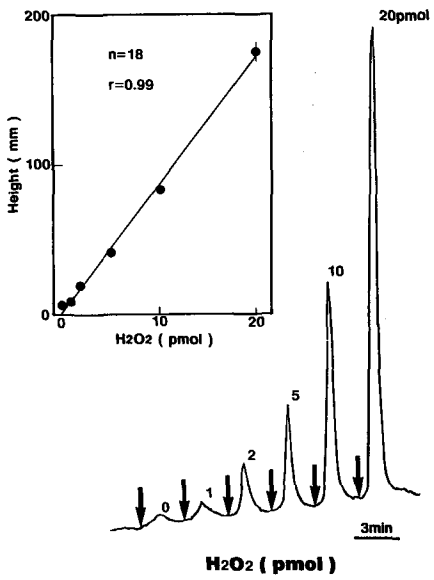


FIG. 5. Measurement of H_2O_2 by the immobilized peroxidase-fluorescence method. Typical measurements of H_2O_2 by the present method and a standard curve (insert) are shown.

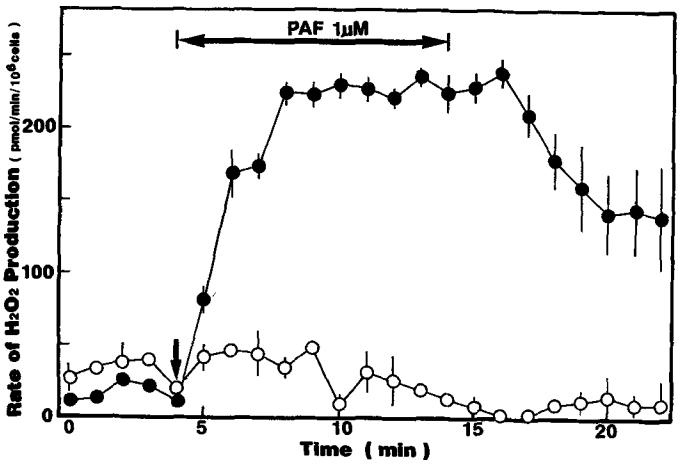


FIG. 6. Effect of extracellular calcium on H_2O_2 production in peritoneal macrophages stimulated by PAF. H_2O_2 production in macrophages stimulated by 1 μM PAF was measured in the absence (-o-) or presence (-●-) of 1 mM extracellular Ca^{2+} as described in Materials and Methods. Values are means ± S.E.M. of three determinations.

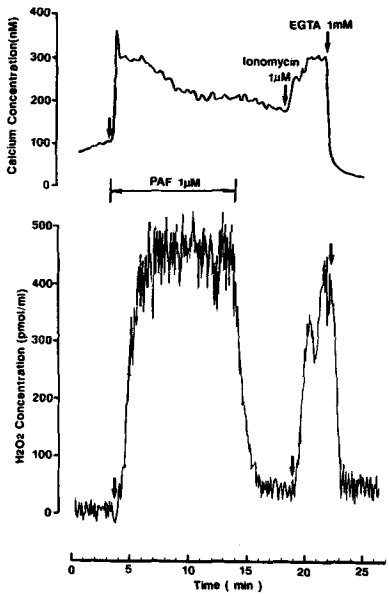


FIG. 7. Simultaneous determination of the calcium signal and H_2O_2 production in peritoneal macrophages. In the presence of external Ca^{2+}, the calcium signal and the rate of H_2O_2 production were directly compared after stimulation with 1 μM PAF and 1 μM ionomycin.

on H_2O_2 production in macrophages is also shown in Figure 6 (open circles). Upon addition of 1 μM PAF, the rate of H_2O_2 production did not increase in the absence of extracellular Ca^{2+}.

Direct comparison of $[Ca^{2+}]i$ and the rate of H_2O_2 production. For this purpose, the superfusates were directly delivered into the immobilized peroxidase column (Fig. 7).

After stimulation with 1 μM PAF, $[Ca^{2+}]i$ increased quickly and reached a peak (350 nM) within 1 min, and then decreased slowly, in a fashion similar to that shown in Figure 2. The rate of H_2O_2 production increased more slowly than the calcium signal and reached a peak within 3 min. Removal of PAF from a superfusion solution decreased H_2O_2 production to the basal level before $[Ca^{2+}]i$ reached the basal level. After stimulation with ionomycin, both $[Ca^{2+}]i$ and H_2O_2 production increased (Fig. 7).

DISCUSSION

The stimulation of cell surface receptors by various specific ligands elicits cellular responses mediated by a second messenger system (22). Macrophages have more than fifty types of receptors, and PAF is a representative stimulant. PAF increases $[Ca^{2+}]i$ in mouse peritoneal macrophages activated by thioglycollate as has been shown with quin2 as a probe (23). In the present study using fura-2, we demonstrated a similar increase in $[Ca^{2+}]i$ in peritoneal macrophages stimulated by PAF. However, the patterns of decrease of $[Ca^{2+}]i$ differed when quin2 and fura-2 were cited as fluorescent probes—with fura-2, $[Ca^{2+}]i$ showed a biphasic decrease, i.e., an initial rapid phase and a subsequent slow one (Fig. 2). On the other hand, with quin2, only a single phase of decrease occurred (12,23). This difference may be due to the lower concentration of fura-2 than of quin2 required in the cells for measuring $[Ca^{2+}]i$ and the higher K_d value of fura-2 than of quin2 for calcium binding (13).

As described above, in the absence of extracellular Ca^{2+}, only one symmetrical sharp peak was seen and a second slow phase of increase in $[Ca^{2+}]i$ was not observed (Fig. 4b). The addition of Ca^{2+} restored the second phase of $[Ca^{2+}]$ increase, suggesting that once PAF receptors are stimulated by PAF, Ca^{2+} movement across the plasma membrane occurs by some unknown mechanism. Thus, we assume that the increase in $[Ca^{2+}]i$ originates from two different sources—the first sharp peak of increase is due to movement of Ca^{2+} from intracellular stores and the second to influx of Ca^{2+} from extracellular medium across the plasma membrane. The idea of two modes of calcium movement in macrophages is consistent with previous observations on a murine macrophage cell line, J774.2 cells (16). In macrophages, the voltage-dependent calcium channel is not observed as it is in other leukocytes (24), and the mechanism of calcium influx remains unknown. One possibility is that extracellular Ca^{2+} enters via non-selective cation channels as has been observed in human neutrophils (25).

It should be also noted that the chemotactic peptide, fMLP, increase $[Ca^{2+}]i$ in peritoneal macrophages (Fig. 2), but not in J774.2 cells (16). We speculate that macrophages consist of multiple populations of cells at different stages of differentiation and that expressions of various receptors occur at different time periods after activation with paraffin, thioglycollate and other stimuli.

It is generally thought that neutrophil activation occurs by increase in $[Ca^{2+}]i$, which is followed by a respiratory burst, and by superoxide and H_2O_2 production (26). As one of the responses of activated macrophages, we measured the production of H_2O_2 using a new assay system in which immobilized peroxidase is used in the fluorometric assay described by Zaitsu and Ohkura (17). Results obtained by this assay using *p*-hydroxyphenylacetate were shown to correspond well to those obtained by the chemiluminescence method (27,28). HPPA was a more sensitive fluorogenic substrate of horseradish peroxidase than was *p*-hydroxyphenylacetate (17). The method is simple, highly reproducible, and can be applied to measure H_2O_2 in fractions. For a more time-resolved assay, the superfusion system was directly connected to an on-line system. PAF stimulation induced a prompt increase in the rate of H_2O_2 production reached a plateau within 5 min after PAF stimulation, and then decreased to the original level upon removal of PAF. Ionomycin also caused H_2O_2 production. This response, a result of the respiratory burst, required the presence of Ca^{2+} in the extracellular medium. This Ca^{2+} dependency of H_2O_2 production has also been observed in polymorphonuclear leukocytes (27,29). We suggest that PAF stimulation first causes the Ca^{2+} response, which is followed by H_2O_2 production in mouse peritoneal macrophages.

In the superfusion system, we were able to measure changes in $[Ca^{2+}]i$ and production of H_2O_2 simultaneously. This system will be useful for measuring H_2O_2 or lysosomal enzymes in superfusates to establish potential relationships between signal transduction and cell response.

ACKNOWLEDGMENT

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Platelet-Activating Factor Stimulates Receptor-Mediated Formation of Reactive Oxygen Intermediates in Human Monocytes[1]

M.G. Pustynnikov*, N.V. Porodenko, O.V. Makarova, A.V. Kozyukov, E.Yu. Moskaleva, A.A. Sokolovsky and E.S. Severin
Research Center of Molecular Diagnostics, USSR Ministry of Health, 113149 Moscow, USSR

Stimulation of production of reactive oxygen intermediates (ROI) was examined in human peripheral blood monocytes by luminol-dependent chemiluminescence. The dose-response curve characterizing the dependence of ROI production on the concentration of platelet-activating factor (PAF) showed that stimulation occurred within a concentration range of 2×10^{-9}M to 5×10^{-6}M. Transformation of the dose-response curve to an Eadie-Hofstee plot indicated that the process is characterized by two K_m values. The K_m value corresponding to the high-affinity branch of the curve is 1.3 ± 0.14 nM. In the same cells, the dissociation constant for the [^{3}H]PAF/receptor complex was determined. The K_d value was 0.8 ± 0.1 nM, which agreed quite well with the high-affinity K_m value obtained in the Eadie-Hofstee plot. The data indicate that stimulation of ROI generation is mediated through PAF binding at specific receptor sites at nanomolar PAF concentrations. Along with the specific receptor-mediated ROI generation, a nonspecific effect of PAF at a high concentration was demonstrated.
***Lipids* 26, 1214–1217 (1991).**

Platelet-activating factor (PAF) is a phospholipid mediator that activates many of the inflammatory cells including eosinophils, neutrophils, platelets, and macrophages (1–4). Among the products that are formed during the activation of these cells, reactive oxygen intermediates (ROI), namely superoxide anion (O_2^-) and hydrogen peroxide (H_2O_2), are particularly important. Active forms of oxygen are involved in the destruction of microbes (5). Convincing evidence also suggests that blood cells respond by the release of O_2^- and H_2O_2, which can be induced by several agonists (6–8). NADPH-oxidase, an enzyme located in plasma membranes, catalyzes singlet electron reduction of O_2 to O_2^-, as well as O_2^- release in macrophages (9).

The ability of PAF to stimulate the formation of ROI in human peripheral blood macrophages has only been demonstrated quite recently (4,10) and it is still not regarded as an established fact. However, such stimulation has not been observed in alveolar macrophages (11). In the present study we evaluated the effect of PAF on the induction of ROI by human monocytes in comparison with similar effects observed in experiments with tetradecanoylphorbol acetate (TPA), a well known stimulator of intracellular oxidative burst.

MATERIALS AND METHODS

Chemicals. Sodium chloride, potassium chloride, disodium phosphate, and potassium dihydrophosphate were obtained from Fluka (Buchs, Switzerland), ethylenediaminetetraacetic acid (EDTA) from Merck (Darmstadt, Germany), Ficoll-Paque from Pharmacia (Uppsala, Sweden), trypan blue, PAF, and TPA from Serva (Heidelberg, Germany), [^{3}H]PAF (a mixture of [^{3}H]C_{16}PAF and [^{3}H]C_{18}-PAF), from Amersham (Little Chalfont, England), and superoxide dismutase (SOD) from Boehringer (Mannheim, Germany).

Cell isolation. Monocytes were obtained from donor blood containing citrate anticoagulant (0.38% aqueous solution of sodium citrate). The blood was placed on a Ficoll-Paque gradient (density 1.077 g/mL), and the mixture was centrifuged for 40 min at $400 \times g$. The monocyte-containing cell fraction from the interface was collected and washed twice in phosphate buffer (137 mM NaCl, 2.7 mM KCl, 8.1 mM Na_2HPO_4 and 1.5 mM KH_2PO_4, pH 7.4) and centrifuged for 15 min at $400 \times g$. The above procedures were performed twice. The monocytes were further purified by attaching them onto the surface of plastic culture dishes. The attached macrophages were washed in phosphate buffer and removed from the surface by intensive shaking in 0.2% EDTA. The viability of the cells was determined using trypan blue (12). The suspended cells were counted by the nonspecific esterase test (13).

Determination of reactive oxygen intermediates. The active oxygen forms were measured by chemiluminescence of luminol (5-amino-2,3-dihydro-1,4-phthalasinedion) (14), which reacts with these radicals during the oxidative burst of phagocytizing cells. Emission was measured with an LKB 1250 luminometer at 37°C (sensitivity of 1mV/cm). Cells (1.4×10^6) were placed into a cuvette at a luminol concentration of $1\text{–}2 \times 10^{-5}$M.

Quantitative characteristics of PAF binding. The dissociation constant (K_d) of the [^{3}H]PAF complex with the receptor was estimated by the radioreceptor method using a Scatchard plot. The reaction mixture containing the cell suspension (1×10^6 cells) and labeled PAF at a concentration range of 0.2 nM to 50 nM were incubated in phosphate-buffered saline (PBS) for 30 min at 37°C. The bound and the free ligands were separated by centrifugation ($3000 \times g$ for 3 min) followed by rinsing. Nonspecific binding was obtained as binding in the presence of 1 μM unlabeled PAF. For competition experiments, the conditions of incubation were the same; the reaction mixture contained [^{3}H]PAF at a concentration of 0.5 nM and unlabeled PAF at a concentration range of 0.5 nM to 1 μM.

[1]Based on a paper presented at the Third International Conference on Platelet-Activating Factor and Structurally Related Alkyl Ether Lipids, Tokyo, Japan, May 1989.

*To whom correspondence should be addressed at Research Center of Molecular Diagnostics, USSR Ministry of Health, Symphero-polsky Blvd., 8, 113149 Moscow, USSR.

Abbreviations: lysoPAF, lyso platelet-activating factor, 1-*O*-hexadecyl-*sn*-glycero-3-phosphocholine; PAF, platelet-activating factor, 1-*O*-alkyl-2-acetyl-*sn*-glycero-3-phosphocholine; ROI, reactive oxygen intermediates; SOD, superoxide dismutase; TPA, tetradecanoylphorbol acetate.

RESULTS

PAF stimulated rapid formation of ROI in macrophages which were assessed by luminol-dependent chemiluminescence (Fig. 1). A maximum level of ROI formation was observed approximately after 1 min. Dose dependence was observed in the PAF concentration range from 2×10^{-9}M to 5×10^{-6}M (Fig. 2), with a maximum effect at 2×10^{-6}M PAF. Figure 3 depicts Eadie-Hofstee transformation of the ROI stimulation curve (its hyperbolic part). The dependence was strikingly non-linear, with K_m being equal to 1.3 nM and 94 nM for the "high" and "low" affinity branches, respectively.

The data on displacement of ^{3}H-labeled PAF, bound to human peripheral blood monocytes, are represented in a semilogarithmic plot in Figure 4. The data are consistent with PAF interaction at one binding site.

The dissociation constant (K_d) for the [^{3}H]PAF/receptor complex on monocytes (K_d) was 0.8 ± 0.1 nM. This K_d value agreed rather well with the high-affinity K_m value obtained in the Eadie-Hofstee plot (Fig. 3). The low-affinity branch may reflect less specific processes occurring in cells which bear no relation to specific PAF receptor binding at corresponding binding sites. Addition of lysoPAF at concentrations of up to 1 μM had no effect.

To determine the origin of the product formed by monocytes in response to PAF treatment, the sample was supplemented with 350 U of superoxide dismutase (SOD). As seen in Figure 1b, no ROI formation was observed that would be indicative of PAF stimulated release of superoxide anion. O_2^- induction can be activated by the soluble stimulus TPA. The corresponding curve is shown in Figure 5. As in the case of PAF, chemiluminescence ceased after the addition of SOD (Fig. 5b), indicating O_2^- release.

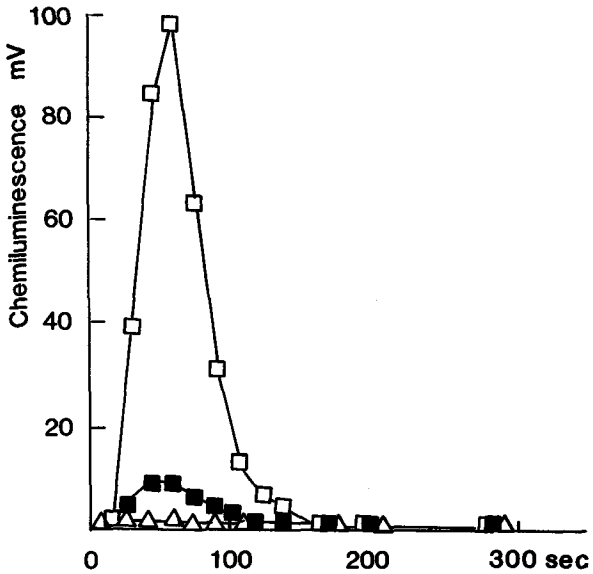


FIG. 1. Effect of PAF and SOD on ROI production in human monocytes. Experiments were performed as described in Materials and Methods; —□— in the presence of PAF (2×10^{-6}M); —■— in the presence of PAF (2×10^{-6}M) and SOD (350 units); —△— in the presence of 1 μM lysoPAF. The results are shown as the means of triplicate determinations from one of three similar experiments.

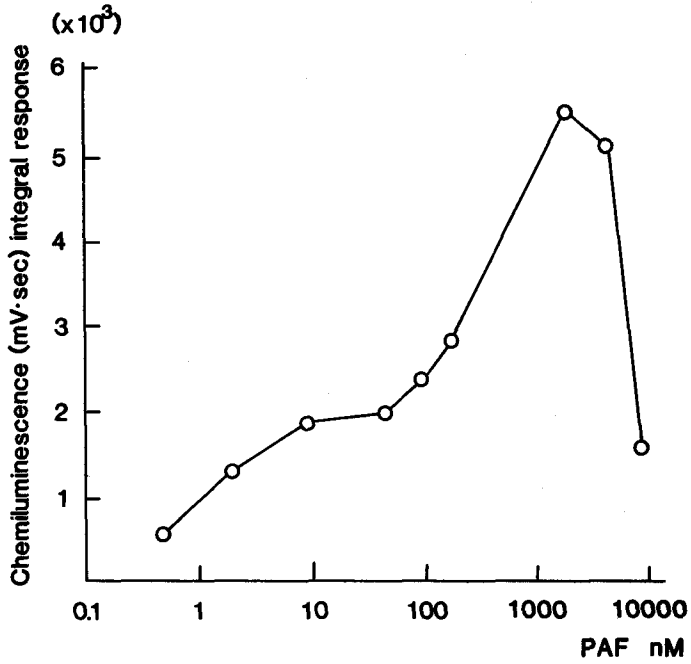


FIG. 2. Effect of increasing dose of PAF on ROI production. The results shown are the means of duplicate determinations from one of three similar experiments.

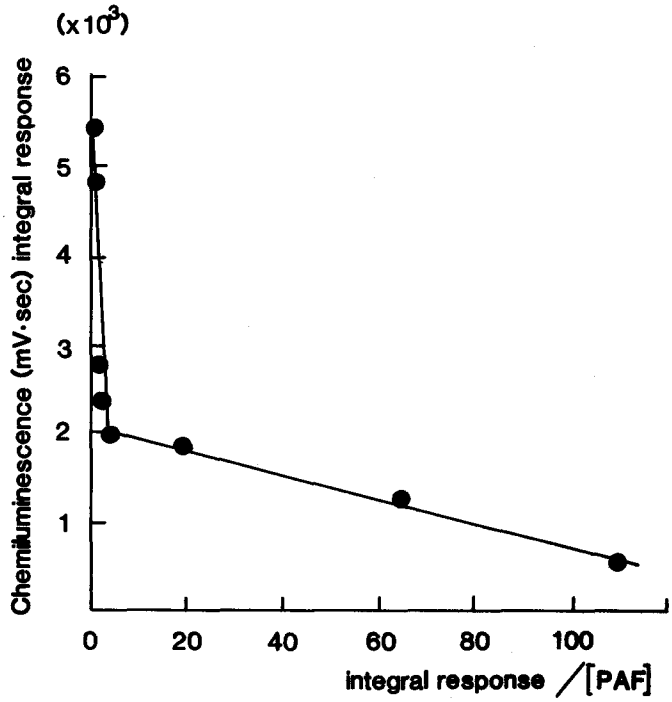


FIG. 3. Dependence of ROI production on PAF concentration in the Eadie-Hofstee plot. Data are the means of duplicate determinations from three separate experiments.

A comparison of the data in Figure 1 and Figure 5 shows that TPA appears to be a more efficient activator of respiratory burst in monocytes (Fig. 6); the kinetic parameters of O_2^- release under the action of PAF and TPA were significantly different.

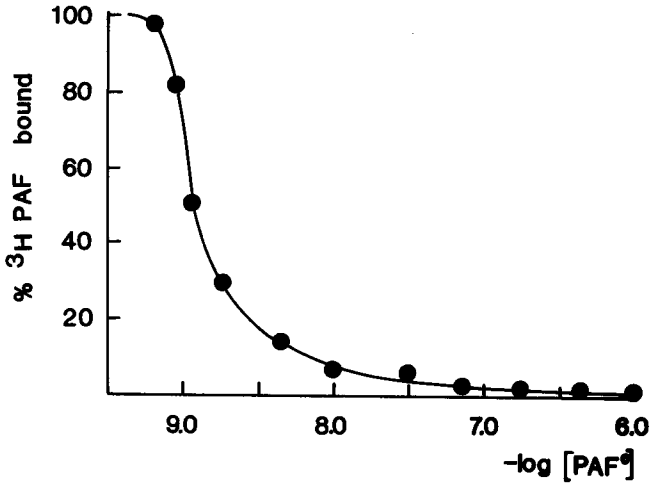


FIG. 4. Competitive displacement of [^{3}H]PAF by nonlabeled PAF. PAF°, nonlabeled PAF. Experiments were performed as described in Materials and Methods. Data are the means of triplicate determinations.

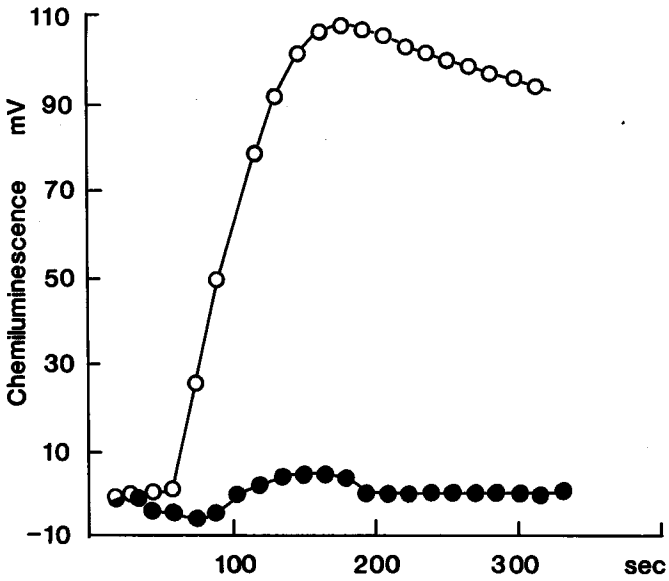


FIG. 5. Effect of TPA and SOD on ROI production in human monocytes; —○— in the presence of TPA (1×10^{-7}M); —●— in the presence of TPA (1×10^{-7}M) and SOD (350 units). The results shown are the means of triplicate determinations from one of three similar experiments.

DISCUSSION

ROI production, being one of the key functions of macrophages, is known to be induced by intracellular stimuli (15). Although these stimuli can be quite diverse, the metabolic pathways of their action within the cell are quite similar. A noticeable stimulation of ROI production, once the macrophages are exposed to TPA treatment, indicates the involvement of protein kinase C in this process (16,17).

Our results have shown that TPA stimulates ROI production more actively than does PAF. This is probably due to a lesser activation of protein kinase C by diacylglycerol than that observed with TPA.

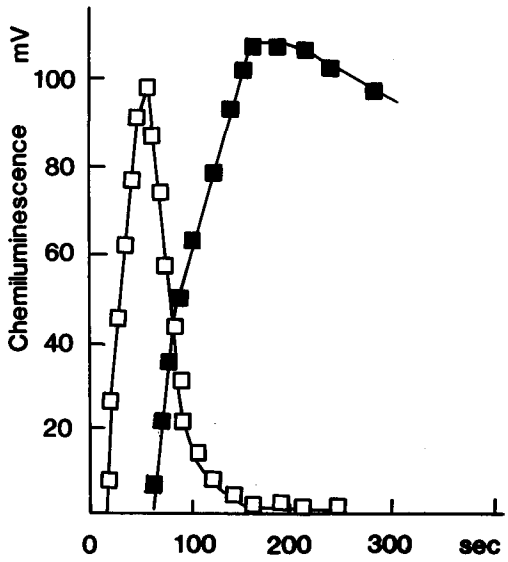


FIG. 6. Stimulation of ROI production in response to PAF and TPA; —□— in the presence of PAF (2×10^{-6}M); —■— in the presence of TPA (1×10^{-7}M). Experiments were performed as described in Materials and Methods.

The estimated PAF concentration generating a half-maximal response of monocytes (1.3×10^{-9}M) correlates rather well with the K_d value obtained for PAF interaction with the specific receptor on the monocytes. This concentration of PAF is substantially lower than indicated by the binding constant in (4) (5×10^{-6}M). We found that chemiluminescence decreases when the PAF concentration exceeds 3×10^{-6}M (Fig. 2).

Our data are consistent with the postulated role of PAF as a stimulator of ROI generation by monocytes which is mediated through PAF receptor binding at specific sites at nanomolar PAF concentrations.

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Calcium Channel Blockade Inhibits Platelet Activating Factor Production by Human Umbilical Vein Endothelial Cells[1]

J.P. Tolins*, A. Melemed, D. Sulciner, K.S. Gustafson and G.M. Vercellotti
University of Minnesota School of Medicine and Veterans Administration Medical Center, Minneapolis, Minnesota 55417

An increase in intracellular calcium level is an important signal in the regulation of cellular responses under normal and pathological conditions. Because two key enzymes in the synthetic pathway of platelet activating factor (PAF), phospholipase A_2 and acetyltransferase, are calcium dependent, we hypothesized that calcium channel blockade may inhibit agonist-induced PAF synthesis. Primary cultures of human umbilical vein endothelial cells (EC), pre-incubated with [^{3}H]acetate, were exposed to thrombin (5 U/mL) and PAF production was quantitated by incorporation of radiolabel into the EC lipid fraction co-migrating with exogenous PAF in thin-layer chromatography. The effect of pre-incubation with calcium channel blockers (verapamil, diltiazem, 10^{-4} M) or buffer was determined. Results (triplicate experiments, * $P<0.05$ vs buffer, † $P<0.05$ vs thrombin) demonstate that pre-incubation with calcium channel blocker markedly inhibits thrombin-induced PAF production (verapamil:buffer 273 ± 122, thrombin 10,735 ± 1524*, thrombin + verapamil 178 ± 91 † cpm/plate; diltiazem:buffer 1097 ± 581, thrombin 15,283 ± 2661*, thrombin + diltiazem 280 ± 56 † cpm/plate). The effect of diltiazem was dose-dependent (% inhibition: 10^{-7} M, 46%; 10^{-5} M, 60%; 10^{-4} M, 98%). Diltiazem also inhibited bradykinin (10^{-8} M) induced PAF synthesis. In calcium-free medium or in the presence of $LaCl_3$ (10^{-3} M), the PAF response of EC to thrombin was blunted (buffer 582 ± 360, thrombin 5394 ± 1069, thrombin + calcium free medium 1055 ± 571, thrombin + $LaCl_3$ 1271 ± 58 cpm/plate). We conclude that calcium channel blockers prevent agonist-induced PAF synthesis, possibly by preventing cellular calcium influx and activation of PAF synthetic enzymes. We speculate that this mechanism may underlie, at least in part, the beneficial effect of calcium channel blockade under various pathological conditions.
***Lipids 26,* 1218–1222 (1991).**

An increase in intracellular calcium concentration has been postulated to be pathogenetically important in diverse forms of cellular injury, such as ischemia, by virtue of calcium-dependent activation of membrane-associated phospholipases, subsequent degradation of the cell membrane, and eventual loss of the barrier properties essential to cell function (1,2). Less severe forms of cellular injury and cellular activation by various endogenous mediators are also associated with increases in intracellular calcium levels and activation of phospholipases (3). In this setting the phospholipids of the cell membrane serve as a storage depot for precursors of a variety of lipid-derived, biologically active mediators, such as arachidonic acid and lysophospholipids, which can be rapidly converted into sets of highly active compounds including eicosanoids, leukotrienes and platelet activating factor (4,5). Thus, an increase in intracellular calcium, either through influx of extracellular calcium or mobilization of intracellular storage pools, links cellular activation with the generation of lipid mediators of inflammation that may play a role in various pathological conditions.

Platelet activating factor (PAF†) has been identified as 1-*O*-alkyl-2-acetyl-*sn*-glycero-3-phosphocholine (6). It is synthesized by various activated cell types, including vascular endothelial cells (7), and has a broad spectrum of inflammatory and hemodynamic actions that may be important in disease states (6,8). Based on *in vitro* studies, it is clear that the synthesis of PAF is dependent on the presence of extracellular calcium (9), and that transmembrane calcium flux is the signal that initiates PAF synthesis (10). This most likely reflects the calcium-dependence of two key enzymes in the PAF synthetic pathway, phospholipase A_2 and acetyltransferase (9,11).

Calcium channel blockers, such as verapamil and diltiazem, are in widespread clinical use for the treatment of hypertension and atherosclerotic cardiovascular disease (12). While they have been demonstrated to block calcium entry via voltage-dependent calcium channels, particularly in vascular smooth muscle cells, the effect of these agents on receptor-operated calcium channels is not as well defined (13). Block and coworkers (14) recently reported that calcium channel blockade inhibited the mitogenic effect of platelet-derived growth factor (PDGF) on vascular smooth muscle cells, in association with a decrease in intracellular calcium accumulation. Furthermore, they suggested that this effect could be due to modulation of the PDGF receptor itself or the receptor-linked calcium channel. Shultz and Raij (15) also recently reported that three different calcium channel antagonists inhibited human mesangial cell proliferation in response to either PDGF or thrombin. They suggested that the calcium antagonists blocked receptor mediated calcium influx in this cell type. We therefore hypothesized that by interfering with receptor-mediated cellular calcium influx, calcium channel blockade might prevent agonist-induced synthesis of platelet activating factor.

Thrombin has a number of biological effects that are mediated by a high affinity cell-surface receptor, rather than its enzymatic action (16). Included in this category is the induction of PAF synthesis by vascular endothelial cells (7,17). This effect is associated with an increase in intracellular calcium concentration that

[1]Based on a paper presented at the Third International Conference on Platelet-Activating Factor and structurally Related Alkyl Ether Lipids, Tokyo, Japan, May 1989.

*To whom correspondence should be addressed at Veterans Administration Medical Center, Renal Section 111-J, 1 Veterans Drive, Minneapolis, MN 55417.

†Abbreviations: BSA, bovine serum albumin; HBSS, Hank's balanced salt solution; EC, human umbilical vein endothelial cells: PAF, platelet activating factor; PDGF, platelet derived growth factor, PGI2, prostacyclin.

is dependent on the presence of extracellular calcium (18). In the current study, we evaluated the effect of calcium channel blockade on thrombin-induced stimulation of PAF synthesis in human umbilical vein endothelial cells (EC) in culture.

MATERIALS AND METHODS

Materials. PAF, bovine serum albumin (BSA), lanthanum chloride ($LaCl_3$), bradykinin and human thrombin were purchased from Sigma Chemical Co. (St. Louis, MO). Hank's balanced salt solution (HBSS) was obtained from Gibco (Grand Island, NY). [^{3}H]Acetate was from New England Nuclear (Boston, MA). Verapamil was purchased from Searle Pharmaceuticals (Chicago, IL). Diltiazem was the generous gift of Marion Laboratories (Kansas City, MO). All materials were endotoxin-free as tested by the Limulus amoebocyte assay (19).

Endothelial cell cultures. EC were harvested from umbilical cords by collagenase digestion as previously described (20). EC plated in 60 × 15 mm tissue culture dishes (Becton-Dickinson Labware, Oxnard, CA) were grown to confluent monolayers (2.5×10^6 cells/plate) and used as primary cultures.

PAF assay. Plates were preincubated with either buffer (HBSS/0.5% BSA) or buffer containing appropriate concentration of calcium channel blocker for 20 min at 37°C. EC were then exposed to [^{3}H]acetate (55 μCi/dish) with or without calcium blocker, either in the presence or absence of thrombin (5.0 U/mL) for 5 min at 37°C. PAF production was then quantified by determining [^{3}H]acetate incorporation into EC PAF. Cells were scraped from the plate and a lipid extraction performed on the 2 mL cell suspension by the method of Bligh and Dyer (21). The suspension was transferred to the extraction solvents, chloroform (2.5 mL) and methanol (5.0 mL), and 25 μg of cold carrier PAF added. The single phase mixture was vortexed for 2 min and broken by the addition of 2.5 mL chloroform and water, with vortexing after each addition. The mixture was filtered (Whatman #2) and the chloroform layer removed. The remaining aqueous layer was washed with 5.0 mL chloroform and the combined chloroform layers dried under nitrogen. The separation and purification of PAF by thin layer-chromatography was accomplished using the method of Pinckard *et al.* (22). Plates precoated with silica gel H were dried for at least 3 hr at 105°C. Following application of the samples, the plates were returned to the oven for an additional 3–5 min and then developed in a solvent tank containing chloroform/methanol/water (65:35:6, v/v/v). Subsequently, plates were air dried and exposed to iodine vapors to locate the standard lipids. The specific position of PAF was identified using exogenous unlabeled PAF standard ($R_f = 0.21$). This area was scraped and radioactivity determined by liquid scintillation counting (Beckman LS230, Beckman Instruments, Inc., Palo Alto, CA). In control studies using [^{3}H]PAF, 87% recovery of label was obtained using this technique. The authenticity of PAF isolated by this technique was documented by sensitivity of the recovered material to PLA_2, and ability to aggregate rabbit platelets, as previously described (23).

Measurement of intracellular calcium concentration in EC monolayers: Effect of calcium channel blockade on thrombin-induced calcium flux. We measured intracellular calcium concentration in intact monolayers of EC, grown on coverslips, with the fluorescent calcium-sensitive probe, fura 2, by the technique previously described by our laboratory (18). EC were preincubated with either diluent or calcium channel blocker (diltizem or varapamil, 100 μg/mL final concentration) for 10 min, and then exposed to thrombin (0.5 U/mL). Intracellular calcium concentration was monitored for five additional minutes. Experimental conditions were performed in duplicate.

Effect of calcium channel blockade on thrombin-induced prostacyclin synthesis. EC were harvested as previously described (20) and plated in 2 cm^2 24-well Costar plates (Costar, Cambridge, MA). EC were grown to confluent monolayers and used as primary cultures. Experiments were performed in triplicate. All wells were rinsed with buffer (HBSS, 25 mM Hepes, 0.05% human serum albumin) and incubated with either verapamil (10^{-4} M) or buffer for 15 min. All wells were then incubated with thrombin (1.0 U/mL) for 10 min. Supernatants were removed and transferred to cold 12 × 75 mm polypropylene tubes containing 10 μg/mL indomethacin, vortexed and frozen at −70°C until assayed. Samples were assayed for the stable prostacyclin metabolite, 6-keto-prostaglandin-F1-α(PGF1-α), with a commercial radioimmunoassay kit (Dupont NEN, Boston, MA).

Statistical analysis. Data are presented as mean ± standard error. Differences between groups were analyzed by one-way analysis of variance and subsequent Scheffe's test. Differences were considered significant for $P<0.05$.

RESULTS

The effect of calcium channel blockade with either verapamil or diltiazem (10^{-4} M) on thrombin-induced PAF generation by EC is shown in Figure 1. Values represent triplicate experiments performed on different days. Thrombin exposure induced marked radiolabel incorporation into EC PAF compared to control cells exposed to buffer alone, reflecting marked stimulation of PAF synthesis. As can be seen, pre-incubation with either verapamil or diltiazem completely inhibited thrombin-induced PAF synthesis. In separate experiments, incubation of EC with this concentration of calcium antagonist (10^{-4} M) did not effect EC viability as assessed by trypan blue exclusion. Exposure of EC to the structurally distinct agonist, bradykinin (10^{-8} M) also induced PAF synthesis, although to a lesser extent than thrombin. This effect was also inhibited by pre-incubation with diltiazem (10^{-4} M), as shown in Figure 2. Inhibition of thrombin-induced PAF synthesis by diltiazem was dose-dependent (Fig. 3), with 50% inhibition occuring between 10^{-7} M and 10^{-6} M. As shown in Figure 4, exposure of EC to thrombin in the absence of calcium in the medium, or with standard buffer in the presence of $LaCl_3$ (10^{-3} M), resulted in significant blunting of the PAF synthetic response. Thus, thrombin-induced PAF synthesis by EC was dependent on the presence of extracellular calcium and

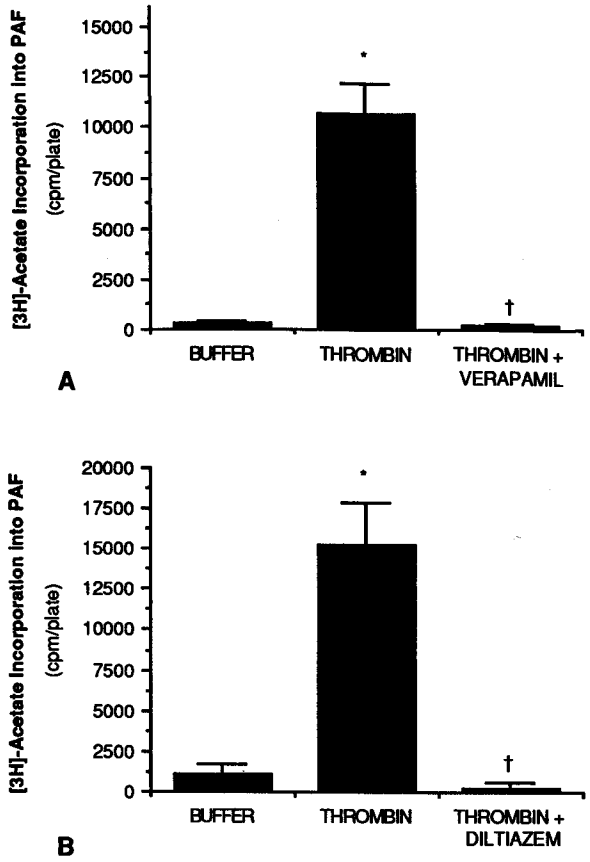


FIG. 1. Effect of calcium channel blockade with verapamil or diltiazem (10^{-4} M) on stimulation of PAF synthesis by thrombin (5.0 U/mL) in endothelial cells. *P<0.05 vs buffer; †P<0.05 vs thrombin.

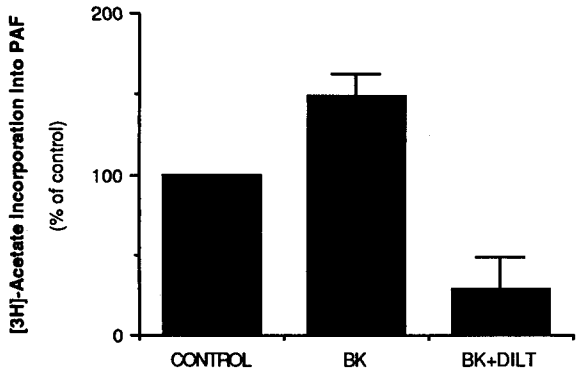


FIG. 2. Effect of calcium channel blockade with diltiazem (DILT, 10^{-4} M) on bradykinin (BK, 10^{-8} M) stimulated PAF synthesis in endothelial cells. Mean of duplicate experiments.

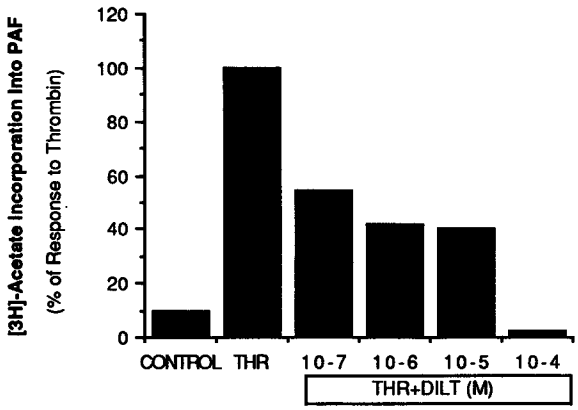


FIG. 3. Effect of calcium channel blockade with diltiazem (DILT) on stimulation of PAF synthesis by thrombin (THR, 5.0 U/mL) in endothelial cells. Diltiazem inhibits thrombin-induced PAF synthesis in a dose-dependent manner. Mean of duplicate experiments.

inhibitable by calcium channel blockade with either the non-specific calcium entry blocker, lanthanum chloride, or the pharmacologic calcium channel blockers, verapamil or diltiazem. The inhibitory effect of diltiazem occurred in a dose-dependent fashion, and this agent also inhibited the PAF synthetic response to a distinct agonist, bradykinin.

As shown in Figure 5, stimulation of EC with thrombin resulted in a rapid rise in intracellular calcium that peaked at 2 min and slowly returned toward baseline over the next 4–5 min, consistent with previous reports from this laboratory (18). Preincubation of the EC with either verapamil or diltiazem markedly blunted this response. Thus, calcium channel blockers, at a concentration that prevented thrombin-induced PAF synthesis, also blunted thrombin-induced increases in intracellular calcium concentration.

If calcium channel blockers prevent thrombin-induced activation of PLA_2 by blunting increases in intracellular calcium concentration, then it would be predicted that synthesis of eicosanoids, in addition to PAF, would also be inhibited by these agents. Consistent with this prediction, preincubation of EC with verapamil markedly inhibited release of prostacyclin into the supernatant after stimulation with thrombin (thrombin vs thrombin + verapamil, 2717 ± 247 vs 968 ± 32 pg PGF1-α/well, P<0.05).

DISCUSSION

The calcium channel blockers are in widespread clinical use in the treatment of hypertension and cardiovascular disease (12). Much attention has focused on the systemic and renal hemodynamic effects of these agents (13,24) and the relationship of these effects to therapeutic benefits. However, given the central role of calcium as a signal in the regulation of diverse aspects of cellular metabolism and, in particular, phospholipid metabolism (3,4), it is not surprising that the importance of the metabolic effects of these agents has become increasingly apparent. The calcium channel blockers have been demonstrated to have protective effects in models of ischemic (25), toxic (26,27) and chronic

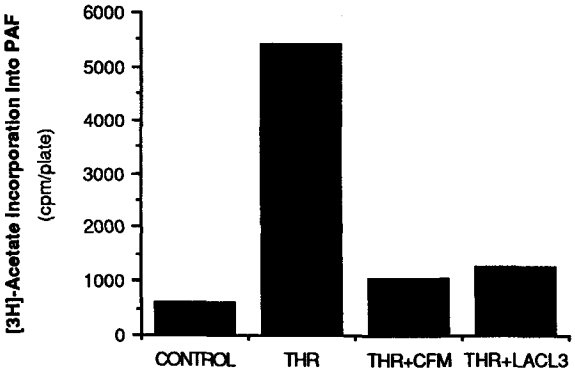


FIG. 4. Effect of calcium-free medium (CFM) or $LaCl_3$ (10^{-3} M) on stimulation of PAF synthesis by thrombin (THR, 5.0 U/mL) in endothelial cells. Mean duplicate experiments.

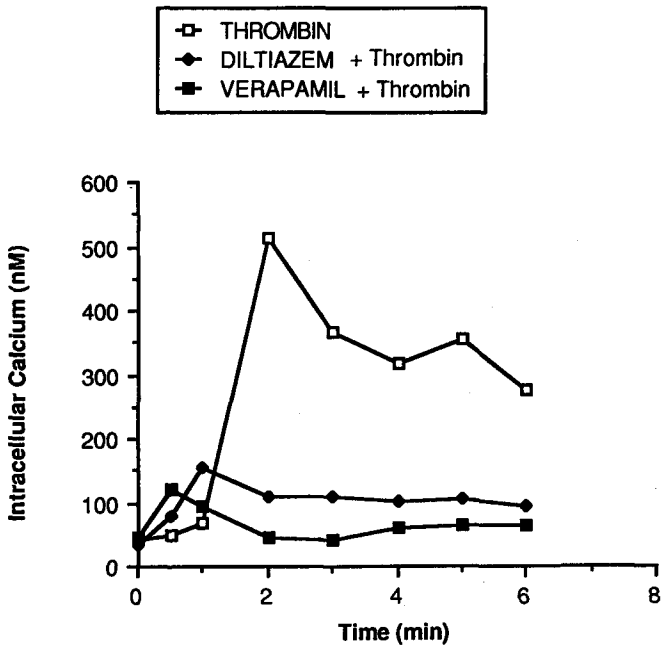


FIG. 5. Effect of calcium channel blockade with verapamil or diltiazem (10^{-4} M) on thrombin-induced increases in intracellular calcium concentration in human umbilical vein endothelial cells. Data points represent the mean of duplicate experiments.

hypertensive renal injury (28,29). Although the mechanism of these protective effects is unclear, prevention of abnormal cellular calcium accumulation, as well as decreased cellular metabolic demands, have been suggested to be important (28,30). An increase in intracellular calcium results in activation of phospholipase A_2 which catalyzes the hydrolysis of the fatty acid ester linkage at position 2 of the phospholipid molecule, generating arachidonic acid and lysoPAF (4). Eicosanoids can be synthesized from arachidonic acid and, furthermore, through action of another calcium dependent enzyme, acetyltransferase, lysoPAF can be converted to PAF (9), with resultant inflammatory and hemodynamic effects that may be important in disease processes (5). By preventing calcium influx in response to biologically relevant mediators, the calcium channel blockers may prevent generation of these inflammatory mediators and thus provide protection from tissue injury in various pathological conditions. Although it has been previously demonstrated that calcium channel blockade blunts agonist-induced prostaglandin synthesis (31,32), the effect of these agents on PAF synthesis has not previously been studied.

In the current study we have demonstrated that pre-incubation with either verapamil or diltiazem markedly inhibits thrombin-induced [^{3}H]acetate incorporation into the EC lipid fraction comigrating with PAF. The effect of diltiazem occurred in a dose-dependent fashion, and this agent also blocked the PAF synthetic response to bradykinin. Removal of calcium from the medium or non-specific blockade of calcium entry with lanthanum chloride also inhibited thrombin-induced PAF synthesis. Furthermore, we have demonstrated that verapamil inhibits EC prostacyclin release after thrombin stimulation. Calcium antagonists, at concentrations that inhibited synthesis of PAF and prostacyclin, also markedly blunted the rise in intracellular calcium concentration observed after stimulation of EC with thrombin. Thus, it is likely that the effect of calcium channel blockade on PAF synthesis is due to the prevention of agonist-induced calcium entry. However, the mechanism whereby the calcium channel blockers exert their inhibitory effect on PAF synthesis cannot be determined with certainty from this study. These agents have been thought to inhibit calcium entry via voltage-dependent calcium channels. Recent evidence suggests that they can interfere with calcium entry due to the binding of agonists to cell receptors, as well. Block *et al.* (14) demonstrated that calcium antagonists inhibited the mitogenic effect of PDGF in vascular smooth muscle cells, and postulated that the calcium channel blockers could modulate the agonist-receptor interaction or interfere with coupling between receptor activation and calcium entry. Similarly, Schultz and Raij (15) demonstrated that calcium channel blockers inhibited glomerular mesangial cell mitogenesis in response to thrombin or PDGF. Calcium channel blockers have also been demonstrated to have PAF-receptor blocking activity on platelets (33). Thus, it is clear that the calcium antagonists can interfere with cellular responses that depend on receptor-mediated calcium fluxes, although the exact mechanism of this effect is unknown.

In summary, calcium channel blockade inhibited thrombin-induced PAF synthesis by endothelial cells, *in vitro*. This suggests an additional, novel mechanism by which calcium antagonists may exert a protective effect in pathological conditions. The exact mechanism of this effect and relevance to clinical use of these agents awaits further study.

ACKNOWLEDGMENTS

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The Effect of Eicosapentaenoic Acid Consumption on Human Neutrophil Chemiluminescence[1]

Philip J. Thompson*[a], Neil L.A. Misso[a], Marion Passarelli[a] and Martin J. Phillips[b]
[a]Department of Medicine, University of Western Australia and [b]Department of Respiratory Medicine, Sir Charles Gairdner Hospital, Nedlands, W.A. 6009, Australia

The effect of eicosapentaenoic acid (EPA) on the inflammatory potential of neutrophils was investigated by supplementing the diets of 12 subjects with 2.16 g of EPA or 12 g of olive oil per day for 4 weeks in a double blind crossover study. Neutrophil function as assessed by luminol enhanced chemiluminescence responses to platelet-activating factor (PAF) and formyl-methionyl-leucyl-phenylalanine (FMLP) was significantly reduced after EPA but not after olive oil consumption in the subjects who consumed EPA first. In contrast, EPA had no significant effect on neutrophil chemiluminescence in the subjects who consumed olive oil first. Dietary supplementation with EPA inhibits neutrophil responses to inflammatory mediators such as PAF while other fatty acids appear to modify the effects of EPA.
Lipids 26, 1223–1226 (1991).

Populations such as the Greenland Eskimos and Japanese have a high dietary intake of fish oil compared to most Western diets and are noted to have a much lower prevalence of coronary artery disease (1) and chronic inflammatory diseases such as asthma, rheumatoid arthritis and psoriasis (2).

These epidemiological observations have led to the hypothesis that the eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA) in oily cold-water fish may offer protection against these diseases. EPA inhibits the uptake of arachidonic acid (AA) into cell membrane phospholipids and competes with AA for metabolism by the cyclooxygenase and lipoxygenase pathways. DHA is an inhibitor of these pathways (3). Metabolites of AA (thromboxane A_2, prostaglandins and leukotrienes) are thought to contribute to the inflammatory process in asthma by recruiting and activating neutrophils, eosinophils and macrophages (4). Platelet-activating factor (PAF), another likely mediator of inflammation in asthma, is derived from 1-*O*-alkyl-2-arachidonoyl-*sn*-glycero-3-phosphocholine, and its formation is closely linked with AA metabolism in neutrophils (5). Dietary supplementation with fish oil increases the proportion of EPA to AA in cell membrane phospholipids (6) and thus could result in a decreased production of PAF and inflammatory eicosanoids as well as the production of less potent analogues such as thromboxane A_3 and LTB_5 (leukotriene B) (3,7).

An important function of stimulated neutrophils is the production of reactive oxygen metabolites (8), a process which can be monitored by the technique of luminol-enhanced chemiluminescence (CL) (9). In this study the effect of dietary supplementation with EPA on the inflammatory potential of neutrophils was assessed by measuring neutrophil CL responses to *in vitro* stimulation with PAF and formyl-methionyl-leucyl-phenylalanine (FMLP) and comparing this with the effect of a placebo in the form of olive oil.

MATERIALS AND METHODS

Twelve healthy volunteers were entered into a double blind balanced crossover study comparing the effects of EPA and olive oil (placebo) on neutrophil chemiluminescence. They were asked to avoid all medication for two weeks prior to entry, and to maintain their normal diets prior to and during the study. The study was of sixteen weeks' duration and divided into 4 four-week intervals. Six subjects (Group 1, 3 males, 3 females aged 24–40 years with a mean age of 33 years) consumed MaxEPA capsules (Lipitac, Reckitt & Colman Products Pty. Ltd., West Ryde, NSW, Australia) in the first four weeks while the other six subjects (Group 2, 3 males, 3 females aged 23–38 years with a mean age of 29.5 years) consumed identical capsules containing olive oil. The dosage was 12 capsules daily, which provided 2.16 g of EPA per day or in the case of olive oil 12 g per day. After 4 weeks all subjects entered a four-week wash-out period when no capsules were taken. In the third four-week period, subjects were crossed over to the alternate capsules, and the study concluded with a four-week wash-out period. At the commencement of the study and after each four-week period (day 0, 28, 56, 85, and 112 of the study), blood was taken for neutrophil isolation.

Neutrophils were isolated by centrifugation of heparin anticoagulated whole blood on discontinuous Percoll (Pharmacia LKB, Uppsala, Sweden) gradients (10). Neutrophils were washed in phosphate buffered saline, counted in a haemocytometer and resuspended in Hanks balanced salt solution buffered with *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid (HBSS-HEPES) at 1.8×10^6 cells/mL.

A 5 mM stock solution of luminol (Boehringer Mannheim, Mannheim, W. Germany) in dimethylsulfoxide (DMSO) was diluted in HBSS to give a 0.5 mM working solution. PAF (1-*O*-hexadecyl-2-acetyl-*sn*-glycero-3-phosphocholine, Sigma Chemical Co., St. Louis, MO) was stored as a 1 mM solution in ethanol at −20°. On the day of use, 0.1 mL of the stock solution was evaporated under N_2 and resuspended in 1 mL of HBSS containing 0.25% human serum albumin to give a 10^{-4} M solution which was further diluted with HBSS to give working solutions of the required concentration. FMLP (Sigma) was stored as a 10^{-2} M solution in 50 μL aliquots of DMSO at

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*To whom correspondence should be addressed at the University Department of Medicine, Queen Elizabeth II Medical Centre, Nedlands, W.A. 6009, Australia.

Abbreviations: AA, arachidonic acid; CL, chemiluminescence; DHA, docosahexaenoic acid; DMSO, dimethylsulfoxide; EPA, eicosapentaenoic acid; FMLP, formyl-methionyl-leucyl-phenylalanine; HBSS-HEPES, Hanks balanced salt solution buffered with *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid; 5-HETE, 5-hydroxyeicosatetraenoic acid; LTB, leukotriene B; PAF, platelet-activating factor.

−70°. Dilutions to give working solutions of the required concentration were made with HBSS.

Chemiluminescence was measured in a LKB 1250 luminometer. The reaction mixture (total vol 0.5 mL) contained 7.5×10^5 neutrophils and 25 μM luminol. The reaction was started by the addition of 50 μL of PAF or FMLP to give the required final concentration. The CL response was monitored for 10–15 min on a digital display/printer unit and chart recorder.

CL responses were measured as the maximum amplitude in mV, and the data were analyzed by calculating mean values (± standard error of mean). Students t-test for paired data with a significance level of $p < 0.05$ was used when comparing data from the same group of subjects on different days.

RESULTS

The luminol-enhanced CL response of neutrophils to PAF and FMLP was biphasic. An initial rapid burst of chemiluminescence which peaked at 1–2 min after the addition of the stimulus was followed by a more gradual rise in CL which reached a maximum at 8–10 min. In this study the maximum amplitude of the initial peak which is associated with the production of extracellular reactive oxygen metabolites (11) was used as a measure of neutrophil CL responses to PAF and FMLP.

Neutrophil CL dose response curves to PAF and FMLP representing the mean responses of the 12 subjects at the commencement of the study are shown in Figure 1. The mean maximum response to PAF was observed at a concentration of 5×10^{-6} M. The mean FMLP response was maximum at 10^{-5} M and was higher than the mean PAF response (14.2 ± 7.3 vs 10.6 ± 2.4 mV). Similar PAF and FMLP concentrations have been used previously in the measurement of neutrophil CL responses and have not been shown to affect cellular integrity (12,13). The FMLP response was more variable at 10^{-5} M than at 10^{-6} M, and for this reason the latter concentration was used when comparing the mean responses on different days.

At the PAF concentration which produced a maximum response, neutrophils from Group 1 subjects who consumed EPA from day 0 to day 28 showed a significant decrease in the mean CL response on day 28 compared to day 0 (4.9 ± 2.9 vs 10.9 ± 4.6 mV, $p < 0.005$) (Fig. 2A). At the end of the first wash-out period (day 56), the CL response had increased to 8.3 ± 3.4 mV which was not significantly different to day 0. There were no significant changes in the CL responses measured after consumption of placebo in the form of olive oil (day 85) or at the end of the study (day 112) (Fig. 2A).

In the Group 2 subjects who consumed placebo capsules from day 0 to day 28, the mean CL response to a maximum PAF concentration was not significantly different on day 28 (6.4 ± 1.3 mV) compared to day 0 (10.3 ± 4.3 mV) (Fig. 2B). However the mean responses on day 85 (8.8 ± 2.0 mV) after EPA consumption and on day 112 (6.3 ± 2.0 mV) were also not significantly different to the response on day 0 or on day 56 (6.9 ± 1.6 mV) prior to EPA consumption.

In response to 10^{-5} and 10^{-6} M PAF, similar trends were observed with a reduction in CL at day 28 in the Group 1 subjects. With 10^{-7} M PAF, the CL responses were so low that meaningful variations with time could not be discerned. For the Group 2 subjects, mean CL responses to PAF concentrations of 10^{-5}, 10^{-6} and 10^{-7} M did not change significantly during the course of the study.

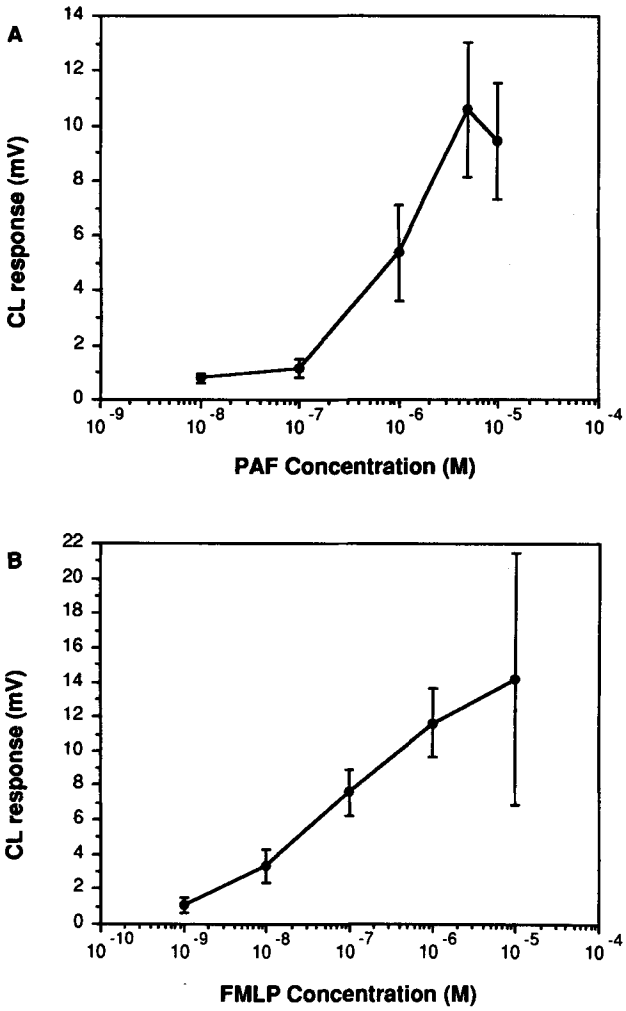


FIG. 1. Dose responses of neutrophil chemiluminescence to PAF (A) and FMLP (B). Each point represents the mean (±SEM) CL response for all 12 subjects on day 0. For each test, PAF or FMLP was added to 7.5×10^5 neutrophils with 25 μM luminol, and the maximum amplitude of the CL response was measured in mV.

Comparisons of the mean CL responses to 10^{-6} M FMLP were used to assess the effect of EPA consumption on neutrophil response to this stimulus (Fig. 3A). In Group 1 subjects, the CL response to 10^{-6} M FMLP was 13.8 ± 2.5 mV on day 0. After EPA consumption this decreased to 7.6 ± 3.3 mV on day 28 and decreased further to 5.7 ± 1.5 mV at the end of the first wash-out on day 56 which was significantly less than day 0 ($p < 0.05$). After subsequent placebo (olive oil) consumption, the mean CL response returned to 12.0 ± 3.6 mV on day 85 and to 13.4 ± 5.2 mV at the end of the study.

Neutrophils from Group 2 subjects who consumed olive oil first gave a mean CL response to 10^{-6} M FMLP of 9.4 ± 3.0 mV on day 0 (Fig. 3B). This did not change significantly after placebo consumption (11.8 ± 5.3 mV on day 28, 11.5 ± 3.9 mV on day 56) and although there was a decrease in the response after EPA consumption

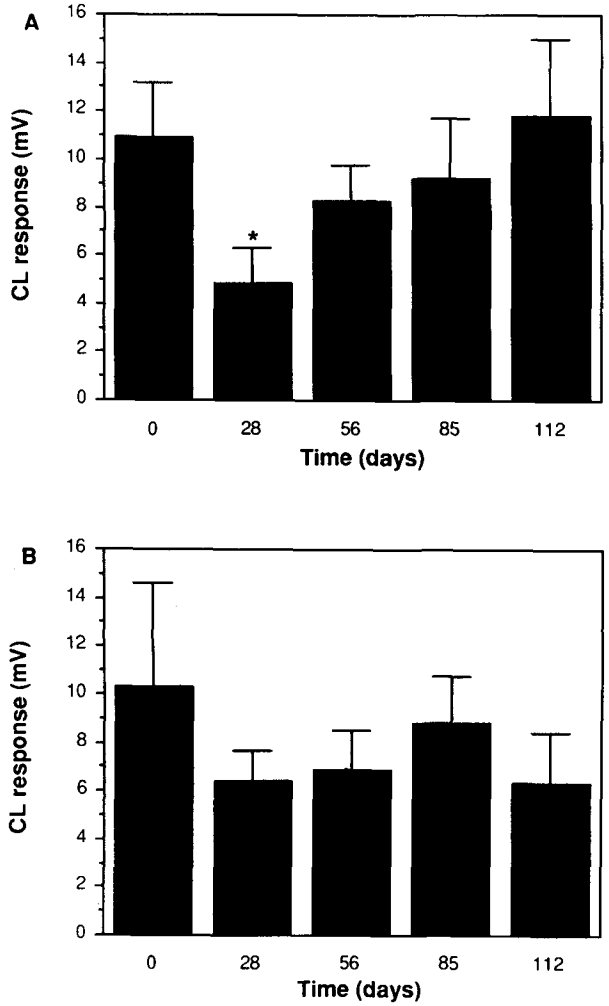


FIG. 2. Mean (±SEM) neutrophil chemiluminescence responses to PAF (5×10^{-6} M) in Group 1 subjects who consumed EPA from day 0 to day 28 (A) and in Group 2 subjects who consumed olive oil capsules from day 0 to day 28 (B). * = $p < 0.005$ compared to day 0 by paired t-test.

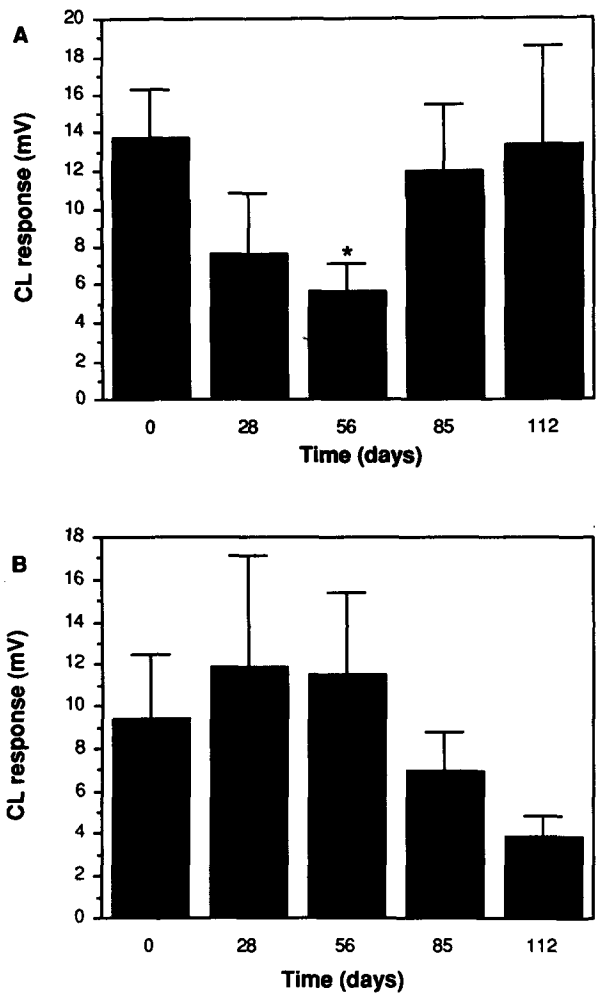


FIG. 3. Mean (±SEM) neutrophil chemiluminescence responses to FMLP (10^{-6} M) in Group 1 subjects who consumed EPA from day 0 to day 28 (A) and in Group 2 subjects who consumed olive oil capsules from day 0 to day 28 (B). * = $p < 0.05$ compared to day 0 by paired t-test.

(7.0 ± 1.8 mV on day 85, 3.9 ± 1.0 mV on day 112), this did not reach statistical significance.

In the Group 1 subjects, CL responses to 10^{-5} and 10^{-8} M FMLP showed similar trends to those described for 10^{-6} M FMLP with CL being reduced on day 56 compared to day 0. With 10^{-7} M FMLP, the trend for a reduction in CL could not be discerned. For the Group 2 subjects, the CL responses to 10^{-5}, 10^{-7} and 10^{-8} M FMLP did not change significantly with time.

DISCUSSION

In the group of six subjects who consumed EPA capsules from day 0 to day 28, there was a significant reduction in the neutrophil CL responses to PAF and FMLP immediately after the consumption of EPA. Consumption of placebo capsules containing olive oil from day 56 to day 85 had no significant effect on neutrophil CL responses to PAF or FMLP in this group. In the other group of six subjects, olive oil consumption from day 0 to day 28 did not produce significant changes in CL response. After EPA consumption from day 56 to 85, the CL response to FMLP, but not to PAF, declined although the change was not statistically significant. Thus the results from the Group 2 subjects have to be regarded as inconclusive although there is some suggestion that olive oil may inhibit EPA suppression of neutrophil chemiluminescence.

Three previous studies have examined the effects of dietary supplementation with EPA and DHA on human neutrophil chemotaxis and LTB_4 production (14-16). Higher doses (3.2-4.0 g) and longer durations (6-10 weeks) were used compared to this study. Neutrophil chemotaxis was reduced by up to 70% (14) and LTB_4 production was decreased, with an increase in LTB_5 production (15,16). In one of these studies, neutrophil function was not altered by consumption of placebo capsules containing olive oil (16). These studies have all assessed neutrophil function by measuring chemotactic responses to various stimuli (LTB_4, FMLP, C5a). The chemiluminescence response investigated in this study monitors the release of reactive oxidant species, an important function of stimulated neutrophils. The chemotactic and chemiluminescence responses may be modified differently by dietary supplementation with EPA. Furthermore the

effect of EPA on PAF-induced neutrophil responses had not been investigated prior to this study. EPA consumption may have differential effects on neutrophil responses to PAF and other stimuli such as FMLP, LTB_4 and C5a.

The normal product of lipoxygenase activity in stimulated neutrophils, LTB_4, may amplify the chemotactic and chemiluminescence responses to other stimuli such as PAF and FMLP. PAF has been shown to stimulate the production of LTB_4 in neutrophils (17,18), and in support of this data the lipoxygenase inhibitor, nordihydroguaiaretic acid, inhibits the neutrophil CL response to PAF (12). CL responses to FMLP have also been linked to lipoxygenase activity (12). Other workers have shown that consumption of 3.2 g of EPA per day for 3 weeks (total dose 67.2 g) resulted in a 500% increase in the content of EPA in neutrophil phospholipids (14). It seems likely that in our study in which the total dose was 60.5 g of EPA (2.16 g per day for 4 weeks) a similar incorporation of EPA into neutrophil phospholipids would have occurred and that this would have caused decreased LTB_4 production, and increased LTB_5 production. Consequently, reduced CL responses to PAF and FMLP as observed in this study would be expected to occur. Incorporation of EPA into the phospholipids of neutrophil membranes may alter the structure and/or fluidity of the membrane such that function of surface receptors, such as those for PAF and FMLP, may be altered. However, in one previous study, evidence for a change in neutrophil membrane fluidity after dietary supplementation with EPA was lacking (14).

In this crossover study, there was some indication that the effect of EPA persisted for up to four weeks after dietary supplementation ceased. Thus, in Group 1 subjects, the mean CL response to FMLP was less on day 56 than on day 28, and in Group 2 subjects the mean FMLP response was less on day 112 than on day 85. This effect was not observed in the mean responses to PAF. In the design of this study, the duration of the wash-out period was determined on the basis that neutrophils are turned over rapidly, with a blood clearance half-time of approximately 7 hr. The fact that the effects of EPA on FMLP-induced CL responses persisted at the end of a four-week wash-out period suggests that the EPA was turned over and reincorporated into maturing cells or possibly incorporated into stem cells during dietary supplementation.

Consumption of placebo capsules which contained olive oil did not alter neutrophil CL in this study, and is in agreement with the lack of effect on chemotaxis as reported previously (16). However prior consumption of olive oil appeared to reduce the effectiveness of EPA in inhibiting neutrophil CL. Oleic acid, the major fatty acid constituent of olive oil (approximately 75%), can be metabolized to eicosatrienoic acid which may be converted to LTB_3 by the lipoxygenase pathway (7). Recent studies (7,19) have suggested that LTB_3 may be as potent as LTB_4 as an inflammatory stimulus. Olive oil also contains linoleic acid (5%) which can be metabolized to AA. Thus consumption of placebo capsules by the Group 2 subjects may have increased the amounts of pro-inflammatory eicosatrienoic acid and AA in their neutrophil membranes thereby reducing the subsequent effectiveness of the fixed dose of EPA. Other fatty acids may also be capable of influencing the effects of EPA, and it has been suggested (20) that the low prevalence of coronary artery disease and inflammatory disorders in Eskimos is due to the combined effects of high dietary EPA and genetically determined high plasma concentrations of dihomo-gamma-linolenic acid.

Olive oil is probably not an ideal placebo because, as this study has shown, it may be metabolized by similar biochemical pathways to those which metabolize EPA. Indeed the question of whether olive oil affects neutrophil function is worthy of investigation. The only ideal placebo for the study of the effects of EPA would be a marine lipid concentrate from which all the EPA is removed, but this is expensive and difficult to produce.

Although the dose (2.16 g) and duration (4 weeks) of EPA supplementation were reduced in this study in comparison with others (3-4 g of EPA for 6-10 weeks) (14-16), the results are in agreement that dietary supplementation with EPA suppresses neutrophil function *in vitro*, and suggest lower doses may be effective. Although several studies have shown EPA has no effect on clinical symptoms when administered to asthmatic subjects (16,21,22), this may reflect clinical trial design. Alternatively, neutrophils may not play the major role in the inflammatory processes of asthma, and the concentration and duration of EPA supplementation needed to influence other inflammatory cell types and mediators involved in the pathogenesis of asthma (4) have yet to be elucidated.

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Role of Platelet-Activating Factor (PAF) in Superoxide Production by Human Polymorphonuclear Leukocytes[1]

Shuji Takahashi*, Toshikazu Yoshikawa, Yuji Naito, Toru Tanigawa, Norimasa Yoshida and Motoharu Kondo

First Department of Medicine, Kyoto Prefectural University of Medicine, Kyoto 602, Japan

The effect of platelet-activating factor (PAF) in superoxide production by human polymorphonuclear leukocytes (PMN) was studied. Cypridina luciferin analog (CLA) dependent chemiluminescence was used to detect superoxide anion radicals. PAF induced superoxide generation in human PMN in a dose-dependent manner. Preincubation with a small amount of PAF (5×10^{-9} M) enhanced PMN superoxide release induced by various stimuli, such as phorbol myristate acetate (PMA), opsonized zymosan (OZ), calcium ionophore (A23187) and *N*-formyl-methionyl-leucyl-phenylalanine (FMLP). The PAF antagonist, CV-6209, inhibited superoxide production induced by PAF, but not that induced by other stimuli. These findings would indicate that PAF may play an important role at inflammatory reaction sites and that CV-6209 may inhibit excessive inflammatory reaction.

Lipids 26, 1227-1230 (1991).

Platelet-activating factor (PAF, 1-*O*-alkyl-2-acetyl-*sn*-glycero-3-phosphocholine) was initially discovered by its ability to aggregate and degranulate platelets *in vitro* (1). PAF, an endogenous phospholipid, which is released from a number of activated cells, including polymorphonuclear leukocytes (PMN), macrophages, monocytes and endothelial cells (2-5), also shows a variety of other biological activities (6). For example, PAF induces hypotension and bronchoconstriction, and it increases vascular permeability. Recently, it has been suggested that PAF stimulates PMN as well as platelets, and causes aggregation, chemotactic motility, superoxide production, and enzyme release (7-9). It has also become apparent that oxygen-derived free radicals, such as superoxide and hydroxyl radicals, play an important role in the pathogenesis of many diseases (10,11).

In the present study, we investigated the role of PAF on superoxide generation from human PMN by means of chemiluminescence using the Cypridina luciferin analog, 2-methyl-6-phenyl-3,7-dihydroimidazo[1,2-a]pyrazin-3-one (CLA) which is highly specific for and sensitive to superoxide anion radicals (12-14).

MATERIALS AND METHODS

Reagents. The following reagents were purchased from Sigma Chemical Co. (St. Louis, MO): phorbol myristate acetate (PMA), zymosan A, *N*-formyl-methionyl-leucyl-phenylalanine (FMLP), calcium ionophore A23187, and diethylenetriaminepentaacetic acid (DETAPAC). Other reagents used were: Ficoll-Paque (Pharmacia AB., Uppsala, Sweden), PAF (Sandoz Pharmaceuticals Ltd., Basee, Switzerland), dimethylsulfoxide (DMSO, Wako Pure Chemical Industry, Osaka, Japan), and PAF antagonist 2-[*N*-acetyl-*N*-(2-methoxy-3-octadecyl-carbamoyloxy-proxycarbonyl)aminomethyl]-1-ethylpyridinum chloride (CV-6209, Takeda Chemical Industry, Osaka, Japan). PAF and CV-6209 were each dissolved in physiological saline at the required concentration and diluted in assay buffer. PMA, FMLP and A23187 dissolved in DMSO, respectively, at 20 μg/mL, 10^{-2} M and 10^{-3} M, were stored at −70°C until use, and diluted with distilled water to the required concentration, and then diluted in assay buffer.

Preparation of human PMN. PMN were isolated from the peripheral blood of normal volunteers by dextran sedimentation followed by Ficoll-Paque separation and hypotonic lysis of contaminating erythrocytes. PMN, 4×10^5 cells/mL, were suspended in Hanks' balanced salt solution (HBSS) at pH 7.4 and kept at 0°C until use. Cell viability was assessed by trypan blue exclusion and exceeded 96%. We examined the influence of preincubation of PMN with PAF or CV-6209 for 10 min on cell viability by this method.

Opsonized zymosan (OZ). Zymosan A in physiological saline (4 mg/mL) was boiled for 1 hr and centrifuged at $1600 \times g$ for 10 min. Sediments were washed twice with HBSS, suspended in fresh human serum (40 mg/mL), and incubated at 37°C for 30 min. The resultant opsonized zymosan pellet was washed twice with HBSS, resuspended in the same solution at a concentration of 30 mg/mL, and stored frozen at −70°C until use.

Measurement of superoxide. A Luminescence Reader (Aloka, Co., Ltd., Tokyo, Japan) was used for the assay of CLA dependent chemiluminescence. The standard reaction mixtures contained 4×10^5 PMN/mL, 1 μM CLA, 0.1 mM DETAPAC, plus stimulus in a total volume of 2 mL HBSS. PMN in HBSS were preincubated at 37°C for 3 min; the reaction was initiated by the addition of PAF as stimulus at a final concentration of 5×10^{-9} M to 5×10^{-4} M. The amount of superoxide produced was determined using the maximal increase of light intensity. In some experiments, after incubation of PMN for 3 min at a low concentration of PAF, which induced production of only a small amount of superoxide, a stimulus was added, and the measured maximal intensity was compared with that produced in the absence of PAF in order to assess the priming effect of PAF. PMA, OZ, FMLP, or A23187 were used as the PMN stimuli at a final concentration of 2 ng/mL, 1 mg/mL, 1×10^{-5} M, or 1×10^{-6} M, respectively. In other experiments, CV-6209 was added to test its effect on superoxide production by stimulated PMN.

Data are given as mean ± SE. Significances between groups were assessed by Student's t-test.

[1]Based on a paper presented at the Third International Conference on Platelet-Activating Factor and Structurally Related Alkyl Ether Lipids, Tokyo, Japan, May 1989.

*To whom correspondence should be addressed at First Department of Medicine, Kyoto Prefectural University of Medicine, Kawaramachi, Hirokoji, Kamigyo-ku, Kyoto 602, Japan.

Abbreviations: CLA, Cypridina luciferin analog; DETAPAC, diethylenetriaminepentaacetic acid; FMLP, *N*-formyl-methionyl-leucyl-phenylalanine; HBSS, Hanks' balanced salt solution; OZ, opsonized zymosan; PAF, platelet-activating factor; PMA, phorbol myristate acetate; PMN, polymorphonuclear leukocytes.

RESULTS

Human PMN stimulated by PAF produced superoxide anion radicals in a dose-dependent manner (Fig. 1). The greatest effect was seen at a PAF concentration of 5×10^{-5} M, whereas there was only a slight effect at a concentration of 5×10^{-9} M. This dose-dependency was observed in all cases examined. The superoxide production by PMN stimulated by PMA, OZ, A23187 or FMLP was enhanced in PMN preincubated with 5×10^{-9} M PAF (Figs. 2 and 3). This enhancement by PAF was much more than the increase that would be induced by 5×10^{-9} M PAF alone. This enhancement was also not seen when the PMN were exposed to PAF (5×10^{-9} M) simultaneously with the second stimulus (data not shown); *i.e.*, PAF needs to be preincubated with PMN prior to the second stimulation to prime the PMN. PAF priming was not dose-dependent (Table 1).

When PMN were first preincubated for 3 min with the PAF antagonist, CV-6209 and then stimulated by PAF, PMN superoxide production induced by PAF was inhibited dose-dependently (Fig. 4). However, CV-6209 did not inhibit superoxide production induced by PMA or A23187 (Table 2), nor by OZ or FMLP (data not shown). CV-6209 at a concentration of 5×10^{-6} M completely abolished superoxide generation by stimulated PMN (Table 2). Viability of PMN exceeded 95% when PMN were preincubated for 10 min with up to 5×10^{-5} M PAF or with up to 5×10^{-7} M CV-6209 (Fig. 5). However, 5×10^{-4} M PAF, or 5×10^{-6} M CV-6209, caused loss of cellular viability (Fig. 5).

DISCUSSION

During the inflammatory process, respiratory burst can be induced in PMN exposed to a variety of biological stimuli. The resultant oxygen-derived free radicals can act as microbicidal agents or, if produced in excess, as initiators or amplifiers of inflammation (15). Among these bioactive substances are soluble stimuli such as PMA, FMLP, or A23187, and particulate stimuli such as OZ. PAF is a mediator of allergic and inflammatory processes (16) and stimulates PMN degranulation, aggregation, oxidative metabolism, and chemotaxis. PAF is also produced by various cells and tissues at inflammatory reaction sites (17). Several studies have demonstrated now the induction of respiratory burst in human PMN by PAF (8,18) and PAF priming of superoxide production by PMN.

In the present study, we also examined the role of PAF in superoxide production by PMN, but used a newly designed chemiluminescence probe, CLA (12-14). We observed that PAF alone induced a dose-dependent burst of superoxide production by human PMN. Preincubation of PMN with small amounts of PAF furthermore enhanced superoxide production when PMN were subsequently exposed to soluble or particulate stimuli. These results, which are mostly consistent with those of previous reports (7-9), suggest that PAF may play an important role in the inflammatory process by enhancing PMN superoxide generation. However, there are some differences between our reults and those of others; *i.e.*, a lack of a dose-dependency of PAF priming, and a lack of an instantaneous priming effect. We speculate that PMN, when incubated with PAF at higher than 5×10^{-9} M concentra-

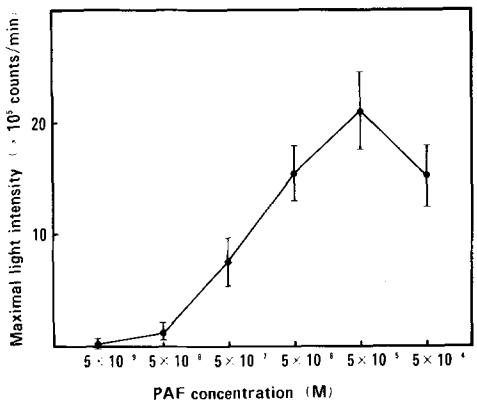


FIG. 1. CLA-dependent chemiluminescence of 4×10^5 PMN stimulated by PAF at various concentrations. Each point represents the mean ± SE of 6 experiments.

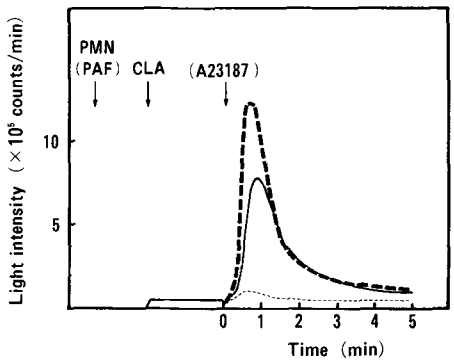


FIG. 2. Effect of PAF on superoxide production by PMN monitored continuously. PMN were preincubated with PAF (5×10^{-9} M) or physiological saline 3 min before the addition of A23187 (1×10^{-6} M). The tracings are reproduced from actual recordings and are representative of the results for four experiments. PAF addition 3 min before A23187, ---- ; A23187, ——— ; PAF, ------ .

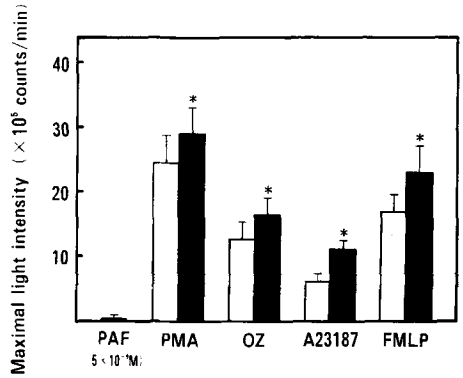


FIG. 3. Effect of PAF on superoxide production by PMN. PMN were incubated with PAF (5×10^{-9} M) or physiological saline (control group) for 3 min at 37°C and stimulated with PMA, OZ, A23187, or FMLP, and maximal light intensity representative of the amount of superoxide production was measured by CLA-dependent chemiluminescence. Each value indicates the mean ± SE of 4 experiments. Open bars represent the control group, and shaded bars represent preincubation with PAF. *$p<0.05$ when compared with the control group.

TABLE 1

Effect of PAF at Varying Concentrations on Superoxide Production by A 23187 Stimulated PMN[a]

PAF concentration (M)	Maximal light intensity (10^5 counts/min)
0	6.8 ± 2.1
5×10^{-9}	12.1 ± 3.8[b]
5×10^{-8}	14.3 ± 4.1[b]
5×10^{-7}	13.0 ± 3.0[b]
5×10^{-6}	8.6 ± 3.3
5×10^{-5}	2.5 ± 2.1[b]
5×10^{-4}	0.8 ± 0.7[b]

[a]PMN were incubated with PAF at varying concentrations for 3 min at 37°C and stimulated with A23187, and maximal light intensity was measured. Each value indicates the mean ± SE of 4 experiments. PAF, platelet-activating factor; PMN, polymorphonuclear leukocytes.

[b]$p<0.05$ when compared with the value in the absence of PAF.

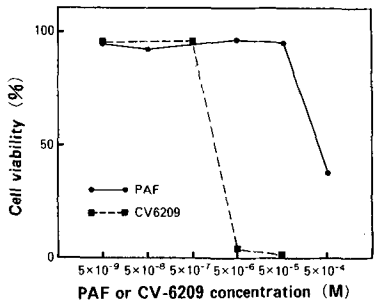


FIG. 5. Effect of PAF or CV-6209 on cell viability examined by trypan blue exclusion. PMN were incubated with PAF or CV-6209 at varying concentrations at 37°C for 10 min, and then trypan blue was added.

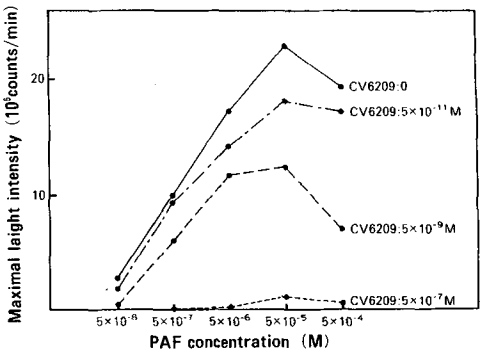


FIG. 4. Effect of CV-6209 on superoxide production by PMN. PMN were incubated with CV-6209 at varying concentration for 3 min at 37°C, stimulated with PAF at varying concentration, and superoxide production was measured. Results shown are representative of four separate experiments.

TABLE 2

Effect of CV-6209 on Superoxide Production by PMA or A23187 Stimulated PMN[a]

CV-6209 concentration (M)	Maximal light intensity (10^5 counts/min)	
	PMA stimulation	A23187 stimulation
0	23.5 ± 5.6	6.6 ± 2.4
5×10^{-11}	21.9 ± 4.9	6.4 ± 1.9
5×10^{-9}	20.8 ± 6.0	6.7 ± 2.3
5×10^{-7}	21.1 ± 5.1	6.8 ± 3.0
5×10^{-6}	0	0

[a]PMN were incubated with CV-6209 at varying concentrations for 3 min at 37°C and stimulated with PMA or A23187, and maximal light intensity was measured. Each value indicates the mean ± SE of 4 experiments. PMA, phorbol myristate acetate; PMN, polymorphonuclear leukocytes; CV-6209, a specific PAF antagonist.

tion, produce a fair amount of superoxide during preincubation. Thus superoxide production induced by a second stimulus may diminish, resulting in a lack of dose-dependency of the priming event. The differences and lack of instantaneous priming may also be explained in part by differences in assay conditions, including the superoxide assay used, preincubation time, or the stimulus concentration chosen. Further studies will be needed to pinpoint the detailed mechanism of priming.

It appears that PAF promotes the oxygen-dependent microbicidal activity of PMN (18) by enhancing superoxide production. However, at excessive levels, PAF may induce superoxide production by PMN in excess of that required, leading to exacerbation of inflammation. In the present study, at concentrations lower than that of PAF, CV-6209, a specific PAF receptor antagonist, dose-dependently inhibited PMN superoxide production induced by PAF. The inhibitory effects of CV-6209 were shown to be specific for PAF (19). So CV-6209 blocked PAF priming, but did not inhibit superoxide production induced in PMN by other stimuli. The PAF antagonist had little effect on the enzymes of the arachidonic acid cascade, on α-adrenergic receptors or calcium channels (19). Cell viability was lost at 5×10^{-6} M CV-6209, but CV-6209 exerted its anti-PAF effect at much lower concentrations that did not affect cell viability. It appears likely that CV-6209 would reduce tissue injury caused by excess PAF.

Our data suggest that PAF plays an important role at inflammatory reaction sites and that CV-6209 should be able to inhibit excessive inflammatory reaction.

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IgA Immune Aggregates Stimulate Platelet-Activating Factor and Superoxide Anion Production by Human Neutrophils. A Comparison with IgG Aggregates[1]

Paloma Hernando, Jesus Egido*, Rosario de Nicolas and Eva Gonzalez
Department of Nephrology, Fundación Jiménez Diaz, Universidad Autonoma-CSIC, Madrid, Spain

We have examined the possibility that human polymorphonuclear cells exposed to IgA immune complexes can mediate the production of platelet-activating factor (PAF) and oxygen radicals. We found that human IgA and IgG immune aggregates stimulated, to a similar extent, PAF and O_2^- production by human polymorphonuclear cells (PMN) in a concentration and time dependent manner. The PAF, that was largely associated with cells, was shown to be identical to synthetic PAF, as determined by physicochemical, chromatographic and enzymatic assay. Furthermore, *de novo* synthesis of PAF by PMN was shown to occur by incorporation of radioactive precursors, such as [^{3}H]acetate. The addition of normal human serum to PMN incubated with IgG aggregates resulted in a significant amount of PAF formation which was not observed with IgA aggregates. By contrast, no change was seen in PMN O_2^- with either aggregates. The preincubation of PMN with cytochalasin B, an inhibitor of phagocytosis, did not affect PAF and O_2^- production by both aggregates. The results suggest that the interaction of PMN with the IgA complexes in blood vessel walls of different tissues can result in the release of lipid mediators, such as PAF and oxygen radicals that could contribute to the inflammatory response.
Lipids 26, 1231–1235 (1991).

Immune complex (IC) deposition in vessel walls generates products of complement activation that increase vascular permeability and recruit polymorphonuclear cells (PMN) from the blood. In addition, it has been suggested that IC can stimulate PMN to release lysosomal proteases and to generate oxygen metabolites (reviewed in ref. 1). It is already well established that IgG-IC deposition in different organs, such as in kidney and lung, can cause tissue injury. Much less information exists in regard to the mechanisms of IgA-mediated tissue injury (reviewed in ref. 2).

IgA seems to play a key role in various human diseases, such as IgA nephropathy, Henoch-Schölein purpura, systemic *lupus erythematosus* and *dermatitis herpetiformis* (reviewed in ref. 3). A model of acute lung injury in rat caused by IgA-IC has been described (4). Recent studies have also shown that IgA-IC, or IgA-opsonized *Stafilococcus aureus,* can stimulate oxygen radical production by various cells (5). This is not surprising since the presence of Fcα membrane receptors (FcαR) in most blood cells has been reported (6).

Platelet-activating factor (PAF: 1-*O*-alkyl-2-acetyl-*sn*-glycero-3-phosphocholine) is a potent biologically active phospholipid that plays a major role in inflammatory reactions. This lipid is synthesized and released by several inflammatory cell types including platelets, neutrophils, macrophages, monocytes, mast cells, eosinophils, and endothelial and mesangial cells (7,8). Although a variety of stimuli, including IgG-IC, opsonized zymosan, calcium ionophore A23187, and C5a, are capable of inducing PAF production, there is no evidence that IgA-IC may also play such a role.

The aim of the present study was to explore the possibility that human PMN exposed to IgA immune aggregates (akin to immune complexes) can mediate the production of PAF and of oxygen radicals. For comparison, we have also tested IgG immune aggregates of the same molecular weight.

MATERIAL AND METHODS

Isolation of proteins. Human IgA and IgG were isolated from the sera of patients with myeloma, as previously described (9). Both the IgA and IgG preparations were found to be free of other immunoglobulins as judged by immunoelectrophoresis and double-immunodiffusion analysis. F(ab')$_2$ fragments of IgG were prepared by pepsin digestion and gel filtration as described by Stanworth and Turner (10). The F(ab')$_2$ preparation was free of intact heavy chain when analyzed by sodium dodecylsulfate polyacrylamide gel electrophoresis (SDS-PAGE) following the method of Laemmli (11).

Preparation of soluble aggregates. Aggregation was brought about by heating 10 mg/mL of IgA or IgG at 63°C in 0.15M Tris-buffered saline (TBS) for 150 or 30 min, respectively. Soluble aggregates of IgA or IgG of 1–2 × 10^6 mol wt were separated from non-aggregates by column chromatography on Ultrogel AcA 22 (LKB Instruments Ltd., Bromma, Sweden) with TBS, pH 7.4, containing 0.5% bovine serum albumin (BSA) to prevent aggregation (12).

Isolation of neutrophils. Human peripheral blood polymorphonuclear leukocytes (PMN) were isolated from the blood of healthy donors using a Ficoll/Hypaque gradient, gelatin sedimentation, and hypotonic lysis of red cells following standard techniques. The cell suspension contained 95 ± 1% PMN without contamination from platelets or erythrocytes.

Measurement of neutrophil PAF production. In most experiments, PAF was determined by bioassay by measuring the release of [^{3}H]serotonin from rabbit platelets as described by Henson (13) with some modifications (14).

[1] Based on a paper presented at the Third International Conference on Platelet-Activating Factor and Structurally Related Alkyl Ether Lipids, Tokyo, Japan, May 1989.

*To whom correspondence should be addressed at Laboratorio de Nefrologia, Fundación Jiménez Diaz, Avda Reyes Católicos 2, 28040 Madrid, Spain.

Abbreviations: BSA, bovine serum albumin; IC, immune complexes; IgA-A, IgA immune aggregates; IgG-A, IgG immune aggregates; O_2^-, superoxide anion; PAF, platelet-activating factor; PMA, phorbol myristate acetate; PMN, polymorphonuclear cells; SDS-PAGE, sodium dodecylsulfate polyacrylamide gel electrophoresis; TBS, Tris-buffered saline; TLC, thin-layer chromatography.

Briefly, 5×10^6 PMN were incubated with various concentrations of immune aggregates, or 10 μM calcium ionophore A23187 (Sigma Chemical Co., St. Louis, MO), in a final volume of 1 mL of HEPES containing BSA 0.25%. After stimulation, the samples were centrifuged at 2000 rpm at 4°C for 10 min. The supernatants were decanted into 1 mL of methanol. The cell pellets were resuspended in 1 mL of methanol, and vortexed. Each sample was extracted twice by the method of Bligh and Dyer (15) and the chloroform phase was dried under a nitrogen stream.

Samples were purified by thin-layer chromatography (TLC) on silica gel plates (Merck, Darmstadt, Germany) using chloroform/methanol/water (1:1:0.9, v/v/v) as solvent. Fractions were scrapped off the plates as narrow zones corresponding to the migration rate of authentic PAF (Sigma), the samples were extracted by two consecutive elutions with methanol, and the extracts were pooled and dried under a stream of nitrogen. Each sample was then dissolved in 0.1 mL of HEPES containing 0.25% of BSA.

PAF was quantified by measuring the release of [^{3}H]-serotonin from labeled rabbit platelets (13). The percentage of release effected by each sample was then compared with a standard curve of the release elicited by known amounts of synthetic PAF (hexadecyl PAF, Sigma). The amount of PAF in each sample was expressed as nanogram equivalents of the synthetic PAF.

In some experiments the production of PAF by stimulated PMN was measured by [^{3}H]acetate (sodium salt) incorporation into PAF (16). In these experiments, the same stimulation protocol was followed, except that the PMN were preincubated with 25 μCi of [^{3}H]acetate (3.6 Ci/mmol, Dupont, Boston, MA) for 10 min before agonist stimulation. The reaction was stopped by placing the cell suspension into glass tubes containing chloroform and 50 mM acetic acid in methanol. Lipids were extracted from stimulated PMN as previously described (15) and the radioactivity associated with the PAF band in TLC was quantitated by liquid scintillation counting (see above). In two experiments, a portion of the purified sample of neutrophil-derived PAF was treated with phospholipase A_2 (from *Crotalus adamanteus,* Sigma) before determining biological activity (17).

Measurement of O_2^- generation. PMN (5×10^6 cells) were incubated in duplicate samples with phorbol myristate acetate (PMA, Sigma) or immune aggregates at various concentrations in the presence of 75 μM ferricytochrome C, type III (Sigma) with or without 10 μg/mL of superoxide dismutase (2700 U/mg) (Sigma) in 1 mL of KRP solution, pH 7.4, for various periods of time up to 30 min (18). Incubations were terminated by placing the tubes in ice, after which they were centrifuged at 4°C for 10 min at 2000 rpm. Cell-free supernatants were decanted and the amount of reduced cytochrome C generated during the incubation was measured at 550 nm in a Spectronic 601 spectrophotometer (Bausch-Lomb, München, Germany). The total amount of cytochrome C present was calculated using an absorbance coefficient of 21.1 $mM^{-1} \times cm^{-1}$ at 550 nm. O_2^- generation is expressed as nanomoles cytochrome C reduced per 5×10^6 PMN.

In some experiments PMN were preincubated for 10 min with 5 μg/mL of cytochalasin B (Sigma) before the addition of immune aggregates.

Statistical analysis. Values are expressed as mean ± standard error of the mean. The Wilcoxon test was employed in all comparisons. Differences were considered significant for $p<0.05$.

RESULTS

Effect of IgA immune aggregates on the synthesis of PAF by neutrophils. Both IgA and IgG aggregates stimulated PAF production by PMN in a dose and time dependent manner (Fig. 1). Although maximal production of PAF was obtained when PMN were incubated with IgG aggregates, the differences were not significant as compared to the levels obtained with IgA aggregates (Fig. 1, top). Maximal accumulation of PAF, with both aggregates ocurred at 60 min (Fig. 1, bottom). The total amount of PAF produced by cells stimulated with aggregates was significantly greater than with nonstimulated cells, and much less than that observed in response to calcium ionophore A23187 (not shown).

The PAF produced in this manner was largely associated with cells and not secreted into the medium. Cell-associated material constituted 70% of the total PAF seen after stimulation.

The bioactive material was confirmed as PAF by use of the structurally unrelated PAF receptor antagonists BN 52021 (Institut Henri Beaufour, Les Plessis Robinson, France) and WEB 2086 (Boehringer Ingelheim, Germany). The release of [^{3}H]serotonin from platelets, induced by synthetic PAF or the "PAF-like material" obtained from stimulated cells, was inhibited when the platelets were preincubated with BN 52021 (10^{-5} M) or WEB 2086 (10^{-6} M). The activity of the "PAF-like material" was also lost after phospholipase A_2 treatment (not shown). In some experiments, the PMN were preincubated with [^{3}H]acetate before the addition of immune aggregates. PMN incorporated [^{3}H]acetate into a polar lipid that co-migrated with authentic PAF in TLC. In these experiments, the maximal incorporation of [^{3}H]acetate was obtained when PMN were incubated with IgG aggregates. For the same dose of protein (150 μg/mL) the percentage increase in relation to basal levels was 128 ± 14 for IgG *vs.* 87 ± 32 for IgA (not significant) (Fig. 2).

In other experiments, we examined PAF production by PMN incubated with IgA or IgG immune aggregates (100 μg/mL) in the presence of normal human serum (100 μL). The addition of human serum to PMN incubated with IgG immune aggregates resulted in a significant amount of PAF production (an increase of 227% in relation to the aggregates alone). This incremental increase was not observed when PMN were incubated with IgA aggregates in the presence of serum (an increase of 22% in relation to the aggregates alone) (not shown).

The preincubation of PMN with cytochalasin B (5 μg/mL), before the addition of immune aggregates, did not affect the total production of PAF from PMN stimulated with both immune aggregates (Table 1).

In other experiments, PMN (5×10^6) were incubated with $F(ab')_2$ of IgG and IgG aggregates for 60 min. At the same dose of protein (100 μg), the $F(ab')_2$ fragments of IgG released a significantly lesser amount of PAF than did the IgG aggregates (67.6 ± 11 pg/5×10^6 cells for $F(ab')_2$ *vs.* 254 ± 49 pg/5×10^6 cells for IgG aggregates) ($p<0.001$). A representative case is shown in Figure 3.

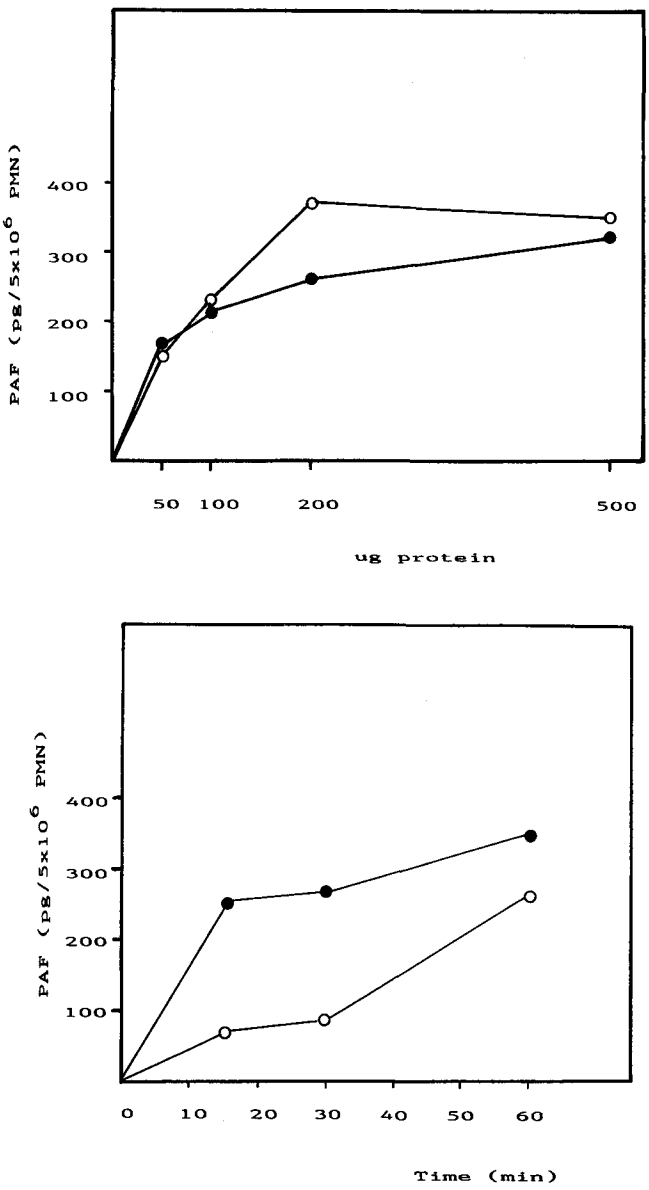


FIG. 1. Production of PAF by human PMN stimulated with IgG (○) or IgA (●) aggregates. Top: Production of PAF by PMN incubated with varying concentrations of IgG or IgA aggregates for 60 min. Total PAF was determined by bioassay, as described in Materials and Methods. Bottom: Time course of PAF production obtained upon IgA or IgG aggregate stimulation. PMN were incubated with 100 μg/mL of IgA or IgG aggregates. Results represent the mean of 6–10 experiments. No differences were found between either aggregate.

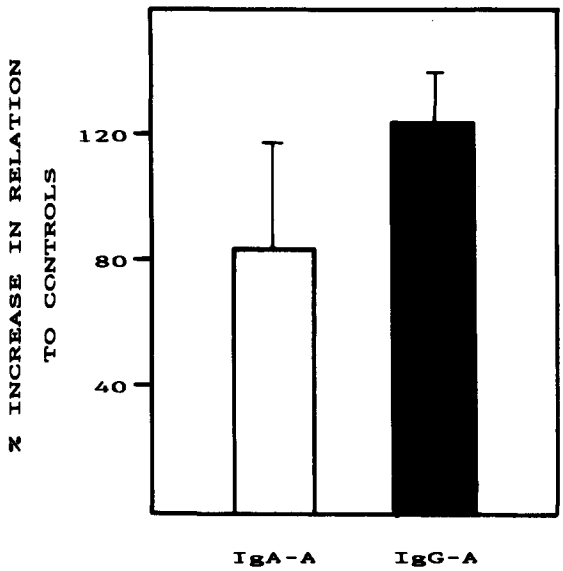


FIG. 2. Production of PAF by human PMN stimulated with IgG or IgA aggregates. PMN were preincubated with 25 μCi of [^{3}H]acetate for 10 min before addition of aggregates. The reaction was terminated as described in Materials and Methods. The data are expressed in percentage increase in relation to that obtained in PMN without aggregates. Mean ± S.D. (n=4), not significant.

TABLE 1

Effect of Cytochalasin B on PAF and O_2^- Production by Neutrophils Stimulated with IgG and IgG Aggregates[a]

Stimulus	PAF (pg/5 × 10^6 cells)	O_2^- (nmol/5 × 10^6 cells)
None	89 ± 65	0.9 ± 0.1
A-IgG[b]	254 ± 49	4.7 ± 0.4
A-IgG + cyto B	200 ± 40	4.3 ± 0.7
A-IgA[c]	216 ± 72	5.2 ± 0.9
A-IgA + cyto B	195 ± 77	5.1 ± 0.7
PMA	ND[d]	24.5 ± 7.0

[a]The doses of both IgG and IgA aggregates were 100 μg/mL and 300 μg/mL for PAF and O_2^- production, respectively. PAF was determined at 60 min of incubation and O_2^- at 30 min (each study was done in separate experiments). Results represent the mean of 6–14 experiments in duplicate.

[b]$p < 0.0025$. [c]$p < 0.0125$. [d]Not detected.

To determine the effect of cell adhesion on PAF production, further experiments were carried out. The PAF production by IgG aggregates was similar in both adherent and suspended cells, except that PAF production was delayed and decreased in adherent PMN. These results are in agreement with those published by Sisson *et al.* (16).

PMN O_2^- generation. In order to determine the ability of IgA and IgG immune aggregates to stimulate O_2^- generation in PMN, the cells were incubated with various concentrations (100–500 μg/mL) of both immune aggregates for different time periods (5,15 and 30 min), and the O_2^- release into the supernatant was measured.

PMN stimulated with immune aggregates produced a significantly higher amount of O_2^- compared to the non-stimulated cells (Table 1). PMA stimulation of PMN produced significantly greater O_2^- generation than did immune aggregates (Table 1). Both IgA and IgG immune aggregates enhanced O_2^- release in a dose and time dependent manner (Fig. 4). Although maximal O_2^- generation was observed when PMN were incubated with IgA aggregates, the differences were not significant (Fig. 4). Stimulation of PMN (5 × 10^6) with F(ab')$_2$ of IgG for 30 min resulted in a significant decrease in O_2^- generation compared with O_2^- production in cells stimulated with identical doses of aggregates [for the same dose of protein, 100 μg/mL, 1.73 ± 0.54 nmol O_2^-/5 × 10^6 cells for F(ab')$_2$ *vs.* 3.0 ± 0.79 nmol O_2^-/5 × 10^6 cells for IgG

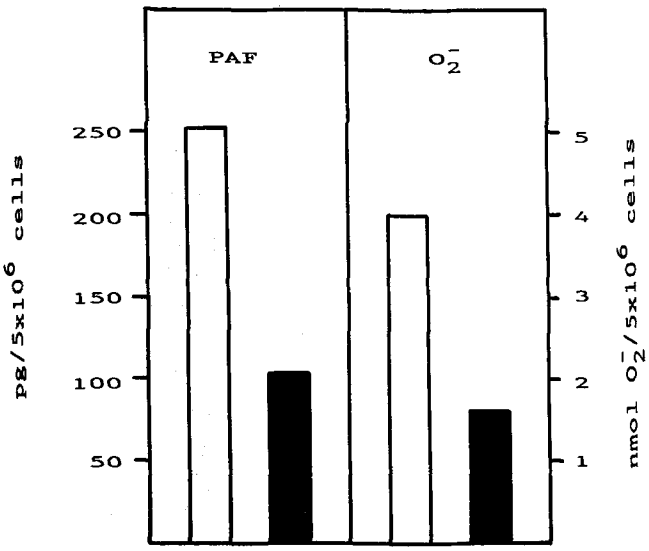


FIG. 3. Production of PAF and O_2^- by PMN stimulated with 100 μg/mL F(ab')$_2$ fragments of IgG (closed columns), obtained as described in Materials and Methods, or (100 μg/mL) IgG aggregates (open columns). A representative case of four different experiments is shown.

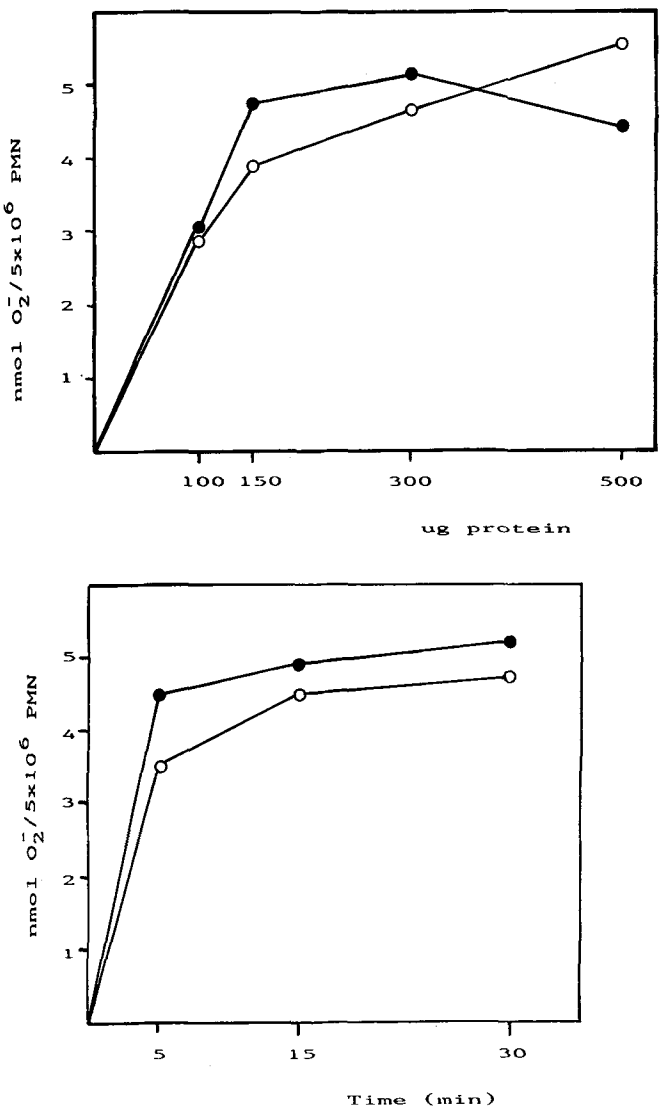


FIG. 4. Effect of IgA (●) and IgG (○) aggregates on O_2^- production by human PMN. Top: Release of O_2^- by PMN incubated with various concentrations of IgA or IgG aggregates for 30 min at 37°C. Bottom: Time course of O_2^- release. PMN were incubated with 300 μg/mL of IgA or IgG aggregates. Each point represents the mean of 10–14 experiments. No differences were found between the two aggregates.

aggregates, $p<0.05$]. A representative case is shown in Figure 3.

The cytochrome C reduction induced by IgG and IgG aggregates was almost completely inhibited by 10 μg/mL superoxide dismutase (never exceeding 0.72 nmol/5 × 10^6 PMN per 30 min) which suggests that the reduction was O_2^- mediated.

In other experiments, we also examined O_2^- generation by PMN incubated with IgA or IgG immune aggregates (100 μg/mL) in the presence of normal human sera. The addition of human sera to PMN did not increase O_2^- generation induced by both immune aggregates (not shown).

Treatment of PMN with cytochalasin B is known to induce a variety of PMN responses. In this study, we found that the release of O_2^- from PMN stimulated with immune aggregates was not increased by pretreatment of PMN with cytochalasin B (5 μg/mL) (Table 1).

DISCUSSION

Our results show that IgA and IgG immune aggregates induce PAF and O_2^- production by human PMN in a dose and time dependent manner. The presence of PAF was determined by bioassay, and in selected experiments by the incorporation of [^{3}H]acetate. Characterization of the labeled and non-labeled lipid obtained under various conditions (see Results) indicated that it was in fact PAF. Furthermore, it was shown that incorporation of [^{3}H]-acetate is an accurate measure of PAF synthesis, as determined by gas chromatography and mass spectrometry (19). Our data suggest that not only IgG, but also IgA, immune complexes can contribute to inflammatory reactions by stimulating PMN to produce PAF and O_2^-. Presumably, the stimulation of PMN by these immune aggregates takes place through the Fc receptor. This conclusion is supported by our studies in which immune aggregates containing the F(ab')$_2$ portion were ineffective in causing PMN activation as defined as production of O_2^- and PAF. Although we have not done the same type of study for IgA, the presence of receptors for IgA (FcαR) has recently been demonstrated on phagocytes from different species (6). In contrast to our results in humans, rat neutrophils exhibited O_2^- production and lysosomal enzyme secretion in response to IgG immune complexes, but not in response to IgA complexes. Interestingly, alveolar macrophages did respond to IgA complexes (20). We do not have a good explanation for this discrepancy, but it is clear that species differences do exist.

PMN treatment with cytochalasin B is known to cause a variety of PMN activation responses (21,22). In the present study, cytochalasin B had no effect on PAF and O_2^- production by PMN stimulated with IgA or IgG immune aggregates. These data suggest that phagocytosis by PMN is not required for immune complexes to stimulate

PAF and O_2^- synthesis, as it is for particulates such as zymosan (1).

Most of the PAF produced by PMN upon stimulation with both classes of immune aggregates remained cell associated quite independently of the time of incubation and the total amount of PAF synthesized. This feature is reminiscent of the processing of PAF by activated human PMN under a variety of conditions, and by other human cells including endothelial cells (23,24).

The addition of normal fresh sera as a source of complement potentiated PAF production by PMN upon stimulation with IgG immune aggregates more significantly than by PMN upon stimulation with IgA immune aggregates. This is not unexpected. Although it has recently been shown that IgA immune complexes activate the alternative pathway of complement, their capacity of triggering C5 cleavage to C5a and C5b is less striking than that of IgG immune complexes (25,26).

Although this is the first observation of PAF production induced by IgA complexes, previous studies have shown the activation of arachidonate metabolism in PMN by aggregated IgA (27). Therefore, the interaction of PMN with IgA immune complexes deposited in blood vessel walls in Henoch-Schölein purpura, or in organs such as lung and kidney, may result in the release of a series of lipid mediators including PAF and arachidonic acid metabolites, as well as oxygen free radicals, that could contribute to the inflammatory response.

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REVIEW

Platelet-Activating Factor and Polyunsaturated Fatty Acids in Cerebral Ischemia or Convulsions: Intracellular PAF-Binding Sites and Activation of a Fos/Jun/AP-1 Transcriptional Signaling System[1]

Nicolas G. Bazan[a,b,d,*], Stephen P. Squinto[a,2], Pierre Braquet[e], Thomas Panetta[c] and Victor L. Marcheselli[b,d]

[a]Departments of Biochemistry and Molecular Biology, [b]Ophthalmology, and [c]Surgery, [d]Neuroscience Center, Louisiana State University Medical Center School of Medicine, New Orleans, Louisiana 70112 and [e]Institut Henri Beaufour, Le Plessis Robinson, France

Platelet-activating factor (PAF) is a lipid mediator formed in the early response of the central nervous system to ischemia or convulsions. Free polyunsaturated fatty acids and arachidonic and docosahexaenoic acids are accumulated along with PAF. Antagonists of PAF have been found to improve cerebral blood flow and partially block the rise in free fatty acids, an effect that may arise by way of inhibition of PAF receptors or stimulation of the reacylation of free fatty acids released upon insult. Three intracellular PAF-binding sites have been identified in rat cerebral cortex. These very high-affinity binding sites are inhibited by PAF antagonists, with certain antagonists exhibiting specificity for a particular binding site. This specificity indicates heterogeneity in these binding sites. Ischemia or stimulation also leads to protooncogene transcriptional activation. Here, we discuss studies with cells in culture showing that PAF promotes transcriptional activation of immediate-early genes. PAF activates the transcription of the immediate-early genes *fos* and *jun*, whose gene products are regulators of the transcription of other genes. Transcription of *fos* is also activated by convulsion or ischemia in the central nervous system. The activation of these genes by PAF can be inhibited by PAF antagonists, and is apparently accomplished by way of an AP-1 transcription regulatory sequence in the promoter region of the target genes. Studies with deletion mutants show that PAF can also exert its activating properties by way of cyclic adenosine-3′,5′-monophosphate-(cAMP) and Ca^{2+}-responsive elements, and suggest that PAF is involved in an interconnected network of cell signaling that may coordinate short-term and long-term responses of cells to stimulus and injury.

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Several neural cell signal transduction systems involve the degradation of membrane phospholipids by phospholipases C and A_2 through various and selective receptor-mediated events. Many products of these enzymes are second messengers. Under pathological conditions (*e.g.* ischemia or convulsions), an overactivation of these events leads to the synthesis and accumulation of biologically active mediators in brain.

Certain phospholipids of synaptic membranes are rapidly deacylated by phospholipase A_2 during convulsions, leading to the accumulation of free arachidonic and docosahexaenoic acids (1–3). Ischemia and other forms of brain injury, such as cryogenic brain edema, also lead to the accumulation of free polyunsaturated fatty acids (PUFA) (1,4–12). Moreover, the ischemia-induced accumulation of free fatty acids preferentially takes place in gray matter as compared with white matter (13). In the retina, an integral part of the central nervous system, similar responses occur during relative anoxia (14), or when exposed to depolarizing K^+ concentrations (15). An enhanced production of lipoxygenase products takes place under these conditions in the retina as well as in synaptosomes. Synaptosomal fractions from rats undergoing bicuculline-induced status epilepticus show that this fraction, and not endoplasmic reticulum, is the prevalent site of phospholipase A_2 activation and accumulation of lipoxygenase reaction products (16).

During reperfusion after ischemia, the renewed availability of oxygen leads to enzyme-mediated oxygenation of arachidonic acid (and perhaps also of docosahexaenoic acid) and to the triggering of lipid peroxidation. In the pathogenesis of brain damage, degradation of certain phospholipids of excitable membranes, effects triggered by the accumulated free polyunsaturated fatty acids, and/or subsequent synthesis of oxygenated metabolites of those fatty acids, may contribute to functional impairment (9,17,18). These early responses of the nervous system may result from overstimulation of receptors and increased intracellular calcium (2,17,19). The membrane phospholipids that are degraded by phospholipases are thought to be ethanolamine plasmalogen (20), inositol lipids (21), or phosphatidylcholine (2,22). The involvement of inositol lipids is further documented since these lipids decrease in ischemia (11) and convulsions while stearoyl-arachidonoyl-*sn*-glycerol increases (1,4,23). The molecular species of choline phospholipids (alkyl-acyl-glycerophosphocholine) that lead to platelet-activating factor (PAF) synthesis may also serve as the source of the free polyunsaturated fatty acids that accumulate in the nervous system under these conditions.

The synthesis of platelet-activating factor (PAF, PAF-

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*To whom correspondence should be addressed at the LSU Eye Center, 2020 Gravier Street, Suite B, New Orleans, LA 70112.

[2]Present address: Regeneron Pharmaceuticals, Inc., Molecular and Cellular Biology Division, Suite 10, 777 Old Saw Mill River Road, Tarrytown, NY 10591.

Abbreviations: AGEPC, 1-*O*-alkyl-2-acetyl-*sn*-glycero-3-phosphocholine; B_{max}, binding sites density; cAMP, cyclic adenosine-3′,5′-monophosphate; CNS, central nervous system; DSE, dyad symmetry element; FFA, free fatty acids; GPC, glycerophosphocholine; HIV-1, human immunodeficiency virus-1; IC_{50}, 50% inhibitory concentration; K_D, rate of dissociation; K_i, inhibitor constant; LTR, long terminal repeat; mRNA, messenger ribonucleic acid; PAF, platelet-activating factor; PLA_2, phospholipase A_2; PMA, phorbol myristate acetate; PUFA, polyunsaturated fatty acids; SRE, serum response element.

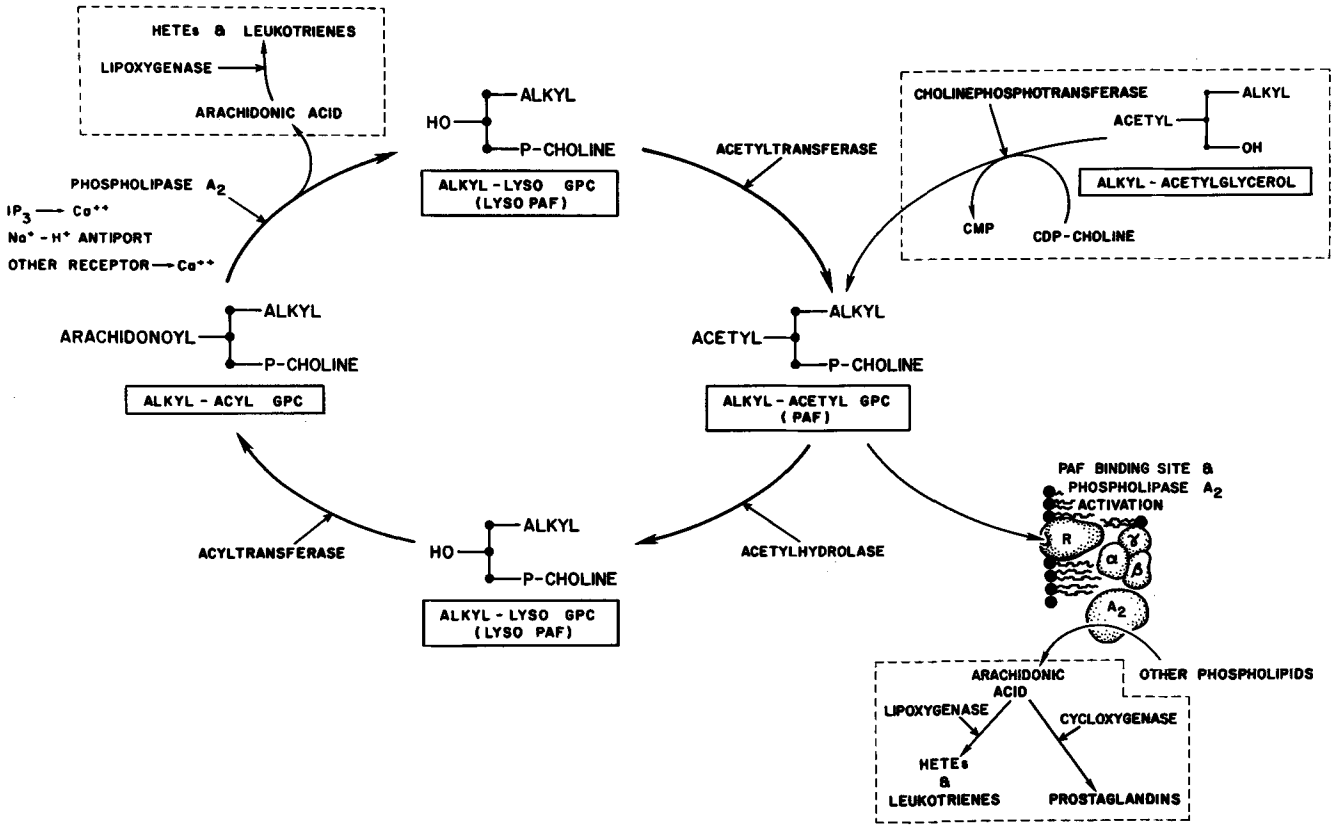


FIG. 1. The PAF (platelet-activating factor) cycle. Synthesis of PAF can involve two different routes. One pathway stems from alkyl-acyl-glycerophosphocholine (alkyl-acyl-GPC) which is enriched in arachidonoyl chains. The first step toward PAF synthesis is the deacylation of alkyl-acyl-GPC by phospholipase A_2 to form lysoPAF, which releases mostly arachidonic acid. Then an acetyltransferase introduces an acetyl group at C-2 of lysoPAF. Effector systems that can modulate the activity of phospholipase A_2 include IP_3-induced calcium mobilization, the Na^+,H^+ antiport (34), and other receptor-mediated events that may enhance intracellular calcium concentration. The second route of PAF synthesis involves the transfer of phosphocholine from CDPcholine to alkyl-acetyl-glycerol. PAF interaction with specific membrane binding sites results in activation of phospholipases and other second-messenger systems. PAF can be metabolized by acetyl hydrolase followed by acylation *via* an acyltransferase or transacylase.

acether, AGEPC, 1-*O*-alkyl-2-acetyl-*sn*-glycero-3-phosphocholine) is triggered in several tissues in response to injury, inflammation, or during immune reactions. Since this synthetic route involves as the initial step a phospholipase A_2 cleavage (Fig. 1), it was suggested that PAF synthesis may be engaged, at least in part, in the generation of free polyunsaturated fatty acids following ischemia or convulsions in the nervous system (24–26). Furthermore, alkyl-acyl-glycerophosphocholine (GPC), upon which phospholipase A_2 acts, is known to be enriched in arachidonoyl groups at *sn*-2 in several cells and tissues although information about neural tissue is not yet available. PAF may also elicit its effects as an agonist activating phospholipases after it has been synthesized during early events after ischemia or convulsions. The other phospholipase A_2 cleavage product, lysoPAF, is subsequently acetylated to form PAF.

Here we summarize studies showing that PAF antagonists restore blood flow during ischemia-reperfusion and that in ischemia or convulsions, the accumulation of free polyunsaturated fatty acids is ameliorated. Because of these observations, the subcellular distribution of PAF-binding sites was studied, and, in addition to sites on synaptic plasma membranes, high-affinity intracellular PAF-binding sites were found in cerebral cortex. Moreover, in human neuroblastoma cells, PAF-induced transcriptional activation of proto-oncogenes was observed. How all of these events and effects may interrelate is discussed here in terms of a working model linking signaling modulators to gene expression.

EFFECTS OF PAF ANTAGONISTS ON CEREBRAL BLOOD FLOW AND ON ENDOGENOUS FREE PUFA DURING ISCHEMIA OR CONVULSIONS

Alkyl-acyl-GPC contains a large proportion of arachidonoyl chains, and its deacylation by phospholipase A_2 may underlie, at least in part, the free polyunsaturated fatty acid (PUFA) changes that occur at the onset of ischemia or convulsions. In Figure 2, possible factors involved in phospholipase A_2 activation and PAF synthesis are indicated. Both free polyunsaturated fatty acids and PAF can have potent biological consequences. One of the effects of the fatty acids released by phospholipase A_2 (PLA_2) is the uncoupling of oxidative phosphorylation. Therefore, the effects of a PAF antagonist systemically administered at the onset of reperfusion after 10 min of bilateral carotid artery ligation in gerbils were studied. It was found that the PAF antagonist BN 52021 improves cerebral blood flow and decreases the

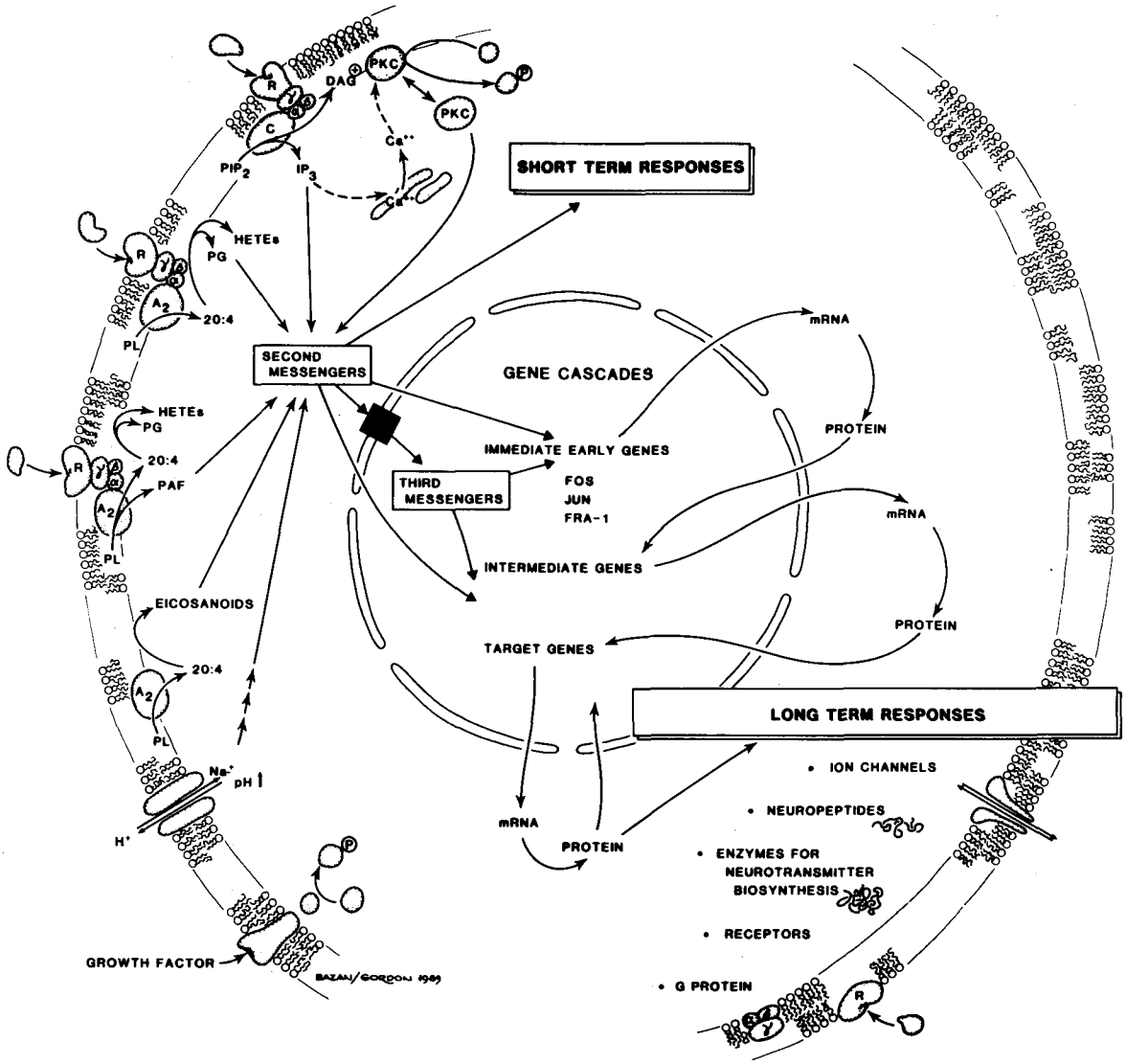


FIG. 2. Outline of the possible events coupling second messengers with long-term responses to cell activation. The events depicted here are a composite of those taking place in several different cells. The cell-signal transduction molecules depicted, on the left of the diagram, on the plasma membrane, lead to the formation of second messengers that in turn bring about short-term responses. These second messengers or mediators are synthesized by receptor-mediated hydrolysis upon interaction of an agonist (*e.g.* hormone, growth factor, neurotransmitter) with a receptor. As a response to injury, ischemia, convulsions, or inflammation, several of these messengers or mediators are accumulated and actively participate in the pathophysiological responses. In the center of the figure, gene cascades are outlined, starting from immediate-early genes, and possible effects of the second messengers are indicated. Target genes may then be engaged in the long-term responses by synthesizing mRNA. Most of the events depicted in the figure occur in neural cells, although how the events are interlinked is not understood. Reprinted with permission from Bazan, N.G., and Bazan, H.E.P. (1990) Ocular Responses to Inflammation and the Triggering of Wound Healing: Lipid Mediators, Proto-Oncogenes, Gene Expression, and Neuromodulation in *New Trends in Lipid Mediators Research,* Bazan, N.G., ed., S. Karger AG, Basel Switzerland, pp. 1-10.

accumulation of free fatty acids (FFA) in brain (24). Moreover, this antagonist also partially blocks the electroconvulsive shock- or ischemia-induced rapid rise in brain free fatty acids (25). The rise in FFA reflects activation of phospholipase A_2, as evidenced by the fact that arachidonic and docosahexaenoic acids are the prevalent fatty acids liberated (1).

Whether the PAF antagonist BN 52021 elicited these effects by "antagonizing" a PAF-binding site remains to be ascertained. However, additional and/or alternative effects are also possible.

The free fatty acids released during central nervous system (CNS) stimulation can also be reacylated and incorporated into membrane lipids. This reuptake may be stimulated by the PAF antagonist BN 52021, which may also facilitate the washout of fatty acids into the bloodstream or the cerebrospinal fluid.

Other events taking place under these conditions are not well-understood. For example, the generation of free radicals is stimulated by PAF and appears to be sensitive to PAF antagonists, and the arachidonic acid cascade, which gives rise to several metabolites through the actions of cyclooxygenase and lipoxygenases, may also be affected.

From this incompletely understood network of events in cerebral injury, steps sensitive to PAF antagonists are

involved thereby implicating PAF as an effector in these reactions. PAF also has been identified as one of the most potent chemotactic agents for eosinophils, which are recruited to sites of injury and produce rapid and profound effects on the vascular system (27).

INTRACELLULAR PAF-BINDING SITES IN CEREBRAL CORTEX

Since PAF accumulates during ischemia or convulsions in brain (28) and because PAF antagonists exert a protective neurological effect under these conditions and reverse some of the neurochemical changes, it was important to define the subcellular distribution of specific PAF-binding sites in brain.

Two distinct PAF-binding sites occur in the microsomal fraction, and a third binding site is present on the synaptic plasma membranes of rat cerebral cortex (29–31). These binding sites are sensitive to the ionic environment, since concentrations of free Ca^{2+} over 10 μM induce a 50% inhibition of PAF binding to microsomal membranes. These membranes also have the highest binding affinity measured for PAF, with a rate of dissociation (K_D) of 22.5 pM (Table 1). This K_D value is in the range of that for accumulated endogenous PAF induced by picrotoxin or bicuculline-induced convulsions (28). Tables 2 and 3 show the 50% inhibitory concentration (IC_{50}) and inhibitor constant (K_i) for different PAF antagonists in microsomal and synaptosomal membranes, indicating different pharmacological responses between those binding sites. In the microsomal fraction, some of the antagonists tested show activity on both binding sites (CV-3988, BN-52115). Meanwhile, the antagonists L-652731 and CV-6209 are specific for only one binding site, indicating heterogeneity in the binding sites (31).

TABLE 1

Rate of Dissociation (K_D) and Binding Sites Density (B_{max}) for PAF Receptors in Microsomes and Synaptic Membranes from Rat Cerebral Cortex[a]

Membranes	K_D	B_{max}
Microsomes	22.5 ± 1.7 pM (4)	82.5 fmole/mg protein
	25.0 ± 0.8 nM (4)	60.0 pmole/mg protein
Synaptic membranes	1.2 ± 0.6 nM (3)	0.06 pmole/mg protein

[a]Values are averages ± SD for (n) independent experiments, using cerebral cortex from 25 rats in each experiment. Curves were fitted by means of an EBDA-LIGAND program (62) up to reach the best-fit model ($p < 0.05$). Subcellular fractionation procedure was presented in detail elsewhere (31). The binding assay was carried out at 25°C for 30 min, 175 μg membrane protein were used in 1 μL assay volume. The buffer was 10 mM tris(hydroxymethyl)-aminomethane (Tris), 5 mM $MgCl_2$, 2 mM ethyleneglycol *bis*(β-aminoethyl ether)*N,N'*-tetraacetic acid (EGTA), 0.025% bovine serum albumin (BSA), 0.1 mM phenyl methyl sulfonyl fluoride (PMSF), 56 title international units (TIU)/L aprotinin, pH 7.6. The labeled ligand was [^{3}H]hexadecyl-2-acetyl-*sn*-glycero-3-phosphocholine (PAF) in a 50 pmole-12 nmole concentration range. A 3-nM concentration was selected as standard. Nonspecific binding was assessed with 1 μM unlabeled PAF. The bond-labeled ligand was recovered by filtration on glass microfiber filter glass/fiber, grade "b" (gf/b) from Whatman Int., Ltd. (Maidstone, England).

TABLE 2

IC_{50} and Equilibrium Dissociation Constant of Unlabeled PAF and Other PAF-Binding, Competitive Inhibitors in Microsomes of Cerebral Cortex[a]

Competitive inhibitor	IC_{50}	K_i
Unlabeled PAF	2.02×10^{-10}	1.29×10^{-11}
CV-3988	1.57×10^{-9}	1.55×10^{-10}
L-652731	2.95×10^{-8}	2.18×10^{-10}
BN-52115	3.70×10^{-8}	6.04×10^{-9}
CV-6209	5.91×10^{-8}	6.66×10^{-10}
BN-50727	3.15×10^{-7}	2.29×10^{-8}
BN-52021	1.62×10^{-6}	1.31×10^{-6}
WEB-2086BS	5.02×10^{-6}	6.05×10^{-8}
BN-50726	6.01×10^{-5}	1.75×10^{-7}

[a]Data expressed as molar concentration were calculated from at least three displacement curves for each inhibitor or unlabeled ligand using an EBDA program (62). Assay conditions were the same as in Table 1.

TABLE 3

IC_{50} and Equilibrium Dissociation Constant (K_i) of Unlabeled PAF and Other Competitive Inhibitors in Synaptic Membranes from Cerebral Cortex[a]

Competitive inhibitors	IC_{50}	K_i
Unlabeled PAF	2.10×10^{-8}	1.20×10^{-8}
BN-50726	1.33×10^{-7}	8.98×10^{-8}
BN-50727	1.46×10^{-7}	1.15×10^{-7}
BN-52021	1.64×10^{-7}	1.79×10^{-7}
CV-3988	2.54×10^{-7}	2.37×10^{-6}
WEB-2086BS	7.93×10^{-7}	1.90×10^{-7}
CV-6209	2.13×10^{-6}	1.64×10^{-6}

[a]Data expressed as molar concentration. Calculations and conditions were the same as Table 1.

The presence of high-affinity PAF-binding sites in the cerebral cortex agrees with the active cellular uptake of PAF (32) and retention in the cell (33). Moreover, in retina, neurotransmitter-mediated PAF synthesis leads to tissue retention rather than release of the lipid mediator (34). An intracellular mediator role has been suggested for PAF (33,35,36), and the discovery of intracellular binding sites supports this contention.

IMMEDIATE-EARLY GENES

Several hormones, growth factors and experimental conditions that elicit activation of many membrane receptors lead to transcriptional activation of immediate-early genes. The messenger system(s) linking the plasma membrane with genomic response are not well-understood (37,38). The rapid activation of immediate-early genes gives rise to proteins that are regulators of transcription of other genes. Two of these genes are c-*fos* and c-*jun*, whose gene products, Fos and Jun, interact with a regulatory sequence, AP-1, found in the promoter region of many genes (39–41).

C-*fos* transcription is activated in response to such conditions as convulsions (42–47) and ischemia (48) in the

central nervous system. C-*fos* messenger ribonucleic acid (mRNA) has also been observed after stimulation of neurotransmitter receptors and membrane depolarization (42). Also during convulsions or ischemia, membrane phospholipids, particularly those linked through phospholipase A_2 and phospholipase C to cell signal transduction events, are targets that generate the potentially active lipid mediators arachidonic acid, docosahexaenoic acid, oxygenated metabolites of arachidonic acid (*e.g.* prostaglandins, hydroxyeicosatetraenoic acids, leukotrienes) and platelet-activating factor. It was postulated, therefore, that a lipid mediator may play a role as a messenger in the transcriptional activation of immediate-early genes (49). PAF was studied as a lipid mediator because i) it accumulates in brain during ischemia or convulsions (28), ii) its synthesis involves a phospholipase A_2 that generates arachidonic acid, iii) PAF antagonists inhibit the accumulation of free polyunsaturated fatty acids in brain triggered by ischemia or convulsions (24,25), and iv) because in addition to cell-surface PAF-binding sites there are very high-affinity intracellular binding sites in brain (29,31).

PAF ACTIVATES TRANSCRIPTION OF C-*FOS* AND C-*JUN*

The effect of PAF on the transcription of the genes c-*fos* and c-*jun* was studied in cultured human neuroblastoma SH-SY5Y cells (49). PAF results in a 7-fold increase of c-*fos* mRNA in 15 min to a maximum of 12-fold, after 30 min. This stimulation is inhibited by pretreatment of the cells by the PAF antagonist BN 52021.

The activation of c-*fos* transcription in neural cells was compared to the effect of PAF on c-*fos* activity in MOLT-4 T-cells. PAF treatment elicits a 2–3-fold increase in c-*fos* transcription, which is also inhibited by BN 52021 pretreatment of the cells. These effects on MOLT-4 T-cells are much smaller, as compared with neuroblastoma cells.

The stimulatory effect of PAF on the transcription of c-*jun* is similar to its effect on the c-*fos* gene in SH-SY5Y cells, and is inhibited by pretreatment with PAF antagonist. Similar to c-*fos*, c-*jun* activation elicited by PAF in T-cells is lower than in the neuroblastoma cells. The c-*jun* response is also much slower than the c-*fos* response, reaching maximum level, an 8-fold increase in transcription, in 60 min, as compared to 30 min for c-*fos*. Moreover, nuclear run-off experiments demonstrate that the PAF effect is a result of transcriptional activation (49).

An 11.5-fold increase in transcription is seen after addition of the phorbol ester phorbol myristate acetate (PMA) to SH-SY5Y cells. When PMA and PAF are added at the same time to these cells, a massive, 27-fold increase in transcription is observed. This synergistic interaction needs to be studied further, since it suggests that PAF may elicit its effect by a protein kinase C-independent pathway.

Cloned deletion mutants were transfected into the SH-SY5Y cells to determine which parts of the promoter gene sequences are necessary to produce the PAF-induced activation of transcription. In cells containing the sequence PF4CAT, transcription is elevated 10-fold by addition of PAF, a response that is inhibited by the PAF antagonist BN 52021. Removal of the sequence DSE, dyad symmetry element, which is an AP-1-like sequence, reduces this effect of PAF. Replacement of the DSE sequence results in a PAF-induced activation of transcription comparable to the PF4CAT control. However, replacement of an AP-1-like sequence does not restore the PAF-induced effect. Replacement of two tandem copies of the DSE from a different mutant results in PAF stimulation comparable to the PF4CAT control. Deletion mutants lacking the Ca^{2+}- and cAMP-responsive elements show no PAF-induced transcription (49).

One question addressed by these studies was whether PAF elicits mRNA production from genes containing AP-1 sequences, because AP-1 regulatory elements are responsive to stimulation by Fos/Jun heterodimers, and PAF activates both *fos* and *jun* transcription (49).

Transcription in SH-SY5Y cells transfected with a plasmid containing four copies of the AP-1 sequence is activated by PAF, but this effect is not observed when the transfecting plasmids do not contain the AP-1 sequence. The specificity of this effect was shown by its inhibition by BN 52021 (49).

Another gene with an AP-1 sequence, human immunodeficiency virus-1 (HIV-1), was assayed for PAF activation. One part of the promoter region of this gene, the LTR (long terminal repeat sequence), contains two AP-1-like regions. When this gene is transfected into SH-SY5Y cells, PAF produces a 6-fold stimulation of transcription, which is blocked by pretreatment with BN 52021 (50).

PAF produces a rapid, transient activation of c-*jun* and c-*fos* proto-oncogenes in SH-SY5Y cells, and a lesser activation in MOLT-4 T-cells. The possibility that this effect represents a receptor-mediated activation is suggested by its inhibition by the PAF antagonist BN 52021. This suggests that PAF may act as a messenger in neural cells, activating transcription in target genes containing an AP-1 sequence within their promoters.

The immediate-early gene c-*fos* is activated by several extracellular signals, including growth factors and serum, by way of the sequence SRE (serum response element). Within the SRE is a sequence, DSE (dyad symmetry element), which binds a serum-response factor. These elements interact in the activation of c-*fos* transcription.

C-*jun* transcription is elicited by both serum factors and growth factors, but the promoter of c-*jun* does not have the SRE (51). Therefore, it is possible that the two genes, c-*fos* and c-*jun*, are activated by way of different promoter regulatory sequences in response to extracellular signals.

PAF seems to exert actions on both the SRE and a cAMP- and Ca^{2+}-responsive sequence in the c-*fos* promoter (52). The latter sequence is necessary, as shown in deletion mutant studies. Therefore the SRE sequence is not needed for the PAF-induced activation of transcription, but nevertheless contributes to the effect of PAF on the gene.

Perhaps the requirement for the Ca^{2+}- and cAMP-sensitive sequences suggests that the effects of PAF involve the release of these messengers in order to activate transcription. The fact that phorbol esters use the SRE sequence for activation of c-*fos* indicates that PMA activates protein kinase C (53). Furthermore, various control mechanisms, including Ca^{2+}, cAMP, and protein kinase C, may be interrelated in the nuclear response to PAF.

The gene products Fos and Jun produce heterodimers that interact with the transcription regulatory sequence

AP-1, present in many genes (41,54,55). As to whether PAF interacts directly with the AP-1 sequence, the transfection assays suggest, in fact, that PAF does interact with the AP-1 sequence. Therefore, PAF may act by way of a specific receptor or receptors to elicit the transcription of a set of immediate-early genes whose protein products may further activate regulatory sequences in AP-1-containing target genes.

MODULATORS OF CELL SIGNALING AND GENE EXPRESSION IN NEURAL CELLS

Some of the lipid mediators that accumulate during the early phases of ischemia or during convulsions may also play a role in linking early responses with long-term changes (56). Figure 2 outlines the possible role of lipid mediators coupling membrane-derived messengers with genomic changes. PAF is one candidate for such a role, since Kornecki and Ehrlich (57) found that PAF modulates neuronal differentiation as well as calcium fluxes. PAF accumulates in the brain during convulsions (28), and PAF antagonists decrease damage and the accumulation of free fatty acids in the brain during ischemia or convulsions (24,25,58).

There is only preliminary evidence that other genes may be activated subsequent to proto-oncogene induction by PAF (59,60). The studies discussed here were conducted using PAF in the micromolar range in cultures maintained in fetal calf serum-containing medium. Therefore it is likely that the concentration of PAF that reaches the cells is even lower, due to binding by lipoproteins and hydrolysis by serum acetylhydrolase. To overcome these effects, nonhydrolyzable forms of PAF were studied and found to be effective in eliciting proto-oncogene expression in the nanomolar range (60,61).

The link between platelet-activating factor—already recognized as intimately involved in the short-term neural responses to trauma and inflammation—and gene expression may contribute to the understanding of neural plasticity and other cellular long-term responses to stimulation or injury.

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Calcium Ion Mobilization in Neuronal Cells Induced by PAF[1]

Elizabeth Kornecki[a,*] and **Yigal H. Ehrlich**[b,2]

[a]Department of Anatomy and Cell Biology, State University of New York Health Science Center, Brooklyn, New York 11203 and the [b]CSI/IBR Center for Developmental Neurosciences, College of Staten Island, City University of New York, Staten Island, New York 10301

We have reported previously that platelet-activating factor (PAF) interacts with the neuronal cell line NG108-15 (neuroblastoma X glioma hybrid) and the pheochromocytoma cell line, PC12. PAF acts on these cells by raising levels of intracellular free calcium ions. In the present report, we extend these studies. PAF induced the vesicular release of adenosine 5′-triphosphate (ATP) from PC12 cells in a dose-dependent manner. The PAF-induced ATP release was inhibited by the PAF antagonists, CV-3988 and CV-6209, and the calcium antagonist prenylamine. The relevance of the interaction of PAF with neuronal cells was investigated further by using brain synaptosomal preparations and primary cortical and neostriatal cells. Nanomolar concentrations of PAF induced calcium transients in aequorin-loaded synaptosomal preparations, and cortical and neostriatal cells were sensitive to the action of PAF. The possible physiological and pathophysiological roles of PAF in brain function are discussed.
Lipids 26, 1243–1246 (1991).

Studies on the effectiveness of certain neuroactive drugs, the triazolobenzodiazepines, as specific platelet-activating factor (PAF) antagonists, have lead to the suggestion that PAF may play a role in the regulation of neuronal function (1). Subsequent studies in our laboratories as well as those of others have provided experimental findings in support of this suggestion. Biosynthesis of PAF in neural tissue was reported by Bussolino *et al.* (2) using preparations of chick retina. The neurotransmitters, acetylcholine and dopamine, stimulated PAF biosynthesis *via* the *de novo* pathway in these preparations (3). More recently, Kumar *et al.* (4) demonstrated PAF biosynthesis in perfused rat brain, and its stimulation by administration of the convulsant drugs bicuculline and picrotoxin. The presence of specific binding sites for PAF was detected in membranes prepared from gerbil brain (5). Several studies utilizing PAF antagonists have implicated PAF in neuropathological processes (6). The mechanism mediating these effects appears to involve activation of phospholipase A_2 (7), possibly as a secondary event to a pathophysiological increase in the level of intracellular free calcium (8).

We have reported previously that in cultured neural cells, PAF can induce an increase in the levels of intracellular free calcium, and stimulate calcium-dependent vesicular secretion of adenosine 5′-triphosphate (ATP) (8). Using PC12 cells, Bussolino *et al.* (9) demonstrated that the PAF-induced increase of intracellular calcium levels is associated with dopamine release. In the present report, we extend our previous findings and demonstrate the effectiveness of the PAF antagonist CV-6209 in blocking these effects. In addition, we report here the effects of PAF on calcium ion mobilization in cells and synaptosomes derived from the central nervous system.

MATERIALS AND METHODS

Reagents. CV-3988, namely 2-methoxy-3-(octadecylcarbamoyloxy)propyl 2-(3-thiazolio)ethyl phosphate, and CV-6209 which is 2-[*N*-acetyl-*N*-(2-methoxy-3-octadecylcarbamoyloxypropoxycarbonyl)aminomethyl]-1-ethylpyridimium chloride were obtained from Takeda Chemical Industries, Ltd. (Osaka, Japan). The CV-3988 solution was heated to 50°C, cooled to room temperature, and the pH adjusted to 7.4. CV-6209 was dissolved in *N*-2-hydroxyethyl piperazine-*N*′-2-ethane sulfonic acid (HEPES) (5 mM) buffer, pH 7.4, containing sodium phosphate (10 mM), NaCl (150 mM), 1mM $MgCl_2$, and 1% glucose. Aequorin was obtained from Dr. John R. Blinks, Department of Pharmacology, Mayo Foundation, (Rochester, MN). The luciferin and luciferase reagent mixture was obtained from ChronoLog (Havertown, PA) and dissolved in water. Prenylamine was obtained from Sigma Chemical Co. (St. Louis, MO). PAF was obtained from Calbiochem Corp. (San Diego, CA) as a pure lyophilized preparation, with chains consisting mainly of C_{16} and C_{18}. A 10 mM stock solution of PAF, dissolved in a solution of 0.35% bovine serum albumin and 0.15M NaCl, was stored frozen at −20°C.

Preparation of PC12 cells for measurement of intracellular calcium and ATP release. Cells of the pheochromocytoma clone PC12 were cultured as monolayers in RPMI 1640 medium containing 10% heat-inactivated horse serum and 5% fetal calf serum as described (10). For the aequorin studies, cells at 80 to 95% confluency were harvested, washed, and resuspended in a HEPES (5 mM) buffer (pH 7.4) containing ethyleneglycol-*bis*(β-aminoethyl ether)*N,N*′-tetraacetic acid (EGTA((5mM), sodium phosphate (10 mM) , NaCl (150 mM), and 1% glucose. The cells were washed and resuspended in the same buffer, then loaded with aequorin according to the dimethyl sulfoxide (DMSO) method of Yamaguchi *et al.* (11). For measuring aequorin signals, aequorin-treated cells were resuspended at a final count of 10^6 cells per milliliter in a buffer containing 1 mM $MgCl_2$ and 1 mM $CaCl_2$. Aliquots (1.1 mL) were placed in cuvettes and examined in a Platelet Ionized Calcium Aggregometer (PICA; ChronoLog) set at 400 rpm and 37°C. Agents tested for inducing changes in intracellular free Ca^{++} were added by injection through a rubber septum, and aequorin luminescence was plotted by a chart recorder.

[1]Based in part on a paper presented at the Third International Conference on Platelet-Activating Factor and Structurally Related Alkyl Ether Lipids, Tokyo, Japan, May 1989.

*To whom correspondence should be addressed at the Department of Anatomy and Cell Biology, State University of New York Health Science Center at Brooklyn, 450 Clarkson Avenue, Box 5, Brooklyn, NY 11203.

[2]Mailing address: College of Staten Island, City University of New York, 120 Stuyvesant Place, Staten Island, NY 10301.

Abbreviations: ATP, adenosine 5′-triphosphate; CNS, central nervous system; DMSO, dimethyl sulfoxide; EGTA, ethyleneglycol-*bis*(β-aminoethyl ether)*N,N*′-tetraacetic acid; HEPES, *N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid; PAF, platelet-activating factor; PAF-acether, 1-*O*-alkyl-2-acetyl-*sn*-glycero-3-phosphocholine.

For measuring ATP release from PC12 cells, cells were harvested and washed in phosphate-buffered saline supplemented with 1 mM $MgCl_2$, 1 mM $CaCl_2$, and 1% glucose. The cells were aliquoted (0.45 mL) into a cuvette and placed into a ChronoLog Lumi Aggregometer set at 37°C and 400 rpm. Agents (10 μL) tested for inducing ATP release were added to cell suspensions containing luciferin/luciferase reagent. ATP release was measured in luminescence units.

Preparation of rat brain synaptosomes. Adult rat brain synaptosomes were prepared using Ficoll-sucrose gradients, essentially as described by Sapirstein *et al.* (12) with the following modification: 8.5% Ficoll layer was used instead of a 7.5% Ficoll layer, and two successive gradients were employed for the isolation of the synaptosomal fraction (P_2B). Synaptosomal preparations at a protein concentration of 7.0 mg/mL were loaded with aequorin according to the DMSO procedure of Yamaguchi *et al.* (11). Aliquots (1.1 mL) of the synaptosomal preparations at a protein concentration of 56.1 μg/mL were placed into cuvettes in a ChronoLog PICA instrument at 37°C under stirring. Reactions were initiated by the injection of PAF (10 μL) into the 1.1 mL synaptosomal preparation through a rubber septum.

Preparation of mouse brain cortical and striatal cells from mouse embryos. CD1 mice were obtained from Charles River Laboratories (Wilmington, MA). Neostriata and cortex were dissected from the brain of 14-day-old mouse embyros and mechanically dissociated, basically as described by Zhang *et al.* (13), and suspended in HEPES-phosphate buffered saline (pH 7.4) containing 1% glucose, 1 mM $MgCl_2$ and 1 mM $CaCl_2$ at a cell count of approximately 10^5 cells/mL. The cells were loaded with aequorin by the DMSO method (11). Cell suspensions (1.1 mL) were aliquoted into cuvettes and placed into a ChronoLog PICA instrument set at 37°C and 400 rpm. Aliquots of PAF (10 μL) were injected into the cell suspension to initiate an aequorin response.

RESULTS AND DISCUSSION

The influx and efflux of calcium through the plasma membrane and the delicately balanced control of the intracellular level of free calcium ions play a crucial role in the maintenance of neuronal physiological activity. In addition, Ca^{++} fluxes control the communication between neurons by regulating the critical process of neurotransmitter release (14). We have shown previously that PAF can induce an increase in intracellular levels of free calcium in cultured cloned neural cells (8). The results suggested that these effects appear to be mediated by the activation of low affinity PAF receptors which are coupled to calcium channels (8). This conclusion is further supported by the results obtained with additional drugs tested in the present study. As shown in Table 1, the PAF antagonist CV-6209 was found to be a potent inhibitor of PAF-induced aequorin signals in PC12 cells, exerting this effect with an IC_{50} value of 1.3 μM. This IC_{50} value was an order of magnitude lower than that obtained for CV-3988 (IC_{50}=20 μM). As can be seen in Table 1, CV-6209 also inhibited the PAF-induced vesicular release of ATP from PC12 cells. As reported previously, the calcium antagonistic drug prenylamine inhibited the PAF-induced increase in intracellular free calcium in PC12 cells (8). We have now found that, in addition, prenylamine also inhibited the PAF-induced ATP release from PC12 cells (Table 1). Based on the observation that the PAF-induced ATP release required the presence of extracellular calcium, we proposed that PAF induces in neural cells a calcium-dependent vesicular secretion (8). The present finding that prenylamine potently inhibits this process further supports this conclusion.

TABLE 1

Comparison of the Effects of Various Drugs on the PAF-Induced Aequorin Response and ATP Release in PC12 Cells[a]

Drugs tested	Aequorin response IC_{50}	ATP release IC_{50}
Prenylamine	5 μM	2 μM
CV-3988	20 μM	30 μM
CV-6209	1.3 μM	6 μM

[a]PC12 cells were prepared in suspension as described in the Methods section. Incorporation of aequorin into PC12 cells was performed in the presence of DMSO as described in the Methods section. Aliquots (1.1 mL) of the cell suspension were tested for their response to PAF in the presence of various concentrations of the drugs indicated above. Cell suspensions were incubated with drugs for 5 min at 37°C prior to addition of PAF (4μM, final concentration). For ATP release measurements, aliquots (0.45 mL) of the cell suspension containing the luciferin/luciferase reagent were incubated for 5 min at 37°C with various concentrations of drugs. PAF was added to initiate the release of ATP.

The effects of PAF on calcium mobilization in neural cells were reported to date using cloned neuronal cell lines originating from neuroblastomas of the peripheral nervous system, or from pheochromocytoma of the adrenal medulla. To determine whether PAF affects calcium mobilization of cells from the central nervous system (CNS), we have used dissociated cells obtained from embryonic mouse brain, and loaded with aequorin using the DMSO method (11). Dissociated cells from two brain regions were tested separately for their responses to PAF: neostriatum and cerebral cortex. Figure 1 shows the effect of various concentrations of PAF on neostriatal cells. Concentrations of PAF below one micromolar induced an aequorin response indicative of calcium mobilization in these cells. Higher concentrations of PAF induced larger aequorin signals with maximal responses to PAF occurring at approximately 9 μM PAF. Dissociated cortical cells also exhibited responses to PAF as shown in Figure 2. The aequorin response of cortical cells to PAF was much higher than that observed with neostriatal cells, and a ten-fold greater sensitivity of cortical cells to PAF was observed. The maximal aequorin response in striatal cells was approximately 80 units (as measured from a chart recorder) as compared to the maximal aequorin response in cortical cells of 550 units measured under identical assay conditions. Maximal aequorin responses to PAF in cortical cells occurred with approximately 12 μM PAF.

The dissociated cell preparations used in the experiments described above contained mixed populations of neuronal and glial cells, and either or both of these cell types may be responding to PAF. To test the responsiveness of neuronal elements to PAF, we have used

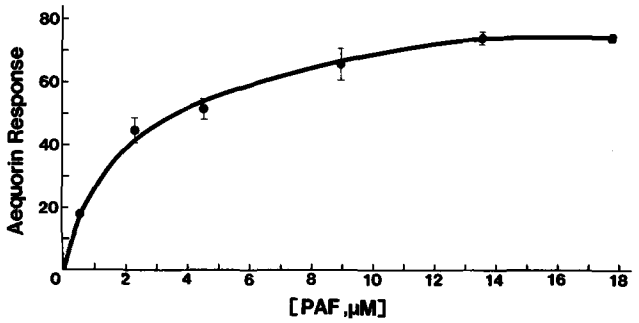


FIG. 1. Effect of PAF on aequorin-loaded neostriatal cells. Mouse embryo neostriata were dissociated and loaded with aequorin as described in the Methods section. Cell suspensions (10^5/mL) were aliquoted into cuvettes and placed in a ChronoLog PICA instrument for measurement of calcium transients. PAF (10 μL) was injected through a rubber septum directly into the cell suspensions. The concentrations of PAF shown above are final. The aequorin response was measured in units of luminescence. Data shown are the means ± S.E.M. for three experiments.

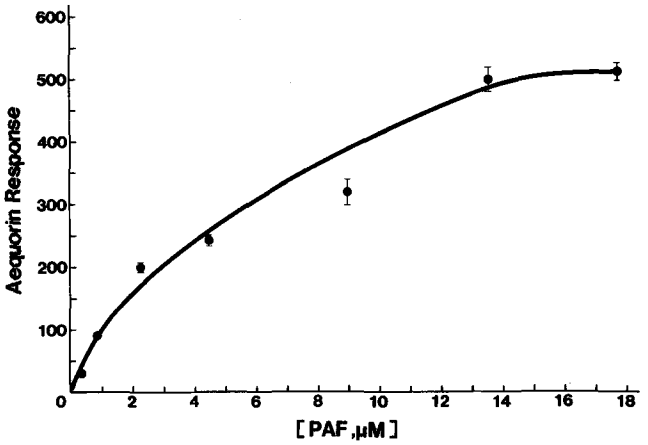


FIG. 2. Effect of PAF on aequorin-loaded cortical cells. Mouse embryo cortices were dissociated and loaded with aequorin as described in the Methods section. Calcium transients resulting from the addition of various concentrations of PAF to aliquots of cortical cell suspensions were measured as described in Figure 1. Data shown are the means ± S.E.M. for three experiments.

preparations of pinched-off nerve endings (synaptosomes) prepared from adult rat brain using density gradient centrifugation. We now report that rat brain synaptosomes preloaded with aequorin also respond to PAF. Table 2 shows the response of adult rat brain synaptosomal preparations to various concentrations of PAF. The synaptosomes responded to PAF with an aequorin signal that could be achieved with PAF concentrations as low as 9.9 nM. Maximal responses to PAF were obtained with approximately 497 nM PAF.

The results presented here confirm the notion based on studies using neuroblastoma and pheochromacytoma cells that PAF can exert regulatory effects on neuronal calcium mobilization and that these effects may be mediated by receptor-operated calcium channels. In addition we demonstrate here for the first time direct effects of PAF on cells derived from the central nervous system of embryonic brain.

Recent studies by Kumar *et al.* (4) provided data

TABLE 2

PAF Induces an Increase in Free Ca^{++} in Rat Brain Synaptosomes[a]

[PAF]	Aequorin response (% maximal)
0	0
9.9 nM	42
249 nM	64
497 nM	100
990 nM	100

[a]Rat brain synaptosomes were prepared and loaded with aequorin using the DMSO method as described in the Methods section. Synaptosomes, 1.1 mL aliquots, were incubated in a cuvette at 37°C under stirring conditions using a ChronoLog PICA instrument. PAF was added to initiate an aequorin response. Final concentrations of PAF are shown above.

indicating that the blood-brain barrier is impermeable to circulating PAF. Thus, physiological effects of PAF on neuronal function would require biosynthesis of PAF within brain tissue. Indeed, such biosynthesis was demonstrated by Kumar *et al.* (4) in adult perfused brain, and by Bussolino *et al.* (2) in retinal preparations. More recently, Bussolino *et al.* (3) demonstrated PAF synthesis in immature retina suggesting a role for PAF in neuronal development and maturation. This suggestion is consistent with our finding that low concentrations of PAF, such as can be synthesized by brain tissue, can induce neuronal differentiation in cultured NG108-15 cells (8). A physiological increase in intracellular levels of free calcium ions may be one of the molecular mechanisms through which PAF influences neuronal development.

Under certain conditions of trauma and injury, or pathophysiological processes which compromise the blood-brain barrier, cells in the central nervous system may become exposed to relatively high levels of PAF which would induce an exaggerated increase in intracellular free calcium levels resulting in neurodegeneration (8). A body of evidence, based primarily on documenting the protecting effects of PAF antagonists, demonstrates the involvement of PAF in cerebral ischemia (15–17). The results reported here, demonstrating that the calcium system of cells derived from the central nervous system are responding to PAF, indicate that also the pathophysiological effects of PAF in the CNS may be mediated by calcium mobilizing systems.

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Role of Platelet-Activating Factor in Aggregation of Leukocytes and Platelets in Cerebral Ischemia[1]

S. Uchiyama*, M. Yamazaki and S. Maruyama
Department of Neurology, Neurological Institute, Tokyo Women's Medical College, Tokyo 162, Japan

Leukocyte and platelet aggregation stimulated with formyl-methionyl-leucyl-phenylalanine (FMLP) was measured in 32 patients with cerebral ischemia and in 15 controls, using a whole blood aggregometer. The increases in impedance and the reductions in leukocyte and platelet counts were significantly greater in stroke patients than in controls. Aggregation was inhibited by oral ticlopidine, but not by oral aspirin. The effects were clearly counteracted by platelet-activating factor (PAF) antagonists, and counteracted in part by a 5-lipoxygenase inhibitor. The results suggest that platelets tend to be activated by PAF and leukotrienes liberated from hyperaggregable leukocytes in patients with ischemic stroke.
***Lipids* 26, 1247-1249 (1991).**

Recent evidence suggests that not only platelets but also leukocytes play an important role in thrombogenesis and other related pathological processes of cerebral ischemia by releasing various chemical mediators such as platelet-activating factor (PAF), leukotrienes, free radicals and cytokines (1-4). We measured leukocyte and platelet aggregation stimulated by the chemotactic peptide formyl-methionyl-leucyl-phenylalanine (FMLP) in patients with cerebral ischemia. We also investigated the effects of various pharmacological agents on the aggregation of leukocytes and platelets in order to determine what mediates leukocyte-platelet interaction.

MATERIALS AND METHODS

The subjects were 26 patients with cerebral infarction (18 males and 8 females, mean age 64 years, range from 40 to 82 years), 6 patients with transient ischemic attacks (4 males and 2 females, mean age 59 years, range from 43 to 78 years), and 15 controls including patients with various neurological disorders other than stroke (9 males and 6 females, mean age 58 years, range from 40 to 79 years). Patients with hypertension, diabetes mellitus, ischemic heart disease, peripheral vascular disease, collagen vascular diseases, infections or other conditions that could possibly affect platelet or leukocyte function were excluded from the control group. The patients had not been treated with any antiplatelet agents, anticoagulants, or fibrinolytic agents for at least 14 days prior to blood collection. Aggregation studies were performed as follows (5-7). Nine mL of venous blood was collected in a disposable plastic syringe containing 1 mL heparin sodium solution at a final concentration of 10 units/mL. The blood was stored for 30 min at room temperature. Then, 5 μL of 1 mg/mL cytochalasin B (Sigma Chemical Co., St. Louis, MO) was added to 975-μL aliquots of heparinized blood placed in plastic cuvettes (Chrono-Log, Havertown, PA). Stirring was continued for 1 min at 900 rpm at 37°C. Changes in electrical impedance (ohms) were measured using a whole blood aggregometer (Chrono-Log C540) 10 min after adding FMLP (Sigma) at a final concentration of 2 μM. Aliquots were collected into an equivalent volume of 1.5% formalin containing 5 mM EDTA both before and 10 min after adding FMLP and were used for measuring leukocyte and platelet counts.

In order to pinpoint the mediator of the interaction between leukocytes and platelets, the effects of various pharmacological agents were studied *ex vivo* and *in vitro*. Aggregation studies were performed before and on the 7th day after starting daily oral administration of either of the following drugs: one Baby's Bufferin® tablet (Banyu Pharmaceutical Co., Tokyo, Japan) containing 81 mg aspirin; 200 mg of ticlopidine hydrochloride (2 tablets of Panaldine®, Daiichi Pharmaceutical Co., Tokyo, Japan), or 100 mg of CV-4151 (Takeda Chemical Industries, Osaka, Japan) (8), a thromboxane A_2 (TXA_2) synthetase and receptor inhibitor. The *in vitro* effects on leukocyte and platelet aggregation stimulated with FMLP of the following compounds were also measured: dazoxiben (Pfizer, London, U.K.), a TXA_2 synthetase inhibitor; ONO-3708 (Ono Pharmaceutical Co., Osaka, Japan) (9), a TXA_2 receptor inhibitor; the PAF antagonists CV-3988 (Takeda) (10) and CV-6209 (Takeda) (11); and AA-861 (Takeda) (12), a 12-lipoxygenase inhibitor.

Student's independent t-test was used to verify the difference between the group of stroke patients and the group of patient controls. Student's dependent t-test was used to verify the difference between before and after medication or upon adding an inhibitor; $p<0.05$ was taken to indicate a significant difference.

RESULTS AND DISCUSSION

The addition of FMLP did not alter the impedance of platelet suspensions, although it slightly increased impedance of leukocyte suspensions. However, FMLP caused a marked increase in the impedance of combined leukocyte-platelet suspensions (Fig. 1). Oda *et al.* (13) and Cöeffier *et al.* (6) had previously shown, using turbidimetric aggregometry, that FMLP induces biphasic aggregation (an immediate weak phase and a delayed strong phase) in a mixture of rabbit polymorphonuclear leukocytes (PMN) and platelets. They found only immediate aggregation when they used PMN alone; no aggregation occurred with platelets alone. These results imply that the aggregation response to FMLP is initiated by leukocytes and is later amplified by platelets (6,13). The electron microscopic study by Cöeffier *et al.* (6) provides some confirmation of this. These authors found that washed rabbit platelet suspensions never showed aggregation while

[1]Based on a paper presented at the Third International Conference on Platelet-Activating Factor and Structurally Related Alkyl Ether Lipids, Tokyo, Japan, May 1989.

*To whom correspondence should be addressed at Department of Neurology, Neurological Institute, Tokyo Women's Medical College, 8-1 Kawada-cho, Shinjuku-ku, Tokyo 162, Japan.

Abbreviations: AA, arachidonic acid; ADP, adenosine diphosphate; FMLP, formyl-methionyl-leucyl-phenylalanine; PAF, platelet-activating factor; PMN, polymorphonuclear leukocytes; TXA_2, thromboxane A_2.

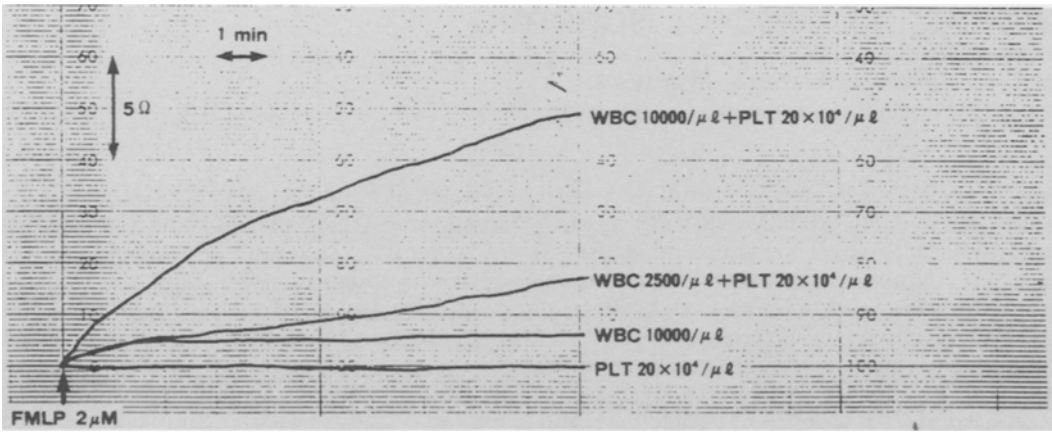


FIG. 1. Impedance changes produced by FMLP in purified platelet and/or leukocyte suspensions.

PMN suspensions showed small aggregates, and large, dense platelet aggregates were formed around the PMN aggregates in mixed suspensions.

We noted that the increase in impedance was significantly greater in patients with ischemic stroke than in controls (8.25 ± 2.03 Ω *vs* 6.77 ± 1.85 Ω, mean ± SD, $p < 0.02$). The percent reductions of leukocytes and platelets were also significantly greater in patients with ischemic stroke than in controls (48 ± 21% *vs* 33 ± 18%, 54 ± 23% *vs* 39 ± 21%, respectively; both $p<0.05$). The results for 1 μM FMLP were similar to those obtained with 2 μM FMLP. The variability of the percent reductions in leukocytes and platelets was large among the subjects with low cell counts, since these data were calculated from the ratio of the cell counts before and after adding FMLP. However, the differences between the two groups were still significant despite this variability. The results imply that leukocyte-platelet aggregation coupling is increased in patients with cerebral ischemia, since there was a significant correlation between the reduction in the leukocyte count and the platelet count in our subjects ($r = 0.64$, $p<0.05$). Arterial thrombi are composed primarily of aggregated platelets, but are also rich in granulocytes (2). Thus, platelets and leukocytes are associated in thrombosis, and their interaction may amplify the course of thrombosis.

Oral aspirin did not clearly affect the aggregation response, which suggests that the arachidonic cyclooxygenase system is not important in leukocyte-platelet interaction (Table 1). In addition, the oral intake of CV-4151, which inhibits TXA_2 synthetase and antagonizes TXA_2 receptors, did not affect the aggregation response (Table 1). Moreover, neither dazoxiben, a TXA_2 synthetase inhibitor, nor ONO-3708, a TXA_2 receptor antagonist, altered the aggregation response *in vitro*. Thus, we concluded that TXA_2 does not participate in leukocyte-platelet interaction.

By comparison, ticlopidine clearly inhibited the aggregation response stimulated with FMLP. We have previously investigated in patients with ischemic stroke the effects of aspirin and ticlopidine on platelet aggregation stimulated with adenosine diphosphate (ADP), arachidonic acid (AA) and platelet-activating factor (PAF) (14). We found that aspirin inhibited platelet aggregation by ADP and AA, but not by PAF, while ticlopidine inhibited platelet aggregation by ADP and PAF but not by AA. These results suggested that the chemical mediator of leukocyte and platelet aggregation was possibly PAF.

In order to confirm this, we studied the effects of the specific PAF antagonists, CV-3988 and CV-6209, on the aggregation response stimulated with FMLP. Preincubation of whole blood samples with 10 μM CV-3988 clearly

TABLE 1

Effects of Inhibitors on FMLP-Induced Aggregation Responses

Inhibitor	Route	Dose	N	Percent inhibition		
				Impedance	Leukocytes	Platelets
Ticlopidine	*ex vivo*	200 mg	5	32 ± 14[a]	24 ± 18[a]	34 ± 21[a]
Aspirin	*ex vivo*	81 mg	4	8 ± 7	2 ± 3	8 ± 8
CV-4151	*ex vivo*	100 mg	4	7 ± 6	3 ± 3	7 ± 6
Dazoxiben	*in vitro*	10 μM	5	10 ± 10	9 ± 8	12 ± 10
ONO-3708	*in vitro*	10 μM	5	13 ± 12	8 ± 7	12 ± 11
CV-3988	*in vitro*	10 μM	7	68 ± 32[b]	57 ± 34[b]	53 ± 28[b]
CV-6204	*in vitro*	10 μM	7	73 ± 27[b]	64 ± 32[b]	58 ± 25[b]
AA-861	*in vitro*	10μM	7	34 ± 20[a]	30 ± 19[a]	29 ± 20[a]

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

inhibited the delayed aggregation response (Table 1). This has already been reported by Oda *et al.* (13) for a rabbit PMN-platelet mixture. In addition, preincubation with CV-6209, which has 100 times the potency of CV-3988, produced a more pronounced inhibition of the aggregation response (Table 1). These results strongly suggest the importance of PAF as a chemical mediator of leukocyte-platelet interaction.

Other candidates for mediating leukocyte-platelet interaction are lipoxygenase-dependent metabolites including leukotrienes (15,16). Cöeffier *et al.* (6) reported that nazafatrom, a lipoxygenase inhibitor, suppressed the aggregation response stimulated with FMLP in rabbit whole blood. We found in the present study that AA-861, a lipoxygenase inhibitor, partially inhibited FMLP-induced aggregation in human whole blood (Table 1). These *in vitro* experiments were carried out with samples from patients or controls. There was no difference in test results between the patients and the controls.

The above results suggest that platelets tend to be activated by PAF and leukotrienes liberated from hyperaggregable leukocytes in patients with ischemic stroke. Inhibition of leukocyte-platelet interactions by drugs may be potentially valuable for treating thrombotic stroke, a disease in which leukocytes may play a pivotal role.

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REVIEW

Cardiovascular Effects of Platelet-Activating Factor[1]

Robert E. Goldstein*, Giora Z. Feuerstein[2], Linda M. Bradley[3], Joseph J. Stambouly[3], Francisco R.M. Laurindo[4] and Nancy J. Davenport[5]

Division of Cardiology, Department of Medicine, and the Division of Neurobiology, Uniformed Services University of the Health Sciences, Bethesda, Maryland 20814-4799

Sudden release of platelet-activating factor (PAF) into the circulation can cause hypotension, tachycardia, and circulatory collapse. To further examine this response, we performed detailed studies of cardiovascular function after PAF administration to young domestic pigs and newborn piglets. Our results indicate that circulatory dysfunction after PAF reflects severe constriction of pulmonary resistance vessels and consequent acute right ventricular failure. Although PAF-induced coronary artery constriction and contractile depression may be complicating problems, left ventricular underperfusion and dysfunction after PAF are mainly the result of systemic arterial hypotension and diminished left ventricular filling. The adverse hemodynamic effects of PAF are accompanied by substantial release of thromboxane A_2 (TxA_2). These effects are mimicked by the TxA_2 agonist U-46619 and partially blocked by specific and nonspecific inhibitors of TxA_2 synthesis (OKY-046 and indomethacin). Even more potent blockade of PAF action is exerted by the TxA_2 receptor blocker, SQ 29,548. Taken together, these findings indicate that severe pulmonary vascular constriction and hemodynamic collapse soon after intravenous PAF are at least partially mediated by PAF-induced TxA_2 release.

Tachyphylaxis to PAF influence has been observed in studies of leukocyte and platelet function. We hypothesized that tachyphylaxis to PAF might also occur in our studies of constrictor responses in pulmonary vessels. Recently, we have examined the capacity of PAF to produce sustained pulmonary vasoconstriction in open-chested, anesthetized newborn piglets. Infusions sufficient to produce 100% increase in mean pulmonary artery pressure after 3 min showed no loss of efficacy when sustained for 30 min. The same was true for infusions of U-46619. Thus, the pulmonary vasoconstrictor influence of PAF or U-46619 is not readily diminished by tachyphylaxis. These findings favor the viewpoint that PAF or TxA_2 release during inflammatory processes could have prolonged adverse actions on pulmonary and systemic circulations.

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Platelet-activating factor (PAF) can exert major effects on cardiovascular function. During inflammatory and hypersensitivity reactions, PAF is likely released by activated granulocytes, macrophages, and platelets (1,2). Certain stimuli, *e.g.*, thrombin exposure, also promote PAF synthesis (3) and possibly PAF release from endothelial cells. Stimulated release of PAF may be an important factor altering circulatory performance during inflammatory states.

PAF affects many cells and tissues concerned with cardiovascular function. *In vitro* studies show that PAF depresses contractility of superfused papillary muscles (4). When given to isolated hearts, PAF causes marked decrease in myocardial contractility and perfusion (5–7). Isolated, precontracted blood vessel segments respond to PAF with endothelium-dependent relaxation (2,8,9). Despite this, PAF often acts to inhibit blood flow. PAF-induced aggregation of circulating granulocytes and platelets can lead to mechanical plugging of microvessels. Moreover, PAF-initiated stimulation of granulocytes and platelets can cause release of a variety of substances with vasoconstrictor influence, *e.g.*, thromboxane A_2 (TxA_2), peptide leukotrienes, and superoxide anion (10–12).

In vivo, PAF administration can induce significant arterial hypotension and even sudden death in several species (13–18). Such exposure to PAF might occur during septic or anaphylactic shock and figure in the pathogenesis of cardiovascular collapse. The primary hemodynamic events underlying PAF-induced hypotension are uncertain. Understanding of these events might elucidate the pathophysiology of PAF-related circulatory disorders. This article will examine the influence of intravenously administered exogenous PAF (1-*O*-hexadecyl-2-acetyl-*sn*-glycero-3-phosphocholine or 16:0-AGEPC) on hemodynamic performance and on changes in a correlated metabolic parameter, the plasma level of thromboxane B_2 (TxB_2), the stable metabolite of TxA_2. The chromatographically pure 16:0-AGEPC administered in our laboratory was kindly provided by Dr. Fred Snyder, Oak Ridge Associated Universities, Oak Ridge, TN.

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The opinions or assertions contained here are those of the authors. They do not reflect the views of the Department of Defense or the Uniformed Services University of the Health Sciences. The experiments reported here were conducted according to the principles set forth in the "Guide for the Care and Use of Laboratory Animals," Institute of Laboratory Animal Resources, U.S. Department of Health and Human Services (NIH Publ. 85-23, 1985).

*To whom correspondence should be addressed at the Division of Cardiology, Department of Medicine, and the Division of Neurobiology, Room A-3060, USUHS, 4301 Jones Bridge Road, Bethesda, MD 20814-4799.

[2] Present address: Smith, Kline and French Laboratories, P.O. Box 1539, King of Prussia, PA 19406-0939.

[3] Present address: Children's Hospital National Medical Center, Washington, D.C.

[4] Present address: Instituto do Coraçáo-FMUSP, São Paulo, Brazil.

[5] Present address: Washington Hospital Center, Washington, D.C.

Abbreviations: CI, cardiac index; CO, cardiac output; LV, left ventricular; LVEDP, LV end-diastolic pressure; MPAP, mean pulmonary artery pressure; MAP, mean systemic arterial pressure; PAF, platelet activating factor (AGEPC); PVR, pulmonary vascular resistance; PVRI, pulmonary vascular resistance index; RAP, mean right arterial pressure; RV, right ventricular; RVEDP, RV end-diastolic pressure; SVR, systemic vascular resistance, TxA_2, thromboxane A_2; TxB_2, thromboxane B_2.

HEMODYNAMIC CHANGES DURING PAF-INDUCED CIRCULATORY COLLAPSE

Earlier observations showed that prolonged PAF-induced hypotension was associated with decreased cardiac output and left ventricular contractility and increased systemic and pulmonary vascular resistances (13,15). In addition, effective arterial blood volume progressively fell and hematocrit rose, suggesting plasma sequestration (13). These observations did not indicate whether such alterations were primary responses to PAF or secondary responses to a PAF-induced, protracted low output state. Less attention has been directed toward the hemodynamic changes at the beginning of PAF-induced circulatory collapse. In particular, pulmonary vascular resistance early after PAF exhibited a severe and sustained rise, along with an early but unsustained peak in pulmonary arterial pressure (15). In several models, pulmonary artery hypertension was at least partially mediated by release of TxA_2 (13–17). The impact of pulmonary vascular alterations on the pathogenesis of PAF-induced circulatory collapse remained uncertain.

We examined the possibility that PAF-induced reduction in cardiac output was primarily the result of acute right ventricular failure (*i.e.*, an acute decrease in forward stroke volume despite elevated right ventricular filling pressures and volumes) and consequent left ventricular underfilling in the setting of severe, sustained increase in pulmonary vascular resistance (PVR). The increase, in turn, would be caused by PAF-induced release of TxA_2. These possibilities were tested in several ways. Initially, we evaluated synchronous pulmonary and systemic vascular changes during PAF administration to open-chested young (10-week-old) pigs (18) or neonatal piglets (19). Then we examined the hemodynamic effects of infused U-46619, a stable TxA_2 analogue. We also compared the actions of PAF and U-46619 with those of mechanical occlusion of the main pulmonary artery. In further experiments we gave PAF to closed-chested pigs before and after blockade of TxA_2 synthesis with the nonspecific cyclooxygenase blocker, indomethacin, or the specific thromboxane synthetase inhibitor, OKY-046, while monitoring plasma levels of TxB_2.

In our first studies, anesthetized, open-chested 25-kg pigs were instrumented for simultaneous recording of contractile function (shortening of distance between implanted ultrasound crystals) in right and left ventricles as well as intracardiac pressures and cardiac output (18). Ten pigs were infused with PAF, 0.04–0.28 nmol/kg/min. Doses were begun at the low end of this range and escalated every 2 min until the appearance of systemic hypotension (mean arterial pressure decrease >50%). PAF infusion resulted in a progressive rise in PVR (Fig. 1). Initially, this was manifested as a rise in mean pulmonary artery pressure (MPAP) with little associated change in cardiac output. As PVR rose further, cardiac output began to fall along with rising systemic vascular resistance (SVR) and declining systemic arterial presssure.

Right ventricular overload during PAF infusion was manifested by increased right atrial pressure, rise in right ventricular segment length (intercrystal distance) at end-systole and end-diastole, and decrease in right ventricular segmental shortening. At this time, left ventricular segmental shortening also decreased. In contrast to right ventricular performance, however, the left ventricular segment length was smaller at end-systole and end-diastole, indicating a diminution in left ventricular dimensions. This was accompanied by a decrease in left ventricular end-diastolic pressure.

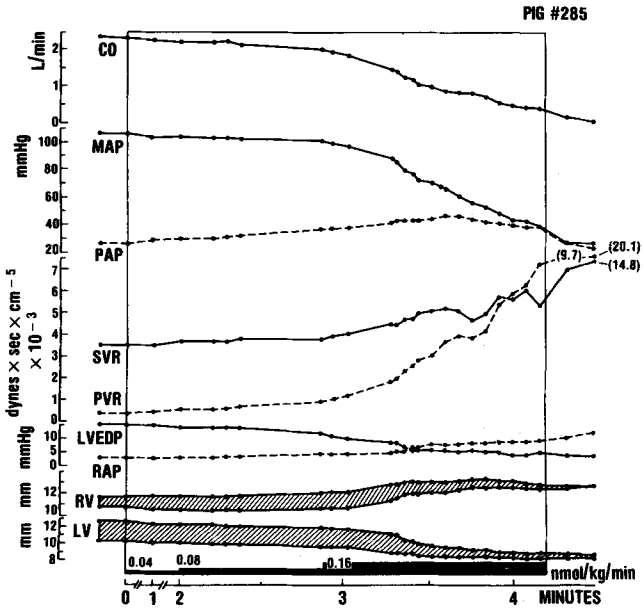


FIG. 1. Representative plot showing sequence of hemodynamic alterations induced by PAF infusion in an open-chested pig. Initial dose of 0.04 nmol/kg/min was increased stepwise to 0.16 (bottom bar). Pulmonary vascular resistance (PVR) rose initially along with an increase in mean pulmonary artery pressure (PAP). Further PVR rise was associated with a decrease in both cardiac output (CO) and PAP. Heart rate was 112–120 beats/min. At peak PAF effect, PVR surpassed systemic vascular resistance (SVR) while CO and mean systemic arterial pressure (MAP) fell markedly. Left ventricular (LV) and right ventricular (RV) segment lengths are each shown by paired lines (upper, end-diastolic length; lower, end-systolic length) separated by shading. RV segment shortening (vertical distance between line pairs) decreased despite elevated preload, as assessed by increase in RV diastolic segment length and mean right atrial pressure (RAP). LV segment shortening also fell during PAF. In contrast to RV performance, LV end-diastolic pressure (LVEDP) and end-diastolic segment length dropped significantly, indicating a marked decrease in LV preload. Reproduced with permission of the American Physiologic Society (18).

Further PAF-induced increase in PVR resulted in peaking and then actual decline of MPAP. Eventually, cardiac output fell rapidly. Despite progressively rising SVR, severe systemic hypotension supervened, often accompanied by transient bradycardia. As segmental shortening continued to fall in each ventricle, the differences between right and left ventricular performance became even more pronounced: right ventricular dimensions and filling pressures (right atrial pressures) continued to rise and left ventricular dimensions and filling pressures continued to fall. Although one animal died with prolonged right ventricular failure, the remaining nine had recovery of systemic arterial pressures to >90% of baseline within 3 min of PAF discontinuation. In the initial recovery phase, MPAP actually rose even as PVR fell from 10,800 ± 2,700 (SE) to 2,100 ± 200 dyne sec cm^{-5}. By 10 min after PAF discontinuation MPAP declined from a peak of 47 ± 2 to 29 ± 5 mm Hg; by 30 min after discontinuation, all parameters had returned to within 10% of baseline values.

The sequence of hemodynamic changes observed with PAF infusion suggested that early-appearing pulmonary vasoconstriction and right ventricular failure were important contributors to PAF-induced shock. Later-appearing decline in cardiac output, underfilling of the left ventricle, and eventual systemic hypotension primarily reflected the consequences of right-sided events.

In four pigs we attempted to reverse PAF-induced left ventricular underfilling and possible peripheral venous pooling by the rapid infusion of large volumes of 0.9% NaCl. This infusion did not mitigate any of the hemodynamic deterioration caused by PAF. On the contrary, it only elevated right ventricular filling pressures without any improvement in right ventricular output. This experience confirmed the concept that PAF-induced circulatory collapse was the result of severe pulmonary vasoconstriction and consequent right ventricular dysfunction, conditions that would not be expected to improve with further increase in already-elevated right ventricular filling pressures. It apparently excluded an important PAF-induced removal of plasma volume from the circulating pool by venodilation or extravasation.

Results similar to data from open-chested animals given PAF infusion were obtained with bolus intravenous administration of PAF to closed-chested animals (18). In 12 young (10-week-old) pigs, a low PAF dose (0.3 nmol) produced only modest rises in PVR (Fig. 2). Larger doses (>0.6 nmol) caused the marked increase in PVR and right atrial pressure and the fall in cardiac output, systemic arterial pressure, and left ventricular end-diastolic pressure seen in open-chested pigs of the same age.

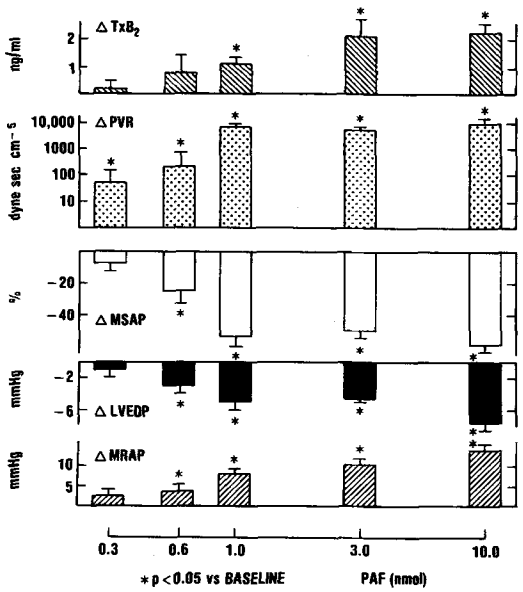


FIG. 2. Effects of PAF boluses in 12 closed-chested pigs. PAF doses were given in random order; each mean (± SE) represents 5–7 replications. Results are expressed as absolute deviation from respective baseline values, except for mean systemic arterial pressure (MSAP), which is given as percent change. Baseline values were not different for each dose (analysis of variance). Variations in PVR are depicted on a logarithmic scale. Peak PAF effects on PVR and MSAP were evident at 1 nmol, although changes in ventricular filling pressures and thromboxane B_2 (TxB_2) levels tended to be successively larger up to the 10 nmol dose. Reproduced with permission of the American Physiologic Society (18).

Thromboxane levels were also measured in animals receiving PAF. Plasma TxB_2 levels rose after PAF in a dose-related manner (Fig. 2). The magnitude of increase in TxB_2 was not consistently correlated with the corresponding change in PVR. However, animals with particularly large increases in PVR had much greater elevations of plasma TxB_2.

Because of the reactivity of neonatal pulmonary vessels and the likelihood of inflammatory pulmonary problems in neonates, additional studies of PAF-induced hemodynamic responses were conducted in 34 open-chested newborn piglets (19). In these animals as little as 0.01 nmol *i.v.* PAF produced increments in pulmonary vascular resistance and pulmonary pressure. At doses of 0.3–1.0 nmol, PVR index increased 15-fold. However, cardiac index fell, left ventricular shortening fraction declined, and systemic vascular resistance index rose only at the highest (1 nmol) dose level. These latter findings may reflect a greater ability of the neonatal right ventricle to maintain pump function despite an augmented pressure load. Our results demonstrated the capacity of PAF to induce pulmonary vasoconstriction in neonates and suggested that this inflammatory mediator may play a role in neonatal shock states that accompany infection and other disorders. Experiments with blocking agents, described subsequently, indicated that PAF-induced pulmonary vasoconstriction in newborns was at least partially mediated by release of TxA_2. In contrast, effective receptor blockade of peptide leukotrienes with LY171883 (an inhibitor of leukotriene C_4) had no influence on PAF action (19). We considered that PAF-induced release of serotonin might figure in the pulmonary vasoconstrictor responses. However, our piglet preparation exhibited no vasoreactivity in response to large intravenous doses of serotonin.

HEMODYNAMIC EFFECTS OF PAF-RELATED INTERVENTIONS

The studies just described suggested that hemodynamic collapse after PAF administration was related to PAF-induced release of TxA_2 and the consequent, marked rise in PVR. These hypotheses were further explored by assessing hemodynamic changes during infused U-46619, a potent and specific TxA_2 receptor agonist, or during mechanical constriction of the main pulmonary artery (18).

U-46619 was administered to six open-chested pigs according to the same protocol used for PAF—initial dosage of 0.3–3.0 nmol/kg/min was increased every 2 min until hypotension supervened. Hemodynamic effects were very similar to those seen with PAF infusion. A progressive, marked rise in PVR (eventually increasing 33-fold) was associated with an initial rise in mean pulmonary artery pressure and then a decline. Falling cardiac output, systemic vascular resistance, and ultimate, severe drop in systemic arterial pressure all appeared with U-46619 infusion, replicating the results seen with PAF infusion. Similarly, both right and left ventricles exhibited decreased segmental shortening in this later phase of infusion with the right ventricle manifesting signs of pressure and volume overloading (rising right atrial pressures and right ventricular segmental dimensions) and the left ventricle showing indications of underloading (falling left

ventricular end-diastolic pressure and left ventricular segmental dimensions). Thus, U-46619 administration caused systemic hemodynamic actions that were essentially the same as the actions of PAF infusions with similar pulmonary vasoconstrictor efficacy.

Because dose equivalence of TxA_2 and U-46619 has not been established, the interpretation of these infusion data is open to question. Nonetheless, the results emphasize the capacity of TxA_2 receptor activation to reproduce the sequence of PAF-induced hemodynamic changes leading to circulatory collapse. TxA_2 release is very likely a contributor to PAF-induced hypotension. If present in sufficient quantity, TxA_2 might well mediate the entire phenomenon.

Stepwise mechanical occlusion of the main pulmonary artery in five open-chested pigs caused a sequence of hemodynamic responses very similar to those observed during infusion of PAF or U-46619. Early changes included a rise in right ventricular systolic pressures with later decline accompanied by a steep fall in cardiac output and systemic arterial pressures. Segmental shortening was markedly reduced in both the left ventricle (12 ± 2% to −0.9 ± 1.6%) and right ventricle (6 ± 1 to 1.5 ± 2.1%). As in the case of infused PAF or U-46619, decrease in myocardial shortening was accompanied by a fall in indices of left ventricular filling (left ventricular end-diastolic pressures and end-diastolic length) and a rise in indices of right ventricular filling (mean right atrial pressure and right ventricular end-diastolic segment length).

The similarity of hemodynamic events after administration of PAF or U-46619 and after mechanical pulmonary occlusion suggests that the observed circulatory actions of these drugs are mainly due to pharmacologically-induced pulmonary vasoconstriction. Additional hemodynamic consequences, including ultimate circulatory collapse during PAF or U-46619 infusion, may reflect relatively non-specific responses to acute, severe rises in right ventricular afterload. The acutely overloaded right ventricle fails to propel blood forward against massive resistance. Backward leakage occurs due to dilation of the tricuspid valve ring. The failing right ventricle cannot sustain flow delivery to the left side of the heart. Although systemic arterial pressure is supported transiently by baroreceptor-mediated compensatory increases in systemic vasoconstriction, severe hypotension ultimately supervenes. Hypotension of any cause is likely to exaggerate this situation by inducing myocardial ischemia and diminished contractility, particularly in the overloaded right ventricle.

In vitro studies have shown that PAF exerts primary depressant effects on cardiac muscle (5–7). The rather close resemblance of PAF infusion and mechanical pulmonary artery constriction suggests that this depressant action plays a relatively modest role in the context of our *in vivo* experiments.

EFFECTS OF MORE PROLONGED EXPOSURE TO PAF

To date, studies of exogenous PAF have only administered the substance as a bolus or brief infusion. During inflammatory disorders, however, PAF release would presumably occur over a more prolonged period. The circulatory effects of sustained PAF administration may be important in assessing the potential role of PAF in shock states. PAF influence might actually disappear with prolonged exposure. Studies of isolated platelets or leukocytes have suggested that PAF exposure rapidly induces tachyphylaxis—PAF unresponsiveness—due to unavailability of PAF receptors (20). Should this phenomenon occur *in vivo,* it would question the relevance of PAF to circulatory disorders that accompany inflammation.

Recently, we have evaluated the capacity of PAF to produce sustained pulmonary vasoconstriction in nine open-chested newborn piglets. After baseline parameters were measured, PAF was administered in rapidly escalating intravenous doses to achieve a 100% increase in mean pulmonary artery pressure within 3 min. The dose that achieved this effect, averaging 0.15 nmol/kg/min, was then maintained for another 30 min. Throughout this period of constant infusion, pulmonary vascular resistance and mean pulmonary artery pressure remain steadily elevated (Fig. 3). Thus, there was no evidence of tachyphylaxis to infused PAF in the intact circulations of newborn piglets. Systemic vascular resistance and other parameters of systemic circulatory function remained undisturbed by the PAF infusion. These data indicated that there was no cumulative circulatory dysfunction during relatively prolonged exposure to PAF. Hemodynamic changes might persist after discontinuation of PAF infusion if these were due to PAF-induced microvessel plugging or other self-perpetuating inflammatory phenomenon. In our newborn piglets all hemodynamic parameters reverted to baseline values within 3 min of stopping PAF infusion. When PAF infusion was repeated at the same dose rate after administration of the selective PAF receptor blocker, SRI 63-441, 5 mg/kg i.v., its effects were entirely eliminated. Moreover, giving SRI 63-441 20 min after initiating a 30

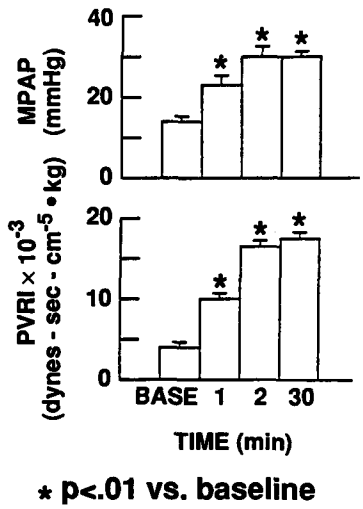


FIG. 3. Time course of MPAP and pulmonary vascular resistance index (PVRI) in nine newborn piglets receiving an intravenous infusion of PAF. Infusion rates were initially adjusted upward to achieve stable doubling of MPAP after 2 min and then maintained constant for the ensuing 30 min. Mean values are shown ± SE. Between 2 and 30 min, both MPAP and PVRI remained constant, indicating that PAF-induced pulmonary vasoconstriction did not exhibit tachyphylaxis. Moreover, the potent PAF infusion did not cause cumulative toxic effects on the neonatal circulation. Measured parameters reverted to baseline as soon as the infusion was halted.

min steady PAF infusion interrupted the pulmonary vasoconstriction induced by PAF. These findings indicated that sustained PAF receptor stimulation was responsible for continuing PAF efficacy and further documented the rapidly reversible nature of PAF action on the pulmonary vasculature.

PAF INHIBITION RESULTING FROM BLOCKADE OF TxA_2

Mechanisms of PAF action may vary depending upon the way in which PAF is tested. When the heart is perfused with cell-free, PAF-containing fluids (5–7), actions mediated by PAF influence on circulating elements are excluded. When distributed *via* the intact circulation, however, PAF is likely to induce release of inflammatory mediators from granulocytes, platelets, endothelium, and other cells in intimate contact with circulating blood. Such mediators may be responsible for important components of PAF action on the intact circulation.

In studies of intravenous PAF administration to intact pigs we found that PAF-related changes in hemodynamic performance were regularly accompanied by increases in plasma TxB_2 (14,18,19). This suggested—but clearly did not prove—that TxA_2 production was involved in PAF-induced pulmonary vasoconstriction and other PAF-induced changes. To further explore the potential mediating role of TxA_2, we evaluated the capacity of blockers of TxA_2 synthesis and TxA_2 receptor activation to alter responses to intravenous PAF (18,19,21).

Cyclooxygenase blockade. Eleven closed-chested pigs were tested with three bolus doses of PAF, 0.1–10 nmol *i.v.* (18). These animals were then given indomethacin, 6 mg/kg, and the dosing sequence repeated. A submaximal PAF dose, 3 nmol, produced severe hemodynamic changes: PVR rose 16-fold and systemic arterial pressure fell by 51%. These changes and an associated rise in plasma TxB_2 were eliminated after indomethacin. The maximal 10 nmol PAF dose caused even greater changes: in the absence of indomethacin, PVR rose 28-fold and systemic arterial pressure fell by 59%. Plasma TxB_2 rose from 0.71 ± 0.18 to 3.03 ± 0.32 ng/mL. After indomethacin, PVR still increased 13-fold and arterial pressure decreased 30% ($p<0.05$ for either change), although these were diminished from pre-indomethacin values. The PAF-induced rise in plasma TxB_2 was eliminated by indomethacin: after this cyclooxygenase blocker, TxB_2 was 0.74 ± 0.18 before PAF and 0.76 ± 0.06 after PAF. Cyclooxygenase blockade with indomethacin proved sufficient to eliminate effects after all but the highest dose of PAF. These data suggest that TxA_2 is an important mediator of the circulatory actions of PAF even when PAF is introduced in amounts sufficient to produce marked hypotension. They also suggest that very-high-dose PAF has an ability to induce pulmonary vasoconstriction independent of cyclooxygenase metabolites, *e.g.*, by direct agonist action on vascular smooth muscle or by induced release of alternative mediators. Lipoxygenase products, such as the peptide leukotrienes might be elaborated when PAF-released arachidonate is shunted to alternative pathways due to cyclooxygenase blockade. Any vasoconstrictor release would be amplified by the absence of vasodilator cyclooxygenase products.

Indomethacin produced similar blockade of PAF action in nine newborn piglets (19). The marked rise in pulmonary artery pressure and PVR induced by PAF, 0.01–0.03 nmol *i.v.*, was eliminated after indomethacin, 6 mg/kg. PAF, 1 nmol, was only partially blocked after indomethacin; systemic effects were not apparent but pulmonary artery pressure and PVR rose significantly. Plasma levels of TxB_2 and 6-keto-$PGF_{1\alpha}$ (the stable metabolite of prostacyclin) showed complete inhibition of synthesis *via* cyclooxygenase pathways after indomethacin, even with the 1 nmol PAF dose.

TxA_2 synthetase blockade. To further evaluate the role of TxA_2 as mediator of PAF effects, we examined the capacity of a selective inhibitor of TxA_2 synthetase, OKY-046, to modulate PAF action in six closed-chested pigs (18). OKY-046 significantly reduced the elevated PVR (by 71%) and attenuated the augmented systemic vascular resistance (by 63%) that were seen when PAF, 1 nmol, was given without OKY-046. Similarly, OKY-046 lowered the increased pulmonary artery pressure (by 22%) and tended to diminish other effects produced by PAF, 0.3 nmol. OKY-046 was not consistently effective against PAF, 3 nmol. OKY-046 did reduce plasma TxB_2, even after PAF, 3 nmol: levels were 0.87 ng/mL with PAF alone and 0.40 with PAF plus OKY-046. Similar reductions in plasma TxB_2 were evident after lower doses of PAF plus OKY-046. In agreement with previous studies, 6-keto-$PGF_{1\alpha}$ increased from 0.06 ± 0.02 to 0.24 ± 0.04 ng/mL after PAF, 1 nmol, indicating PAF enhancement of prostacyclin production. The same PAF dose during thromboxane synthetase inhibition with OKY-046 increased 6-keto-$PGF_{1\alpha}$ to 0.50 ± 0.08 ng/mL, significantly ($p<0.05$) greater than levels after PAF alone (22).

In our studies OKY-046 was effective in blocking the hemodynamic actions and rise in plasma TxB_2 after PAF. These results provide further support for the hypothesis that circulatory actions of PAF are mediated to a substantial degree through PAF-induced release of TxA_2. OKY-046 did not appear to block PAF as effectively as indomethacin. This may reflect a lesser ability of OKY-046 to prevent TxA_2 synthesis, although our values for plasma TxB_2 do not consistently favor this viewpoint. It should be noted that thromboxane synthetase inhibitors may increase levels of TxA_2 precursor metabolites, PGG_2 and PGH_2, substances that can exert significant TxA_2 agonist properties. Increased production of PGG_2 and PGH_2 may explain dose-related increases in pulmonary vasoconstriction after PAF and OKY-046 despite attenuation of TxB_2 levels.

Evidence of an augmented rise in prostacyclin production was seen when PAF was accompanied by OKY-046. This may represent a redirection of arachidonate from synthesis of TxA_2 to synthesis of prostacyclin, a novel occurrence *in vivo*. It is possible that OKY-046 could convert local production of vasoconstrictor and platelet-aggregator substances such as TxA_2 to local production of the vasodilator and platelet-disaggregator, prostacyclin. Although such redirection may have value in other contexts, it is noteworthy that PAF accompanied by OKY-046 caused more (rather than less) pulmonary vasoconstriction than PAF accompanied by indomethacin and the presumed elimination of both prostacyclin and TxA_2 synthesis.

TxA_2 receptor blockade. Simultaneous administration of the thromboxane receptor antagonist SQ 29,548

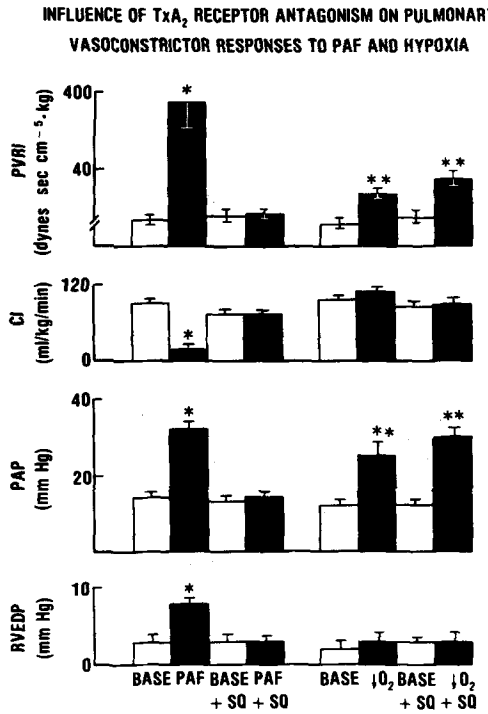


FIG. 4. Influence of thromboxane A_2 (TxA_2) receptor antagonism on pulmonary vasoconstriction due to PAF or hypoxia ($\downarrow O_2$). Both PAF, 0.1 nmol/kg, and hypoxia caused significant rises in pulmonary artery pressure (PAP) and PVRI when compared to baseline. PAF administration also caused right ventricular end-diastolic pressure (RVEDP) to increase and cardiac index (CI) to decrease. Baseline values were not different before and during infusion of the TxA_2 blocker, SQ 29,548. TxA_2 receptor antagonism completely blocked PAF-induced pulmonary vasoconstriction and the related fall in CI; hypoxic pulmonary vasoconstrictor responses remained intact. Results are expressed as absolute means ±SE. PVRI data are multiplied by 10^{-3} and displayed on a logarithmic scale. BASE, baseline; BASE+SQ, baseline during SQ 29,548 infusion; PAF+SQ, PAF during SQ 29,548; $\downarrow O_2$+SQ, hypoxia during SQ 29,548. *p<0.05 *vs.* BASE; **p<0.01 *vs.* BASE. Reproduced with permission of the International Pediatric Research Foundation (21).

appeared to block the circulatory effects of PAF at least as effectively as any of the agents tested to date (21). Thirteen open-chested newborn piglets were given PAF doses (0.1 nmol/kg *i.v.* bolus) causing near-maximal hemodynamic response. The same PAF dose was repeated during administration of SQ 29,548 (0.05 mg/kg/min *i.v.*) or its vehicle. The first PAF dose markedly raised PVR from 9.4 ± 1.4 to 340 ± 66 dynes sec cm^{-5}·kg with concomitant reductions in cardiac index (91 ± 5 to 15 ± 5 mL/kg/min) and systemic arterial pressure (62 ± 4 to 37 ± 4 mm Hg). Repeat dosing with PAF plus vehicle produced the same hemodynamic response. Repeat dosing with PAF plus SQ 29,548, however, completely eliminated these hemodynamic changes (Fig. 4). PAF-induced rises in TxB_2 and 6-keto-$PGF_{1\alpha}$ were unaffected by the TxA_2 receptor blocker. In the absence of PAF, SQ 29,548 had no effect on our preparation. Additional studies of blocker specificity showed that SQ 29,548 was a highly effective inhibitor of the TxA_2 mimetic agent, U-46619, but did not alter pulmonary vasoconstrictive actions of hypoxia or leukotriene D_4.

The PAF-inhibitory efficacy of SQ 29,548 provides further support for the concept that TxA_2 is an important mediator of the circulatory actions of PAF *in vivo*. TxA_2 receptor blockade may be a particularly potent intervention because there is no concomitant interference with synthesis of prostanoid vasodilators, as seen with cyclooxygenase inhibition and no enhancement in levels of precursors with TxA_2 agonist activity, as is likely the case with thromboxane synthetase inhibition.

CONCLUSIONS

During inflammatory states, activated leukocytes, platelets, or other cells may release PAF in amounts sufficient to alter cardiovascular function. This possibility is suggested by the capacity of PAF inhibitors to attenuate endotoxic shock (23–26) as well as the ability of exogenous PAF to produce shock when administered by vein to intact animals (13–18).

Assuming that PAF delivery by vein has pathophysiologic importance, we examined mechanisms by which PAF might induce hypotension. Our results in intact animals indicate that pulmonary vasoconstriction is a major mediator of PAF-induced hypotension. This pulmonary vasoconstriction leads to right heart failure, manifested as declining cardiac output accompanied by increased right ventricular filling pressures and overstretched right ventricular myocardium as well as reduced right ventricular shortening fraction. Inadequate flow delivery to the left side of the circulation causes left ventricular underfilling, seen as reduced filling pressures and volumes as well as declining contractility. Although rising systemic vascular resistance can transiently counter the effects of reduced cardiac output, eventually hypotension supervenes.

The sequence just described was seen in neonatal as well as older animals. The prominent pulmonary vascular response to PAF was evident in sustained infusions as well as brief bolus presentations. These findings suggest that PAF may figure in disorders of the very young and that tachyphylaxis to PAF, a common observation in isolated cells and tissues, is not seen in circulatory responses.

It should be noted that experiments in our laboratory used only a single molecular species, 16:0-AGEPC, in evaluating the actions of PAF. Other closely related molecular species, such as 18:0-AGEPC, may have somewhat different effects on the cardiovascular system.

The circulatory effects of PAF appear to involve the release of TxA_2. Pulmonary vasoconstriction and other manifestations of circulatory distress after PAF are regularly accompanied by rises in the TxB_2, the stable metabolite of TxA_2, in rough proportion to the extent of hemodynamic change. Administration of a TxA_2 mimic, U-46619, produces hemodynamic changes very similar to those of PAF. Most importantly, specific and non-specific blockers of TxA_2 synthesis and a blocker of TxA_2 receptors each had the capacity to abridge or totally interrupt the circulatory actions of PAF. The cardiovascular effects of PAF may ultimately involve release of other inflammatory mediators, *e.g.*, lipoxygenase products. However, our findings identify a preponderant role for TxA_2. They suggest that pharmacologic strategies for interrupting the synthesis or receptor agonist actions of TxA_2 may well prove as effective as PAF blockers in attenuating the cardiovascular consequences of PAF exposure.

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REVIEW

Platelet-Activating Factor in Cardiovascular Stress Situations[1]

Reuven Rabinovici, Tian-Li Yue and Giora Feuerstein*
Cardiovascular Pharmacology, SmithKline Beecham Laboratories, King of Prussia, Pennsylvania 19406-0939

Since the elucidation of its chemical structure two decades ago, platelet-activating factor (PAF) has emerged as an important mediator of various cardiovascular stress situations. Most notably, PAF was implicated as a key factor in the septic shock syndrome, based on the similarities between endotoxin and PAF biological effects, the elevation of circulating and tissue levels of PAF during endotoxemia, and the protective effect of PAF antagonists in the septic state. In addition, accumulating data suggest the involvement of PAF in the pathophysiological processes associated with ischemia, hemorrhage and trauma, where PAF exerts its effects directly on cells and blood elements or indirectly through interactions with other mediators such as cytokines and prostaglandins. Nevertheless, the relative contribution of PAF to the pathophysiological processes in endotoxemia is still unknown and should await further investigations. The primary aims of this chapter are: to delineate the effects of PAF on the cardiovascular system, to summarize the data which suggest the involvement of PAF in stress situations of the cardiovascular system, and to identify areas where future experimental efforts should be focused.
***Lipids 26,* 1257–1263 (1991).**

Platelet-activating factor (PAF, PAF-acether, AGEPC, APRL) was first found to be released from rabbit basophils through an IgE-dependent mechanism (1), and its chemical structure was later identified as 1-*O*-alkyl-2-acetyl-*sn*-glycero-3-phosphocholine (2,3). The synthetic preparation of PAF and the introduction of specific PAF antagonists contributed profoundly to the accelerated research on PAF biology and pathophysiology. Consequently, within a short period of time PAF has become recognized as one of the major mediators of inflammatory and tissue injury reactions, including various shock states.

The purpose of this chapter is to give an overview on the state of the art of PAF in the cardiovascular system with special emphasis on the role of PAF in the pathophysiology of septic, traumatic and hemorrhagic shock.

PAF METABOLISM

PAF is generated by various organs and cells. The predominant cells shown to produce PAF are macrophages, neutrophils, natural killer lymphocytes, platelets, and endothelial cells (4–6). Kidney (7), heart (8,9), brain (10,11), lung (12), and intestine (13) are the predominant sites for PAF synthesis. However, in every cell where PAF production has been shown, it follows the basic scheme illustrated in Figure 1. PAF synthesis occurs in the intracellular compartment (14–16), whereas its inactivation occurs in both the cell (17,18) and the plasma (19–21). PAF is stored within cellular membranes in the form of its precursor, alkylacyl-glycerophosphocholine (alkylacyl-GPC). The stimulation-dependent action of phospholipase A_2 on this precursor results in the production of lyso-PAF, an inactive intermediate substance. The lysoPAF is then metabolized to PAF by the acetyl transferase system. Some of the newly produced PAF is inactivated within the cell and converted back to alkylacyl-GPC via an opposing pathway which includes the sequential catalytic activity of acetylhydrolase and acyltransferase. Part of those PAF molecules that reach the circulation are inactivated rapidly in the plasma through the conversion to lysoPAF, a process catalyzed by plasma acetylhydrolase. This reaction was suggested to determine the short half-life of PAF, <30 min (20), and to explain the short duration of its low-dose hypotensive effect (22). The remaining circulating PAF interacts with its cellular receptor to induce biological response, and then after leaving the receptor and crossing the membrane, is inactivated by the intracellular pathway.

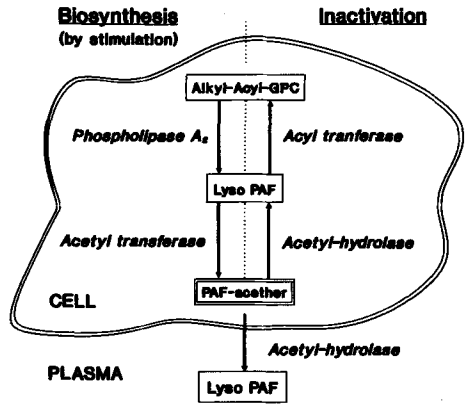


FIG. 1. The metabolic cycle of PAF.

CARDIOVASCULAR EFFECTS OF PAF

Systemic hemodynamic. PAF, when administered systemically, induces a prominent hypotensive effect in all species studied (23–27). Nevertheless, this effect was most thoroughly investigated in the rat (Table 1). The PAF-induced hypotension has several characteristics: i) it is dose-dependent (20), ii) its action is very rapid and reaches maximal effect within 30–60 sec (28,29), iii) recovery time is dose-dependent, and basal mean arterial pressure is

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*To whom correspondence should be addressed at Cardiovascular Pharmacology, SmithKline Beecham Laboratories, P.O. Box 1539, King of Prussia, PA 19406-0939.

Abbreviations: GM-CSF, granulocyte-monocyte colony-stimulating factor; IL, interleukin; MDF, myocardial depressant factor; PAF, platelet activating factor; TNF, tumor necrosis factor.

TABLE 1

Hypotension and Recovery Time of MAP to Regain Basal Values, Induced by Intravenous Administration of PAF in the Rat

Study author	Reference	Dose (nmol/kg)	Δ MAP	Time of max effect	RT (min)[a]
Chang *et al.*	(30)	0.02	−23%	1 min	—
Sanchez-Crespo *et al.*	(115)	1.0	−44 mmHg	—	4.6
		4.8	−92 mmHg	—	20.5
		19.1	s.d.[b]		
Doebber *et al.*	(29)	0.6	−70 mmHg	30 sec	10-12
Terashita *et al.*	(116)	1.9	−52%	1 min	60
Handley *et al.*	(117)	0.2	−39%	few sec	3.2
Siren and Feuerstein	(28)	0.1	0	—	—
		0.3	−9%	30 sec	5
		1.0	−35%	30 sec	60
		3.0	−47%	30 sec	—
Zukowska-Grojec *et al.*	(27)	1.3	−48 mmHg	1 min	5
Feuerstein *et al.*	(26)	0.7	−36%	—	—
		3.3	−57%	—	—
		16.7	−76%	—	—

[a]RT, recovery time. [b]Sudden death = s.d.

obtained within 5-10 min (28,29), and iv) it does not show tachyphylaxis (29,30). Although the mechanisms involved in PAF-induced hypotension are not yet fully understood, accumulating data suggest that its actions on the heart, peripheral vasculature and microcirculation play a key role in the pathogenesis of this biological response. These specific actions will be discussed below. Both PAF-induced increase in pulmonary vascular resistance (31,32) and PAF-induced bronchoconstriction (33) contribute to hypoxemia and hence reduction in oxygen delivery to essential organs such as the heart. Ultimately, this process may result in reduced cardiac output and shock. The platelet's sensitivity to PAF was suggested to mediate the PAF-induced bronchoconstriction (33). However, the species-specificity of the PAF-induced thrombocytopenia in guinea pigs (34) but not in the rat (35), might indicate that the vasodepressor response is independent of the respiratory or hematologic effects of PAF. In addition, studies on PAF-induced hypotension ruled out renin, vasopressin, and angiotensin II inhibition, central mechanism, and α-adrenergic blockade as important contributing factors (36).

The PAF-induced hypotension is usually associated with tachycardia. Several observations support the possibility that this response reflects a hypotension-triggered sympathetic reflex: i) hypotension induced by PAF was accompanied with elevated levels of plasma catecholamines (26,27), ii) β-adrenergic receptor blockers completely prevented the tachycardic response (26,27), and iii) pithed rats in which the spinal cord and central nervous system were completely disrupted did not elevate their heart rate in response to PAF administration (26).

Cardiac. There is still considerable debate in regard to the effect of PAF on the coronary circulation, and its systemic hemodynamic significance. While some *in vitro* studies using guinea pig, dog or rat isolated perfused hearts demonstrated PAF-induced coronary vasoconstriction (9,37-40), other studies failed to report such response (41). *In vivo* studies, conducted in the domestic pig and dog, showed an initial increase in coronary flow (due to vasodilatation) followed by a decrease in flow (due to coronary constriction) (42-44). The coronary vasoconstrictive response to PAF seem to be mediated, at least in part, by thromboxane A_2 since it was associated with selective thromboxane B_2 production, and was blocked by the cyclooxygenase inhibitor indomethacin. The relative contribution of the coronary flow changes produced by PAF to the overall reduction in myocardial performance is difficult to assess in view of the overall cardiac pathophysiology induced by PAF via several different types of cardiovascular dysfunction.

In respect to myocardial function, some controversy exists as to whether PAF exerts a direct negative inotropic effect on the cardiac muscle. Several *in vivo* and *in vitro* studies which supported this possibility were not conclusive since they did not completely separate the direct effect of PAF on the myocardium from its effects on the coronary and intra-mural hemodynamics: i) Low-dose PAF infused intra-coronary in the domestic pig caused substantial reduction in the myocardial contractility at times where no changes in mean arterial and left ventricular pressures were recorded (45,46). In the rat, however, PAF reduced cardiac index only at doses which produced hypotension (28). ii) PAF reduced the contractile force of several isolated perfused heart preparations (9,40,47,48). However, other investigators, using models which could reasonably exclude neural, humoral and hemodynamic mechanisms, also suggested a direct effect on the myocardium—PAF compromised the myocardial function of human cardiac muscle strips (49), and of paced non-coronary perfused left atrium and ventricular papillary muscle (9,50).

The possibility that PAF might be involved in direct myocardial dysfunction was also obtained in ischemia-reperfusion models; in rabbits, PAF administration during the post-ischemic reperfusion period augmented the decrease in cardiac performance (51), and PAF was found to be released in significant amounts during the initial reperfusion period (52). PAF was also elevated in lipid extracts from ischemic baboon heart 24 hr after ligation of

the left anterior descending artery (53). Recently, PAF was suggested to play a role in the "stunned myocardium" syndrome following ischemia and reperfusion (54). Pharmacological studies with several PAF antagonists also support the possible direct effect of PAF on cardiac functions. For example, BN 52021, BN 52020, kadsurenone and L-652,731 attenuated the PAF-induced decrease in myocardial contractility and coronary flow in the perfused guinea pig heart (55–59).

The pathogenic mechanisms of PAF-induced myocardial dysfunction are poorly understood. However, interference by PAF with Na^+ conductance (46) or with Ca^{2+} flux through the slow Ca channels (47,60) was suggested.

PAF was found to interfere with the cardiac conducting system, and to produce lethal arrhythmias (24). Moreover, purkinje fibers of guinea pigs demonstrated PAF-induced electrophysiological changes such as abnormal automaticity and decreased action potential amplitude (61). Interestingly, similar effects were found in the papillary muscle as well.

Peripheral vasculature. The effect of PAF on blood vessels has not yet been established, mainly due to the following factors: i) the direct *in vivo* effect of PAF on blood vessels is masked by the profound sympathetic vasoconstrictor reflex triggered by the PAF-induced hypotension; ii) PAF effects on blood vessels seem to be dose- and species-dependent. However, if selected organs are perfused under constant pressure with low-dose PAF, the common response is vasodilatation. This response was demonstrated in the coronary circulation of the domestic pig (42) and the dog (43). Moreover, vasodilatory response of the kidney, mesenteric and hindquarters vessels (measured by chronically implanted miniaturized doppler probes) was produced by intravenous doses of PAF which had no or minimal systemic effects (25). In addition, mesenteric vasodilatation was also produced following topical application of PAF (62). The vasodilatory effect of PAF was also confirmed in an *in vitro* study of isolated rat kidneys (63). Nevertheless, other studies reported PAF-induced vasoconstriction in both *in vivo* and *in vitro* preparations such as the pig pulmonary vessels (64–66) and dog renal vessels (67,68). This response was shown in some models to be thromboxane A_2 and peptidoleukotriene dependent (31,42,69).

Microcirculation. PAF was shown to increase the permeability of the microcirculation in all species studied (35,70,71) including humans (72). This phenomenon was shown in several models including intra-dermal PAF injection (71), and ^{125}I (30) and dye concentration measurement (73) following i.v. PAF injection. The change in capillary permeability leads to hemoconcentration and decrease in intravascular volume. Although some studies suggested that the contracted intravascular volume contributes to the PAF-induced hypotension and decrease in cardiac output (70), it is possible that the hypotension and the permeability defect are two independent events. The latter hypothesis is supported by the fact that hypotension occurs at lower doses and is immediate and reversible (74), whereas the extravasation response requires 4–10 min to peak and several hours to reverse (75–78).

The mechanism by which PAF increases the permeability of the microcirculation is unknown. However, PAF was shown to disrupt endothelial cell junctions and to cause vacuolization (71). Generally, PAF exerts its effects directly on the microcirculation through interaction with endothelial wall receptors (78,79). The permeability defect is augmented by histamine, associated with prostaglandin I_2 production (80) and increased Ca^{2+} fluxes (81), and might involve polymorphonuclear leukocyte adhesion and activation (82). Since PAF and several other phospholipid mediators can induce the release of each other, it is possible that positive self-generated feedback cycles contribute to the pathogenic mechanism by which PAF damages the microcirculation. For example, PAF induces peripheral blood-derived monocytes to produce tissue necrosis factor (TNF), and enhances the endotoxin-induced TNF synthesis.

These effects are prevented by various PAF antagonists (83). Furthermore, PAF was shown to modulate the production of interleukin 1 (IL-1) and IL-2 from rat monocytes (84) and lymphocytes (85). On the other hand, TNF and IL-1 (but not IL-2, IL-3, IL-6 and colony-stimulating factor) induce PAF synthesis by cultured endothelial cells (86). In addition, TNF can prime PAF-induced superoxide generation by human neutrophils (87), and granulocyte-monocyte colony-stimulating factor (GM-CSF) facilitates PAF synthesis by human polymorphonuclear leukocytes (88). Interestingly, GM-CSF, in combination with IL-3, also induced monocytes to produce TNF (89). Although the regulatory mechanisms of this feedback network under physiological conditions and in shock states are unknown, several endogenous inhibitors of PAF, TNF, IL-1 and IL-2 have been reported (87). The fact, that endothelial cells were shown to produce and metabolize PAF (90) in a PGI_2-regulated process, may suggest another positive feedback cycle in the pathogenicity of PAF.

Blood cells. The PAF-platelet interactions, which probably play a key role in the pathogenicity of PAF, are not clearly understood. Controversy exists as to: i) the effect of PAF on platelet count—PAF consistently induced thrombocytopenia in the rabbit (55,91) or guinea pig (24) but not in the rat (35). The thrombocytopenic response to PAF may reflect PAF-induced platelet aggregation (92), PAF-induced platelet redistribution due to pulmonary platelet recruitment (93), or a combination of these two mechanisms. ii) the mechanism of PAF effect on platelets—PAF was hypothesized to mediate a "third pathway of platelet aggregation" since neither its production by stimulated platelets nor its effects were ADP or arachidonic acid-dependent (92). However, several recent studies cast doubt on this possibility. For example, several PAF antagonists failed to prevent thrombin-induced aggregation of aspirin-treated ADP-depleted platelets (94). Despite the possible role of arachidonic acid metabolites and ADP in the activation of platelets by PAF, it seems that PAF acts through a specific mechanism since it activates aspirin-treated or ADP-depleted platelets (95). Platelets activated *in vitro* by PAF secrete a variety of secondary mediators such as serotonin, leukotrienes, prostaglandins and oxygen radicals. Moreover, the PAF-stimulated platelets form a propagating thrombus (96) which is surrounded by leukocytic infiltration over the adjoining vacuolized epithelium. Thus, it seems that PAF-activated platelets interact with other blood elements which produce and secrete inflammatory mediators. The end point of these processes is tissue injury or thrombus formation.

Systemic administration of PAF leads to leukopenia in most species studied (24,55,91). However, PAF-induced leukocytosis following high *in vivo* doses was reported as well (97). These effects are associated with leukocyte activation and cell margination. On the other hand, stimulated neutrophils (98), monocytes (99), macrophages (100), mast cells (101), and eosinophils (98) produce PAF. Of great importance is the fact that PAF-induced permeability capillary defect (88), pulmonary injury (102) and anaphylactic reactions (103) were all associated with leukocytic activation and aggregation. Therefore, it is possible that leukocytes are activated directly by PAF or indirectly by platelets (see above), and so facilitate ultimate tissue injury.

PAF IN SHOCK STATES

Septic and endotoxic shock. PAF was recently implicated as one of the major mediators of septic and endotoxic shock. However, there is only little biochemical evidence that PAF is produced during specific organ or systemic pathophysiological processes. This is the result of the short half-life of PAF (hydrolyzed rapidly in the plasma) combined with the limitations of the techniques employed for PAF extraction and assay. However, elevated levels of PAF (measured by a rabbit platelet aggregation bioassay after partial purification) were found during *E. coli* endotoxemia in rats (29). The delayed appearance of PAF following the endotoxin administration (at 10 min following the injection of endotoxin but not at 1.5 min) correlated with the LPS-induced hypotension, which only began 3 min following injection. Since PAF decreases systemic arterial pressure immediately, it is conceivable that the delayed elevation of circulating PAF level reflects the time required for PAF synthesis and secretion. Furthermore, enhanced release of PAF (measured by the [^{3}H]serotonin release from washed rabbit platelets) from spleen lymphocytes and peritoneal cells taken from rats exposed to *E. coli* peritonitis was also reported (104). Significant amounts of PAF in the peritoneal protein-rich exudate could be obtained already at 30 min after the inoculation of *E. coli,* while PAF in peritoneal and splenic leukocytes was elevated only at 2 hr following the injection of endotoxin. The endotoxin-induced elevation of PAF levels in the peritoneal and splenic cells occurred before the PAF-induced blood volume depletion, suggesting that hemoconcentration and volume contraction are secondary to PAF production. Additional biochemical evidence for the production of PAF during endotoxemia was presented by Chang *et al.* (30) who reported a three-fold elevation in plasma PAF, and a ten-fold elevation in whole lung homogenate PAF, 20 min following intraperitoneal injection of Salmonella enteritidis. It is important to note that these levels persisted for at least 2 hr. In the kidney, PAF values 90 min after endotoxin administration were significantly increased over the controls (30). Both the baseline and post-endotoxin renal PAF levels were markedly lower than those found in the lung. The persistent elevation of plasma PAF levels might cause other organs to experience prolonged PAF exposure, although no direct PAF production took place in the specific organ. Interestingly, vinblastine-induced leukocyte depletion did not reduce the endotoxin-induced PAF production in the lung. These data, taken together with the *in vivo* and *in vitro* reports of PAF release from injured pulmonary endothelial cells (105,106), suggest that these cells play a major role in PAF production during septic shock, and negate a neutrophil source for the accumulation of PAF in the lungs.

While only few studies actually measured circulating PAF levels in septic shock, most of the data concerning the role of PAF in endotoxemia were obtained by *in vivo* experiments with PAF antagonists. The majority of the studies investigated the effect of various PAF antagonists on LPS-induced hypotension (Table 2). Other studies, using a large variety of PAF antagonists, have shown protection or reversal of endotoxin-induced leukopenia, thrombocytopenia and hemoconcentration. For example, FR-900452, tested in anesthetized rabbits (107), and the specific PAF antagonist BN 52021, injected to anesthetized guinea pigs (108), were shown to attenuate the endotoxin-induced thrombocytopenia. Moreover, ONO 6240 partially prevented the leukopenia induced by endotoxin in conscious sheep (109). The same antagonist was also shown to abolish the *E. coli*-induced decrease in cardiac output observed in conscious sheep (109). A recent preliminary study demonstrated a protective effect of CV-3988 against endotoxin-induced low cardiac output and increased peripheral resistance. Blood flow to essential organs such as heart, kidney and splanchnic bed was also substantially improved over the non-treated septic group (110). Only few reports extended their study to end points which included survival rate (Table 3). These studies, which utilized several PAF antagonists, demonstrated improved survival rates in the PAF antagonist-treated groups. However, it should be noted that all these studies evaluated the effect of pretreatment rather than treatment with PAF antagonists.

Hemorrhagic and traumatic shock. The role of PAF in mediating traumatic shock is supported by several recent studies conducted in rats subjected to the Noble-Collip trauma; the most direct evidence for PAF production during traumatic shock was provided by Stahl *et al.* (111) who reported that peritoneal fluid from traumatized or PAF-infused rats, analyzed by high performance liquid chromatography (HPLC), contained a phospholipid which had a similar retention time to authentic PAF, and aggregated washed rabbit platelets. This aggregation was blocked by a specific PAF antagonist, CV-6209. Other studies utilizing the same traumatic shock model reported beneficial effects of various PAF antagonists. For example, combined treatment with CV-6209 and PGE_1 improved survival and decreased the accumulation of myocardial depressant factor (MDF, 112). Moreover, three structurally different PAF antagonists—WEB 2086, L-652,731 and CV-6209—increased the post-trauma mean arterial pressure and survival rate (113). In addition, PAF antagonists had salutary effects in a hemorrhagic shock model (114), although no direct biochemical evidence is available at the present time on the release of PAF in this model.

SUMMARY

PAF has been recognized as an important mediator of septic, traumatic and hemorrhagic shock. However, while there is a wide spectrum of pharmacological evidence (i.e., studies with PAF antagonists) to support the central role

TABLE 2

Summary of PAF Antagonists Which Protect Against Endotoxin-induced Hypotension

Antagonist	Reference	Dose, mode and time of administration	Species	Anesthesia	Endotoxin	Degree of prevention/reversal
ONO-6240	(109)	i.v.[a] 3 mg/kg 15 min before LPS,[b] and 0.1 mg/kg/min maintenance	sheep	conscious	*E. coli* i.v. 1 μg/kg	complete
SRI 63-441	(117)	i.v. 0.18 mg/kg (ED_{50}) 1 min after LPS	rat	pentobarbital	*E. coli* i.v. 5 mg/kg	complete
SRI 63-072	(118)	i.v. 1 mg/kg 6–8 min after LPS	rat	pentobarbital	*E. coli* i.v. 15 mg/kg	complete
kadsurenone	(29)	i.v. 2.2 μmole/kg 6 min after LPS, and 0.9 μmole/kg maintenance	rat	nembutal	*E. coli* i.v. 50 mg/kg	partial
WEB 2086	(119)	PO[c] 1–10 mg/kg 60 min before, and i.v. 0.1–5 mg/kg 5 min before LPS	rat	pentobarbital	*E. coli* i.v. 15 mg/kg	complete
CV 3988	(116)	i.v. 5 mg/kg 5 min before, i.v. 0.05–1 mg/kg 7–10 min after LPS	rat	pentobarbital	*E. coli* i.v. 15 mg/kg	complete
CV 3988	(30)	i.v. 10 mg/kg 5 min before, and i.v. 5 mg/kg 50 min after LPS	rat	conscious	Salmonella enteritidis IP[d] 20 mg/kg	partial
BN 52021	(108)	i.v. 1 and 6 mg/kg 15 min before LPS	guinea pig	pentobarbital	Salmonella typhimurium i.v. 0.3 mg/kg	partial
L-652,731	(120)	i.v. 500 nmol 6 min after LPS, and i.v. 25 nmol/min maintenance (beginning 8 min after LPS)	rat	nembutal	*E. coli* i.v. 50 mg/kg	partial
BN 50739[e]		IP 10 mg/kg 30 min before LPS	rat	conscious	*E. coli* i.v. 14.4 and 43.2 mg/kg	partial

[a]Intravenous = i.v. [b]LPS—lipopolysaccharide. [c]PO—per os. [d]IP—intraperitoneal. [e]Rabinovici *et al.* (in preparation).

TABLE 3

Summary of PAF Antagonists Which Protect Against Lethality in Animals Exposed to Septic Shock

Antagonist	Reference	Dose, mode and time of administration	Species	Endotoxin	Survival before drug	(%) after drug
CV 3988	(30)	i.v.[a] 10 mg/kg 5 min before LPS,[b] and 5 mg/kg 50 min after LPS	rat	Salmonella enteritidis IP[c] 20 mg/kg	0 at 24 hr	64
CV 3988	(116)	i.v. 10 mg/kg with LPS	rat	*E. coli* i.v. 5 mg/kg	33 at 20 hr	71
BN 52021	(73)	SC[d] 5 mg/kg 15 min before LPS	rat	Salmonella enteritidis i.v. 5 mg/kg	+45% vs control at 24 hr	
BN 52021	(121)	i.v. 5 mg/kg 15 min before LPS	dog	*E. coli* i.v. 1 mg/kg	0 at 72 hr	100
WEB 2086	(119)	PO[e] 10 mg/kg 60 min before, and i.v. 10 mg/kg with LPS	rat	*E. coli* i.v. 7.5 mg/kg	0 at 24 hr	100
BN 50739[f]		IP 10 mg/kg 30 min before LPS	rat	*E. coli* i.v. 14.3 mg/kg	0 at 7 days	100

[a]Intravenous = i.v. [b]LPS—lipopolysaccharide. [c]IP—intraperitoneal. [d]SC—subcutaneously. [e]PO—per os. [f]Rabinovici *et al.* (in preparation).

of PAF in these pathophysiological processes, there is only a handful of reports that provided direct chemical evidence (i.e., measurement of PAF in biological material). Therefore, future experimental efforts should focus on the facilitation of the available methods that directly measure PAF, and on their utilization in shock states.

In addition, therapeutic efficacy has only been established in prophylactic endotoxemic studies, where PAF antagonists were given prior to the bolus administration of a single endotoxin. Therefore, at the present time there is no data on the capacity of PAF antagonists to reverse hemodynamic, metabolic and biochemical derangements produced by endotoxins in addition to improved survival. Furthermore, one should notice that the endotoxemic models may not fully represent septic shock where multiple toxins in addition to bacterial growth confound the disease process.

Finally, it is important to keep in mind that multiple mediators (e.g. leukotrienes, catecholamines, prostaglandins, MDF, etc.) are produced during these complex pathological conditions. It is therefore conceivable that the pathogenic process of the different shock states reflects the actions and interactions of these multiple mediators. Consequently, future studies should be conducted to establish the relationships between the various vasoactive mediators. Moreover, the therapeutic strategy should be based on multiple sites of actions.

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Studies on the Role of Platelet-Activating Factor in Blood Pressure Regulation[1]

Katsuhiko Sakaguchi[a], Shigeto Morimoto[a,*], Fuminori Masugi[a], Shuichi Saeki[a], Toshio Ogihara[a], Kouji Yamada[b] and Isao Yamatsu[b]

[a]Department of Geriatric Medicine, Osaka University Medical School, Fukushima-ku, Osaka 553 and [b]Tsukuba Research Laboratories, Eisai Co., Ltd., Tsukuba, Ibaraki 300-26, Japan

Circulating levels of 1-*O*-hexadecyl-2-acetyl-*sn*-glycero-3-phosphocholine (C_{16}PAF) in human subjects were measured by gas chromatography/mass spectrometry using negative ion chemical ionization. The mean (± S.D.) circulating C_{16}PAF levels in patients with essential hypertension (18.1 ± 5.3 pg/mL, n=16) were not significantly different from those in normotensive subjects (17.2 ± 7.2 pg/mL, n=14). During a salt balance study, high salt intake (20 g/day) significantly increased the circulating level of C_{16}PAF, and changes in circulating C_{16}PAF significantly and positively correlated with changes in mean arterial blood pressure ($r=0.47$, $p<0.05$). Changes in C_{16}PAF also correlated with changes in creatinine clearance ($r=0.55$, $p<0.05$), but did not correlate with changes in plasma sodium concentration, plasma chloride concentration and plasma volume. An intravenous injection of 50 μg of human atrial natriuretic peptide (hANP) decreased circulating C_{16}PAF levels from 20.0 ± 2.7 to 13.9 ± 2.4 pg/mL of blood (n=10, $p<0.01$) in healthy subjects. The data appear to indicate that C_{16}PAF levels are changed by salt intake-induced mild increase in blood pressure, and that hANP may be an endogenous factor which lowers circulating C_{16}PAF.

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Platelet-activating factor (PAF) is an alkyl ether lipid with diverse biological activities. The composition of the alkyl group of PAF in human neutrophils has been reported to be 59% C_{16}PAF (1-*O*-hexadecyl-2-acetyl-*sn*-glycero-3-phosphocholine), 18% C_{18}PAF (1-*O*-octadecyl-2-acetyl-*sn*-glycero-3-phosphocholine) and 23% $C_{18:1}$PAF (1-*O*-octadecenyl-2-acetyl-*sn*-glycero-3-phosphocholine) (1). The pathophysiological role of PAF is not yet clearly understood, which has been due partly to a lack of sensitive methods to measure changes in endogenous PAF levels. Recently, a highly sensitive method to measure C_{16}PAF in circulating blood has been developed utilizing gas chromatography/mass spectrometry (GC/MS) with negative ion chemical ionization (NICI) after conversion of PAF to its pentafluorobenzoyl derivative (2).

PAF has potent hypotensive activity (3). We previously reported that a PAF-like substance, as well as an unknown factor which inhibited the aggregation of platelets, were found in renal venous blood of patients with renovascular hypertension after percutaneous transluminal renal angioplasty (PTRA) (4). We also suggested that PAF may participate in blood pressure regulation following PTRA in these patients (4). It is known that PAF decreases the glomerular filtration rate (GFR), urine volume (UV) and urinary sodium (UNa) excretion in animals *in vivo* (5,6). A decrease in GFR by PAF was also confirmed in perfused kidney (7). By contrast, human atrial natriuretic peptide (hANP) increases GFR, UV and UNa excretion and inhibits glomerular contraction induced by PAF (8). Although stimuli such as antigen-induced immunoglobulin E, calcium ionophore A23187 and thrombin activate PAF biosynthesis, no endogenous factor such as hANP has been reported to inhibit PAF synthesis.

In the present paper, we describe the effects of dietary salt on circulating C_{16}PAF levels, on the role of PAF on systemic blood pressure regulation, and on the suppressive effect of hANP on circulating C_{16}PAF in humans.

MATERIALS AND METHODS

Subjects and study protocol. Thirty-seven healthy human subjects (20 males, 17 females, mean ± S.D. age of 47 ± 9 years) and 16 untreated patients with essential hypertension (10 males, 6 females, WHO classifications I and II, 46 ± 9 years) with a mean blood pressure of 163 ± 13/97 ± 12 mm Hg were used for the basal study in which we measured circulating C_{16}PAF levels. Blood samples were collected at 8:00 a.m. after overnight fasting. Blood was also obtained at 4:00 p.m. from eight healthy subjects to study circadian rhythm.

The sodium balance study was performed in 12 patients (8 males and 4 females, 44 ± 8 years, 167 ± 12/98 ± 9 mm Hg) out of the 16 hypertensive patients with essential hypertension. These patients had a normal salt diet, a low salt diet, or a high salt diet, with a sodium chloride intake of 10, 2 and 20 g/day, respectively, for 14 days. Blood samples were obtained early in the morning of the last day of each period, and urine samples were collected over a 24-hr period on the last day.

Ten healthy male volunteers (mean age ± S.D., 27 ± 3 years) were used for the hANP injection study. Subjects started the study with a void urine bladder and were placed at rest for 1 hr before hANP injection. hANP (Peptide Institute, Osaka, Japan; in 50 μg/mL of saline) was injected intravenously, and the C_{16}PAF levels in blood were monitored. Blood was collected before and 1 hr after hANP injection.

Measurement of C_{16}PAF. Fresh blood (5 mL) was immediately mixed with 30 mL of ice-cold methanol for the assay of C_{16}PAF. The resulting slurry was centrifuged at 10,000 × *g* for 10 min at 4°C. The supernatant was used

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*To whom correspondence should be addressed at Dept. of Geriatric Medicine, Osaka University Medical School, Fukushima-ku, Osaka 553, Japan.

Abbreviations: ANP, atrial natriuretic peptide; C_{16}PAF, 1-*O*-hexadecyl-2-acetyl-*sn*-glycero-3-phosphocholine; C_{18}PAF, 1-*O*-octadecyl-2-acetyl-*sn*-glycero-3-phosphocholine; $C_{18:1}$PAF, 1-*O*-octadecenyl-2-acetyl-*sn*-glycero-3-phosphocholine; GC/MS, gas chromatography/mass spectrometry; GFR, glomerular filtration rate; hANP, human atrial natriuretic peptide; PAF, platelet-activating factor, 1-*O*-alkyl-2-acetyl-*sn*-glycero-3-phosphocholine; PTRA, percutaneous transluminal renal angioplasty; UNa, urinary sodium excretion; UV, urine volume.

for the C_{16}PAF assay (2). Briefly, 2 mL of fresh blood was immediately mixed with 30 mL of ice-cold methanol and centrifuged at 3,000 × *g* for 20 min. Three mL of the methanol layer were mixed with 1 mL of distilled water and 4 mL of chloroform. The lower phase was collected and evaporated, the residue dissolved in chloroform and then applied to a Bond Elute SI column (Analytichem International, Harbor City, CA) for collection of the C_{16}PAf fraction. The PAF fraction was then incubated with 0.8 U of phospholipase C, 72 mM $CaCl_2$ and 50 mM Tris-HCl (pH 7.5) at 37°C for 60 min. The hydrolyzed mixture was extracted by the method of Folch and was evaporated, the residue was treated with 3 mM pentafluorobenzoyl chloride in distilled acetonitrile, and incubated at 35°C for 60 min, after which time the derivatization was complete. After evaporation, the residue was dissolved in ethyl acetate and injected into the gas chromatograph/mass spectrometer. The detection limit of PAF in the system was 5 pg, and the intraexperimental coefficient of variation was 3.8% (2).

Other measurements. Serum levels of Na^+, Cl^-, creatinine, and total protein, and the urinary level of creatinine were measured using a Technicon autoanalyzer (Technicon Co., Tarrytown, NY).

Statistical analysis. All data are expressed as mean ± S.D. The data were analyzed by paired Student's *t*-test.

RESULTS

Stability of C_{16}PAF in blood samples. Figure 1 shows the time-dependent decrease in measured C_{16}PAF levels of blood samples before mixing with ice-cold methanol. Thirty percent of C_{16}PAF was rapidly degraded within 30 seconds. Subsequent data were therefore obtained on blood samples which had been immediately mixed with ice-cold methanol.

Circulating levels of C_{16}PAF in healthy subjects. The circulating level of C_{16}PAF in 37 healthy subjects ranged from 5 pg/mL of blood to 25 pg/mL of blood, with a mean value of 18.4 ± 6.2 pg/mL (Table 1). The mean circulating

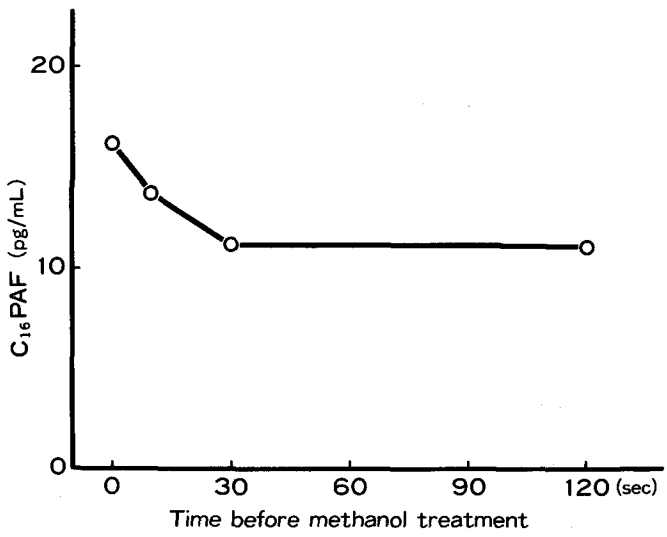


FIG. 1. Time-dependent decrease of C_{16}PAF levels in blood samples before mixing with ice-cold methanol. The C_{16}PAF levels were determined for samples of the same origin differing in pretreatment time before addition of ice-cold methanol.

TABLE 1

Circulating Levels of C_{16}PAF in Healthy Human Subjects and Hypertensive Patients

	N	C_{16}PAF (pg/mL blood)
Total subjects	37	18.4 ± 6.2
Younger subjects (<60)	22	18.5 ± 4.6
Elderly subjects (≥60)	15	18.3 ± 6.3[a]
Male subjects	20	18.5 ± 5.4
Female subjects	17	18.4 ± 5.4[b]
Male subjects	8 (a.m. 8:00)	18.4 ± 5.1
	(p.m. 4:00)	18.4 ± 5.0
Blood pressure difference		
Normotensives	14	17.2 ± 7.2
Hypertensives	16	18.2 ± 5.0

[a]Values are not significantly different from values in younger subjects.

[b]Values are not significantly different from values in male subjects.

C_{16}PAF levels in younger subjects (<60 years old) at 18.5 ± 4.6 pg/mL of blood did not differ from those in elderly subjects (≥ 60 years old) at 18.3 ± 6.3 pg/mL. The mean circulating levels of C_{16}PAF in male subjects at 18.5 ± 7.1 pg/mL of blood also did not differ from those in female subjects at 18.4 ± 6.4 pg/mL (Table 1). A comparison of the circulating values at 8:00 a.m. with those at 4:00 p.m. in eight healthy subjects showed no circadian rhythm of C_{16}PAF levels (Table 1).

Circulating levels of C_{16}PAF in hypertensive patients. The mean values of circulating C_{16}PAF in hypertensive patients were 18.1 ± 5.0 pg/mL of blood; in age- and sex-matched normotensive subjects (9 males and 5 females, 46 ± 9 years) the corresponding values were 17.2 ± 7.2 pg/mL of blood. The difference was not statistically significant (Table 1).

Dietary salt intake and C_{16}PAF levels. The effects of dietary salt intake on haematocrit, plasma total protein, plasma creatinine, UV, urinary excretion of creatinine and on mean blood pressure in the 12 patients with essential hypertension are shown in Table 2. The high salt diet significantly decreased the values of haematocrit, plasma total protein, and plasma creatinine, and significantly increased the values of UV and mean blood pressure in the patients compared to those on a normal and/or low salt diet (Table 2). The mean circulating level of C_{16}PAF slightly, but not significantly, decreased with dietary salt depletion, and significantly ($p<0.01$) increased with the high salt diet (Fig. 2). During the salt balance study, changes in circulating C_{16}PAF levels in individual patients correlated significantly with both changes in mean blood pressure ($r=0.47$, $p<0.05$; Fig. 3) and with changes in creatinine clearance (($r=0.55$, $p<0.05$; Fig. 4), as the latter were effected by changes in dietary sodium. However, there was no significant correlation with changes in plasma sodium concentration ($r=0.32$, NS), chloride concentration ($r=0.39$, NS), or plasma volume ($r=0.34$, NS) (Fig. 5).

Effect of hANP injection on circulating C_{16}PAF levels. The effect of hANP on circulating C_{16}PAF levels was determined in 10 normotensive subjects. Circulating C_{16}PAF levels significantly decreased from 20.0 ± 9.0 to

TABLE 2

Plasma Parameters During Sodium Balances[a]

	Dietary salt		
	Normal	Low	High
Haematocrit (%)	33.4 ± 5.7	36.4 ± 4.8[b]	32.1 ± 6.7[c]
Plasma total protein (g/dL)	6.7 ± 0.4	7.2 ± 0.6[b]	6.7 ± 0.6[c]
Plasma creatinine (mg/dL)	0.94 ± 0.30	1.20 ± 0.41[d]	0.81 ± 0.26[e]
Urinary volume (mL/day)	1033 ± 38	1108 ± 33	1247 ± 32[d]
Urinary creatinine (mg/day)	848 ± 211	886 ± 173	1080 ± 199
Mean blood pressure (mm Hg)	108.7 ± 6.5	103.3 ± 6.5[b]	112.8 ± 7.8[b,c]
Creatinine clearance (mL/min)	62.8 ± 15.6	51.2 ± 12.3	92.6 ± 11.5

[a]Significant difference: normal *vs.* low or high; [d]p<0.05; [b]p<0.01; significant difference: low *vs.* high; [e]p<0.05, [c]p<0.01.

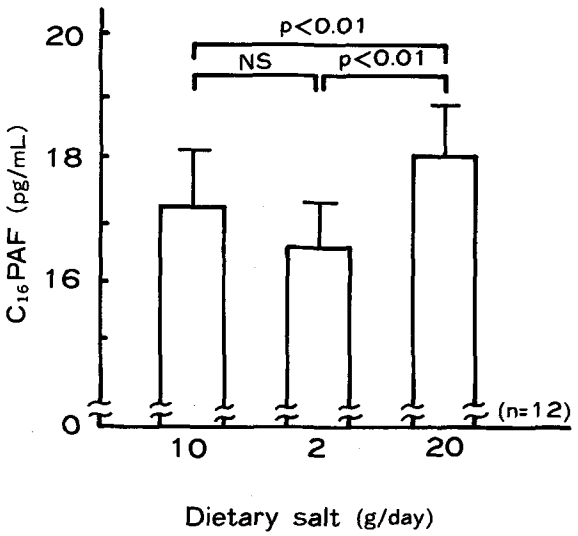


FIG. 2. Changes in circulating C_{16}PAF levels caused by dietary salt intake in twelve patients with essential hypertension (8 males and 4 females, 44 ± 8 years). The patients had normal salt diet, low salt diet, or high salt diet with sodium chloride levels of 10, 2 or 20 g/day, respectively, for each 14 days.

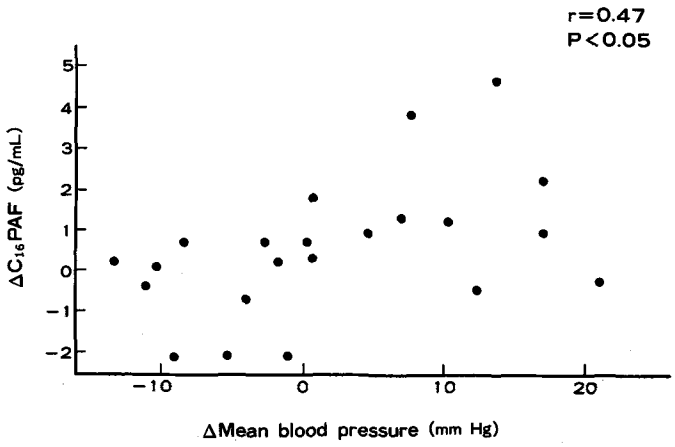


FIG. 3. Relation between changes in circulating C_{16}PAF levels and changes in mean arterial blood pessure in twelve patients with essential hypertension during the salt balance study.

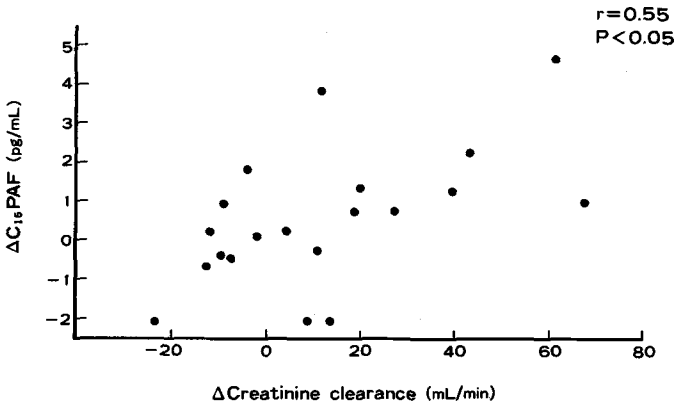


FIG. 4. Relation between changes in creatinine clearance and changes in circulating C_{16}PAF in twelve patients with essential hypertension during the salt balance study.

13.9 ± 5.9 pg/mL of blood (p<0.01) upon hANP injection (Fig. 6).

DISCUSSION

Circulating levels of C_{16}PAF in normal subjects were found to be 18.4 ± 6.2 pg/mL of blood, or 3.7×10^{-11} M, based on plasma volume. *In vitro*, 10^{-7} M PAF activates human platelets (9). Therefore, the concentration of circulating C_{16}PAF is less than one thousandth of the concentration that would activate platelets. Moreover, circulating C_{16}PAF levels were similar for different age- and sex-groups. The data indicate that a small but steady level of C_{16}PAF is present in circulating blood and that this PAF concentration is too low to activate circulating platelets in healthy human subjects. A circadian rhythm in circulating PAF levels was not observed, although the levels were measured only for two time points per day. This may indicate that plasma cortisol levels, which affect phospholipase A_2 activity, do not influence circulating C_{16}PAF levels. Plasma-free PAF is thought to be rapidly hydrolyzed to lysoPAF by acetylhydrolase (10). As shown in Figure 1, a portion of measurable PAF was rapidly degraded within 30 seconds, but the remaining portion of PAF was quite stable beyond that time. This may suggest that more than half of the total PAF present in blood

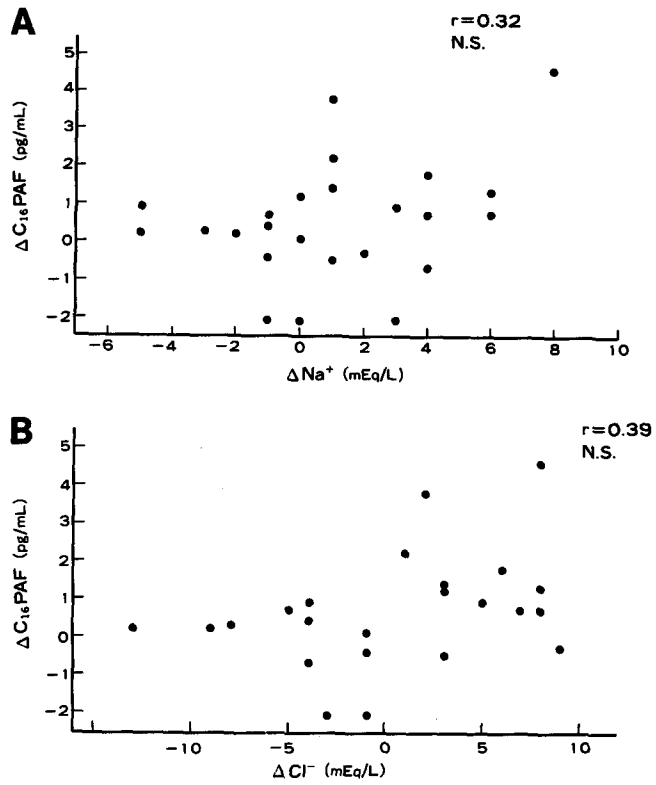


FIG. 5. Relation between changes in C_{16}PAF levels and changes in plasma sodium concentration (A), plasma chloride concentrations (B) and plasma volume (C) in twelve patients with essential hypertension during the salt balance study.

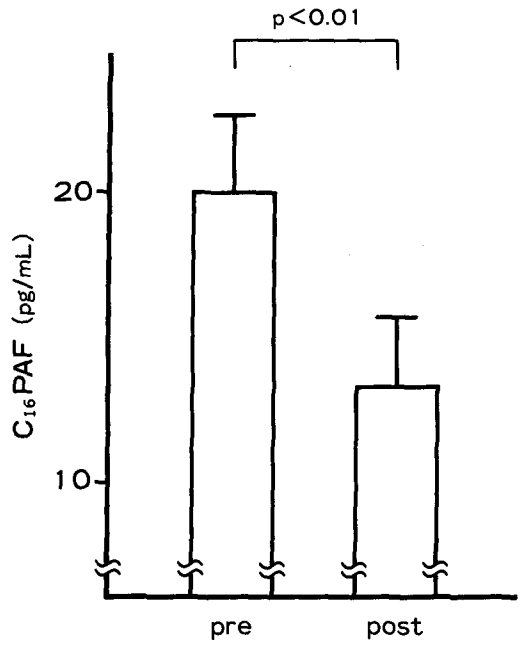


FIG. 6. Effect of hANP on C_{16}PAF levels in 10 normal subjects. A saline solution of hANP (50 μg) was injected into 10 normal volunteers and C_{16}PAF levels in circulating blood were determined before and 60 min after hANP injection.

would exist in a bound or cell-associated form. It is known that newly synthesized PAF is not readily released from cells (11,12). Another explanation for the persistent levels of PAF beyond 30 seconds might be the limited concentration or activity of acetylhydrolase *ex vivo*. Although the effect of dietary salt on circulating PAF levels was rather small, the changes in circulating C_{16}PAF levels effected by the salt balance significantly correlated with the changes in mean arterial blood pressure. This may indicate that free PAF in the circulation directly affects systemic blood pressure.

PAF has been shown to affect intrarenal hemodynamics (5,6). Infusion of PAF into a renal artery resulted in the reduction of GFR and renal plasma flow (5,6). On the other hand, atrial natriuretic peptide (ANP) has been reported to inhibit glomerular contraction induced by PAF (8). Moreover, we have shown for the first time that injection of a natriuretic dose (50 μg) of hANP decreases circulating PAF levels. These results suggest that hANP-induced modulation of GFR or UNa excretion may be mediated through its effects on PAF synthesis. ANP increases prostaglandin production in the kidney *in vivo* (13), but has no effect on renal prostaglandins *in vitro* (14). This would indicate that hANP does not decrease phospholipase A_2 activity, and therefore, that this is not a mechanism by which PAF is decreased. Cholinephosphotransferase activity may contribute to the control of basal PAF levels, but we do not have data in support of this hypothesis. Further work is needed to elucidate the precise mechanism(s) for the ANP-induced decrease in the circulating PAF and to pinpoint the relationship between the hANP-induced decrease in circulating C_{16}PAF levels and other circulatory blood-pressure related factors such as renin, angiotensin II, aldosterone, and calcitonin gene-related peptide.

In conclusion, PAF levels in circulating blood are increased by high dietary salt intake and are decreased by exogenous hANP injection, suggesting that dietary salt and hANP may be regulators of circulating PAF levels.

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Intravascular Release of a Platelet-Activating Factor-Like Lipid (PAF-LL) Induced by Cigarette Smoking[1]

Tada-atsu Imaizumi*
Department of Pathologic Physiology, Institute of Neurological Diseases, Hirosaki University School of Medicine, Hirosaki, 036, Japan

To assess the role of platelet-activating factor (PAF) in smoking-induced vascular injury, the effect of cigarette smoking on PAF-like lipid (PAF-LL) in plasma was studied. The subjects were 12 young smokers (22 ± 1.3 years old), 13 young non-smokers (22 ± 2.1 years old), 14 older smokers (59 ± 9.6 years old), and 11 older non-smokers (60 ± 8.7 years old). Lipids were extracted from 5 mL of plasma and then were separated by thin-layer chromatography. The fraction with the same migration as authentic PAF was recovered and tested for the ability to aggregate human polymorphonuclear neutrophils (PMN). The activity was identified as PAF-LL because it was inactivated by phospholipase A_2, and because the effects on PMN were blocked by CV-3988, a competitive antagonist of the PAF receptor. PAF-LL was detected in plasma from 3 young non-smokers (23%), 5 young smokers (42%), 3 older non-smokers (27%) and 11 older smokers (79%). The incidence of the detection of plasma PAF-LL in older smokers was significantly higher than those in young non-smokers ($p<0.01$) or in older non-smokers ($p<0.05$). In young smokers, the acute effect of smoking was also studied. Plasma PAF-LL was detected in all of the 12 subjects immediately after smoking a cigarette, in sharp contrast to the incidence of 42% before smoking. Plasma β-thromboglobulin was highest in older smokers, and it increased significantly after smoking a cigarette in young smokers. Smoking is associated with increased production of PAF or a similar lipid, which may play an important role in the pathogenesis of smoking-induced vascular diseases.
Lipids 26, 1269–1273 (1991).

Cigarette smoking is an important risk factor of atherosclerotic disease (1), and platelet activation is thought to be one of the mechanisms by which smoking induces vascular injury (2,3). However, the precise mechanisms by which cigarette smoking exerts its adverse effect have not been elucidated.

Platelet-activating factor (PAF) is a phospholipid autacoid with a variety of activities. PAF induces aggregation of human platelets in the concentration range of 10^{-9}–10^{-8}M (4) by interaction with a specific receptor (5), and has been implicated in the basic mechanism of platelet aggregation. PAF is produced by and active on platelets (4), leukocytes (6) and vascular endothelial cells (7,8), and it may be one of the crucial mediators in the interactions of these cells. Therefore, PAF may play a role in the pathogenesis of thrombosis and vascular disorders.

The rationale for this study was that PAF may be involved in the pathogenesis of smoking-induced vascular disease. This hypothesis was addressed by examining plasma for a PAF-like lipid (PAF-LL) following both chronic and acute cigarette smoking. Plasma β-thromboglobulin (β-TG), an indicator of *in vivo* platelet activation (9), and platelet aggregability were also measured in order to observe the relation between PAF-LL and platelet function.

MATERIALS AND METHODS

Study design. First, the chronic effect of cigarette smoking was examined. For this purpose, four groups of healthy male volunteers were chosen, *i.e.*, 13 young non-smokers with an average age of 22 ± 2.1 years (mean ± SD), 12 young smokers (22 ± 1.3 years old), 11 older non-smokers (60 ± 8.7 years old) and 14 older smokers (59 ± 9.6 years old). Young smokers had smoked an average of 15 ± 5.6 cigarettes a day for 3.5 ± 1.7 years, and older smokers had smoked 18 ± 4.7 cigarettes a day for 37 ± 12.9 years. The young volunteers were recruited from university students, and the older ones were selected from participants in an annual screening for circulatory diseases. The dietary habit and constitution of these subjects were about average for Japan. They had no abnormalities by detailed history and physical examination including blood pressure, electrocardiogram and blood chemistry. All had abstained from medications for at least 4 wk prior to this study. Venous blood was obtained early in the morning, after the subjects had fasted and refrained from smoking for at least 9 hr.

The study on the acute effect of cigarette smoking was conducted in the same group of young smokers. After the first blood drawing, they had a 15-min rest and then smoked one filtered cigarette containing 1.1 mg nicotine and 1.6 mg tar (Nihon Tobacco, Tokyo, Japan) within 3 min. They were instructed to inhale deeply. Immediately after smoking the cigarette, another venous blood sample was obtained.

Extraction of plasma PAF-LL. Blood was taken into a plastic tube containing 15% (v/v) acid-citrate dextrose solution. The lipids were extracted from 5 mL plasma by the method of Folch *et al.* (10) and separated by thin-layer chromatography (TLC) on a silica gel plate (Silica gel 60, Merck, Darmstadt, Germany) using chloroform/methanol/acetate/water (50:25:8:4, by vol) as a solvent system. The area of silica gel with the same migration as authentic PAF (1-*O*-alkyl-2-acetyl-*sn*-glycero-3-phosphocholine, Sigma, St. Louis, MO) was scraped off, and the lipids were re-extracted and dissolved in 250 μL of 20 mM phosphate-buffered saline (PBS), pH 7.4, containing 2.5 mg/mL human serum albumin (HSA; Sigma). This solution was tested for the activity to aggregate human polymorphonuclear neutrophils (PMNs) according to the method described by Prescott *et al.* (8).

[1]Based on a paper presented at the Third International Conference on Platelet-Activating Factor and Structurally Related Alkyl Ether Lipids, Tokyo, Japan, May 1989.

*To whom correspondence should be addressed at Department of Pathological Physiology, Institute of Neurological Diseases, Hirosaki University School of Medicine, 5 Zaifucho, Hirosaki, 036, Japan.

Abbreviations: β-TG, β-thromboglobulin; HSA, human serum albumin; PAF, platelet-activating factor; PAF-LL, platelet-activating factor-like lipid; PBS, phosphate-buffered saline; PLA_2, phospholipase A_2; PMN, polymorphonuclear neutrophil(s); PPP, platelet-poor plasma; PRP, platelet-rich plasma; TLC, thin-layer chromatography.

PMN aggregation. PMN were separated from heparinized venous blood of healthy non-smoking subjects using Ficoll-Paque according to the method of Böyum (11). PMN were finally suspended in Hanks' balanced salt solution containing 2.5 mg/mL HSA. The number of white cells was adjusted to 0.5–1 × 10^7/mL. The PMN aggregation studies were performed by the light transmission method (12). To 200 μL of PMN suspension in a siliconized cuvette, 50 μL of 25 μg/mL cytochalasin B (Sigma) was added and the mixture was stirred at 37°C for 10 min. Then, 50 μL of the TLC fraction of the plasma lipid or authentic PAF was added, and the changes in light transmission were recorded. Aggregation of PMN was observed, in a concentration-dependent manner, with 10^{-9}–10^{-7} M authentic PAF.

Characterization of PAF-LL. For the characterization of the PMN aggregating activity in plasma, the effects of phospholipase A_2 (PLA_2) and a PAF receptor antagonist were examined. PLA_2 treatment was according to the method of Blackwell *et al.* (13). The TLC fraction with PMN aggregating activity was incubated with 77 U/mL PLA_2 from porcine pancreas (Sigma) at 37°C for 1 hr. After the incubation, lipids were extracted and tested for the ability to aggregate PMN as described above.

The effect of CV-3988, a specific antagonist of the PAF receptor (14), was tested by treating the PMN with 10 μM CV-3988 for 10 min prior to the addition of the TLC-purified plasma lipid fraction.

Measurement of plasma β-thromboglobulin. Venous blood was obtained without stasis by the two syringe technique. Blood was taken into a glass tube containing sodium citrate, citric acid, theophylline, adenosine and dipyridamole (Diatube H®, Diagnostica Stago, Asnières, France), and mixed immediately. The samples were kept on ice for at least 15 min and then were centrifuged for 30 min at 2,500 × *g* at 4°C. Plasma β-TG was estimated by enzyme-linked immunosorbent assay (15) using a commercially available kit, Asserachrom β-TG (Diagnostica Stago).

Platelet aggregation. Platelet aggregation was performed by the method of Born (16). Blood samples for platelet aggregation were collected with 1/10 vol of 3.8% trisodium citrate. The blood was centrifuged at 100 × *g* for 15 min and platelet-rich plasma (PRP) was obtained. The remaining blood was then centrifuged at 900 × *g* for 10 min to yield platelet-poor plasma (PPP). The platelet count of PRP was adjusted to 300–400 × 10^3/mm^3 with PPP. The aggregating agents used were 2 μM ADP (Sigma) and 40 ng/mL PAF (Sigma). Aggregation was expressed as a percentage of maximal light transmission.

Statistical analyses. Statistical analyses were performed using Fisher's exact probability test, Student's t-test and paired t-test.

RESULTS

Detection and characterization of PAF-LL in plasma. The separation of PAF-LL from total plasma lipids by TLC is shown in Figure 1. The PMN aggregating activity in plasma was always detected in a single fraction which showed the same migration as authentic PAF. This PAF-LL was almost completely inactivated by treatment with 77 U/mL PLA_2 for 1 hr (Fig. 2). Aggregation of PMN by the plasma PAF-LL was inhibited by pretreating the PMN with 10 μM CV-3988 (Fig. 3).

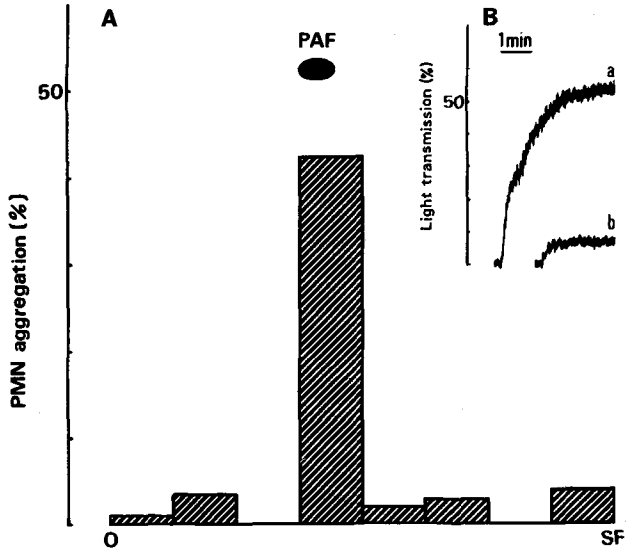


FIG. 1. Plasma PMN aggregating activity separated by TLC. (A) PMN aggregating activity of plasma lipid fractions separated by thin-layer chromatography. Areas of silica gel 1 cm in length were scraped off and the lipids were extracted and tested for the PMN aggregation activity. The migration of authentic PAF is marked as a black eclipse. O: origin, SF: solvent front. (B) Typical patterns of PMN aggregation induced by active (a) and inactive (b) fractions.

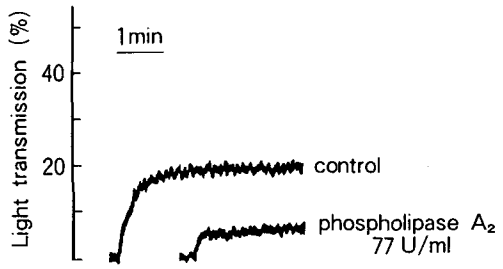


FIG. 2. Effect of phospholipase A_2 (PLA_2) on PMN aggregating activity. The fraction with PMN aggregating activity was incubated with 77 U/mL PLA_2 or buffer at 37°C for 1 hr. After the incubation, lipids were extracted and tested for the PMN aggregation.

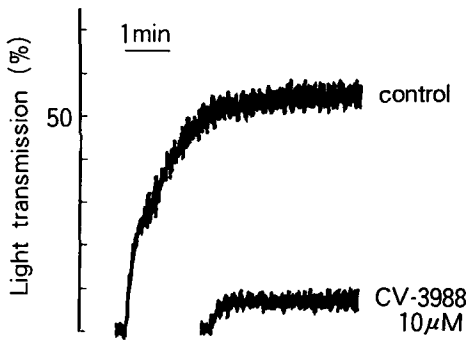


FIG. 3. Effect of CV-3988 on PMN aggregation. PMN were treated with 10 μM CV-3988 of buffer for 10 min prior to the addition of the TLC-purified plasma lipid fraction.

Chronic effect of cigarette smoking. The incidence of the PMN aggregating activity in each of four groups is shown in Table 1. The activity was detected in plasma from 3 young non-smokers (23%), 5 young smokers (42%), 3 older non-smokers (27%) and 11 older smokers (79%). The incidence of PMN aggregating activity in older smokers was significantly higher than that in young non-smokers ($p<0.01$), or older non-smokers ($p<0.05$). However, there was no significant difference between young smokers and young non-smokers.

The levels of plasma β-TG and platelet aggregability in four groups are shown in Table 2. Plasma β-TG was highest in older smokers, and lowest in young non-smokers. There was a statistically significant difference between plasma β-TG levels in young non-smokers and those in older non-smokers ($p<0.01$) or older smokers ($p<0.05$). The platelet aggregability induced by ADP in young smokers was significantly higher than that in young non-smokers ($p<0.05$) or older smokers ($p<0.01$). On the other hand, there was no such difference in the platelet aggregability in response to PAF. Platelet aggregability with both agonists in older smokers was the lowest in four groups.

Acute effect of cigarette smoking. The incidence of PMN aggregating activity in plasma samples from young smokers before and after smoking is shown in Table 3. The activity was detected in plasma from 5 to 12 subjects before smoking, and in all of 12 plasma samples obtained after smoking. The occurrence of the PMN aggregating activity in plasma after smoking was significantly more frequent than that before smoking ($p<0.01$).

Acute effects of cigarette smoking on platelet function are shown in Table 4. Plasma β-TG increased significantly after smoking ($p<0.05$); however, platelet aggregability was not affected.

TABLE 1

Incidence of PMN Aggregating Activity in Plasma from Young Non-Smokers, Young Smokers, Older Non-Smokers and Older Smokers

		PMN aggregating activity (+)	(−)		
Young non-smokers	(n = 13)	3	10		
Young smokers	(n = 12)	5	7		b
Older non-smokers	(n = 11)	3	8	a	
Older smokers	(n = 14)	11	3		

[a]$p<0.05$, [b]$p<0.01$, based on Fisher's exact probability test.

TABLE 2

Plasma β-Thromboglobulin (β-TG) and Platelet Aggregability in Young Non-Smokers, Young Smokers, Older Non-Smokers and Older Smokers

	Plasma β-TG (IU/mL)	Platelet aggregability 2μM ADP (%)	40/ng/mL PAF (%)
Young non-smokers	30 ± 11.1[a,b]	37 ± 19.1[c]	48 ± 21.9
Young smokers	39 ± 14.5	55 ± 21.2[c,d]	47 ± 28.7
Older non-smokers	43 ± 9.1[a]	40 ± 18.5	39 ± 19.3
Older smokers	45 ± 21.0[b]	33 ± 16.8[d]	34 ± 23.6

Mean ± S.D. are shown. [a]$p<0.01$, [b]$p<0.05$, [c]$p<0.05$, [d]$p<0.01$, based on Student's t-test.

TABLE 3

Incidence of PMN Aggregating Activity in Plasma Before and After Smoking a Cigarette in Young Smokers

	PMN aggregating activity (+)	(−)	
Before smoking	5	7	a
After smoking	12	0	

[a]$p<0.01$, based on Fisher's exact probability test.

TABLE 4

Acute Effects of Cigarette Smoking on Platelet Function in Young Smokers[a]

	Before smoking	After smoking	p
Plasma β-TG (IU/mL)	39 ± 14.5	50 ± 20.2	<0.05
Platelet aggregability			
2μM ADP (%)	55 ± 21.2	52 ± 20.3	n.s.[b]
40 ng/mL PAF (%)	47 ± 28.7	54 ± 26.4	n.s.

[a]Statistical analyses are by paired t-test.
[b]Not significant, n.s.

DISCUSSION

The most widely used method for measuring PAF is the bioassay utilizing the aggregation or secretion response of rabbit platelets (17). However, at present there is no absolutely quantitative technique for the assay of PAF in biological materials. The assay also has to be sensitive enough to permit the quantitation of trace amounts of PAF. In the present study, the author employed a bioassay based on the aggregation of human PMN as described previously (8,12). Although this bioassay is a little less sensitive than the method based on rabbit platelets, authentic PAF did induce PMN aggregation in the concentration range of 10^{-9}–10^{-7} M, which covered the changes in plasma PAF-LL levels induced by cigarette smoking in most cases.

The occurrence of the PMN aggregating activity in plasma was more frequent in habitual smokers than in non-smoking controls. In the experiment on the acute effect, the PMN aggregating activity was detected in the plasma of each of 12 volunteers after smoking a cigarette. This PMN aggregating activity in plasma had the same migration in TLC as synthetic PAF, and it was inactivated by pretreatment with PLA_2 and antagonized by CV-3988. These results suggest that cigarette smoking induces intravascular release of PAF, or of a closely related phospholipid, as an acute effect. The accumulative acute effect may bring about the increased plasma PAF-LL levels observed in chronic smokers.

PAF is inactivated by PAF acetylhydrolase, an enzyme which metabolizes PAF (18). In consideration of the relatively high activity of PAF acetylhydrolase in plasma (19,20), the presence of PAF-LL in plasma is somewhat surprising. However, it has been reported that PAF in plasma is carried complexed with lipoproteins (21) or a binding protein (22), which may prevent the conversion of PAF to lysoPAF.

PAF is produced by various cell types including cultured type II alveolar epithelial cells (23). Although the source of plasma PAF-LL is unknown, the possibility exists that it may be derived from leukocytes, platelets, lung, and vascular endothelial cells. However, considering the direct effect of cigarette smoke inhaled into the lung, it is likely that alveolar epithelial cells are one of the major sources of PAF-LL observed after smoking.

Caramelo *et al.* (24) have detected a small amount of PAF in the plasma of healthy humans and rats, and suggested the kidneys as the source since plasma PAF was undetectable in anephric patients and nephrectomized animals. Increased levels of PAF in blood have been reported in patients with cold urticaria (25), sepsis (26), drug-induced allergic reactions (27) and liver cirrhosis (28). It also has been shown that blood PAF is higher in experimental animals with renal allograft rejection (29), endotoxin-induced hypotension (30) and intestinal ischemia (31). These facts suggest that leukocytes and platelets serve as the sources of PAF in blood; these cells may also account for the increase in plasma PAF-LL induced by smoking. The activation of platelets by smoking is also consistent with the observation of increased plasma β-TG levels found after smoking a cigarette. This increase was closely associated with the occurrence of PAF-LL in plasma.

Numerous investigations have dealt with *in vitro* platelet aggregation to assess the effects of cigarette smoking on platelet function. However, they have produced inconsistent results. Fitzgerald *et al.* (32) pointed out that *in vitro* studies of platelet aggregation are inherently limited in the information that they can provide concerning the effects of cigarette smoking on platelet function. Nowak *et al.* (3) observed elevated levels of urinary metabolites of thromboxane in habitual smokers and concluded that platelet activation *in vivo* was induced by chronic smoking. In the present study, although the effects of cigarette smoking on platelet aggregability were not clear, the aggregability in older smokers was lowest. This suggests that platelets obtained from older smokers have decreased sensitivity to agonists, perhaps due to the repeated stimulation by cigarette smoking.

A recent study by Stremler *et al.* (33) showed that an oxidized phospholipid was a substrate for the PAF acetylhydrolase in plasma. It will be of interest to determine whether these oxidized lipids have bioactivities and chemical properties similar to PAF, since cigarette smoking has been suggested to induce oxidation of lipoproteins (34).

In the present study, the author has used TLC only for the isolation and characterization of PAF. Therefore, the phospholipid with PMN-aggregating activity was considered a PAF-like lipid (PAF-LL). This PAF-LL will be characterized further by high-performance liquid chromatography (HPLC) or gas chromatography/mass spectrometry (GC/MS).

In conclusion, cigarette smoking induces enhanced production of PAF, or of a PAF-like lipid, which may play an important role in the pathogenesis of smoking-induced vascular disease.

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Platelet-Activating Factor (PAF) Induces Contraction of Saponin-Skinned Smooth Muscle of Coronary Artery[1]

A.I. Soloviev[a,*] and **P. Braquet**[b]

[a]A.A. Bogomoletz Institute of Physiology, Kiev-24, 252024, Ukraine, U.S.S.R. and [b]H. Beaufour Institute, F-92350, Le Plessis Robinson, France

The effect of platelet-activating factor (PAF) on the development of isometric tension by saponin-skinned coronary artery was studied. PAF caused two types of contractions of coronary smooth muscle cells (SMC): (i) rapid, transient (phasic) contractions of SMC were induced by inositol 1,4,5-trisphosphate dependent Ca^{2+} release from sarcoplasmic reticulum (SR); (ii) slow sustained (tonic) contractions were induced by increase in Ca^{2+}-sensitivity of the contractile apparatus of SMC by protein kinase C activation. The present results support the hypothesis that, in SMC of coronary artery, PAF receptors are located not only on the plasma membrane, but also on the SR membranes.

Lipids 26, 1274–1276 (1991).

Spasm of the large epicardial coronary arteries is one of the main causes of cardiac pathology, *i.e.* angina pectoris, myocardial infarction, disturbances in heartbeat, and sudden death. Angiographic tests on patients during anginal attacks have shown that coronary spasm is the reason for onset of heart pain and the development of myocardial infarction (1). The cellular mechanisms of coronary spasm have not yet been established. But it is well documented that the main determinants of vasospasm are intrinsic to the vascular wall. Among the potential endogenous mediators of coronary vasospasm, special attention has recently been given to platelet-activating factor (PAF), a phospholipase A_2-sensitive phospholipid identified as 1-*O*-alkyl-2-acetyl-*sn*-glycero-3-phosphocholine.

PAF can cause dose-dependent contraction of coronary arteries and a significant decrease in coronary blood flow (2,3) and may be one of the mediators of coronary spasm under hypoxic conditions (4). In addition, PAF binds to specific receptors on the plasma membrane, which induces the activation of phospholipase C, the degradation of phosphatidylinositol 4,5-bisphosphate, and the formation of inositol 1,4,5-trisphosphate (Ins P_3) and diacylglycerol (DG) (5).

We have shown that PAF can contract not only intact vascular smooth muscle strips but also skinned smooth muscle cells (SMC) which have been made hyperpermeable by the cholesterol-precipitating agent saponin.

The purpose of the present investigation was to study the mechanisms by which PAF affects skinned SMC of coronary arteries.

MATERIALS AND METHODS

Materials. Na_2ATP and EGTA were obtained from Calbiochem Behring Corporation (La Jolla, CA). Saponin was from Merck (Darmstadt, Germany). Tris-HCl, procaine, caffeine, and rotenone were purchased from Serva (Heidelberg, Germany). Triton X-100 (alkylphenylpolyethyleneglycol) was from Ferak (Berlin, Germany). Ruthenium red was from Fluka (Buchs, Switzerland), PAF was from Novabiochem (Läufelfingen, Switzerland), BN 52021 was from H. Beaufour Institute (Le Plessis Robinson, France), and polymyxin B was obtained from MinMed-Bioprom (Kiev, Ukraine, U.S.S.R.). Sodium vanadate was from Reachem (Sverdlovsk, Russia, U.S.S.R.), and 1-(5-isoquinolinylsulfonyl)-2-methylpiperazine (H-7) was obtained from Sigma (St. Louis, MO).

Skinned artery strip preparation and tension measurements. Circular strips of isolated porcine coronary artery, 3 mm long and 150 to 200 μm wide, were used in the experiments. Each artery strip was suspended, in a tissue bath, between a stationary hook and a force displacement transducer coupled to a physiograph. The artery strips were lengthened sufficiently to produce a passive force of approximately 4 mN and allowed to stress-relax for 60–90 min. The bathing medium (modified Krebs bicarbonate buffer) contained (in mM): NaCl, 133; KCl, 4.7; $NaHCO_3$, 16.3; NaH_2PO_4, 1.38; $CaCl_2$, 2.5; $MgCl_2$, 1.2; glucose, 7.8; pH 7.4. Following equilibration and relaxation in Krebs bicarbonate buffer, the strips were exposed to the so-called relaxing solution, pH 6.8, containing (in mM): KCl, 130; $MgCl_2$, 5; Tris-HCl, 20; ethyleneglycol-*bis*(β-aminoethylether)tetraacetic acid (EGTA), 4; Na_2ATP, 3.3; and dithiothreitol, 0.5, at a temperature of 20°C for 20 to 30 min. To obtain the skinned fibers, strips were treated with the same relaxing solution, containing 0.05–0.1 mg/mL of saponin for 20–30 min. The concentration chosen and the duration of the saponin action on the artery strips depended on whether the sarcoplasmic reticulum (SR) needed to be preserved or could be destroyed. The presence of intact SR in skinned SMC was evaluated by its ability to contract in response to caffeine.

The contractions of skinned SMC were produced by the activating solution. This solution had the same composition as the relaxing solution, except that EGTA was substituted for by Ca^{2+}/EGTA. The free Ca^{2+} concentration in the activating solution was determined using a computer program that solved the equations for multiple binding constants. Contractions were elicited by increasing the Ca^{2+}/EGTA ratio to obtain the desired free Ca^{2+} concentration in the range of 1 nM to 10 μM while keeping the total EGTA concentration at 5 mM. The apparent binding constant for the Ca^{2+}/EGTA complex was assumed to be 10^6 M^{-1}. The binding constant of Na_2ATP

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*To whom correspondence should be addressed at A.A. Bogomoletz Institute of Physiology, Ukrainian Academy of Sciences, Bogomoletz Str. 4, 252024 Kiev-24, Ukraine, U.S.S.R.

Abbreviations: DG, diacylglycerol; EGTA, ethyleneglycol-*bis*(β-aminoethylether)tetraacetic acid; Ins P_3, inositol 1,4,5-trisphosphate; PAF, platelet-activating factor, 1-*O*-alkyl-2-acetyl-*sn*-glycero-3-phosphocholine; SMC, smooth muscle cells; SR, sarcoplasmic reticulum.

for Mg^{2+} was found to be 4×10^3 M^{-1}. The ionic strength of the solution was kept constant by changing the concentration of KCl. Finally, the Ca^{2+} concentration in the activating solution was expressed as pCa (negative logarithm of Ca^{2+} concentration).

The maximum isometric tension developed by skinned preparations at a free Ca^{2+} concentration of 10^{-5}M averaged 80% of the tension elicited by 130 mM K^+ for the preskinned (intact) preparations.

RESULTS

The minimal free Ca^{2+} concentration in the activating solution, leading to a marked increase in the level of isometric tension of the skinned artery strips, is 2×10^{-7} M. The maximal tension of SMC is attained at 7×10^{-6} M Ca^{2+}. In this case, the curve of "pCa-tension" shows a typical S-shaped pattern, and the Michaelis-Menten constant (Km) is 8.2×10^{-7} M (Fig. 1).

Figure 2 shows that PAF induces dose-dependent contraction of skinned SMC with intact SR in Ca^{2+}-free solution (SR was prior loaded with Ca^{2+} in buffer solution containing 3×10^{-7} M Ca^{2+}). When the SMC are preconstricted with Ca^{2+}, PAF is capable of causing additional contraction with an amplitude that can reach 25–100% of the Ca^{2+}-induced contraction. These contractions are rapid and transient (phasic) at the low Ca^{2+} concentration (Fig. 3 B) and slow and sustained (tonic) at the maximal Ca^{2+} concentration in the activating solution (Fig. 3 A).

The PAF-induced phasic contraction of the skinned artery strips significantly decreases, or even disappears, after treatment of SMC with ruthenium red (3×10^{-6} M) or procaine (5×10^{-3} M), which block Ca^{2+}-release from the SR. Upon destruction of SR by saponin or Triton X-100, and also after depletion of the SR Ca^{2+}-stores by caffeine, PAF induces only slow tonic contraction. The maximal amplitude of this contraction is about one-quarter of that of the PAF-induced contraction of SMC with intact or non-depleted SR (Fig. 4).

In our experiments, preliminary incubation for 10–50 min with lithium ions (10^{-3} M), which inhibit both inositol 1-monophosphatase and G protein, led to a significant decrease (by 75%) in the amplitude of PAF-induced phasic contraction.

Sodium vanadate, an inhibitor of Ca^{2+}-ATPase in SR, reduced at 5×10^{-4} M the amplitude of PAF-induced

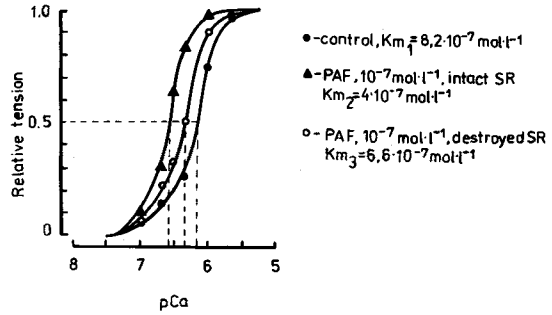


FIG. 1. The effect of PAF on the contractions of saponin-skinned coronary artery strips with intact or damaged sarcoplasmic reticulum ("pCa-tension" relationships). Vascular preparations initially resting in relaxing solution ($Ca^{2+} < 5 \times 10^{-8}$ M) were contracted by replacing the original solution with solutions of progressively higher free Ca^{2+} concentration to which PAF (10^{-7} M) was added. Closed circles, control, Km_1, 8.2×10^{-7} M; control strip (no PAF) had intact SR. Open circles, PAF, damaged SR, Km_2, 6.6×10^{-7} M. Triangles, PAF, intact SR, Km_3, 4×10^{-7} M. Symbols represent means ± SEM (n = 11).

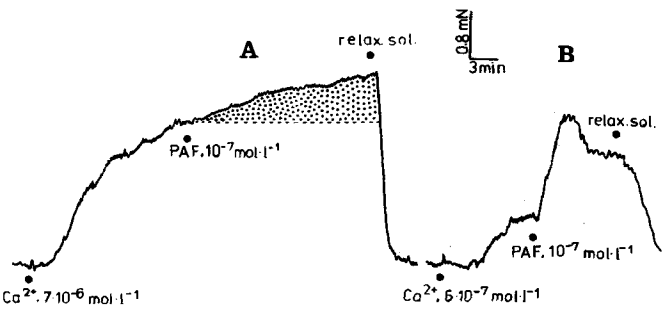


FIG. 3. Effects of PAF on isometric tension in skinned coronary artery strips preconstricted by low (B) and high (A) Ca^{2+} concentration in the activating solution.

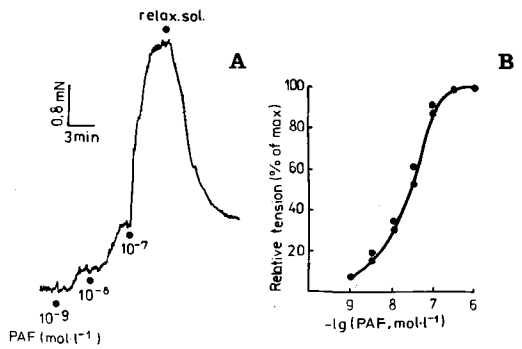


FIG. 2. Contractile responses to PAF of saponin-skinned coronary artery strips with intact sarcoplasmic reticulum after Ca^{2+} loading of SR. (A) the force recording of an artery strip illustrating the PAF concentration dependence of force development; (B) the "dose-response" curve for artery strips treated with different concentrations of PAF. Symbols on panel B represent means ± SEM (n = 9).

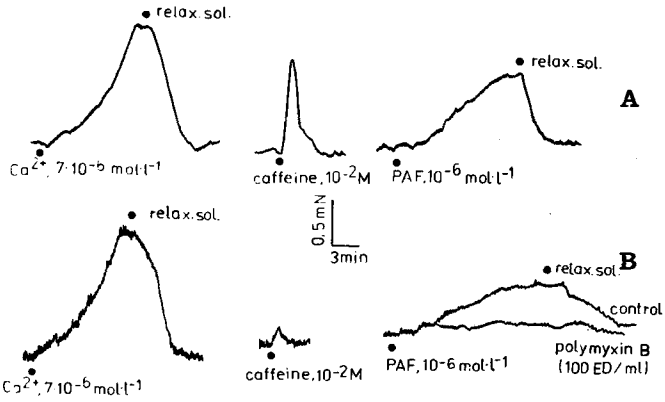


FIG. 4. Contractile responses to PAF of saponin-skinned coronary artery strips before (A) and after (B) the destruction of sarcoplasmic reticulum by Triton X-100. The presence of intact SR in SMC was evaluated by its ability to contract in response to caffeine (10^{-2} M).

contraction by not more than 10–15%. Inhibitors of mitochondrial oxidative phosphorylation, such as KCN (0.5–1.0 mM) or rotenone (0.5–1.0 mM), did not affect the amplitude of PAF-induced contraction.

Figure 1 shows the effect of PAF on typical "pCa-tension" curves for a skinned coronary artery with intact and destroyed SR. As can be seen from Figure 1, a sigmoidal relationship exists between the calcium ion concentration in the medium and force development in skinned fibers. When 10^{-7} M PAF is added to the medium, the force/calcium relationship shifts to the left, *i.e.* to higher pCa values, indicating that calcium sensitivity of the myofilaments had increased.

We have also shown that polymyxin B and H-7 (2×10^{-5} M), putative inhibitors of protein kinase C, completely suppressed the sustained tonic contraction of skinned coronary artery strips induced by PAF (Fig. 4 B). PAF-induced contraction of the SMC was inhibited by BN 52021, a specific blocker of PAF receptors. But even at a very high concentration of BN 52021 (2×10^{-4} M), the amplitude of the PAF-induced contraction decreased not more than 2-fold.

DISCUSSION

It is well known (5) that after binding to its receptor, PAF activates a yet undefined receptor protein, which in turn activates the guanine nucleotide binding protein (G protein) causing GTP hydrolysis. The activation of G protein could lead to two concomitant events: (i) Induction of phospholipase C which can trigger phosphoinositide breakdown and produce Ins P_3 and DG, both of which are classical second messengers. Ins P_3 would induce release of Ca^{2+} from SR and cause tension development (6). On the other hand, DG is involved in protein kinase C activation resulting in the phosphorylation of cellular proteins that could, in turn, contribute to the modulation of various physiological processes, including changes in Ca^{2+}-sensitivity of the SMC contractile machinery; (ii) Internalization of the PAF/receptor complex and transmembrane movement of PAF.

Thus, PAF induced dose-dependent contractions of the skinned coronary SMC. The contractions were rapid and transient at low Ca^{2+} concentration and slow (tonic) at maximal Ca^{2+} concentrations. Ruthenium red or procaine, drugs which block Ca^{2+} release from SR, and destruction of the SR by Triton X-100 led to a significant decrease in the amplitude of PAF-induced rapid (phasic) contraction of SMC. Pretreatment of SMC with lithium ions caused the same effect on expression of PAF-induced phasic contraction. The data suggest that this type of PAF-induced contraction is mediated *via* Ins P_3-induced Ca^{2+} release from intracellular stores and that its main source is the SR.

Sodium vanadate, an inhibitor of Ca^{2+}-ATPase in SR, reduced the amplitude of PAF-induced phasic contraction by not more than 15%. Hence, the release of Ca^{2+} from SR after PAF treatment is caused by the increase in calcium permeability of the SR membrane under influence of Ins P_3 rather than by the inhibition of Ca^{2+}-ATPase. PAF-induced phasic contraction of SMC is not related to Ca^{2+} release from mitochondria since KCN or rotenone had no effect on this contraction.

PAF shifts the "pCa-tension" relationship to the left, *i.e.* to higher pCa values, which indicates that the Ca^{2+}-sensitivity of contractile proteins in SMC had increased. The increased sensitivity of the contractile proteins of SMC to Ca^{2+} may be linked to protein kinase C activation (7,8). It is known that protein kinase C maintains its activity in skinned SMC, because it usually binds to the plasma membrane in the presence of increased cytosolic Ca^{2+}. Since the skinning process takes place in a Ca^{2+}-free solution in the presence of EGTA, it is unlikely that this membrane-associated enzyme loses its activity during treatment of SMC plasma membrane with detergent.

It has been established (8) that the activation of protein kinase C may lead to contraction in coronary artery SMC, which is linked to an increase in Ca^{2+} sensitivity of the contractile proteins. Actually, polymyxin B or H-7, which are protein kinase C inhibitors, completely supressed the PAF-induced sustained tonic contraction.

Thus, our results are consistent with the hypothesis that the contraction of skinned epicardial coronary artery strips induced by PAF occurs through at least two mechanisms: (i) Ins P_3-induced release of Ca^{2+}, stored in the SR, and (ii) activation of protein kinase C and increase in the Ca^{2+}-sensitivity of the contractile machinery. These two mechanisms are closely interrelated; the formation of Ins P_3 upon binding of PAF with specific receptors and the synthesis of DG, which is capable of activating protein kinase C, occur simultaneously. The data also suggest that PAF receptors are not only located on the plasma membrane of the SMC, but also on the membrane structures of the SR.

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REVIEW

Role for Platelet-Activating Factor in Asthma[1]

K.F. Chung* and **P.J. Barnes**

Department of Thoracic Medicine, National Heart & Lung Institute and Royal Brompton National Heart & Lung Hospital, London SW3 6LY, United Kingdom

Recent studies of the effects of platelet-activating factor (PAF) on human and animal airways would support a putative role for this lipid mediator in asthma. PAF can induce many aspects of the clinical and pathological features seen in asthmatic airways such as airway oedema, eosinophil accumulation in the airway wall, and bronchial hyperresponsiveness. PAF has potent activity as a chemotactic agent and as an activator of eosinophils, which are prominent cells in asthmatic airways, through the activation of specific surface receptors. The interaction between PAF and eosinophils may be crucial in the pathogenesis of bronchial hyperresponsiveness in asthma. A role for PAF in asthma can now be studied using the recently developed antagonists of the PAF receptor. ***Lipids 26*, 1277-1279 (1991).**

Over the past five to six years, there has been considerable interest in the possibility that platelet-activating factor (PAF) may contribute to the pathophysiology of asthma. This increasing interest is partly due to the observations that PAF can induce many of the clinical and pathological features of asthmatic airways, such as airway oedema, eosinophil accumulation into the airway wall and bronchial hyperresponsiveness. In this review, we will examine some of the effects of exogenously administered PAF on the airways. One unique property of PAF is its potent activity as a chemotactic agent and as an activator of eosinophils, which are prominent cells in asthmatic airways. We hypothesize that this interaction between PAF and eosinophils may be crucial in the pathogenesis of bronchial hyperresponsiveness in asthma. Finally, we will review some recent data concerning the effect of specific PAF antagonists in disease models related to asthma.

EFFECT OF PAF ON AIRWAYS

Airway narrowing. PAF is a potent bronchoconstrictor *in vivo* but exerts negligible direct contractile effects on airway smooth muscle preparations *in vitro* (1-4). This suggests that PAF induces airway narrowing by indirect mechanisms *in vivo*, such as the release of smooth muscle constricting mediators. Another potential mechanism underlying the airway narrowing may be the induction of airway oedema secondary to airway microvascular leakage. Human airway smooth muscle contracts in response to PAF in the presence of platelets *in vitro* (5); whether similar responses can be obtained in the presence of other cells, such as neutrophils or eosinophils, is not known. In man, PAF aerosol induces bronchoconstriction within 2-3 min of inhalation (1); facial flushing occurs transiently and is maximal at 5 min. Rapid tachyphylaxis develops in response to the bronchoconstrictor effect of PAF. Airway responsiveness to PAF aerosol is approximately 100-fold lower than that to methacholine (1). Mild asthmatic subjects, who are hyperresponsive to methacholine, do not appear to be more sensitive to PAF as compared to normal subjects (6).

The role of cells and mediators in PAF-induced bronchoconstriction in man remains unclear. Prostacyclin infused at a concentration required to inhibit PAF-induced platelet aggregation *ex vivo* does not inhibit PAF-induced bronchoconstriction, suggesting that platelets do not contribute to this response (7). Although histamine may mediate PAF-induced wheal formation in the skin, it is unlikely to do so in the airway (8).

Airway microvascular leakage and oedema. PAF is one of the most potent inducers of microvascular leakage throughout the respiratory tract of the guinea pig (9,10). This effect is independent of circulating platelets or of eicosanoid generation (9). An immediate and a delayed increase in airway microvascular leakage of radiolabeled albumin has been observed upon intratracheal administration of PAF, associated with a concomitant exudation of plasma proteins across the airway epithelium (11). Whether PAF-induced airway microvascular leakage and oedema results in airway narrowing *in vivo* is difficult to establish directly. In man, PAF-induced bronchoconstriction is only partially inhibited by a dose of a β-agonist that completely blocks a similar degree of bronchoconstriction induced by the smooth muscle constrictor, methacholine (12). It is possible that the response which is resistant to the β-agonist represents the effect of oedema.

Effect on circulation and bronchoalveolar cells. When inhaled by normal and asthmatic subjects, PAF induces a profound transient neutropenia, with a rebound neutrophilia after 15 min (6,12). No significant decrease in platelet count was observed. The decrease in circulating neutrophils may result from haemodynamic factors such as slowing of pulmonary blood flow induced by PAF; in addition, PAF has been shown to cause an increase in the rigidity of cell membrane structures leading to the trapping of cells within the pulmonary capillary bed. In man, circulating neutrophils become hypodense within 15 min of inhalation, as a result of an increase in cell volume, probably indicating neutrophil activation which would predispose toward an increased adherence to the endothelium (13). Following PAF inhalation, circulating platelets do not show desensitization of PAF-induced aggregation *ex vivo*; by comparison the release of chemiluminescence from circulating neutrophils by PAF is reduced at 90 min (14). Thus, *ex vivo* platelet desensitization to PAF cannot be used as evidence against endogenous PAF release.

Four hours after PAF inhalation, there is an approximate 2-fold increase in neutrophil recovery in bron-

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*To whom correspondence should be addressed at the Department of Thoracic Medicine, National Heart and Lung Institute, Dovehouse Street, London, SW3 6LY, United Kingdom.

Abbreviations: LysoPAF, lyso platelet-activating factor, 1-*O*-alkyl-*sn*-glycero-3-phosphocholine; PAF, platelet-activating factor, 1-*O*-alkyl-2-acetyl-*sn*-glycero-3-phosphocholine.

choalveolar lavage fluid without any significant increase in eosinophil counts in normal volunteers (13). In allergic subjects, intradermal injections of PAF causes a selective cutaneous accumulation of eosinophils (15), raising the possibility of a selective response of eosinophils of allergic subjects to PAF.

Bronchial hyperresponsiveness. PAF induces an increase, sometimes, prolonged, in bronchial responsiveness to methacholine and histamine in several species including man (1,4,16–18). Normal subjects demonstrate a mean 3-fold increase in bronchial responsiveness at 3 days, with a continuing effect for 1–3 wk (1). By comparison, asthmatic subjects do not show an overall increase in airway responsiveness (6). The lack of response observed in asthmatic subjects may reflect the difficulty in further enhancing inflammatory mechanisms that are maintaining bronchial hyperresponsiveness in the asthmatic and that would not be present in the normal subject. The mechanisms underlying PAF-induced bronchial hyperresponsiveness are of interest and may involve eosinophil accumulation and interaction with airway epithelial cells (see below).

INTERACTION OF PAF AND EOSINOPHILS

Human eosinophils, of all types of circulating cells, possess the PAF precursor, alkylacylglycerophosphocholine, in highest abundance (19). Purified human hypodense eosinophils, which are found in increasing amounts in the circulation of asthmatic patients (20), release PAF after IgE- (but not IgG-) mediated activation (21). PAF is a potent activator of human eosinophils. Specific binding of the labeled PAF antagonist [^{3}H]WEB 2086 has been described on isolated human eosinophils, with similar affinity and number of binding sites as on human neutrophils (22). Activation of eosinophils by PAF results in an early rapid rise in intracellular calcium followed by the release of granule-associate enzymes such as eosinophil peroxidase (23,24). Incubation of guinea pig eosinophils with PAF for 2 hr resulted in a significant reduction in the density of these cells (25), thus reproducing the hypodensity of circulating eosinophils found in asthma. Electron microscopy of eosinophils, after incubation with PAF, revealed degranulation of dense-core vesicles, with some increase in cell volume (25). PAF also induces a dose-dependent enhancement of eosinophil cytotoxicity (26) and is one of the most potent chemotactic and chemokinetic mediators for eosinophils *in vitro* (27); it also promotes the adhesion of neutrophils and eosinophils to vascular endothelial cells (28).

This interaction between PAF and eosinophils may be crucial in the pathogenesis of the bronchial hyperresponsiveness of asthma and may be self-perpetuating and self-enhancing. Our hypothesis is that release of granule-associated enzymes such as eosinophil peroxidase, eosinophil cationic protein and major basic protein from the eosinophil induced by PAF may result in damage to airway epithelium, which is another pathological hallmark of asthma (29). Epithelial damage may in turn contribute to bronchial hyperresponsiveness by several mechanisms. These include loss of a putative epithelial-derived relaxant factor, or activation of exposed airway afferent nerves by mediators, or the production of inflammatory mediators (30). We recently incubated guinea pig tracheal segments with eosinophils *in vitro* and found extensive epithelial damage and reduction in ciliary beat frequency when PAF was added to the preparation (31). This effect was not seen with lyso platelet activating factor (lysoPAF). PAF on its own had no cytotoxic effect on the epithelium. These results suggest that epithelial damage may occur through the interaction of PAF with eosinophils.

PAF ANTAGONISTS

Specific and potent PAF antagonists are now available for studying the role of PAF in asthma (32). Several studies have been reported on the effect of PAF antagonists in animal models concerned with induced bronchial hyperresponsiveness and late phase responses. For example, WEB 2086, a triazolobenzodiazepine PAF antagonist, inhibits the late response after challenge with Ascaris antigen in allergic sheep, without affecting the early response (33). BN 52021, the ginkgolide PAF antagonist, inhibits ovalbumin-induced bronchial hyperresponsiveness, and eosinophil recovery in bronchoalveolar lavage fluid in ovalbumin-sensitized guinea pigs (34). These observations suggest that PAF may be involved in the pathogenesis of bronchial hyperresponsiveness through the recruitment and activation of circulating eosinophils.

To date, there are virtually no studies on the effects of PAF antagonists on the late phase response or the bronchial hyperresponsiveness after allergen challenge in man. Such studies are currently being conducted. The ginkgolide mixture BN 52063 has recently been shown to inhibit the late phase cutaneous response to allergen in atopic subjects without significantly altering the early wheal response, probably indicating an inhibitory effect of BN 52063 on eosinophil infiltration into the skin (35). Unfortunately, this oral dose of BN 52063 resulted in only a small inhibitory effect against PAF-induced bronchoconstriction in normal volunteers (36). PAF antagonists delivered directly as aerosols to the airways may be more effective. Chronic dosing with the PAF antagonists BN 52063 was associated with beneficial effects in patients with asthma. However, further clinical studies are needed with more potent PAF antagonists.

CONCLUSIONS

Much remains to be understood about the biology of PAF and about its possible role in the inflammatory process in asthma. The interactions of PAF with other mediators, such as those of the lipoxygenase and cyclooxygenase pathways, need to be further clarified. Regulation of the metabolism of PAF in normal and inflamed tissues has to be elucidated, and the localization of PAF receptors in the activation of normal and asthmatic subjects may contribute to our understanding of the mechanism of action of PAF. Interactions between PAF and eosinophil may be crucial in the pathogenesis of bronchial hyperresponsiveness of asthma. We need such information for the interpretation of the results of PAF antagonists in asthma and in planning the best use of PAF antagonists as possible antiasthma agents. The role of PAF and the potential use of PAF antagonists in asthma remain to be elucidated.

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REVIEW

The Contribution of Platelet-Activating Factor to Allergen-Induced Eosinophil Infiltration and Bronchial Hyperresponsiveness[1]

C.P. Page*
Department of Pharmacology, King's College, University of London, London SW3 6LX, United Kingdom

Platelet-activating factor (PAF) is an ether-linked phospholipid having a range of biological properties of relevance to our understanding of the pathogenesis of bronchial asthma and related allergic diseases. In particular the ability of PAF to induce eosinophil activation and recruitment has received considerable attention. Since in experimental animals PAF and allergen-induced eosinophil infiltration are dependent upon platelet activation, it is suggested that platelet activation may be an important component of the allergic process. The ability of PAF antagonists to inhibit various aspects of the allergic response has been demonstrated in a number of animal models but not all of them have been extended into man. *Lipids 26,* **1280–1282 (1991).**

Eosinophil infiltration into airway tissue (1–3) and bronchial hyperresponsiveness (1–3) are now recognized as central features of bronchial asthma. Eosinophils and their products such as major basic protein and eosinophil cationic protein have been implicated in the pathogenesis of bronchial hyperresponsiveness, in part because they initiate damage to the respiratory epithelium (4–6) suggested as being an essential element of the physiological control of airway smooth muscle responsiveness (7). Despite the fact that many endogenous substances have been shown to be active as chemotactic agents for eosinophils including interleukin-5 (IL-5) (8), eosinophil chemotactic factor of anaphylaxis (ECF-A), and formyl methionyl leucyl phenylalanine (FMLP) (9), very little is known about the mechanisms whereby eosinophils are recruited into the lung following exposure of an allergic subject to an appropriate antigen. It is therefore of considerable interest that platelet-activating factor (PAF) has now been identified as a most potent endogenous chemotactic agent for eosinophils *in vitro* (9) as well as having the ability to induce eosinophil recruitment *in vivo* (10–13).

Additionally, PAF is now recognized as one of the few endogenous mediators capable of eliciting bronchial hyperresponsiveness in both experimental animals (11, 14–17) and in man (18,19). This article will review the contribution of PAF to allergen-induced eosinophil infiltration and bronchial hyperresponsiveness.

[1]Based on a paper presented at the Third International Conference on Platelet-Activating Factor and Structurally Related Alkyl Ether Lipids, Tokyo, Japan, May 1989.

*Mailing address: Department of Pharmacology, King's College, Chelsea Campus, Manresa Road, London SW3 6LX, United Kingdom.

Abbreviations: ECF-A, eosinophil chemotactic factor of asophylaxis; FMLP, formyl methionyl leucyl phenylalanine; IgE, immunoglobulin E; IL-5, interleukin-5; PAF, platelet-activating factor, 1-*O*-alkyl-2-acetyl-*sn*-glycero-3-phosphocholine.

ALLERGEN-INDUCED EOSINOPHIL INFILTRATION

Exposure of sensitized animals to an appropriate allergen results in an inflammatory response which is associated with the infiltration of leukocytes (primarily eosinophils) (11,12,20). Such animal models have therefore been used as possible models of allergic asthma as eosinophil infiltration is known to accompany the exposure of allergic subjects to allergen (1,2). Also, leukocyte infiltration into the lung of allergic animals has been shown to be reduced by pretreatment with clinically useful anti-allergic and anti-inflammatory drugs, such as disodium cromoglycate or glucocorticosteroids (20,21). A number of groups have reported that PAF antagonists will inhibit allergen-induced eosinophil infiltration into the lungs of allergic animals, measured both histologically (12) and by analysis of bronchoalveolar lavage fluid (11). Other investigators have reported that PAF antagonists are capable of inhibiting allergen-induced leukocyte infiltration associated with late-onset airways obstruction in allergic guinea pigs (22), sheep (23) and rabbits (24). It should be noted, however, that some investigators could not demonstrate any beneficial effect of PAF antagonists in models of allergen-induced eosinophil infiltration at concentrations that would inhibit PAF-induced eosinophil infiltration in the same species (25,26). The reasons for these discrepancies are presently not clear.

While corresponding studies have yet to be undertaken in man, PAF is known to induce the recruitment of eosinophils into the skin of allergic (3,27) but not normal subjects (12,28) suggesting that allergic eosinophils respond in a manner different from normal eosinophils, a conclusion borne out by recent *in vitro* data (29). PAF release is also known to accompany the late-onset cutaneous allergic response following i.d. allergen (30), a response believed to parallel in many respects the pulmonary late-onset response to allergen challenge (31) and known to be associated with eosinophil infiltration in both the skin (12,31) and the lung (1,2,31). It is therefore of interest that recently one of the PAF antagonists, BN 52063, has been reported to reduce the late-onset response to cutaneous allergen challenge (32) at a dose thought to be reasonably selective as a PAF antagonist in man (33).

ALLERGEN-INDUCED BRONCHIAL HYPERRESPONSIVENESS

Exposure of sensitized animals to an appropriate allergen results in increased bronchial hyperresponsiveness anything from 1 hr (11,20,34) to 24 hr and beyond (1,11,20) in a manner analogous to that observed in allergic asthmatics following exposure to allergen (1). Many of the mediators implicated in the pathogenesis of asthma (35) have been put forward because of their propensity to

induce bronchoconstriction. One of the interesting properties of PAF is its ability to induce long lasting bronchial hyperresponsiveness to other spasmogens in a number of species including man (14–21). The release of PAF into the lung following allergen exposure may therefore contribute to allergen-induced bronchial hyperresponsiveness. Such a conclusion is supported by experiments from a number of laboratories reporting that a variety of PAF antagonists will inhibit allergen-induced bronchial hyperresponsiveness in a number of experimental models, including the allergic rabbit (24), sheep (23) and guinea pig (11,26). Although PAF has been shown by some investigators (18), but not all (14), to induce bronchial hyperresponsiveness in man, early clinical studies with WEB 2086 have not shown any reduction in allergen-induced hyperresponsiveness in allergic subjects (36).

MECHANISM OF PAF AND ALLERGEN-INDUCED EOSINOPHIL INFILTRATION AND BRONCHIAL HYPERRESPONSIVENESS

It has been shown recently that PAF and allergen-induced eosinophil infiltration in the guinea pig is platelet-dependent because it can be significantly reduced by pretreatment of animals with a cytotoxic anti-platelet antibody or anti-platelet drugs (12). Similar observations have been made in allergic rabbits (37) and normal rabbits (38) although it is not yet known whether platelet dependent eosinophilia is a characteristic of human allergic disease. However, it is now recognized that human platelets can release eosinophil chemotactic factors such as platelet factor 4 and certain lipoxygenase metabolites of arachidonic acid metabolism (39). Furthermore, platelets are a good source of a variety of other chemoattractants such as platelet-derived growth factor and transforming growth factor beta, but it is not known whether these materials have chemotactic activity for eosinophils (39).

Likewise, PAF-induced hyperresponsiveness has been shown to be platelet-dependent in both the guinea pig (14) and rabbit (38), and very recently allergen-induced bronchial hyperresponsiveness in allergic rabbits has been shown to be significantly reduced by pretreatment of the rabbits with a cytotoxic anti-platelet antibody (37). The contribution of platelets to human asthma has been both postulated (39) and questioned (40), but to date there is no evidence for allergen-induced hyperresponsiveness in man being platelet-dependent as the definitive experiments required to prove this hypothesis are difficult to perform in man. Inhalation of PAF by normal volunteers is not associated with overt thrombocytopaenia (41) and this has led to the argument that platelets may not be involved in PAF-induced pulmonary effects in man (41). However, similar observations have been reported in the rabbit (38), yet removal of platelets from the rabbits reduces the ability of PAF to influence both hyperresponsiveness and eosinophil infiltration (38). This suggests therefore that we may have to find more subtle ways of evaluating platelets as inflammatory cells analogous to the experiments of others who have shown that immunoglobin E (IgE)-dependent activation of platelets is not associated with overt aggregation of platelets, but nonetheless is a physiological way of activating platelets which can be inhibited by anti-allergy drugs (reviewed in ref. 42).

CONCLUSION

It would therefore seem likely that platelets can contribute to PAF and allergen-induced bronchial hyperresponsiveness making these cell types legitimate targets for future anti-allergy drugs. While the precise relationships between these cell types remain to be resolved, it would seem prudent to investigate this area more fully, particularly as it relates to human allergic disease.

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Platelet Activating Factor-Induced Pulmonary Accumulation of ^{111}Indium-Oxine Labelled Neutrophils in Anesthetized Guinea Pigs[1]

Ursula Hultkvist-Bengtsson[2], Gary P. Anderson[3], Madeleine Kings[4] and John Morley*
Preclinical Research, Sandoz AG, Basel, Switzerland

The thoracic accumulation of neutrophils labelled with ^{111}Indium-oxine in response to infusion of platelet activating factor (PAF, 18 ng/kg/min × 5 min, i.v.) was studied using an automated isotope monitoring system (AIMSplus) in anesthetized guinea-pigs. Loss of cell associated radioactivity *in vitro* was less than 1% over 4 hr. Labelled neutrophils maintained their functional capacity (oxidative response to the cell stimulants N-formyl-L-methionine-L-leucine-L-phenylalanine and phorbol myristate acetate) and >95% viability (ethidium bromide/acridine orange stain) *in vitro*. Total thoracic radioactivity increased significantly from baseline in response to PAF with a slight tachyphylaxia in the neutrophil-accumulation after a repeat PAF infusion. The highest ratios of radiolabel (tissue/blood) were found in the spleen >> liver > lung.
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Polymorphonuclear leukocytes (PMNL), particularly neutrophils, are participants in the acute inflammatory response of the lungs to platelet activating factor (PAF) in experimental animals. Intratracheal instillation of PAF in rabbits causes dose-related structural derangement of the vascular endothelium and bronchial epithelium associated with aggregation of neutrophils within alveolar capillaries and, to a lesser extent, accumulation in alveolar septa (1). In the guinea-pig aggregates of neutrophils, often in association with platelets, within pulmonary arterioles and the submucosal microcirculation are evident histologically within 1 min of intravenous injection of 100 ng/kg PAF (2). In human volunteers, inhalation of PAF causes a transient, marked neutropenia (3).

Previous investigations of the responses of radiolabelled neutrophils to inflammatory mediators have concentrated on skin lesions or peritoneal accumulation *in vivo*, or on neutrophil adhesion to cultured endothelium obtained from large vessels *in vitro*. However, the lung is a highly specialized organ and the properties of the pulmonary vascular endothelium are likely to differ markedly from endothelium in other vascular beds. Currently it is difficult to isolate and culture endothelial cells *in vitro* from the lung microvasculature, a critical tissue in inflammatory reactions. Novel approaches are therefore required to investigate the interactions of the lung and leukocytes in living animals. The objective of the present study was to adapt the techniques of external monitoring of radiolabelled platelets (4) to the investigation of PAF-induced pulmonary neutrophil accumulation in the anesthetized guinea-pig.

MATERIALS AND METHODS

Animals. Male albino guinea-pigs (Dunkin-Hartley, KFM, Füllinsdorf, Switzerland), 400–500 g body weight, have been used in this study. The animals were initially anesthetized with a combination of ketamine (30 mg/kg s.c.) and xylazine (3 mg/kg i.m.). Anesthesia was maintained throughout the experiment by intravenous infusion of ketamine (15 mg/kg/hr). Catheters were inserted into two external jugular veins (for cell and drug administration) and one carotid artery (for blood pressure recordings, Statham-Gould P23 pressure-transducer and Buxco, model 6 analyzer). Body temperature was monitored rectally and maintained between 37–38°C by a heating pad.

Cell isolation and labelling. About 40 mL of blood was taken by heart puncture into citrate buffer from ether anesthetized donor animals. The blood was added to a mixture of Hypaque/methylcellulose and the red blood cells were allowed to sediment. The leukocyte rich plasma was removed and washed in HBSS (Hanks' balanced salt solution) buffered with HEPES containing bovine serum albumin (BSA). Centrifugation on a discontinuous Percoll gradient was used to separate the neutrophils from the other leukocytes. The resulting neutrophils were routinely checked for purity (differential counts) and viability using ethidium bromide/acridine orange stain (Becton-Dickinson, San Jose, CA). Functional testing of the neutrophils was performed repeatedly during the course of the methodological development using the nitroblue tetrazolium reduction (NBT) test on 1×10^6 cells/well in microtiter plates (Costar, Badhoeredorp, The Netherlands). This test provides an accurate spectrophotometric index of cell function and is based on the reduction of phagocytosed NBT dye to insoluble blue formazan during stimulus induced oxidative burst causing an increase in optical density (5).

^{111}Indium-oxine (^{111}In-ox: 25 μ Ci/3 × 10^6 cells) was used for radio-labelling of the neutrophils (incubated for 10 min at 37°C). About 10×10^6 neutrophils were injected into each recipient animal. Labelling efficiency and stability (radio-activity ratio of supernatant to cell pellet) were routinely checked. In two animals, the presence of labelled neutrophils in the blood was checked over two hr after cell injection.

External detection of radioactivity. An automated isotope monitoring system (AIMSplus Mumed Ltd., London, England) was used to evaluate the ^{111}In-ox

*To whom correspondence should be addressed at Preclinical Research, Sandoz AG, 386/325, CH-4002 Basel, Switzerland.
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[2]Present address: Gambro AB, P.O. Box 10101, S-220 10 Lund, Sweden.
[3]Present address: CIBA GEIGY AG, R-1056.2.17, CH-4002 Basel, Switzerland.
[4]Present address: Hoffmann La Roche, PPR/RN 52/110, CH-4002 Basel, Switzerland.

Abbreviations: AIMSplus, automated isotope monitoring system; BSA, bovine serum albumin; FMLP, *N*-formyl-L-methionine-L-leucine-L-phenylalanine; PAF, platelet activating factor; PMA, phorbol myristate acetate; PMNL, polymorphonuclear leukocytes; NBT, nitroblue tetrazolium reduction.

neutrophil accumulation in the lung. This system previously has been described in detail by Smith *et al.* (4). Four experiments were undertaken in parallel using labelled cells from the same batch. An NaI crystal scintillation detector was positioned over the thorax of a spontaneously breathing guinea-pig placed supine. Ten min after the injection of labelled neutrophils, when the cells were circulating, the spectrometer was normalized to count 10,000 cps (^{111}In-ox measured at 245 keV), in order to compare results between animals injected with slightly different amounts of radioactivity. Basal count rates were monitored for a further 30 min prior to challenge. The radioactivity was monitored continuously and count rates were stored on an IBM AT computer.

PAF administration. Two infusions of PAF (1-*O*-hexadecyl-2-acetyl-*sn*-glycero-3-phosphocholine; Nova Biochem, Läufelfingen, Switzerland) were given i.v. by a precision pump in a dose of 18 ng/kg/min for 5 min with 1-hr interval between doses. PAF was dissolved in ethanol and diluted in saline containing 0.25% BSA (final ethanol concentration <0.005%). The BSA-saline vehicle was used as a control infusion.

In vitro *gamma scintillation of dissected organs.* At the end of the experiment, the animals were killed by an overdose of pentobarbital and dissected. Samples (lung, liver, spleen, heart, kidney, ileum, skin, urine, plasma, bone marrow, whole blood) were counted in a well counter (LKB compugamma scintillator) and the ratio between the organ activity and the whole blood activity was calculated.

Statistics. Data are presented as mean ± SD. One-way analysis of variance (ANOVA) over time was used for statistical evaluation within the treatment and control groups.

RESULTS

The cell isolation technique utilized gave a >90 ± 5% pure neutrophil population with a viability of 95 ± 5%. Microscopic examination of the isolated neutrophils revealed that the cells comprised a single cell suspension. The phagocytic and metabolic functions of the isolated neutrophils were evaluated by the NBT-assay, indicating that the cells were still responsive to a stimulus and were not desensitized by *in vitro* handling. This was indicated by a dose-dependent free radical release induced by PMA (7.4×10^{-10}–10^{-6} M, increase in optical density at 540 nm up to 135% from the control value) and FMLP (1×10^{-10}–10^{-6} M, increase in optical density at 540 nm up to 82% from the control value).

Labelling efficiency was found to be> 50%, comparing the activity in the cell pellet with total added activity. The loss of activity from the cells *in vitro* was found to be <1% over a period of four hours. About 90% of the total activity injected into the animals was associated with the cells.

Total thoracic radioactivity in our experimental model represents the net pulmonary retention and efflux of radiolabelled neutrophils. Initially there was a quick release of labelled neutrophils from the lung that slowed after about 25 min from the time of injection. About 35% of the initial ^{111}In-activity had left the thorax region after 25 min. The presence of labelled neutrophils measured as ^{111}In-activity in peripheral blood was found to stabilize after 10 min at about 50% of the peak value determined just after cell injection. The response of radiolabelled neutrophils to intravenous PAF (18 ng/kg/min) is shown in Figure 1. Infusion of PAF caused an acute increase in thoracic radioactivity that peaked at 5 min and fell below pre-infusion levels over a 20-min period. Repeated doses of PAF confirmed that the neutrophils remained functional during the experiment and were only slightly desensitized to the agonist. The first PAF-infusion caused a transient decrease in basal blood pressure (base value 48 ± 8 mmHg) of 30% after 5 min. The second infusion induced a similar but deeper hypotension (45% of base-line) at 6 min.

The highest ratios of radiolabel (tissues/whole blood) in the samples were found in the spleen (52.4 ± 18.8), liver (21.4 ± 8.2) and lung (11.3 ± 9.0). The other samples showed ratios of less than 3.

DISCUSSION

The principal findings of this study are that ^{111}In-ox labelled neutrophils retain functional capacity and viability, and circulate in peripheral blood. In response to intravenous infusion of PAF, the neutrophils accumulated in the lungs of the guinea pigs. The use of external radiation detection of labelled cells has allowed the kinetics of this response to be monitored accurately in real time.

Under the experimental conditions employed in this study the neutrophils became only slightly desensitized to a repeat infusion of PAF. The data suggest that the accumulation of neutrophils in the thorax cannot be accounted for by removal of PAF-damaged neutrophils by the pulmonary circulation. A similar maintenance of functional capacity and lack of tachyphylaxis to leukotriene B_4-induced pulmonary accumulation of ^{111}In-tropolonate labelled neutrophils in the guinea pig *in vivo* has been reported (6). The organs with the highest radioactivity counts in the present study were the spleen and the liver, a finding consistent with the physiological role of these organs as the major clearance sites of neutrophils from the circulation (7). Our experiments cannot resolve whether neutrophils (which are normally short-lived) were damaged by the isolation and labelling procedures and were cleared by the spleen and liver prior to PAF infusion, or were removed from the circulation after PAF exposure. Under normal conditions, the lungs comprise the major pool of blood neutrophils, which may account for the relatively high basal radioactivity of this organ.

The mechanism of neutrophil accumulation after PAF infusion is not known. It is unlikely that the increase in thoracic radioactivity observed after infusion of PAF was an artifact secondary to an increase in thoracic blood volume since PAF infused at the same dose under identical conditions did not cause an increase in thoracic radioactivity when guinea pigs were injected with ^{111}In-labelled erythrocytes, a cell unresponsive to PAF and therefore suitable for use as a marker of thoracic blood volume (4).

Several possible mechanisms may cause neutrophils to be retained within the pulmonary vasculature in response to PAF with a time course consistent with the results of the present study. Firstly, PAF causes direct aggregation of neutrophils *in vitro* (8). Trapping of such aggregates within the microvasculature and their subsequent

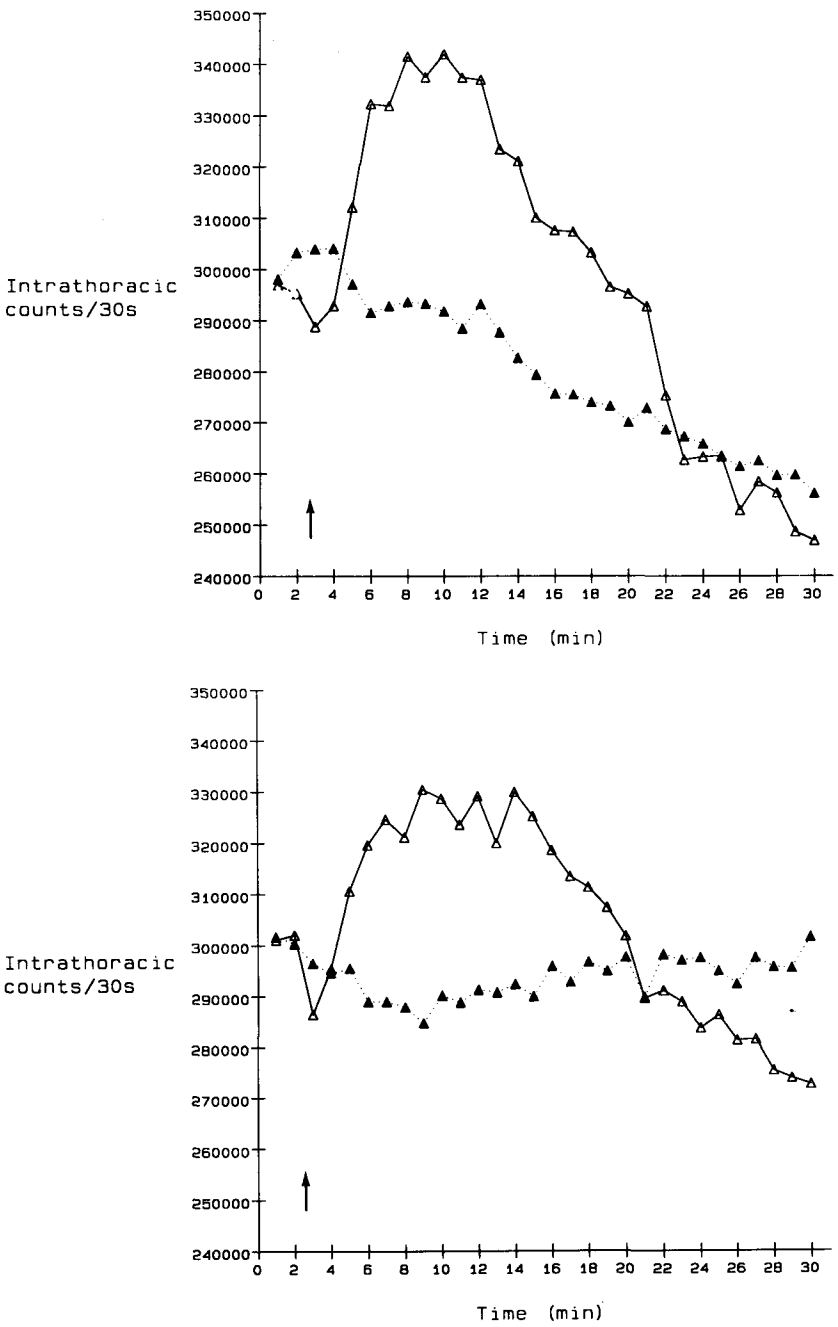


FIG. 1. Effect of i.v. PAF-infusion (18 ng/kg/min, 5 min) on thoracic accumulation of radiolabelled neutrophils. Points represent the mean group value. Top: First infusion (at arrow); Δ-Δ, PAF n=9 p<0.001; ▲-▲, BSA n=7 p<0.001. Bottom: Second infusion (at arrow); Δ-Δ, PAF n=9 p<0.001; ▲-▲, BSA n=6 n.s. One-way analysis of variance (ANOVA) over time was used within the treatment and control groups.

disassociation would account for both the peak and decline in radioactivity but may not explain the fall below the pre-challenge baseline count rate. The present study does not exclude the possibility that PAF simultaneously recruits circulating neutrophils into the lungs and causes detachment of pooled neutrophils altering the ratio between labelled and unlabelled cells that are retained within the pulmonary vasculature. Secondly, PAF also causes platelets to aggregate and to be retained within the lungs (4). Histological sections of guinea pig lungs exposed to PAF have revealed intravascular co-aggregates of neutrophils and platelets (2). Since platelets stimulated by PAF *in vivo* under similar experimental conditions to those of the present study spontaneously aggregate and disaggregate with a time course similar to that observed for neutrophils in the present study (4), it is possible that the increases in thoracic radioactivity we observed represent the trapping of neutrophils in intravascular platelet

aggregates. Finally, investigations into the mechanisms of neutrophil/endothelium interactions have revealed the existence of inducible adhesion molecules, termed leukocyte integrins, which may mediate rapid adhesion of neutrophils to endothelium in response to chemotactic stimuli (9). The contribution of such molecules to the effects of PAF observed in the present study remains to be determined.

Since the present studies suggest that PAF-induced accumulation of neutrophils in the lungs is a transient event, but histological evidence (2) indicates that neutrophils leave the vascular compartment rapidly after stimulation, several important questions remain to be answered. It will be of interest to determine whether inhibition of transient neutrophil accumulation can affect emigration of this cell type from intravascular spaces and whether those cells that emigrate through the endothelium are part of the marginated pool rather than newly recruited neutrophils. Further investigations into the mechanisms and implications of pulmonary neutrophil accumulation in response to PAF are in progress in this laboratory.

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Platelet Activating Factor Induced Respiratory Mucosal Damage[1]

Ken-ichi Hisamatsu*, Tetsuya Ganbo, Tsutomu Nakazawa and Yoshihiko Murakami
Department of Otorhinolaryngology, Yamanashi College of Medicine, Tamaho-cho, Nakakoma-gun, Yamanashi 409-38, Japan

Human sinus mucosal specimens from eight normal individuals were exposed to platelet activating factor (PAF) at concentrations ranging from 10^{-6} M to 10^{-11} M in a humidified CO_2 chamber at 37°C. The mucosal surface of the specimens was recorded on video tapes and magnified 2,500 times on a 19-inch television (TV) monitor. Ciliary activity of each ciliated cell was photoelectrically measured on the TV monitor in real time. PAF induced mucosal damage which resulted in a coarse profile including ciliostasis and exfoliation of epithelial cells. The length of the incubation period in which the initial coarse profile occurred on the mucosal surface inversely correlated with the concentration of exposed PAF ranging from 10^{-6} M to 10^{-10} M with $r = -0.712$ ($p < 2 \times 10^{-4}$). Both the control medium and 10^{-8} M lysoPAF showed no effect on ciliary activity or mucosal surface alteration even after 24 hr of exposure. Significant ciliary inhibition was noted after 6 hr of exposure to PAF at concentrations of 10^{-8} M and 10^{-10} M ($p < 0.05$). After 10 hr of exposure, significant ciliary inhibition ($p < 0.01$) was noted at all concentrations. Inhibition occurred in a time- and dose-dependent manner. The length of the incubation period in which initial ciliostatis occurred and the level of PAF concentration showed an inverse correlation with $r = -0.918$ ($p < 10^{-6}$). These results indicate that PAF is cytotoxic to human respiratory mucosa.

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Although various types of chemical mediators have been proposed in inflammatory disorders, there are few reports on their cytotoxic effect on the respiratory mucosa. In addition, there has only been limited research on inflammatory mediators and/or factors which have the capacity to damage the respiratory mucosa, *i.e.*, neutrophil-derived elastase, superoxide, and eosinophil granule cationic proteins.

Platelet activating factor (PAF) is thought to be an important chemical mediator in respiratory diseases, because it has various potent biological activities such as vasodilation, increased blood vessel permeability, enhanced mucosal gland secretion, and contraction of smooth muscles. Furthermore, PAF induces an accumulation of eosinophils (1) which have the capacity to generate more PAF (2), as well as leukotriene C_4 (3) and superoxide (4). Therefore, PAF has been thought to be involved in the pathogenesis of respiratory allergy through eosinophil-induced mucosal epithelial damage which leads to nonspecific hyperresponsiveness (5).

Although various pharmacological activities of PAF have been reported to date, there is little information on the effect of PAF on the mucosal epithelium.

The mucociliary transport system is an important respiratory defense mechanism. However, in patients suffering from chronic inflammation of the airway, infiltration of the respiratory mucosa by inflammatory cells can be observed. The system's dysfunction results in mucus being retained which contains a considerable number of inflammatory cells. Furthermore, these cells release PAF and LTB_4 resulting in a vicious cycle where the number of inflammatory cells is increased which further increases the release of mediators. This enhances and causes long-lasting clinical symptoms of the respiratory tract.

In the present paper we describe PAF-induced ciliary inhibition of normal human paranasal sinus mucosa *in vitro*. Ciliostasis and mucosal damage are also described. The lowest PAF concentration to induce mucosal damage in our experiments was 10^{-10} M, which is approximately the same as what occurs in nasal secretions (6).

MATERIALS AND METHODS

Mucosal specimens and testing solution. The mucosal specimens were obtained at the time of surgery from the paranasal sinuses of eight patients who had suffered trauma to that region as a consequence of traffic or sporting accidents. None of the eight patients were smokers or suffered from nasal diseases prior to the accidents.

The specimens were transferred to Eagle's minimal essential medium (MEM) containing Kanamycin, 60 mg/L and 20% fetal calf serum (FCS). They were rinsed in the culture medium and cut into pieces approximately 3 × 5 mm. The specimens were then transferred onto a collagen layer in 35 × 10 mm culture dishes which were prepared with collagen for tissue culture and maintained in Eagle's MEM under 5% CO_2 at 37°C.

Three solutions were used in the study. The PAF solution was made by dissolving PAF(C_{16}) in methanol at a concentration of 10^{-2} M. This solution was then diluted with Eagle's MEM without FCS from 10^{-6} M to 10^{-11} M. The 10^{-8} M lysoPAF(C_{16}) solution and the control solution of 10^{-8} M methanol / Eagle's MEM without PAF were prepared in the same manner.

Recording of the mucosal surface profile. The procedure for observation and recording of the mucosal surface profile was described in detail elsewhere (7). Briefly, the experiments were performed under 5% CO_2 at 37°C using a humidified CO_2 chamber equipped with an

[1]Based on a paper presented at the Third International Conference on Platelet-Activating Factor and Structurally Related Alkyl Ether Lipids, Tokyo, Japan, May 1989.

*To whom correspondence should be addressed at Department of Otorhinolaryngology, Yamanashi College of Medicine, 1110 Shimogato, Tamaho-cho, Nakakoma-gun, Yamanashi 409-38, Japan.

Abbreviations: ECP, eosinophil cationic protein; EDN, eosinophil-derived neurotoxin; EPO, eosinophil peroxidase; FCS, fetal calf serum; MBP, eosinophil granule major basic protein; MEM, minimal essential medium; PAF, platelet activating factor; VTR, video tape recorder.

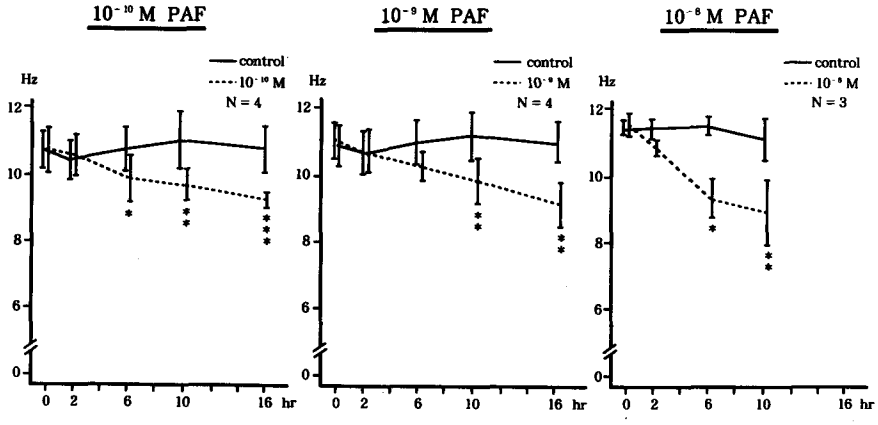


FIG. 1. PAF induced ciliary inhibition at concentrations of 10^{-10} M and higher in the time-dependent manner. Changes in the ciliary activities after exposure to PAF at concentrations of 10^{-10} M (N=4), 10^{-9} M (N=4) and 10^{-8} M (N=3) were analyzed using an analysis of variance for repeated measures. Ciliary activity after exposure to PAF at concentrations of 10^{-10} M, 10^{-9} M and 10^{-8} M significantly decreased after 6 hr, 10 hr and 16 hr of exposure in a time- and dose-dependent manner when compared with time 0 (zero) values, with the exception of 10^{-9} M at 6 hr. There was no significant change in the ciliary activity of each control group. *: $p<0.05$, **: $p<0.01$, ***: $p<0.001$

TABLE 1

Ciliary Activity of the Control and Test Groups at the Onset[a]

Group	N[b]	Hz (mean±SD)	
Control	4	11.0	0.54
10^{-6} M PAF	4	11.0	0.66
Control	3	11.3	0.20
10^{-8} M PAF	3	11.3	0.31
Control	4	10.9	0.51
10^{-9} M PAF	4	10.7	0.59
Control	4	10.8	0.45
10^{-10} M PAF	4	10.8	0.53
Control	3	10.9	0.38
10^{-11} M PAF	3	10.8	0.28
Control	3	11.2	0.12
10^{-10} M lysoPAF	3	11.3	0.14

[a]There were no significant differences at the onset of exposure to PAF between the ciliary activities of each control and test group nor among the test groups.

[b]"N" = the number of experiments performed.

inverted phase-contrast microscope and a television (TV) camera connected to a video tape recorder (VTR) system. The mucosal surface profile was recorded on a video tape and it was magnified approximately 2,500 times on a 19-inch TV screen. The magnified width of a single ciliated cell was about 25 to 30 mm on the screen. Ciliary activities of the individual cells on the TV monitor were measured photoelectrically. If the number of ciliostatic cells increased to more than 25% of all observed cells, the recording was discontinued. Portions of the mucosal specimens used in this study were examined under a light microscope. Only mucosal specimens which appeared to be normal, without inflammatory cell infiltration and with intact surfaces, were used for the tests.

Alterations of mucosal surface profiles were observed and categorized into three groups: i) coarse profile—early phase of exfoliation (may or may not include some exfoliated cells); ii) exfoliation—where xfoliation is clearly observed; iii) ciliostasis—a complete cessation of ciliary movement.

Statistical analysis. Student's t-test was used to compare the ciliary activity of each control specimen with the test group at the onset of exposure to PAF and lysoPAF. Analysis of variance for repeated measures was employed for the effect of PAF on the ciliary activity at concentrations of 10^{-8} M, 10^{-9} M and 10^{-10} M, and changes in the ciliary activity in the time course were analyzed by Scheffé's multiple comparison at the $p<0.05$ (*), $p<0.01$ (**) and $p<0.001$ (***) levels. Common regression lines were established between PAF levels and the length of the incubation period for the appearance of: i) coarsening of the mucosal surface and ii) initial ciliostasis. The correlation coefficients between PAF concentration and the length of the incubation periods were calculated.

RESULTS

Effect of PAF exposure on the mucosal surface profile. Mucosal damage which showed coarse profile, ciliostasis, and exfoliation of the epithelial cells was observed following exposure to PAF at concentrations ranging from 10^{-6} M to 10^{-10} M. PAF exposure caused mucosal cell swelling, increasing the volume of their cell body, resulting in coarsening mucosal surface profile and eventually exfoliation. The incubation period required for coarse profile to initially appear showed an inverse correlation with the concentration of PAF ranging from 10^{-6} M to 10^{-10} M with $r = -0.712$ ($N = 18$, $p< 2\times10^{-4}$). The regression equation was calculated

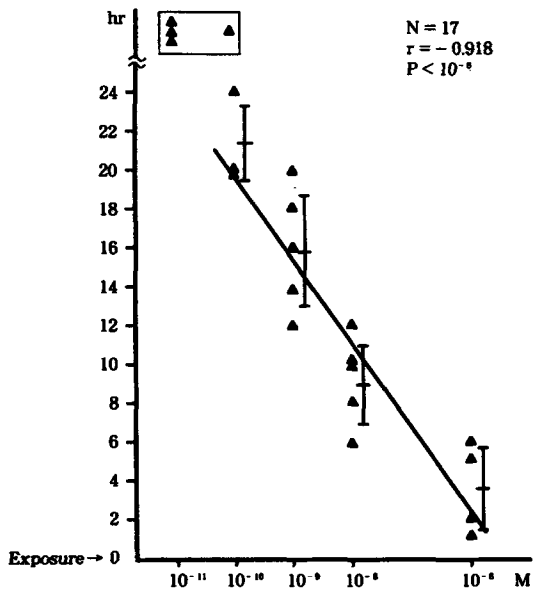


FIG. 2. The length of the incubation period for ciliostasis to initially occur and the PAF concentration showed an inverse correlation with r = − 0.918 (N = 17, p<10⁻⁶). The regression equation is Y = − 4.435 $\log_{10}$X + 24.261. Even after 24 hr, PAF failed to cause ciliostasis at a concentration of 10^{-11} M in all three test groups and in one of four test groups at 10^{-10} M (These groups are indicated inside the rectangle and were not considered in the statistical analysis).

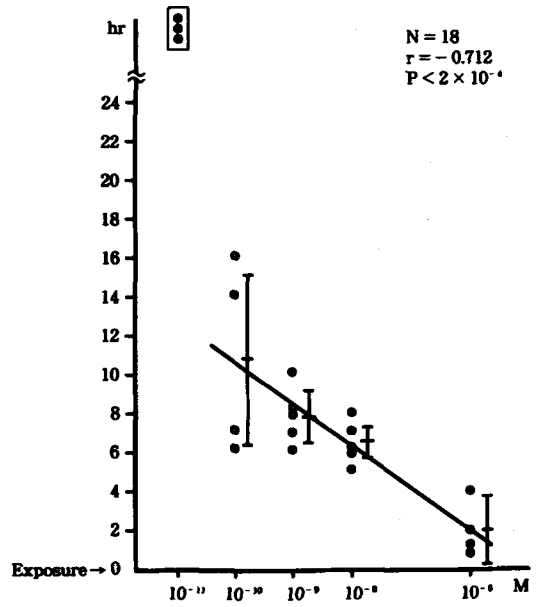


FIG. 3. PAF induced epithelial damage showing coarse profile and exfoliation at concentrations of 10^{-10} M and higher on human sinus mucosal specimens from normal individuals. The length of the incubation period for coarse profile of the mucosal surface to initially occur inversely correlated with the concentration of PAF with r = − 0.712 (N = 18, p<2 × 10^{-4}). PAF failed to cause mucosal damage at a concentration of 10^{-11} M even after 24 hr of exposure (These groups are indicated inside the rectangle and were not considered in the statistical analysis.).

between the incubation period (Y) and the PAF concentration (X): [Y = 10.682 + B $\log_{10}$X; B = −2.109, −3.278 < B < −0.812 (p = 0.01)]. PAF failed to cause alterations of mucosal surface at a concentration of 10^{-11} M even after 24 hr (Fig. 3).

LysoPAF/Eagle's MEM at a concentration of 10^{-8} M and the control groups showed no effect on mucosal surface profile throughout the experiments.

Effects of PAF on ciliary activity of human nasal sinus mucosa. Ciliary activity was measured to investigate cell viability of the ciliated cells of mucosal specimens. There were no significant differences at the onset of exposure to PAF between each control group and test group nor among the test groups (Table 1).

After 2, 6, 10, and 16 hr of exposure to PAF at concentrations of 10^{-8} M to 10^{-11} M, ciliary activity was analyzed in a time-dependent study. After 6 hr of exposure, significant ciliary inhibition ($p<0.05$) was noted at concentrations of 10^{-8} M and 10^{-10} M. After 10 hr of exposure, significant ciliary inhibition ($p<0.01$) was noted at all concentrations (Fig. 1). Ciliary inhibition was not noted in test groups with 10^{-11} M PAF and 10^{-8} M lysoPAF even after 24 hr of exposure.

The correlation between the incubation period required for ciliostasis to initially appear and PAF concentration was analyzed in 17 experiments and showed an inverse correlation with r = −0.918 ($N = 17, p < 10^{-6}$). The regression equation between the incubation period (Y) and the exposed PAF concentration (X) [Y = 24.261 + B $\log_{10}$X; B = −4.435, $-5.894 < B < -2.976$ ($p = 0.01$)] showed a linear relationship. The results clearly indicate that PAF induced slow reacting ciliary inhibition in a time- and dose-dependent manner at concentrations of 10^{-10} M or higher (Fig. 2).

DISCUSSION

One of the important clinical characteristics of respiratory allergic diseases is nonspecific hyperresponsiveness of the respiratory tract. Symptoms, such as sneezing attacks, hyperrhinorrhea, nasal obstruction, asthmatic attacks, etc. are even easily provoked by non-specific irritations. Therefore, it is necessary to understand the mechanisms of hyperresponsiveness of the respiratory tract for the clinical management of allergic patients.

Epithelial damage exposes sensory nerve endings resulting in mucosal nonspecific hyperresponsiveness. In addition it has been suggested that axon reflexes (8) exist at the sites of the exposed sensory nerve endings releasing neuropeptides such as substance P and calcitonin gene-related peptide, which may cause hypersecretion, smooth muscle contraction and also extravasation resulting in mucosal edema. Following epithelial damage, it is also suggested that putative epithelial-derived relaxant factor (9,10) and secretory inhibitory factor (11) might be reduced resulting in hyperresponsiveness of the lower respiratory tract. Thus, the mucosal epithelial damage and dysfunction are thought to be attributable not only to the disturbance of the muco-ciliary transport system resulting in exacerbation and sustained clinical symptoms, but also to the nonspecific hyperresponsiveness of the respiratory tract.

PAF is a unique chemical mediator because PAF inhalation causes not only immediate symptoms and sustained airway hyperresponsiveness but also long-lasting inflammation and eosinophilia, in animals (12) and humans (13). Eosinophil accumulation in the respiratory mucosa (14,15) and secretions (16,17) are also one of the distinctive clinical characteristics of respiratory allergic diseases.

Hyposensitivity of the airway to β-adrenergic agonists is a common characteristic found in allergic patients. It has been noted that the incubation of human trachea and lung tissue with PAF decreased the β-adrenergic bronchodilator responsiveness (18). Furthermore, in brain tissue, PAF decreased the density of β-adrenergic receptors (19). This suggests there is a mechanism by which PAF induces hyperresponsiveness by interfering with receptor sites of organ tissues. It is also suggested that PAF acts as a potent regulator of arachidonic acid metabolism and *vice versa* (20,21). Therefore, other inflammatory mediators such as leukotorienes and prostaglandins may possibly mediate PAF actions *in vivo*; however, they do not sustain airway hyperresponsiveness by themselves (22,23).

Presently it is believed that non-specific hyperresponsiveness of the respiratory tract is caused as a consequence of the inflammation following allergen challenge, *i.e.* the late phase response which is characterized by eosinophilic inflammation (24). This hypothesis has been further supported by the following advances in research on PAF and eosinophils. It has been reported that PAF is the most potent eosinophil chemotactic agent, with activity approximately one-hundred times stronger than that of eosinophil chemotactic factor of anaphylaxis released from mast cells following allergen challenge (25).

Eosinophils predominantly generate PAF (26) although various other inflammatory cells such as neutrophils, monocytes, platelets and macrophages also generate and relase PAF (2,27–29). Thus, there seems to be a "vicious cycle" between PAF and eosinophils. PAF activates eosinophils to release LTC_4 and superoxide. Eosinophil activation and degranulation are also possibly regulated by other inflammatory cells such as macrophages (30), T-cells (31), mast cells (32), basophil (31), etc. Recent studies have shown that eosinophils have cytotoxic proteins such as eosinophil granule major basic protein (MBP) (33), eosinophil cationic protein (ECP) (34), eosinophil peroxidase (EPO) (35) and eosinophil-derived neurotoxin (EDN) (36) which are released as a reaction to allergen challenge and/or other stimuli (31). MBP deposition and mucosal damage to the respiratory tract mucosa were observed in chronic sinusitis and/or asthmatic patients (15). Our recent study on MBP cytotoxicity showed human MBP induced human sinus mucosal damage *in vitro* at concentrations from one to ten μM which were achieved *in vivo* (7).

In the present study, living ciliary epithelium were investigated before and after exposure to PAF *in vitro*. Ciliary activity was measured, since it seems to be the best parameter for ciliated cell viability. One of the advantages of departing from the traditional forms of measurement and using our video tape recorder (VTR) system, which records mucosal surface profile in real time, is that the time course of up to 15 ciliated cells per mucosal specimen could be measured throughout the experimental period. Furthermore, since each single cell was magnified 2,500 times, we could easily observe morphological changes (*i.e.* swelling of the cell body which resulted in coarsening mucosal surface and eventually cell exfoliation, and ciliostasis) (7).

Although the mucosal specimens came from 8 normal subjects, all specimens showed a smooth profile, and data obtained showed no significant differences in ciliary activities at the onset of the experiment between controls and tested mucosa nor among the test groups. Furthermore, there were no significant changes in ciliary activity in the controls throughout the experiments, creating a high internal validity.

Cytotoxicity of PAF was noted *in vitro* in a time- and dose-dependent manner (Fig. 1, Fig. 2 and Fig. 3), whereas lysoPAF showed no effect on ciliary activity, nor caused mucosal surface alterations. Although PAF induces a slow reactive effect on sinus mucosa and possibly changes to lysoPAF during culture with mucosal tissues, we feel there is little if any possibility that second messengers induced the mucosal dysfunction and damage obtained in the present study because of the absence of inflammatory cells. In another study, we showed that after one-hour exposure to 10^{-8} M PAF followed by irrigation of the culture medium, ciliary inhibition and mucosal damage occurred. These data suggest that PAF exerts its impact on the mucosal epithelium within one hour of exposure, followed by slow reactive damage.

In order to verify that PAF exposure specifically induced ciliary inhibition and epithelial cell damage, we investigated the effect of CV-3988, a specific PAF receptor antagonist, on the slow reactive phenomenon following PAF exposure. CV-3988 completely inhibited PAF-induced slow reactive mucosal damage (Ganbo, T., and Hisamatsu, K., unpublished data). These results suggest that PAF directly induced mucosal damage through the receptor site of the respiratory mucosal cells.

The mechanism of PAF-induced cell damage is not known in detail. However, swelling of the cell body was consistently observed on the TV monitor during the process of mucosal surface alteration. This is possibly the result of an influx of extracellar liquid into the cell. Furthermore, in a previous paper we had reported that PAF induced ultrastructural alteration of epithelial cells, such as atypical vacuoles, swollen mitochondria, and increasing secondary lysosomes prior to significant ciliary inhibition and mucosal surface alteration (37).

In the present study, the lowest concentration of PAF that induced the mucosal damage appears to be 10^{-10} M. This is particularly interesting in light of a recent study that achieved an approximate concentration of 10^{-10} M in nasal washings from patients with allergic rhinitis (6).

Based on the data obtained in this study, we propose that PAF cytotoxicity may contribute to the dysfunction of the airway muco-ciliary transport system in inflammatory respiratory diseases. Especially in respiratory allergies, eosinophil-derived PAF may contribute to mucosal epithelial damage leading to nonspecific mucosal hyperresponsiveness and persistency associated with cytotoxicity of eosinophil cationic proteins. The results from the present study support that PAF cytotoxicity may also contribute to the mechanisms which induce tissue damages *in vivo* which have been reported recently

(38,39). Details of the mechanism of PAF-induced cytotoxicity and the regulation of cytotoxicity *in vivo* remain to be elucidated.

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Platelet-Activating Factor Detected in Bronchoalveolar Lavage Fluids from an Asthmatic Patient[1]

Tsugio Horii[a], Hitoshi Okazaki[a], Minoru Kino[a], Yohnosuke Kobayashi[a], Kiyoshi Satouchi[b,*] and Kunihiko Saito[b]

[a]Department of Pediatrics and [b]Department of Medical Chemistry, Kansai Medical University, Moriguchi, Osaka 570, Japan

It recently has been recognized that platelet-activating factor (PAF) may be a mediator of asthma exacerbation. We had the opportunity to analyze bronchoalveolar lavage fluids from an asthmatic infant, which were characterized by neutrophil infiltration. The patient's lungs were washed on three occasions with saline during asthmatic attacks. PAF was found in each case on the basis of its ability to cause the immediate aggregation of washed rabbit platelets. The PAF detected was equivalent to 1-1.4 pmol of 1-*O*-hexadecyl-2-acetyl-*sn*-glycero-3-phosphocholine, three quarters of which were recovered in cell-associated form. By contrast, we did not detect PAF in bronchoalveolar exudates from patients with laryngeal stenosis or with respiratory distress syndrome. LysoPAF, the direct precursor as well as initial metabolite of PAF, was also analyzed after being converted to PAF by acetylation. There was a wide variation in the amount of lysoPAF present in individual patients, suggesting that lysoPAF levels cannot be taken as an indicator for the presence of PAF.

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Platelet-activating factor (PAF) is attracting considerable attention as a potential mediator of bronchial asthma (1). Several findings suggest that PAF is involved in human asthma. Alveolar macrophages from asthmatic patients release PAF when stimulated with specific antigens (2). In addition, human inhalation of PAF as an aerosol causes an immediate dose-dependent bronchoconstriction, as well as an increase in nonspecific bronchial responsiveness (3). In bronchial provocation tests to allergens in asthmatic patients, BN 52063, a specific antagonist to PAF, significantly ameliorated early bronchoconstriction and showed a tendency to inhibit residual bronchial hyperreactivity (4). However, there has been no report of PAF being detected in bronchoalveolar lavage fluids (BALF) from asthmatic patients.

In relation to the development of lung function, we have analyzed surfactant phospholipids with special attention being paid to the perinatal period (5). The present study was undertaken to examine whether PAF can be detected in BALF from asthmatic patient.

MATERIALS AND METHODS

Chemicals. 1-*O*-Hexadecyl-2-acetyl-*sn*-glycero-3-phosphocholine (1-*O*-hexadecyl-2-acetyl GPC) and 1-*O*-hexadecyl GPC were purchased from Novabiochem AG (Läufelfingen, Switzerland). 1-Palmitoyl, 1-stearoyl and 1-oleoyl GPC were from Sigma Chemical Co. (St. Louis, MO). Acyl analogues of PAF were synthesized from acyl GPC by reaction with acetic anhydride in the presence of perchloric acid as catalyst (6). Bovine serum albumin (BSA) was obtained from Sigma. Silica Gel 60 and *tert*-butyldimethylchlorosilane were from Merck (Darmstadt, Germany) and Applied Science (State College, PA), respectively. Calcium ionophore A23187 (IoA23187) was from Calbiochem (San Diego, CA). Formyl-methionyl-leucyl-phenylalanine (fMLP) was obtained from the Protein Research Foundation (Osaka, Japan). IoA23187 and fMLP were each dissolved in dimethylsulfoxide at a concentration of 1×10^{-3} M. fMLP was diluted ten-fold with saline. CV-3988 and L-652,731 were kindly donated by Takeda Pharmaceutical Co. (Osaka, Japan) and Merck (Rahway, NJ), respectively. Phospholipase C (EC 3.1.4.3) was prepared from *Bacillus cereus* culture medium (7). All other chemicals were of reagent grade.

Patients. BALF and bronchoalveolar exudates were obtained from patients which were in our hospital for medical attention. These included an 11-month-old boy with the clinical symptoms of asthma of the non-atopic type, and a 4-month-old girl with laryngeal stenosis, but without loss of lung function. BALF was obtained from these patients by washing the lungs with a small volume of sterile saline by tracheal cannula. Other patients included newborn infants with respiratory distress syndrome (RDS) and one 14-year-old patient with pneumonia. Bronchoalveolar exudates were recovered from these patients by suction with a tracheal cannula.

Aliquots of BALF and lung exudates in saline were treated with May-Geimsa's stain before characterizing the inflammatory cells by microscopic examination (8). In the cases of asthma and pneumonia, neutrophils were observed; however, the exudates from patients with stenosis and RDS were essentially free of infiltrated inflammatory cells except for mononuclear cells. In each case, the remaining exudate was immediately subjected to lipid extraction.

Extraction and purification of PAF. Total lipids were extracted using the one- and two-phase systems described by Bligh and Dyer (9). Phospholipid content in extracts was determined by the method of Bartlett (10). Total lipids in minimal volumes of chloroform were then subjected to silicic acid column chromatography. Neutral lipids were eluted with chloroform, and phospholipids were recovered thereafter by methanol elution. More than 95% of lipid phosphorus was thus recovered. The total chloroform fraction and one-tenth of the methanol fraction were subjected to bioassay for PAF, but none of the preparations showed platelet aggregating activity. The remaining methanol

[1]Based on a paper presented at the Third International Conference on Platelet-Activating Factor and Structurally Related Alkyl Ether Lipids, Tokyo, Japan, May 1989.

*To whom correspondence should be addressed at Department of Food Science and Technology, Faculty of Engineering, Fukuyama University, Fukuyama 729-02, Japan.

Abbreviations: BALF, bronchoalveolar lavage fluid; BSA, bovine serum albumin; IoA23187, Calcium Ionophore A23187; fMLP, formyl-methionyl-leucyl-phenylalanine; GPC, *sn*-glycero-3-phosphocholine; PAF, platelet-activating factor; PC, phosphatidylcholine; RDS, respiratory distress syndrome; SIM, selected ion monitoring; *t*-BDMS, *tert*-butyldimethylsilyl, TLC, thin-layer chromatography.

fractions were subjected to preparative thin-layer chromatography (TLC) on Silica Gel 60 plates together with a standard mixture of lysoPC, sphingomyelin and phosphatidylcholine (PC), and the plates were developed with chloroform/methanol/water (65:35:6, v/v/v). One-cm sections from the origin to the solvent front (16 fractions) were scraped off the plates and the lipids were extracted (9). The extracts were suspended in an appropriate volume of 2.5 mg BSA/mL saline solution. Recovery of PAF by TLC was examined by selected ion monitoring (SIM) (11). When 50 ng of 1-*O*-hexadecyl-2-acetyl GPC and 25 μg of standard phospholipid mixture were applied together to a TLC plate, the recovery, which was determined by measuring the ratio of the ions at *m/z* 117 of acetic acid and *m/z* 120 of perdeuteroacetic acid due to exogenously added internal standard after TLC, was 74 ± 4% (mean ± S.D., n=4). In the present experiment, PAF amounts were calculated using samples purified by TLC and are expressed without correction.

Isolation of neutrophils. Neutrophils were obtained from three healthy adult male volunteers. The isolation procedure, which involved dextran sedimentation, Ficoll gradient centrifugation and hypotonic treatment to lyse erythrocytes, was described previously (12). Both purity and viability of the polymorphonuclear leukocytes were greater than 97%.

PAF bioassay. Washed rabbit platelets were prepared by the method of Pinckard *et al.* (13). PAF was detected on the basis of its ability to cause the immediate aggregation of washed rabbit platelets which was monitored in an aggregometer (Nikko Hematracer PAT-4A, Osaka, Japan). PAF equivalents were determined as described (8), using 1-*O*-hexadecyl-2-acetyl GPC as standard.

Distribution of PAF. To determine the proportions of PAF that remained associated with cell components or were released, fresh BALF in 8 mL saline was centrifuged at 1,600 × g for 15 min at 0°C. The resulting sediment was suspended in 8 mL of cold saline. Both suspension and supernatant were extracted to isolate the PAF fraction which was bioassayed.

Stimulation of human neutrophils. Human neutrophils from citrated peripheral blood were suspended in Tyrode's gelatin solution, pH 7.2, containing 1.3 mM Ca^{2+} at a concentration of 5×10^6/mL. An aliquot was stimulated with 1×10^{-7} M of fMLP or 2.5×10^{-6} M of IoA23187 at 37°C. PAF production was measured at 2.5 min for fMLP and 15 min for IoA23187.

Acetylation of lysoPAF. LysoPAF[2] was measured as PAF after acetylation. The TLC fraction corresponding to lysoPAF was treated at room temperature overnight with 100 μL of acetic anhydride and 100 μL of pyridine (14). After evaporation of the reagent, the residue was suspended in an appropriate volume of 2.5 mg BSA/mL saline. The efficiency of conversion of 1-*O*-hexadecyl GPC to 1-*O*-hexadecyl-2-acetyl GPC was 76.5 ± 10.5% (mean ± S.D., n=6).

Selected ion monitoring. After conversion, the PAF was further subjected to hydrolysis with phospholipase C. The product was then quickly converted to *t*-BDMS derivatives and analyzed using a JEOL JMS-DX 300 instrument equipped with a JMS 3100 computer. The ion at *m/z* 117 was selected for quantitation. The conditions for gas chromatography and mass spectrometry were as described previously (6). The ionization energy was 70 eV.

RESULTS

Detection of PAF in BALF from asthmatic patient. Phospholipid fractions from lung exudates were applied to preparative TLC plates, and each 1-cm fraction was extracted and bioassayed for its ability to elicit platelet aggregation. With BALF from the asthmatic patient, the fraction which migrated directly below the sphingomyelin standard caused immediate and irreversible aggregation of washed rabbit platelets (Fig. 1). Moreover, the aggregating activity was almost completely lost by pretreatment of the platelets with 5×10^{-6} M CV-3988 or 5×10^{-7} M L-652,731, both of which are specific PAF receptor antagonists (Fig. 2).

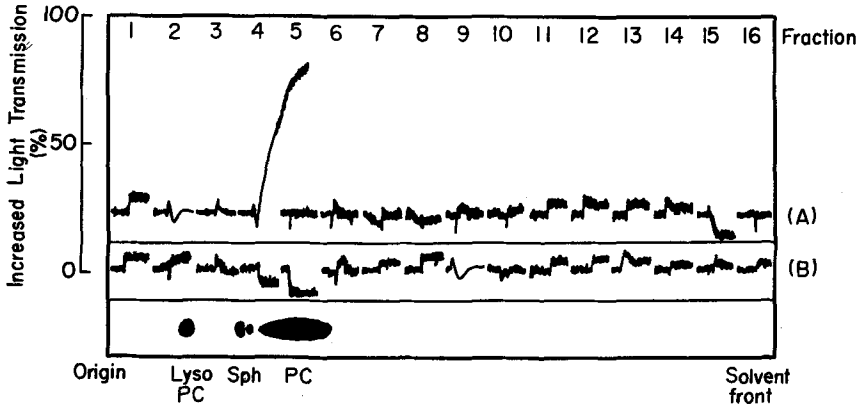


FIG. 1. Thin-layer chromatograms of phospholipid fractions from patients with asthma (A) and laryngeal stenosis (B). Phospholipid fractions, corresponding to 0.53 (A) and 2.41 μmol (B), were applied to Silica Gel 60 plates, developed, and extracted. One-cm fractions from the origin to the solvent front (16 fractions) were eluted and suspended in 200 μL of 2.5% BSA/saline. Ten μL of each fraction was used for bioassay.

[2]The lysoPAF fraction is a mixture of 1-acyl and 1-alkyl species. Upon acetylation, PAF and its 1-acyl analogue are produced, the latter being the major component but exhibiting only negligible biological activity.

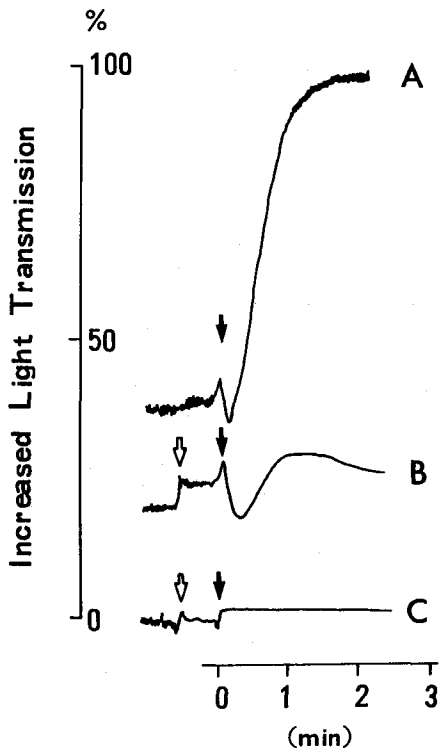


FIG. 2. Platelet aggregation induced by the fraction corresponding to PAF from the asthmatic patient A and its inhibition by antagonist B or C. Open arrows indicate the time when the antagonist L-652,731, B, or CV-3988, C, were added to platelets at the final concentrations of 5×10^{-7} M and 5×10^{-6} M, 1 min prior to the addition of stimuli (closed arrow).

BALF was obtained during three asthmatic attacks that occurred within about three months; in all cases, a similar degree of PAF activity was observed. In contrast, little or no PAF activity was observed in the BALF obtained from the patient with laryngeal stenosis. This was also true of the materials recovered by suction from the bronchoalveolar space of the infants with RDS (Table 1).

TABLE 1

PAF and LysoPAF Detected in Lung Exudates

	Age	Phospholipid (μmol)	PAF[a] (fmol)	LysoPAF (pmol)	Diagnosis
Case 1-1	11 M	1.63	1,206	77	Asthma
Cast 1-2		1.06	1,049	1,060	
Case 1-3		1.56	1,383	128	
Case 2-1	4M	4.84	*b*	53	Laryngeal
Case 2-2		2.48	50	744	stenosis
Case 3	Newborn	0.63	*b*	*b*	RDS
Case 4	Newborn	1.42	*b*	192	RDS
Case 5	Newborn	1.97	35	1,005	RDS

[a]1-*O*-Hexadecyl-2-acetyl GPC equivalents.

[b]Below detection threshold.

Distribution of PAF detected in BALF from asthmatic patient. PAF was detected in the supernatant and sediment fractions (327 fmol and 1,056 fmol, respectively) on the basis of its behavior in TLC and against antagonists.

PAF was also detected in the lung exudate from the patient with pneumonia. A level of PAF equivalent to 827 fmol of 1-*O*-hexadecyl-2-acetyl GPC was recovered from the sediment fraction, in which neutrophils were detected by microscopic examination. No PAF was detected in the bronchoalveolar exudates from patients with RDS. Moreover, the phospholipid distributions were quite different (Table 2).

PAF from stimulated human neutrophils. From 1×10^7 neutrophils, PAF activity equivalent to 272 ± 113 fmol (mean ± S.E., n=5) for fMLP and 57 ± 19.2 pmol (mean ± S.E., n=8) for IoA23187 was detected. This was expressed as 2,656 fmol and 556 pmol/μmol neutrophil phospholipids, respectively.

Characterization of lysoPAF. LysoPAF, which is both a direct precursor as well as an initial metabolite of PAF, was analyzed as PAF after acetylation. LysoPAF was more abundant than PAF, as is shown in Tables 1 and 2.

TABLE 2

Distribution of PAF and LysoPAF in Lung Exudates

		Age	Phospholipid (μmol)	PAF[a] (fmol)	LysoPAF (pmol)	Diagnosis
Cases 1-3	sup[b]	11 M	0.46	327	84	Asthma
	sed		1.10	1,056	44	
Case 6	sup	14 Y	1.06	*c*	111	Pneumonia
	sed		1.81	827	47	
Case 7	sup	Newborn	0.48	*c*	188	RDS
	sed		0.04	*c*	*c*	

[a]1-*O*-Hexadecyl-2-acetyl GPC equivalents.

[b]In the supernatant fraction, a second platelet aggregating activity was observed on a TLC fraction migrating close to the solvent front. The aggregating pattern induced by this fraction was quite different from that of PAF; there was a delay in the induction of aggregation.

[c]Below detection threshold.

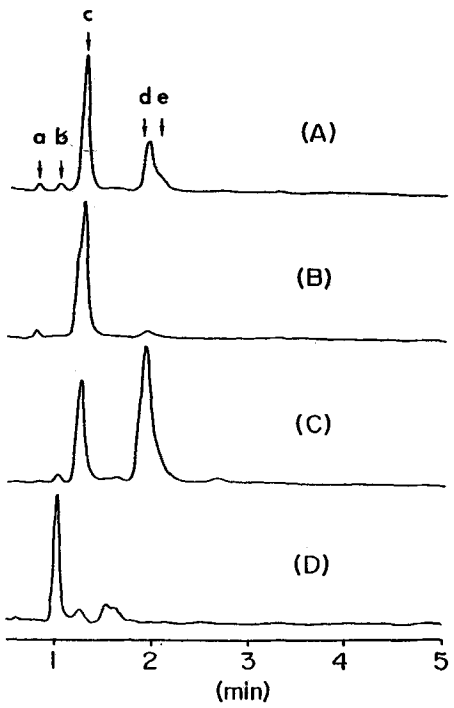


FIG. 3. Selected ion monitoring of lysoPAF. Lyso PAF fractions from patients with asthma (A) and laryngeal stenosis (B), and from human neutrophils (2.5×10^7 cells) (C) were converted to PAF by acetylation. The resulting PAF, as well as the PAF fraction from neutrophils (D), were subjected to hydrolysis with phospholipase C and derivatized. The ion derived from acetic acid at *m/z* 117 was used for selected ion monitoring. The elution times of 1-myristoyl (a), 1-hexadecyl (b), 1-palmitoyl (c), 1-oleoyl (d) and 1-stearoyl (e) derivatives are shown by arrows from left to right in ascending order.

The composition of lysoPAF was analyzed by selected ion monitoring (SIM). The lysoPAF fraction consisted mainly of the 1-acyl analogue with the major component being 1-palmitoyl GPC (Fig. 3). This was evident not only in the lung exudate from the patient with laryngeal stenosis but also in BALF from the asthmatic patient. Though minute in quantity, 1-*O*-hexadecyl species were detected in lysoPAF from the asthmatic patient as well as in the IoA23187-stimulated neutrophils.

DISCUSSION

Bronchial asthma, especially in children, has been thought of solely as an immunologic disorder involving degranulation of mast cells, triggered by antigen *via* an IgE-dependent mechanism. However, not all pathological aberrations in asthmatic patients can be accounted for by allergy (15). The possibility that inflammation is involved in the pathogenesis of asthma was demonstrated by the increased numbers of inflammatory cells in BALF from patients with asthma. We had the opportunity to obtain BALF that had been infiltrated by neutrophils from a child clinically diagnosed as having bronchial asthma. Of the chemical mediators, PAF is now suspected as one of the causative factors in asthma, because it is produced by neutrophils under stimulation (16) and is one of the most potent chemotactic factors of human eosinophils (17,18). In this context, we examined BALF from the asthmatic patient for PAF activity; PAF activity was detected in each of the three times when the patient's lungs were washed with saline during asthmatic attacks. In contrast, we did not detect significant PAF activity in the lung exudates from patients with laryngeal stenosis or RDS, the exudates being essentially free of infiltrated inflammatory cells in both cases.

The amount of PAF detected in the asthmatic patient was calculated as 1–1.4 pmol equivalents of 1-*O*-hexadecyl-2-acetyl GPC. Only one-fourth the amount of PAF was recovered from the supernatant fraction. Assuming that the volume of exudate was 100 μL, the concentration of PAF would be 3.27×10^{-9} M at most, which is insufficient to cause the accumulation of eosinophils (17). Furthermore, the patient was medicated with dexamethazone, which is known to decrease the number of eosinophils (19). The low PAF concentration for chemotaxis and the medication may have been the reasons why we did not detect any significant amount of eosinophils in the airway space. Eosinophils are characteristic inflammatory cells in the late phase of asthma (15).

In BALF from the asthmatic patient, about 1 pmol of PAF per μmol phospholipids was detected. PAF production by human peripheral neutrophils under fMLP stimulation was maximum after incubation for 2.5 min, and the amount was 2.7 pmol per μmol neutrophil phospholipid. Taken together, the PAF amount detected in the asthmatic patient was fairly high.

LysoPAF is a precursor of PAF as well as a metabolite, so it is widely expected that lysoPAF functions as an indicator for the presence of PAF. However, the amounts of lysoPAF varied in the present experiments. Furthermore, unstimulated neutrophils themselves had substantial amounts of lysoPAF; *i.e.*, 530 ± 65 pmol/1 × 10^7 neutrophils (mean ± S.E., n=5). Chemical analyses of the lysoPAF fraction from patients with laryngeal stenosis revealed that this fraction consisted mainly of the 1-palmitoyl analogue [Fig. 3(B)]. The molecular species of the lysoPAF fraction from neutrophils stimulated with IoA23187 also consisted mainly of the 1-acyl form, but the pattern was quite different from that observed in laryngeal stenosis [Fig. 3(C)]. Interestingly, the molecular species of PAF from the same neutrophils consisted mainly of the 1-alkyl form [Fig. 3(D)]. In lysoPAF from asthmatic patient, the percentage of palmitoyl species was decreased compared with that in laryngeal stenosis [Fig. 3(A)], possibly reflecting the contribution of neutrophils in the presence of lung surfactant.

In conclusion, PAF was detected in lung exudates that had been infiltrated with neutrophils from patients clinically diagnosed as having asthma and pneumonia. PAF was mainly cell-associated, as was PAF produced by human neutrophils (12). In contrast, PAF was not detected in the lung exudates that were essentially free of inflammatory cells. This observation is consistent with previous findings (20) that PAF commonly appears in lesions with inflammatory cells accumulation.

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Platelet-Activating Factor (PAF) in Allergic Diseases: Inhibitory Effects of Anti-Allergic Drugs, Ketotifen and Three Kampo Medicines on PAF Production[1]

Tsuneyoshi Nakamura[a,*], Motoaki Kuriyama[a], Keiko Ishihara[a], Yukio Matsumura[b] and Terumasa Miyamoto[a]

[a]Department of Internal Medicine and Physical Therapy, School of Medicine, University of Tokyo, 7-3-1, Hongo, Bunkyo-ku, Tokyo and [b]Matsumura Clinic and Research Institute for Asthma and Immunological Diseases, 5-52-2, Higashinippori, Arakawa-ku, Tokyo, Japan

Platelet-activating factor (PAF) is considered an important mediator in allergic and inflammatory reactions. To further investigate the role of PAF in allergic diseases, we examined the *in vitro* and *ex vivo* effect of anti-allergic drugs on PAF production by human neutrophils. Ketotifen and three Kampo medicines, which are widely used in the treatment of allergic diseases in Japan, inhibited PAF production by normal human neutrophils dose-dependently, whereas disodium chromoglycate (DSCG) and tranilast did not have any inhibitory effect. Ketotifen also suppressed *ex vivo* PAF production by neutrophils from healthy subjects. PAF production decreased from 40.5 ± 5.0 units to 21.6 ± 4.7 units after oral administration of 2 mg of ketotifen per day for one week. We also measured the concentration of lysoPAF in serum from patients with Japanese cedar pollinosis during the pollen season and followed the effect of ketotifen on lysoPAF levels in the serum from these patients. We found that the concentration of lysoPAF, a precursor and degradation product of PAF, in patients with cedar pollinosis (87.8 ± 8.7 units) was significantly higher than in healthy subjects (54.7 ± 7.7 units). After two weeks, lysoPAF levels decreased significantly (82.6 units to 41.3 units) only in the group treated with nasal aerosol and ketotifen. These results suggest that PAF may play a role in allergic diseases and that the clinical effects of anti-allergic drugs may at least partially be due to their anti-PAF action. *Lipids 26,* 1297–1300 (1991).

Platelet-activating factor (PAF, 1-*O*-alkyl-2-acetyl-*sn*-glycero-3-phosphocholine) has been recognized as an important mediator in hypersensitivity and inflammation (1). Evidence also suggests that PAF is involved in the pathogenesis of allergic diseases, especially of bronchial asthma in which PAF induces profound bronchospasms, increased vascular permeability, and leukocyte infiltration into the respiratory tract (2,3). Furthermore, the inhalation of PAF by experimental animals has been shown to cause bronchoconstriction and accumulation of inflammatory cells (4,5). We also reported that PAF may participate in the late asthmatic response following antigen inhalation challenge in patients with bronchial asthma (6).

Japanese cedar pollinosis was first reported by Horiguchi and Saito (7). The number of patients affected by this allergy has increased steadily over the years, and between the end of February and early April nearly 10% of the Japanese population suffer from this disease. The main symptoms are sneezing, rhinorrhea, nasal obstruction, anosmia, conjunctivitis, dermatitis, and throat irritation (7,8). The symptoms suggest that Japanese cedar pollinosis is an allergic disease.

In the present study, we examined the effects of anti-allergic drugs on PAF production by human neutrophils. We also measured serum lysoPAF levels of patients with Japanese cedar pollinosis during the pollen season and observed the changes that occurred after treatment with an anti-allergic drug.

MATERIALS AND METHODS

Materials. Neutrophils were separated from heparinized blood by dextran sedimentation. Contaminating lymphocytes were eliminated on a discontinuous gradient, and red blood cells by hypotonic shock. Ten million neutrophils were suspended in Tyrode-BSA (bovine serum albumin) solution to measure PAF production *in vitro*.

Disodium chromoglycate (DSCG), tranilast and ketotifen were kindly donated by Fujisawa Pharmaceutical Co. (Osaka, Japan), Kissei Pharmaceutical Co. (Matsumoto, Japan) and Sandoz-Japan Pharmaceutical (Tokyo, Japan), respectively. Kampo medicines (Shousaikoto, Shouseiryuto and Saibokuto) were donated by Tsumura Co. (Tokyo, Japan). Synthetic PAF was purchased from Funakoshi Pharmaceutical Co. (Tokyo, Japan).

DSCG, ketotifen and the three Kampo medicines were dissolved in phosphate-buffered saline (PBS), tranilast in 1% of $NaHCO_3$, and then diluted with PBS to the appropriate concentrations. The calcium ionophore A23187 (Calbiochem-Behring, La Jolla, CA) was dissolved in dimethylsulfoxide at 1 mg/mL and stored at −20°C until used.

Two milligrams of ketotifen per day were orally administered to ten healthy subjects for 21 days. Neutrophils were collected from these subjects at 0, 7 and 21 days after administration of Ketotifen to measure *in vitro* PAF production.

Venous blood samples were obtained by venipuncture from 30 untreated patients with cedar pollinosis during the Japanese pollen season. The subjects (8 males and 22 females, 19–57 years of age) were diagnosed as having Japanese cedar pollinosis based on their symptoms, disease history, serum IgE levels, number of eosinophils, positive skin test and/or RAST to Japanese cedar pollen. Serum was immediately separated and then stored at −20°C until assayed for lysoPAF.

[1]Based on a paper presented at Third International Conference on Platelet-Activating Factor and Structurally Related Alkyl Ether Lipids, Tokyo, Japan, May 1989.

*To whom correspondence should be addressed.

Abbreviations: BSA, bovine serum albumin; DSCG, disodium chromoglycate; lysoPAF, lyso platelet-activating factor, 1-*O*-alkyl-*sn*-glycero-3-phosphocholine; PAF, 1-*O*-alkyl-2-acetyl-*sn*-glycero-3-phosphocholine; PBS, phosphate-buffered saline.

Eighteen blood samples, taken in pairs, were obtained before and after the two-week-therapy with nasal beclomethasone dipropionate aerosol, with or without ketotifen treatment (2 mg/day).

Production of PAF by human neutrophils. PAF production by human neutrophils was stimulated as reported by Satouchi *et al.* (9). Briefly, 10×10^6 neutrophils suspended in Tyrode-BSA solution were stimulated with 10 μg/mL of calcium ionophore A23187 for 30 min at 37°C. The supernatants were collected and then stored at −20°C until assayed for PAF.

Acetylation of lysoPAF samples. Acetylation was done using methods reported previously (6,10). Briefly, 100 μL of serum was diluted with 500 μL of methanol and centrifuged at 3,000 rpm for 5 min to sediment protein. Total lipids were extracted from the supernatant by the method of Bligh and Dyer (11). One milliliter of chloroform solution containing the lipids was treated with 0.2 mL of acetic anhydride in the presence of 0.02 mL of perchloric acid for 60 sec at room temperature. The reaction was stopped by adding sodium bicarbonate, and the mixture was centrifuged at 3,000 rpm for 5 min. After a second lipid extraction, the chloroform layer was stored at −20°C until PAF activity was measured.

Measurement of PAF activity. PAF activity was assayed by measuring the aggregation of washed rabbit platelets in an aggregometer (Niko Hematracer, pat. 4A; Niko Biochemical Co., Tokyo, Japan) (6,9). For this purpose, 200 μL of washed rabbit platelets (4 to 6×10^6 mL) suspended in Tyrode-BSA solution were placed in aggregometer cuvettes. Twenty microliters of sample were added to the cuvettes, and platelet-aggregating activity was determined by following changes in light transmission. Platelet aggregating activity was inhibited by CV 3988 (kindly donated by Takeda Chemical Co., Osaka, Japan), a specific PAF antagonist (12). PAF activity was expressed in units calculated from a standard curve obtained for synthetic PAF at different concentrations. One unit was equivalent to 1 ng/mL of synthetic PAF (6).

RESULTS

Inhibitory effects of anti-allergic drugs on PAF production. DSCG, tranilast and ketotifen at concentrations lower than 1 μg/mL did not have any inhibitory effect on *in vitro* PAF production by human neutrophils. DSCG and tranilast did not inhibit *in vitro* PAF production at any of the concentrations measured (10 ng/mL to 100 μg/mL). In contrast, ketotifen inhibited PAF production significantly and in a dose-dependent manner at concentrations higher than 1 μg/mL (Fig. 1).

We also examined the effects of the Kampo medicines, Shousaikoto, Shouseiryuto and Saibokuto, on *in vitro* PAF production by human neutrophils. Figure 2 shows that these three Kampo medicines inhibited *in vitro* PAF production dose-dependently. However, there was no significant difference in the inhibitory effect between the three drugs.

Table 1 shows the changes in PAF production by human neutrophils that occurred after oral administration of ketotifen. PAF production decreased in six out of nine subjects at one week and five out of ten subjects at three weeks after administration of ketotifen. Oral administration of 2 mg of ketotifen, a dose used clinically for the

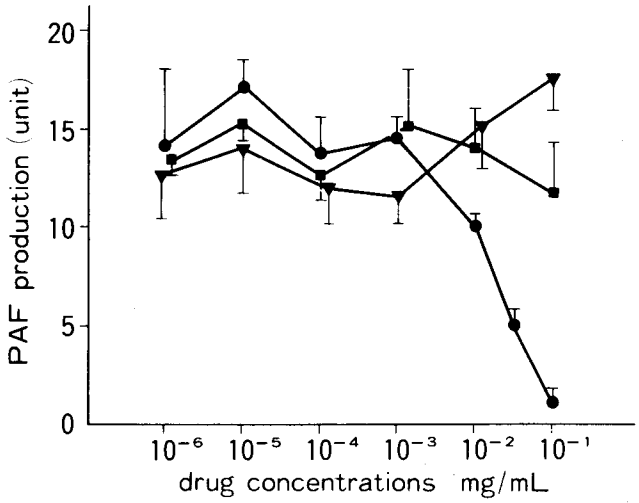


FIG. 1. The inhibitory effects of the anti-allergic drugs DSCG (■), tranilast (▲) and ketotifen (●) on *in vitro* PAF production by human neutrophils. Percent production equals PAF production with drug *vs.* PAF production without drug × 100. The results shown are representative of five different experiments.

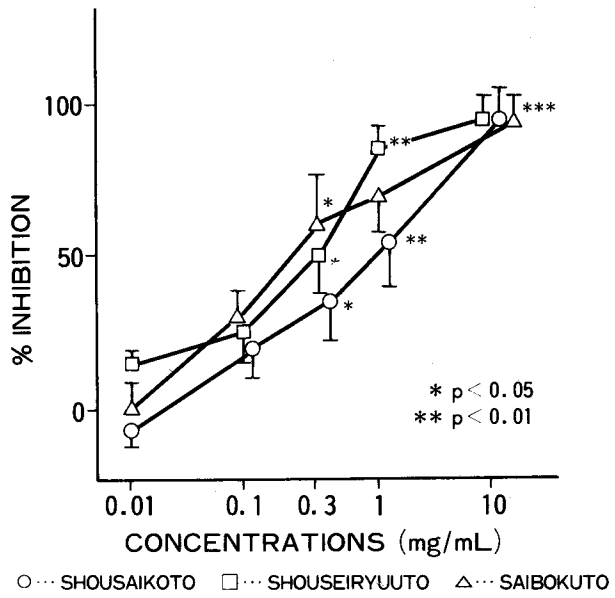


FIG. 2. The inhibitory effects of the Kampo medicines, Shousaikoto (○), Shouseiryuto (□) and Saibokuto (△) on *in vitro* PAF production by human neutrophils. Percent inhibition equals 100 minus PAF production with drug *vs.* PAF production without drug × 100. *P<0.05; **P≤0.01; ***P<0.001. The results shown are representative of five different experiments.

treatment of patients with bronchial asthma, significantly inhibited *ex vitro* PAF production by human neutrophils.

LysoPAF levels in patients with Japanese cedar pollinosis. The serum levels of lysoPAF in untreated patients with Japanese cedar pollinosis were 87.8 ± 8.7 units (mean ± SEM), which was significantly higher than that in healthy subjects (54.9 ± 7.7 units, $p < 0.05$) as shown in Figure 3. Eighteen patients with Japanese cedar pollinosis were divided into two groups; one group was treated with nasal beclomethasone dipropionate aerosol and ketotifen

TABLE 1

Changes in PAF Production by Neutrophils After Oral Administration of 2 mg/Day of Ketotifen to Healthy Subjects[a]

Case		Before	1 Week	3 Weeks
			units/mL	
I.I.	27 M	65.6	38.4	35.2
S.K.	35 M	41.6	N.D.	33.6
M.M.	33 M	46.4	41.6	30.4
T.A.	37 M	28.8	28.8	38.4
S.A.	33 M	63.2	60.8	32.8
S.T.	43 M	50.2	44.8	59.2
K.N.	29 M	34.4	44.8	44.8
U.M.	29 M	23.2	13.6	25.6
S.N.	25 M	38.4	21.6	11.2
Y.S.	37 M	12.8	21.6	19.2
		40.5 ± 5.0	21.6 ± 4.7	33.0 ± 4.0

[a]Two milligrams of ketotifen per day were each orally administered to 10 healthy subjects for 21 days. Neutrophils were collected from these subjects at 0, 7 and 21 days after administration of ketotifen for measuring *in vitro* PAF production. Ten million neutrophils were stimulated with 10 μg/mL of calcium ionophore A23187, and PAF activity was assayed by measuring the aggregation of washed rabbit platelets.

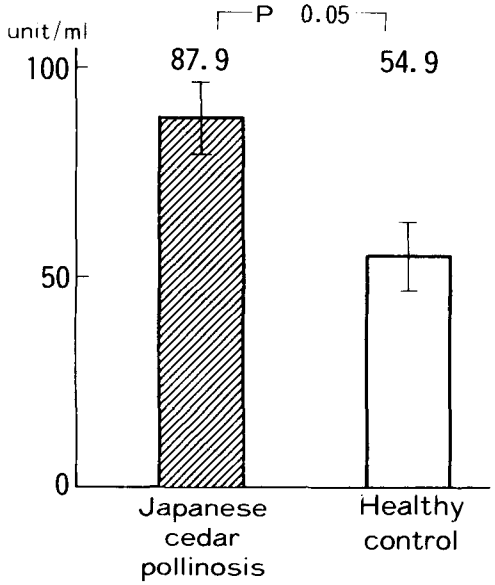


FIG. 3. Serum lysoPAF levels of patients with Japanese cedar pollinosis and of healthy subjects during the pollen season were measured by platelet aggregation assay after acetylation of lysoPAF to PAF.

(2 mg/day) (n = 13), the other group was treated with nasal aerosol only (n = 5). The lysoPAF levels decreased from 82.6 units to 41.3 units ($p < 0.05$) after the two-week therapy with nasal aerosol and ketotifen. However, the level of lysoPAF did not change markedly in the group without ketotifen (from 97.2 units to 70.5 units; not significant) (Fig. 4).

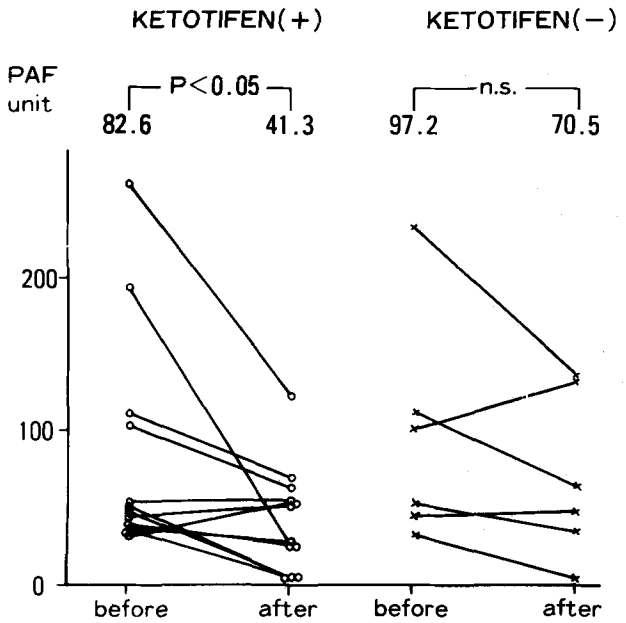


FIG. 4. Decrease in serum lysoPAF levels after treatment with ketotifen. Patients with Japanese cedar pollinosis were divided into two groups. One group was treated with nasal aerosol and ketotifen for 2 weeks (n = 13). The other group was treated with nasal aerosol only (n = 5).

DISCUSSION

Evidence suggests that PAF plays a role in the pathogenesis of allergic diseases, especially of bronchial asthma (1–5). In the present study, we examined the pharmacological effects of the anti-allergic drugs, DSCG, tranilast and ketotifen, on *in vitro* PAF production by human neutrophils, as these three drugs are widely used for the treatment of patients with allergic diseases. Our data show that ketotifen inhibited calcium ionophore-induced PAF production dose-dependently, whereas DSCG and tranilast had no inhibitory effect. The three drugs are known to inhibit histamine release from mast cells and slow-reacting substance of anaphylaxis (SRS-A) from neutrophils and basophils. Our results, however, indicate that there may be distinct differences in the modes of pharmacological action that suppress the allergic reactions.

We also examined the effect of the Kampo medicines, Shousaikoto, Shouseiryuto and Saibokuto, on *in vitro* PAF production by human neutrophils. The precise pharmacological mechanisms of these Kampo medicines are still obscure, but extensive experience in Japan and other Asian countries has established their clinical effectiveness for the treatment of allergic and liver diseases. Our results show that the three Kampo medicines inhibit *in vitro* PAF production dose-dependently and that there is no difference in inhibitory efficacy among these drugs. These Kampo medicines consist of seven to ten major natural components. Kanzo (a grass powder, glycryrrhiza), which shows a strong inhibitory effect on *in vitro* PAF production (data not shown), is a major common component of the three Kampo medicines. This may explain why the three Kampo medicines showed very similar inhibitory activity.

The *in vitro* experiments led us to initiate an oral anti-allergic drug administration trial with healthy subjects. Two milligrams of ketotifen, the usual clinical dose for the treatment of patients with allergic diseases, were administrated to ten healthy subjects for three weeks. PAF production by neutrophils was significantly suppressed after the one-week administration of ketotifen, and this suppression was sustained after three weeks of administration. This suggests that ketotifen inhibits not only *in vitro*, but also *in vivo* PAF production by human neutrophils.

It is of interest, therefore, to examine whether PAF plays a role in Japanese cedar pollinosis. We measured lysoPAF in serum from patients with Japanese cedar pollinosis, as PAF is rapidly metabolized to lysoPAF by the acetylhydrolase present in serum (13,14). To measure the lysoPAF levels, we acetylated the biologically inactive lysoPAF to PAF and measured its platelet aggregating activity. As the major metabolite of PAF in serum is lysoPAF, the level of lysoPAF present in serum may be considered to reflect the level of PAF produced (15). Based on the increased levels of lysoPAF found in serum from patients with Japanese cedar pollinosis, one may conclude that PAF may play a role in the pathogenesis and the clinical manifestation of this disease. Vascular activation and eosinophil accumulation due to PAF could easily account for the symptoms of nasal discharge and conjunctivitis associated with this disease. We compared the serum levels of lysoPAF from patients with Japanese cedar pollinosis before and after treatment with the anti-allergic drug, ketotifen, and nasal aerosol for two weeks. The lysoPAF levels were significantly decreased in the group treated with nasal aerosol and ketotifen. This also suggests that ketotifen inhibits *in vivo* PAF production and that the improvements of clinical symptoms may be partially due to its anti-PAF action.

Further studies are needed to evaluate the clinical significance of PAF in the pathogenesis of allergic diseases. However, our present results strongly suggest that PAF participates in allergic diseases and that the pharmacological benefit of anti-allergic drugs are partly due to their anti-PAF action. Our observations also may provide a new insight into the pathogenesis and the treatment of allergic diseases.

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We thank Dr. K. Satouchi for valuable discussions and Miss A. Furuya for excellent technical assistance in measuring PAF.

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Effect of the Selective PAF Antagonist SM-10661 on an Asthmatic Model. 1. Effect on Passive Anaphylactic Bronchoconstriction in Guinea Pigs[1]

Masako Uchida, Noriaki Imanishi*, Toshinari Sugasawa and Shigeaki Morooka
Research Laboratories, Sumitomo Pharmaceuticals Co., Ltd., Osaka 554, Japan

The effect of SM-10661, a selective antagonist of platelet-activating factor (PAF), on passive anaphylactic bronchoconstriction was examined in guinea pigs. A challenge of ovalbumin to passively sensitized guinea pigs induced bronchoconstriction, which peaked at 4 min. When SM-10661 was administered intravenously 2 min before ovalbumin challenge, bronchoconstriction was inhibited dose-dependently with an ID_{50} of 68 mg/kg. In guinea pigs pretreated with 15 μg/kg mepyramine which is a suboptimal dose, antigen-induced bronchoconstriction peaked at 4–6 min, but was inhibited by SM-10661 with an ID_{50} of 21 mg/kg. When guinea pigs were pretreated intravenously with 2.5 mg/kg mepyramine, 1 mg/kg indomethacin and 0.01 mg/kg propranolol, the antigen-induced bronchoconstriction peaked at 6 min. SM-10661 inhibited the response with an ID_{50} of 45 mg/kg. Histamine- and leukotriene D_4-induced bronchoconstrictions were unaffected by up to 100 mg/kg SM-10661. Ovalbumin challenge of minced lungs from passively sensitized guinea pigs triggered the release of leukotrienes and histamine. SM-10661 had no effect on the antigen-induced release of peptide leukotrienes or histamine up to 10^{-4} M. These results indicate that SM-10661 may be a useful tool to investigate the role of PAF in antigen-induced anaphylactic bronchoconstriction.
***Lipids 26,* 1301–1304 (1991).**

Platelet-activating factor (PAF) is a phospholipid released from a variety of cell types in response to various stimuli (1). PAF has been causally implicated in several diseases, including shock, ischemia, inflammation and renal failure (2,3). PAF induces bronchoconstriction, non-specific increase in bronchial hyperreactivity and inflammatory cell recruitment to bronchoalveolar lavage (2,3), which suggests that PAF may play an important role in asthma. PAF antagonists have been used to examine PAF involvement in active and/or passive anaphylaxis in guinea pigs. PAF antagonists, BN-52021 and WEB-2086, inhibited antigen-induced bronchoconstriction (3,4–6), and antigen-induced releases of leukotriene D_4, thromboxane A_2 and histamine from perfused guinea pig lungs. Unfortunately, these compounds do not inhibit PAF alone, they also inhibit antigen-induced release of histamine and leukotriene. Therefore, it was not known whether the bronchoconstriction was induced only by PAF, and not by other factors.

We developed a selective, orally active PAF-receptor antagonist, SM-10661 ((±)-*cis*-3,5-dimethyl-2-(3-pyridyl)thiazolidin-4-one hydrochloride) which inhibits PAF induced rabbit platelet aggregation dose-dependently (IC_{50} 3.9 μM), and dissociates specific [^{3}H]PAF binding to rabbit platelets (7,8). *In vivo*, the responses induced by externally administered PAF, such as hypotension, death, bronchoconstriction and bronchial hyperreactivity, were inhibited by SM-10661 (ID_{50} 10–30 mg/kg p.o., 1–3 mg/kg i.v.) (7,8).

In the present study, we have examined the effect of SM-10661 (7,8) on passive anaphylactic bronchoconstriction in sensitized guinea pigs and on antigen-induced release of histamine and peptide leukotrienes from minced guinea pig lung *in vitro*.

MATERIALS AND METHODS

SM-10661 ((±)-*cis*-2-(3-pyridyl)-3,5-dimethylthiazolidin-4-one HCl), indomethacin and propranolol were synthetized, and fenoterol was extracted from Berotec-Tablets® (Dainippon Pharmaceuticals Co., Osaka, Japan). Rabbit anti-egg albumin antiserum (IgG type) and phenidone were purchased from Seikagaku Kogyo Co. (Tokyo, Japan) and Nakarai Chemicals, Ltd. (Osaka, Japan), respectively. Egg albumin (grade V), mepyramine maleate, and gallamine triethiodide were from Sigma Chemicals Co. (St. Louis, MO), and leukotriene D_4 was purchased from Daiichi Chemicals, Ltd. (Tokyo, Japan).

Studies on passive anaphylactic shock in guinea pig. Hartley male guinea pigs weighing 430 to 550 g (Japan SLC, Inc., Shizuoka, Japan) were sensitized with rabbit anti-egg albumin anti-serum (0.85 mg/kg) 24 hr before the antigen challenge according to the method of Lagente *et al.* (9). Passively sensitized animals were anesthetized with sodium pentobarbital (30 mg/kg i.p.) and sodium phenobarbital (120 mg/kg i.p.).

An intratracheal cannula was inserted into trachea and indwelling catheters were inserted into the left carotid artery and left jugular vein. Animals were ventilated with air supplemented with O_2 (1 L/min) using a small respirator (Harvard, Boston, MA) set at a stroke volume of 5–7 mL and a rate of 60 breaths/min. Then involuntary respiration was arrested by the administration of gallamine (10 mg/kg, i.m.). To monitor live and dead, mean arterial pressure (MAP) was measured continuously with a pressure transducer (Physiological Pressure Transducers 1280, Hewlett-Packard, Palo Alto, CA) connected to an amplifier (Hewlett-Packard) and recorder (Yokogawa Electric, Tokyo, Japan). Bronchoconstriction was measured according to the method of Konzett and Rössler (10). The rate of bronchoconstriction was measured as an increase in lung overflow with a bronchospasm transducer (Ugo Basile, Varse, Italy) from a side arm off the tracheal cannula, and expressed as a percentage of maximum bronchoconstriction obtained by clamping off the tracheal cannula. Bronchoconstriction values after antigen challenge

[1]Based on a paper presented at the Third International Conference on Platelet-Activating Factor and Structurally Related Alkyl Ether Lipids, Tokyo, Japan, May 1989.

*To whom correspondence should be addressed at Research Laboratories, Sumitomo Pharmaceuticals Co., Ltd., 1-98, Kasugade-Naka, 3-Chome, Konohana-ku, Osaka 554, Japan.

Abbreviations: MAP, mean arterial pressure; PAF, platelet-activating factor; phenidone, 1-phenyl-3-pyrazolidone, SM-10661, (±)-*cis*-2-(3-pyridyl)-3,5-dimethylthiazolidin-4-one HCl.

and maximum bronchoconstriction values were recorded with unirecorder (Model 7050, Ugo Basile).

Sensitized guinea pigs were divided into 3 groups. Animals in Group 1 were used for the experiment under no pretreatment. Animals in Group 2 or 3 were pretreated with a mixture of mepyramine (2.5 mg/kg i.v.), indomethacin (1.0 mg/kg i.v.) and propranolol (0.01 mg/kg i.v.) or mepyramine (15 μg/kg i.v.), respectively, 3 min before intravenous administration of SM-10661 or saline which was adjusted to pH 2 with 1 N HCl. Then ovalbumin (0.1 mg/kg) was injected 2 min later.

To investigate the effect of SM-10661 on LTD_4 (2 nmol/kg) or histamine (8 μg/kg)-induced bronchoconstriction, we used normal guinea pigs with pretreatment of a mixture of mepyramine (2.5 mg/kg i.v.), indomethacin (1.0 mg/kg i.v.) and propranolol (0.01 mg/kg i.v.), or without pretreatment, respectively.

Studies on the release of histamine and peptide leukotrienes from minced lung. Hartley guinea pigs were sensitized as described above, and the lungs were removed and perfused with ice-cold Tyrode's solution through the pulmonary artery. Lung parenchymal strips were dissected to about 0.5 × 0.5 × 1 mm with a tissue chopper (Mickel Laboratory Engineering Co., Guildford, England). Minced lungs (about 100 mg) were preincubated in 1 mL Tyrode's solution at 37°C for 5 min. And various drugs were added to these suspensions and incubated for 10 min. Then 0.01 μg/mL ovalbumin was added and incubation was continued for 15 min. The ice-cold suspensions were filtered through surgical gauze. The filtrate was centrifuged at 3000 × *g* for 15 min at 4°C, and histamine and leukotrienes were measured as described below.

Spectrofluorometric histamine assay. A 1-mL aliquot of the supernatant was mixed with 0.03 mL of 70% perchloric acid. After centrifugation for 10 min at 3000 × *g*, the supernatants were assayed for histamine according to the method of Shore *et al.* (11).

Radioimmunoassay of peptide leukotrienes. An aliquot of supernatant was assayed for total peptide leukotrienes using a [^{3}H]LTC_4, D_4, E_4 Radioimmunoassay Kit (Amersham, London, England).

Statistical analysis. The values in the figures represent the mean ± SEM. Individual differences between the control group and groups treated with drugs were analyzed by Student's t-test.

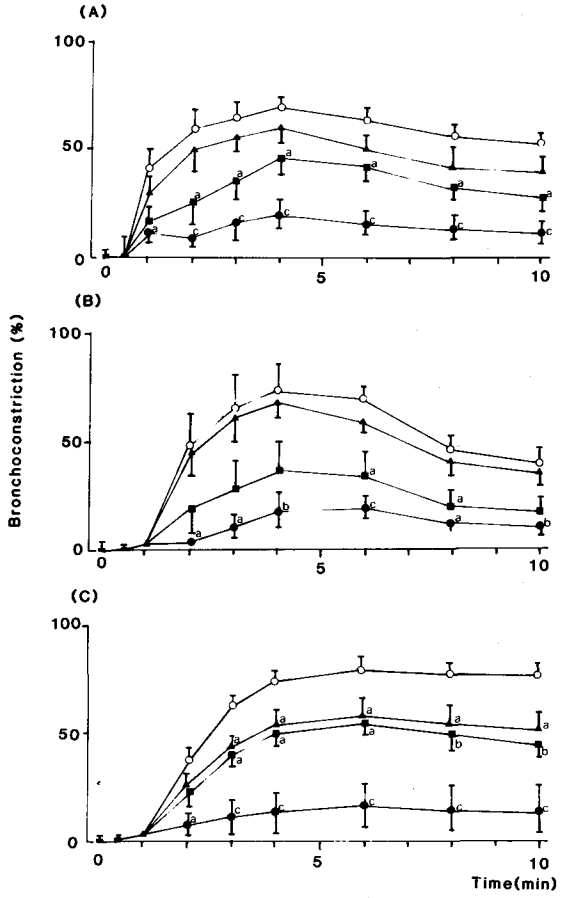


FIG. 1. Effect of SM-10661 on ovalbumin-induced bronchoconstriction in sensitized guinea pigs. (A) SM-10661 at 30 mg/kg (▲), 50 mg/kg (■), or 100 mg/kg (●), or saline (○; control) was given intravenously 2 min before antigen challenge. (B) After pretreatment with mepyramine (15.0 μg/kg i.v.), SM-10661 at 10 mg/kg (▲), 20 mg/kg (■), or 30 mg/kg (●) or saline (○; control) was given intravenously 2 min before antigen challenge. (C) After pretreated with mepyramine (2.5 mg/kg i.v.), indomethacin (1.0 mg/kg i.v.) and propranolol (0.01 mg/kg i.v.), SM-10661 (10 mg/kg (▲), 30 mg/kg (■), 100 mg/kg (●) or saline (○; control) was given intravenously 2 min before antigen challenge. Each value represents the mean ± SEM of 5 to 15 animals. Statistical significance; a: $p<0.05$, b: $p<0.01$, c: $p<0.001$ as compared to the control group.

RESULTS

Effect of SM-10661 on in vivo *passive anaphylactic shock in guinea pigs.* An injection of 0.1 mg/kg ovalbumin to passively sensitized guinea pigs induced bronchoconstriction which peaked at 4 min (71.0 ± 8.9%, n = 15). When SM-10661 was administered intravenously 2 min before ovalbumin challenge, bronchoconstriction was inhibited dose-dependently. Inhibition reached more than 70% when 100 mg/kg of SM-10661 was injected and ID_{50} was 68 mg/kg (Fig. 1 (a), Fig. 2).

Figure 3 shows the effect of the intravenously administered H_1-antagonist, mepyramine, on antigen-induced bronchoconstriction. Mepyramine inhibited maximal constriction of bronchi dose-dependently with an ID_{50} of 21 μg/kg and delayed the onset of bronchoconstriction.

The effect of SM-10661 on antigen-induced bronchoconstriction in guinea pigs previously treated with 15 μg/kg mepyramine was investigated to rule out the partial effect of histamine. Bronchoconstriction peaked at 4–6 min (70.0 ± 5.9 %, n = 6) but was inhibited 71.8% by 30 mg/kg SM-10661 with an ID_{50} of 21 mg/kg (Fig. 1 (b), Fig. 2).

Furthermore, when guinea pigs were pretreated intravenously with a mixture of 0.01 mg/kg propranolol, 1.0 mg/kg indomethacin and 2.5 mg/kg mepyramine to rule out the interference from other potential mediators of bronchoconstriction, antigen-induced bronchoconstriction peaked at 6 min (84.0 ± 5.4%, n = 13), and SM-10661 inhibited it with an ID_{50} of 45 mg/kg (Fig. 1 (c), Fig. 2).

Histamine- and leukotriene D_4-induced bronchoconstrictions were unaffected by SM-10661 up to 100 mg/kg i.v. (Fig. 4).

Effect of SM-10661 on antigen-induced release of histamine and peptide leukotrienes from minced lungs of passively sensitized guinea pigs. Ovalbumin challenge of minced lungs from passively sensitized guinea pigs

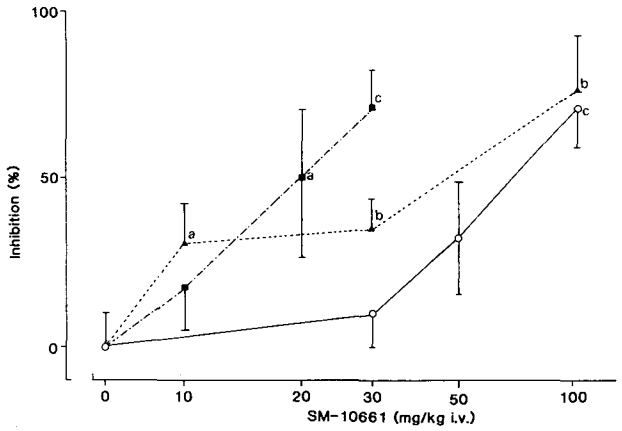


FIG. 2. Dose-dependent inhibitory effect of SM-10661 on antigen-induced bronchoconstriction in guinea pigs. SM-10661 was given to non-treated guinea pigs (○), and guinea pigs pretreated with mepyramine (15 μg/kg i.v.) (■), or mepyramine (2.5 mg/kg i.v.), indomethacin (1.0 mg/kg i.v.) and propranolol (0.01 mg/kg i.v.) (▲). Each value is the mean ± SEM calculated from Figure 1. Statistical significance; a: $p<0.05$, b: $p<0.01$, c: $p<0.001$ as compared to the control group.

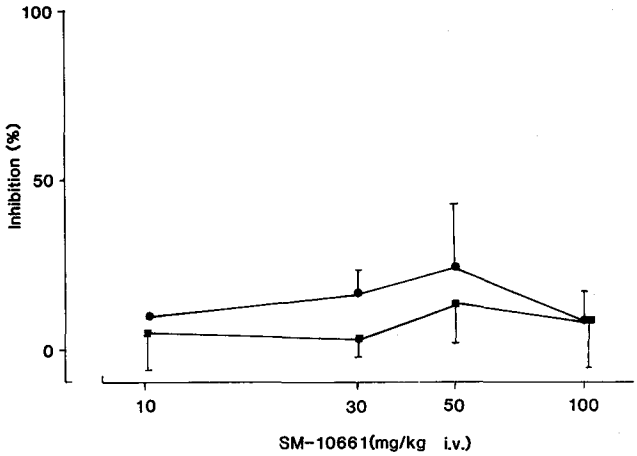


FIG. 4. Effect of SM-10661 on histamine and leukotriene D_4-induced bronchoconstriction. SM-10661 was administered intravenously 2 min before histamine (■) or leukotriene D_4 (●) to guinea pigs. Animals were pretreated with a mixture of mepyramine (2.5 mg/kg i.v.), indomethacin (1.0 mg/kg i.v.) and propranolol (0.01 mg/kg i.v.). Each value represents the mean ± SEM of 5 to 12 animals.

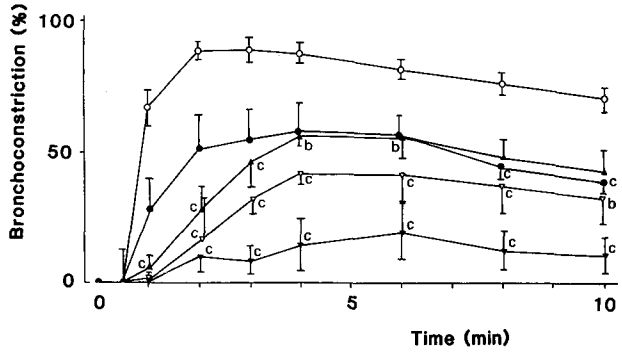


FIG. 3. Effect of mepyramine on ovalbumin-induced bronchoconstriction of sensitized guinea pigs. Mepyramine at 5 μg/kg (●), 10 μg/kg (△), 15 μg/kg (▲), 20 μg/kg (▽), or 50 μg/kg (▼) or saline (○) were administered intravenously 2 min before antigen challenge. Each value represents the mean ± SEM of 4 to 14 animals. Statistical significance; a: $p<0.05$, b: $p<0.01$, c: $p< 0.001$ as compared to the control group.

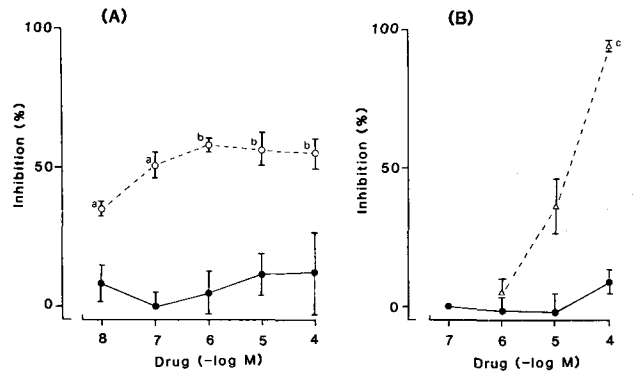


FIG. 5. Effects of SM-10661, fenoterol or phenidone on ovalbumin-induced release of histamine (A) and peptide leukotrienes (B) from minced lungs of sensitized guinea pigs. Dissected peripheral lung strips were preincubated in 1 mL Tyrode's solution at 37°C for 5 min. SM-10661 (●), fenoterol (○) or phenidone (△) were added and the suspensions were incubated for 10 min. Then 0.01 μg/mL ovalbumin was added and the suspensions were incubated at 37°C for 15 min, cooled on ice and filtered, and the filtrates were centrifuged. Histamine and peptide leukotriene levels in supernatants were measured by spectrofluorometric assay and radioimmunoassay, respectively. Each value represents the mean ± SEM of 3 samples. Statistical significance; a: $p<0.05$, b: $p<0.01$, c: $p<0.001$ as compared with the control group.

triggered the release of peptide leukotrienes and histamine. Fenoterol, a β_2-stimulant, significantly inhibited the release of histamine at concentrations above 10^{-8} M. Phenidone, a lipoxygenase inhibitor, significantly impaired the release of peptide leukotrienes at 10^{-4} M. However, SM-10661 had no effect on antigen-induced release of peptide leukotrienes or histamine up to 10^{-4} M (Fig. 5).

DISCUSSION

The present study demonstrated that the selective PAF antagonist, SM-10661, inhibits antigen-induced bronchoconstriction, but does not inhibit histamine- and leukotriene D_4-induced bronchoconstriction. These results indicate that PAF may play an important role in allergic asthma.

Intravenously administered SM-10661 inhibited antigen-induced bronchoconstriction with an ID_{50} of 68 mg/kg. Although guinea pigs are commonly used to study anti-asthmatic drugs, these animals respond differently than humans. For example, histamine is released in guinea pigs from lung and extra-lung sources and plays a major role in systemic anaphylaxis; therefore, anti-histamines are effective on anaphylaxis. By contrast, histamine H_1-antagonists are not effective in the treatment of clinical asthma (12–17). In our study, guinea pigs were pretreated with mepyramine to diminish the role of histamine.

Mepyramine inhibited antigen-induced bronchoconstriction dose-dependently, and at 50 μg/kg i.v., the bronchoconstriction was almost completely suppressed. In animals treated with a suboptimal dose of mepyramine (15 μg/kg i.v.), SM-10661 inhibited antigen-induced bronchoconstriction with an ID_{50} of 21 mg/kg. The inhibition by SM-10661 of bronchoconstriction in guinea pigs pretreated with mepyramine was stronger than that without mepyramine. These results may indicate that PAF produced by antigen challenge potentiates histamine-induced bronchoconstriction. The administration of PAF is reported to induce the bronchial hyperreactivity to histamine (18). In addition, antigen-induced bronchial hyperreactivity is inhibited by PAF antagonists (19–21).

SM-10661 inhibited antigen-induced bronchoconstriction with an ID_{50} of 45 mg/kg in animals pretreated with a mixture of mepyramine (2.5 mg/kg i.v.), indomethacin (1.0 mg/kg i.v.) and propranolol (0.01 mg/kg i.v.). This pretreatment was carried out to potentiate endogenous leukotriene-induced bronchoconstriction by antagonizing the effect of histamine, and inhibiting the cyclooxygenase pathway as well as the bronchodilating action of endogenous catecholamine (22). The inhibitory effect of SM-10661 on antigen-induced bronchoconstriction under these pretreatments was somewhat more pronounced than under no treatment. Considering that SM-10661 inhibited neither leukotriene D_4-induced bronchoconstriction nor the antigen-induced release of peptide leukotrienes, PAF may also be involved in bronchoconstriction under these pretreatments.

BN-52021 inhibited passive anaphylaxis in guinea pigs (3–5) and significantly reduced the anaphylactic release of histamine, leukotriene D_4 and thromboxane A_2 from isolated perfused lungs of passively sensitized guinea pigs (3,4). Another PAF antagonist, WEB-2086, also inhibited not only bronchoconstriction in both passively and actively sensitized guinea pigs, but also the antigen-induced release of histamine and thromboxane A_2 from perfused guinea pig lungs (6).

PAF is known to induce the release of peptide leukotrienes from isolated perfused rat lung (23), guinea pig lung (24) and human eosinophils (25). Thus, the inhibitory effects of BN-52021 and WEB-2086 on anaphylactic bronchoconstriction in guinea pigs may be due to the inhibition of their release.

However, whether PAF induces the release of histamine from lung mast cells at physiological concentrations is not clear (4,26,27). SM-10661 had no effect on the antigen-induced release of histamine from lung parenchyma, but fenoterol inhibited the release. Furthermore, PAF had no effect on histamine release from rat peritoneal mast cells at concentrations of 10^{-11} to 10^{-7} M, and SM-10661 did not inhibit antigen-induced release at concentrations of 10^{-8} to 10^{-5} M (Imanishi, N., unpublished data).

SM-10661 had no effects on antigen-induced release of peptide leukotrienes. Differences in the inhibition of leukotriene release by PAF antagonists may be due to differences of the pharmacological properties of the antagonists used. Also Pretolani *et al.* (26) used isolated perfused lungs to evaluate the effects of WEB-2086, whereas we used minced lungs. In our experiment, phenidone inhibited the release of peptide leukotrienes, and phenidone is known to inhibit antigen-induced bronchoconstriction in guinea pigs pretreated with mepyramine, indomethacin and propranolol (28,29). The inhibitory effect of phenidone on bronchoconstriction appears therefore to be associated with its inhibitory effect on the release of leukotrienes. Our results suggest that SM-10661 inhibits bronchoconstriction without inhibiting leukotriene release and that PAF may play an important role in anaphylactic bronchoconstriction other than that induced by leukotrienes.

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Effect of the Selective PAF Antagonist SM-10661 on an Asthmatic Model. 2. Effect on Antigen-Induced Dual Asthmatic Response and Infiltration of Leukocytes into Airways in Actively Sensitized Conscious Guinea Pigs[1]

Toshinari Sugasawa*, Noriaki Imanishi and Shigeaki Morooka
Research Laboratories, Sumitomo Pharmaceuticals Co., Ltd., Osaka 554, Japan

The effect of a selective platelet-activating factor (PAF) receptor antagonist, SM-10661, on antigen-induced dual asthmatic response and leukocyte infiltration into the airways of actively sensitized conscious guinea pigs was investigated. The animals were pretreated with mepyramine maleate (10 mg/kg i.p.) and then challenged with ovalbumin. The inhalation of ovalbumin caused an immediate decline in specific airway conductance (sGaw) which peaked at 5 min after the challenge. sGaw gradually returned to the baseline 6 hr after the challenge. After the early asthmatic response (EAR), a second phase change, a late asthmatic response (LAR) in sGaw peaking at 17–20 hr was observed. When 1% w/v disodium cromoglycate was inhaled for 2 min on two occasions, EAR was not affected, but LAR was significantly inhibited. Oral administration of 50 mg/kg fenoterol (30 min before challenge) significantly inhibited EAR, but had no significant effect on LAR. SM-10661 administered orally 1 hr before the challenge inhibited EAR dose-dependently (50% inhibitory dose (ID_{50}); 59 mg/kg, but was even more effective against the LAR (ID_{50}); 13–16 mg/kg). When 30 mg/kg of SM-10661 was administered orally 6 hr after ovalbumin challenge, LAR was completely inhibited. The number of total cells, macrophages and eosinophils in bronchoalveolar lavage fluids rose significantly 17 hr after antigen challenge compared to that observed after saline challenge. The number of neutrophils and lymphocytes also increased in response to the ovalbumin challenge, but not significantly. SM-10661 (30 mg/kg) administered orally 1 hr before the challenge significantly inhibited the increase in total cells, macrophages and eosinophils. By comparison, when 30 mg/kg SM-10661 was administered orally 6 hr after the challenge, these increases were not significantly inhibited. These results suggest that PAF is involved in EAR and LAR, and that SM-10661 may be a useful therapeutic agent for the treatment of allergic asthma.
***Lipids 26,* 1305–1309 (1991).**

Inhalation of specific antigen causes immediate bronchoconstriction (early asthmatic response, EAR) in patients with allergic asthma. After the first response, a late asthmatic response (LAR) occurs in some allergic asthmatics within 4 to 8 hr and reaches a maximum within 6 to 12 hr (1,2). Although the pathophysiological mechanisms underlying LAR are not clear, the response is thought to be related to inflammatory reactions which are accompanied by infiltration of inflammatory cells, such as eosinophils, into the airways (3–5). The reactions are accompanied by bronchial hyperresponsiveness, *i.e.* an increase in bronchial responsiveness to nonspecific stimulation, such as inhaled histamine and methacholine (6,7).

Platelet-activating factor (PAF, 1-*O*-alkyl-2-acetyl-*sn*-glycero-3-phosphocholine) is a membrane-derived phospholipid that is a potent inflammatory mediator with various biological activities (8). PAF activates leukocytes and mononuclear cells (9–12) and induces bronchoconstriction (13) and bronchial hyperresponsiveness (14). Furthermore, PAF is generated during pulmonary anaphylaxis (15,16). Recently, several animal models have been developed in which late responses occur in the airways after exposure to allergens (17–22) and PAF antagonists have been effective in reducing these responses (23–25).

In the present investigation, we studied the effect of the selective PAF-antagonist, SM-10661 (26,27) on antigen-induced dual asthmatic response and the role of PAF on late asthmatic response in guinea pigs.

MATERIALS AND METHODS

Materials. SM-10661 was synthesized by Sumitomo Pharmaceuticals Co. Ltd. (Osaka, Japan). Mepyramine maleate and ovalbumin grade III were purchased from Sigma Chemical Co. (St. Louis, MO). Sodium pentobarbital and disodium cromoglycate were purchased from Pitman-Moore (Washington Crossing, NJ) and Fujisawa Pharmaceuticals Co. (Osaka, Japan), respectively. Fenoterol HCl was extracted from Berotec-Tablets™ (Dainippon Pharmaceuticals Co., Osaka, Japan). SM-10661 and fenoterol were dissolved in saline.

Sensitization. Male Hartley guinea pigs weighing 430 to 550 g (Japan SLC, Inc., Shizuoka, Japan) were sensitized by exposure to aerosolized ovalbumin (2% w/v in saline) for 5 min on day 0 and day 7, respectively. The aerosol (particle diameter; about 5.0 μm) was generated by an ultrasonic nebulizer (NE-U11B, Omuron, Tokyo, Japan) at 1.5 mL/min. The animals were used 7 days after the second sensitization. Total arterial blood from 6 of the sensitized animals was obtained *via* an abdominal aorta under diethyl ether anesthesia. Antibody titer was measured by homologous passive cutaneous anaphylaxis

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*To whom correspondence should be addressed at Research Laboratories, Sumitomo Pharmaceuticals Co., Ltd., 1-98, Kasugade-Naka 3-Chome, Konohana-ku Osaka 554, Japan.

Abbreviations: EAR, early asthmatic response; ID_{50}, 50% inhibitory dose; LAR, late asthmatic response; PAF, platelet-activating factor; Pao, airway opening pressure; Pbox, box pressure; PCA, passive cutaneous anaphylaxis; Raw, airway resistance; sGaw, specific airway conductance; Vo, tidal air flow; Vtg, thoracic gas volume.

(PCA) according to the method described by Watanabe and Ovary (28).

Drug treatment and antigen challenge. Actively sensitized guinea pigs were fasted for about 24 hr before antigen challenge, and were pretreated with a H1-receptor antagonist mepyramine maleate (10 mg/kg i.p.) 1 hr before antigen challenge. Fenoterol (50 mg/kg) or SM-10661 (10–100 mg/kg) was orally administered 30 min or 1 hr before the challenge, respectively. Disodium cromoglycate (1% w/v) was inhaled for 2 min at both 15 min before and 6 hr after the challenge. In the other experiment, SM-10661 (30 mg/kg) was orally administered 6 hr after the challenge. The animals were placed in constant-volume pressure-compensated whole-body plethysmograph, which was made according to the method of Amdur and Mead (29) with slight modifications (Fig. 1). The animals were challenged for 5 min with 2% ovalbumin (control) or saline (negative control) aerosol 5 min after measuring the baseline of pulmonary function.

Measurements of pulmonary function. Specific airway conductance (sGaw) was measured according to the method of Hutson *et al.* (21,30) with slight modifications. Conscious animals were placed in the plethysmograph, with head and body compartments separated by a neck restrainer, then the head was inserted into a face mask.

Three transducers were set in the apparatus, a pneumotachograph (TV-241T, Nihon Koden, Tokyo, Japan) connected to a differential air pressure transducer (TP-602, Nihon Koden) to measure tidal air flow (Vo) and two pressure transducers (P10EZ, Nihon Koden) to measure box pressure (Pbox) and airway opening pressure (Pao). With the port open, Vo was recorded while breathing air trapped within the box. After 3 to 4 breaths, the port was clamped at the end of expiration, then Pbox and Pao were measured simultaneously during 3 to 4 breaths. Triplicate measurements were repeatedly performed. The mean values of three breaths were inserted into the following equations to calculate thoracic gas volume (Vtg), airway resistance (Raw) and sGaw. Using the mean of three values of each parameter, lung function was defined at each time point (Equations 1–3).

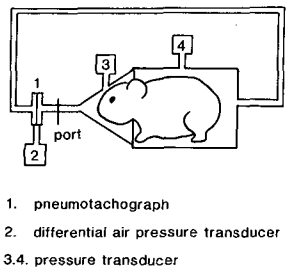
$$Vtg = (Pbox\ (a - w))/(b \times Pao) - c\ (mL) \quad [1]$$

$$Raw = (b \times Pao)/Vo - d\ (cm\ H_2O\ s/mL) \quad [2]$$

$$sGaw = 1/(Raw \times Vtg)\ (/cm\ H_2O\ s) \quad [3]$$

where a = box volume (1566.6 mL), b = correction factor for differences in temperature inside and outside of the animal (1.045), c = dead space volume of face mask (116 mL), d = apparatus resistance at no clamping (0.211 cm H_2O s/mL), w = body weight (g). Airway obstruction was expressed using sGaw as the percentage of change from the prechallenge baseline because the parameter included the difference of lung volume in each animal.

Cytological examination. Other sensitized animals were used for cytological examination. The animals were anesthetized with sodium pentobarbital (50 mg/kg i.p.) and killed by exsanguination from the abdominal aorta. The trachea was exposed, cannulated with a polyethylene tube connected to a syringe. Both lungs were lavaged with 5 mL of sterile saline at 37°C instilled through the tracheal cannula. After 3 min, the lavage fluid was recovered by gentle aspiration. A second lavage was performed with an additional 5 mL of saline. These fluids were combined, cooled to 4°C, and filtered through a stainless mesh (100 μm). The recovery was more than 70% of the total saline introduced. The bronchoalveolar lavage fluid was centrifuged at 200 × *g* for 5 min at 4°C, and red blood cells were lysed using 0.14 M NH_4Cl buffered with 0.017 M Tris (adjusted to pH 7.2 using 6 N HCl). The cells were washed twice with Ca^{2+}-free Dulbecco and Vogt buffer (31), then resuspended in 1 mL of the same buffer. Total leukocyte counts were measured from an aliquot of cell suspension using a microcell counter (CC-150 Toa Medical Co., Kobe, Japan). Differential leukocyte counts were performed using May-Grünwald-Giemsa staining (30). Three hundred cells were counted and classified as macrophages, neutrophils, eosinophils and lymphocytes.



1. pneumotachograph
2. differential air pressure transducer
3.4. pressure transducer

FIG. 1. Whole-body plethysmograph used to measure pulmonary function in actively sensitized, conscious guinea pigs. Port represents area where tubing is clamped. The directions for use of this apparatus are described in the text.

Statistical analysis. All values in the figures are expressed as the mean ± SEM. Significant differences from the control group were determined using Dunnett's test or Student's t-test.

RESULTS

The effect of SM-10661 on early and late phase bronchoconstriction. The sera of 6 actively sensitized guinea pigs showed positive reaction at 4 hr-homologous passive cutaneous anaphylaxis (PCA) (titer; about 1:512 dilution), but no reaction at 7 days-homologous PCA. The titer at 4 hr-PCA was not affected by heat treatment at 56°C for 3 hr (data not shown). The mean baseline values ± SEM of the 52 sensitized guinea pigs for Raw, Vtg and sGaw were 739.1 ± 39.7 (cm H_2O:s/L), 20.4 ± 1.2 (mL) and 0.084 ± 0.004 (/cm H_2O·s), respectively.

The effects of saline and ovalbumin challenge on sGaw in sensitized animals are shown in Figure 2. Saline provocation produced small changes in sGaw, which were not significantly different from the baseline value. In contrast, inhalation of ovalbumin caused an immediate decline in sGaw of −67.3 ± 6.3% of the baseline value 5 min after the challenge. This change was significantly different from the baseline value ($p<0.01$). sGaw gradually returned to the baseline 6 hr after the challenge. A second phase change in sGaw was observed at 17–20 hr, which showed a significant decrease of −38.8 ± 7.2% at 17 hr ($p<0.05$) and of −38.2 ± 7.8% at 20 hr ($p<0.05$). Between the saline and ovalbumin challenged groups, significant differences

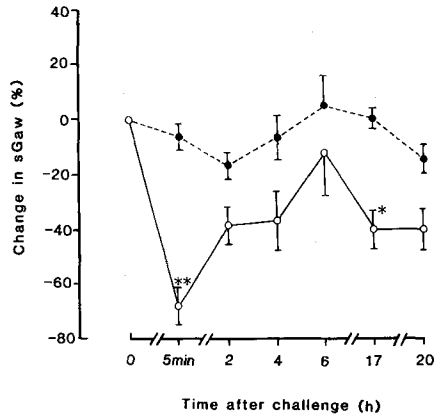


FIG. 2. Effects of challenge with saline (●; n = 5) or 2% ovalbumin (O; n = 10) on specific airway conductance (sGaw) in sensitized guinea pigs. Each point represents the mean ± SEM. Statistical significance: *p<0.05, **p<0.01.

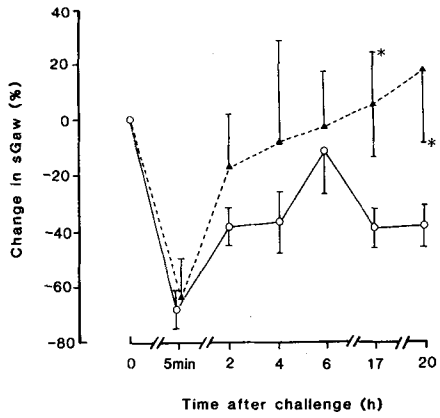


FIG. 3. Effect of disodium cromoglycate on the changes in specific airway conductance (sGaw) after inhalation challenge of 2% ovalbumin in sensitized guinea pigs. An aerosolized solution of 1% w/v disodium cromoglycate was inhalated for 2 min at 15 min before and 6 hr after challenge (▲; n = 5). The control animals (O; n = 10) received saline at the same time. Each value represents the mean ± SEM. Statistical significance: *p<0.05.

were observed at 5 min (p<0.01) and at 17 hr (p<0.05).

When guinea pigs inhaled 1% w/v disodium cromoglycate for 2 min (at both 15 min before and 6 hr after antigen challenge), EAR was not affected at 5 min after antigen challenge, but LAR was completely inhibited at 17 hr (p<0.05) and 20 hr (p<0.05) (Fig. 3).

Oral administration of 50 mg/kg fenoterol 30 min before antigen challenge significantly inhibited EAR measured up to 6 hr after antigen challenge (p<0.05-0.01), but had no significant effect on LAR at 17 hr and 20 hr (Fig. 4).

The effects of orally administered SM-10661 (1 hr before the challenge) on the dual asthmatic response are shown in Figure 5. EAR was inhibited by 10, 30 and 100 mg/kg SM-10661 at 5 min after antigen challenge with ID_{50} 59 mg/kg; this protection was significant (p<0.01) at the 100 mg/kg dose. LAR at 17 hr and 20 hr after antigen challenge was also inhibited dose-dependently by SM-10661 with ID_{50} values of 16 and 13 mg/kg, respectively. This inhibition was significant (p<0.05-0.01) at doses of 30 mg/kg or more. When administered 6 hr after antigen challenge, SM-10661 (30 mg/kg p.o.) almost completely inhibited LAR. The difference at 17 hr between the control and treatment groups was significant (p<0.01) when compared with the percent changes of sGaw from pretreatment values (values 6 hr after the challenge) (Fig. 6).

The effect of SM-10661 on antigen-induced changes of cellular content of bronchoalveolar lavage fluids. Figure 7 illustrates the total and differential leukocyte counts 17 hr after ovalbumin or saline challenge. When compared to the saline challenge, ovalbumin significantly (p<0.01) stimulated the increase in the number of total cells from (54.0 ± 6.34) × 10^5 to (127.3 ± 13.33) × 10^5 cells. The numbers of macrophages and eosinophils also rose significantly from (51.1 ± 5.49) × 10^5 to (96.9 ± 7.02) × 10^5 cells (p<0.01) and from (0.66 ± 0.237) × 10^5 to (11.9 ± 2.29) × 10^5 cells (p<0.01), respectively. The increase in neutrophils and lymphocytes was also stimulated, but not significantly, by ovalbumin challenge, from (0.37 ± 0.186) × 10^5 to (4.1 ± 1.28) × 10^5 cells and from (1.8 ± 0.87) × 10^5 to (14.3 ± 6.61) × 10^5 cells, respectively. SM-10661

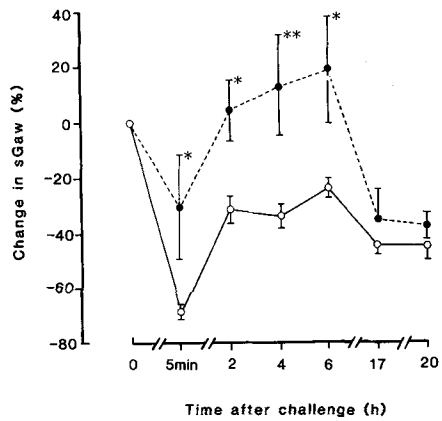


FIG. 4. Effect of fenoterol on the changes in specific airway conductance (sGaw) after inhalation challenge of 2% ovalbumin in sensitized guinea pigs. Fenoterol, 50 mg/kg, was given orally 30 min before challenge (●; n = 5). Control animals (O; n = 19) received distilled water at the same time. Each value represents the mean ± SEM. Statistical significance: *p<0.05, **p<0.01.

(30 mg/kg) administered orally 1 hr before the challenge significantly inhibited the rise in total cells, macrophages and eosinophils, by 87.9%, 81.2% and 79.2% (p<0.01), respectively. The number of lymphocytes was also reduced, but not significantly. When 30 mg/kg SM-10661 was administered 6 hr after the challenge, the increase in total cells, macrophages and eosinophils was slightly inhibited, and the lymphocyte count was reduced by the same level as with the SM-10661 pretreatment; these inhibitions were not significant.

DISCUSSION

In patients with dual allergic asthmatic responses, the early response is inhibited by cromoglycate and β2-adreno-

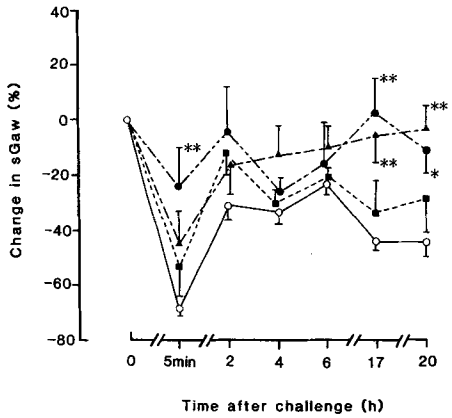


FIG. 5. Effect of SM-10661 on the changes in specific airway conductance (sGaw) after inhalation challenge of 2% ovalbumin in sensitized guinea pigs. SM-10661 (10 mg/kg (■; n = 6), 30 mg/kg (▲; n = 6), 100 mg/kg (●; n = 5)) was given orally 1 hr before challenge. Control animals (○; n = 19) received distilled water at the same time. Each value represents the mean ± SEM. Statistical significance: *p<0.05, **p<0.01.

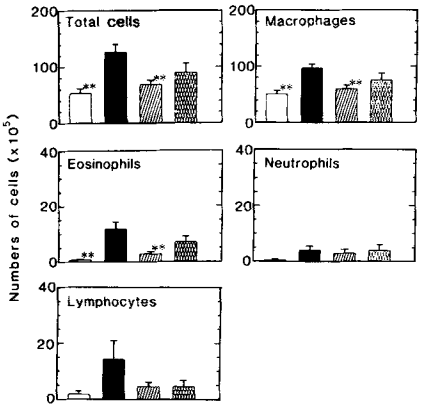


FIG. 7. Effect of SM-10661 on the changes in absolute numbers of total cells and differential cells in bronchoalveolar lavage fluids in sensitized guinea pigs 17 hr after inhalation challenge of 2% ovalbumin. SM-10661, 30 mg/kg, was given 1 hr before (hatched column; n = 10) or 6 hr after (cross-hatched column; n = 10) challenge. Control animals (solid column; n = 12) received distilled water at the same time. Saline (open column; n = 10) was challenged instead of 2% ovalbumin. Each column represents the mean ± SEM. Statistical significance: **p<0.01.

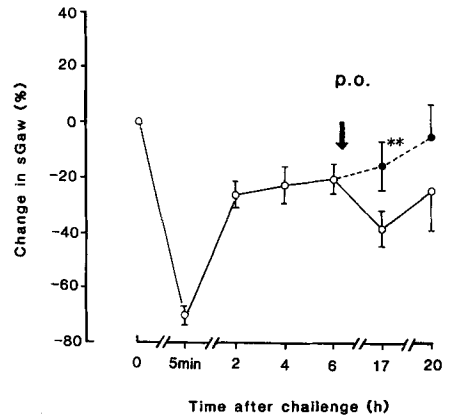


FIG. 6. Effect of SM-10661 (post-treatment) on the changes in specific airway conductance (sGaw) after inhalation challenge of 2% ovalbumin in sensitized guinea pigs. SM-10661, 30 mg/kg, was given orally 6 hr after challenge (●; n = 6). Control animals (○; n = 6) received distilled water at the same time. Arrow indicates the time of administration. Each value represents the mean ± SEM. Statistical significance: **p<0.01.

ceptor agonists, but not by corticosteroids (1, 32–34). Both late asthmatic response and associated airway hyperresponsiveness are inhibited by cromoglycate and corticosteroids, but not by β2-adrenoceptor agonists (1,32–34). Our observations with cromoglycate and a β2-adrenoceptor agonist, fenoterol, on late response agree with those of others.

Hutson *et al.* (30) reported that cromoglycate was effective on both the early and the late responses, but that the β2-adrenoceptor agonist, albuterol, was effective only on the early response in an asthmatic model similar to ours. These authors suggested that their model might be IgE-mediated because cromoglycate worked so well. However, cromoglycate had no effect on the early response in our model at the same dose and time of treatment. Cromoglycate was reported to be effective against IgE-mediated, but not IgG-mediated immediate anaphylactic bronchoconstriction in guinea pigs (35–37). In our model, the sera of the actively sensitized guinea pigs showed positive reaction at 4 hr-passive cutaneous anaphylaxis (PCA) when treated at 56°C for 3 hr, but no reaction at 7 days-PCA. These results indicate that our model was mainly IgG_1-mediated, thus explaining why cromoglycate had no effect on early bronchoconstriction in our studies. The differences in results of our study and previous ones may have been due to the differences in the concentrations of ovalbumin solution and inhalation times during sensitization between our model (2% for 5 min) and others (1% for 3 min).

The present study demonstrated that the selective PAF receptor antagonist, SM-10661, attenuated antigen-induced early and late asthmatic responses in guinea pigs. SM-10661 is a specific PAF receptor antagonist (26), and has no effect on either histamine- or leukotriene D_4-induced bronchoconstriction in guinea pigs (27). The latter also indicates that SM-10661 does not act as bronchodilator.

Hutson *et al.* (25) also reported that a PAF antagonist, WEB-2086, was effective on both early and late asthmatic responses in a model similar to ours. Interestingly, SM-10661 was more effective on the late asthmatic response (50% inhibitory dose (ID_{50}); 13–16 mg/kg) than on the early response (ID_{50}; 59 mg/kg). Treatment with SM-10661 6 hr after antigen challenge completely inhibited the late response, which was similar to the observations of Hutson *et al.* (25). This suggests that PAF is involved in both EAR and LAR, but plays a more important role in the late phase bronchial obstruction.

Late asthmatic response is thought to be related to inflammatory reactions. The analysis of the inflammatory cells recovered from bronchoalveolar lavage suggests that the allergen provocation induced infiltrations of leukocytes into the bronchial lumen, which is thought to injure the mucosa and the submucosa of the bronchi (3–5). The bronchoalveolar lavage technique was also used in this study to examine the local change of cellular composition in the airway lumen. Although there are some differences between the cellular composition of the lavage fluid and lung tissue itself, the lavage fluid is thought to reflect the inflammatory change of the airway lumen (38). The present study also demonstrated that antigens induced the infiltration of inflammatory cells such as eosinophils into the bronchial lumen during the late asthmatic response. SM-10661, at a sufficiently effective dose against late bronchial obstruction, inhibited cellular infiltration into the airway lumen when administered before antigen challenge. Post-treatment with SM-10661 inhibited late asthmatic response, but had no significant effect on cellular infiltration. This suggests that PAF, generated soon after antigen challenge, may induce the migration of inflammatory cells, and that PAF, generated after EAR, may trigger late phase airway obstruction.

Cromoglycate has been demonstrated to inhibit the activation of eosinophils and neutrophils by PAF and other mediators (39,40). In the present study, cromoglycate was effective on IgG_1-mediated late response. The inhibitory effect on the late response may be mediated *via* the inhibition of PAF-induced activation of the inflammatory cells.

PAF antagonists are capable of inhibiting late phase responses in rabbits and sheep (18,23,24,40–43). Using a sheep model, Abraham (43) carefully examined the effects of PAF and leukotriene antagonists. The author suggested that a large component of PAF induced responses in sheep may be mediated *via* leukotrienes (43). Peptide leukotrienes produced by PAF after EAR may induce late phase bronchoconstriction. Further studies are needed to determine whether LAR is mediated by leukotrienes in our model. Nevertheless, SM-10661 may be a useful therapeutic agent for the treatment of allergic asthma.

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REVIEW

The Role of Platelet-Activating Factor (PAF) in Experimental Glomerular Injury[1]

A. Ortiz, M. Gomez-Chiarri, J.L. Lerma, E. Gonzalez and J. Egido*
Department of Nephrology, Fundacion Jimenez Diaz, 28040 Madrid, Spain

Platelet-activating factor (PAF) is a potent autacoid that participates in inflammation and other pathophysiological processes. In this review we deal with recent evidence suggesting that PAF is a mediator that is released early during glomerular injury. PAF can be synthesized in the glomerulus by infiltrating intrinsic glomerular cells. Normal glomeruli produce PAF upon stimulation, and glomerular PAF synthesis is increased in a variety of experimental glomerulopathies. The local infusion of PAF into the renal artery of isolated blood-free kidneys induces proteinuria. PAF attracts and activates inflammatory cells. Glomerular mesangial, endothelial and epithelial cells are also targets for PAF. Therapy with specific PAF receptor antagonists has prevented or reduced proteinuria and improved glomerular inflammation in several experimental models of proliferative glomerulonephritis and minimal change nephrosis. However, the beneficial effect of administration of PAF antagonists once proteinuria is fully developed has been minimal. PAF may also play a role in the recruitment of inflammatory interstitial cells. *Lipids 26,* **1310–1315 (1991).**

During the past decade it has been demonstrated that the renal glomerulus and its various cell types are active sites of synthesis of lipid compounds with vasoactive and proinflammatory activities. The first class is generally referred to as eicosanoids and consists of metabolites of arachidonic acid cyclooxygenation (prostaglandins and thromboxanes) and lipoxygenation (hydroxyeicosatetraenoic acids, leukotrienes and lipoxins). Enhanced glomerular synthesis of these lipids has been described in a variety of clinical and experimental forms of immune complex glomerulonephritis and non-immune experimental nephrosis (1,2). However, the contribution of these mediators to glomerular dysfunction remains unresolved and controversial.

The second class of lipid proinflammatory mediators of interest in glomerulonephritis are the alkyl ether glycerophospholipids, of which platelet-activating factor (PAF) has been the most studied. Recent data strongly suggest that PAF may be a mediator that is released early in glomerular injury, initiating the production of other mediators and expanding the possibility of further tissue damage (2-4). Thus, PAF induces an enhancement of vascular permeability (5) and proteinuria when injected into the abdominal aorta of rabbits (6), as well as into isolated perfused rat kidneys (7,8). Furthermore, treatment with PAF receptor antagonists induced improvement in proteinuria and in glomerular lesions in immune and nonimmune models of nephritis (9-11).

PAF may also be involved in proliferative, immune-mediated and non-proliferative, nonimmune-mediated experimental nephritis. Examples of proliferative, immune-mediated nephroses include acute serum sickness (rabbit), chronic serum sickness (rat), murine genetic lupus, nephrotoxic nephritis, *in situ* immune complex nephritis (rat) and hyperacute graft rejection (rabbit). Non-proliferative, non-immune-mediated nephroses include adriamycin nephropathy and aminonucleoside of puromycin nephropathy.

SOME BIOLOGICAL ASPECTS OF PAF

PAF is a potent autacoid that participates in physiopathological responses, such as inflammation, allergy, shock, and thrombosis (12). It was originally found to be released by antigen-sensitized basophils (13), but has since been shown to be produced by a variety of inflammatory cells, including neutrophils, monocytes, eosinophils, macrophages and platelets (12), as well as by endothelial (14) and mesangial cells (15). PAF is not known to be stored, and in many cells it is synthetized only after cell activation (16). However, the amount of PAF secreted varies considerably from cell to cell (17). PAF acts *via* receptors on the membranes of responsive cells (18). The binding of PAF to its receptor initiates a variety of intracellular responses, including stimulation of GTPase activity and activation of phospholipase C and protein kinase C (18). PAF also stimulates cells to release free arachidonic acid and thus induces formation of eicosanoid metabolites that may be responsible for some observed actions attributed to PAF (18). In addition, PAF induces further PAF synthesis in various cells (19,20). In addition to activating platelets, PAF activates polymorphonuclear leukocytes, monocytes and macrophages, attracts inflammatory cells, increases vascular permeability, and induces hypotension and decreased cardiac output (12). It should be noted that PAF is 1000-fold more potent than histamine in increasing vascular permeability (21). Several antagonists of PAF receptors have been described (12) and tried in experimental diseases, allowing insight into the pathophysiological role of PAF. Some of these antagonists have been employed to treat human diseases (22–24). However, a potent and selective antagonist of PAF synthesis is still lacking.

PAF PRODUCTION BY THE KIDNEY

The release from rabbit renal slices of a bioactive material with PAF characteristics was demonstrated after stimulation with antigen (25). PAF bioactivity was also recovered from the venous effluent of isolated rat kidneys perfused with the calcium ionophore A23187 (26). PAF production

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*To whom correspondence should be addressed at Fundación Jiménez Díaz, Avda Reyes Católicos 2, 28040 Madrid, Spain.

Abbreviations: PAF, platelet-activating factor, PAN, aminonucleoside of puromycin; PG, prostaglandin; Tx, thromboxane.

has been shown to occur in isolated glomeruli and in suspensions of rat medullary cells and tubules (27). In the glomeruli, mesangial cells are a major source of PAF (15, 28,29), but endothelial and infiltrating inflammatory cells can also synthesize PAF (12). Pathophysiological stimuli known to induce PAF synthesis by mesangial cells include endotoxin, phagocytic opsonized substances, angiotensin II, cytokines such as IL-1 and TNF, C3a, PDGF, and PAF itself (15,30–33). The synthesis of PAF by glomerular epithelial cells has not been demonstrated (4).

Human and rat mesangial cells possess intracellular and predominantly extracellular acetylhydrolase activity that rapidly degrades PAF, generating lysoPAF, thus protecting the cells from the deleterious effects of PAF (28,29).

RENAL EFFECTS OF PAF

In vivo systemic administration of PAF induces decreased renal plasma flow, a decrease in glomerular filtration rate and decreased urine output when compared to kidney hypoperfusion (34). Sodium reabsorption is increased along the nephron (34). Increased capillary permeability with loss of intravascular volume to the interstitial space plays a significant role in the hemodynamic effects of PAF (34).

The local administration of PAF avoids the systemic effects. In isolated blood-free perfused kidneys, PAF increased prostaglandin and thromboxane (Tx) production and reduced glomerular filtration (8,35). Direct PAF infusion into the renal artery of anesthetized dogs led to a dose-dependent and reversible decrease in glomerular filtration, urinary flow and sodium excretion (3). There were no systemic hemodynamic changes, nor alterations in the functional parameters of the contralateral, nonperfused kidney. Treatment with indomethacin exacerbated the decline in glomerular filtration rate, suggesting that the vasoconstrictory effect of PAF was partially compensated by the action of vasodilatory prostaglandins (3). In contrast, although intraarterial PAF had similar effects in the rat, the changes were abolished by cyclooxygenase inhibitors, and treatment with a thromboxane synthase inhibitor led to increased glomerular filtration rates and renal plasma flow (36).

As it previously has been demonstrated for inflammatory and endothelial cells, cultured rat or human mesangial cells incubated with PAF display an early release of arachidonic acid *via* phospholipase A_2 stimulation, synthesis of prostaglandins, activation of phospholipase C, increase in intracellular calcium, and release of reactive oxygen species (37–41). PAF also seems to induce mesangial cell contraction, as evidenced by its capacity to cause shape changes in cultured mesangial cells and to reduce the surface area of glomeruli (37,38). This effect was exacerbated after prostaglandin synthesis blockade and reversed by PGE_2 (37).

Cultured endothelial cells express PAF in the outer membrane after stimulation with thrombin, and cell-associated PAF has been shown to be a potent promoter of neutrophil adhesion to these cells *in vitro* (42). PAF also induced changes in the shape of endothelial cells through rearrangement of the cytoskeleton, which leads to increased permeability to proteins *in vitro* (43). Finally, we recently have shown a direct cytotoxic effect of PAF on cultured rat glomerular epithelial cells (44).

PAF INDUCES PROTEINURIA

The injection of PAF into the renal artery of rabbits provoked structural and functional changes (6). In histological studies, an accumulation of neutrophils and platelets was noted. Glomerular endothelial cells appeared swelled and the number and size of the fenestrae were increased. Cationic proteins released by platelets and neutrophils were fixed to the glomerular capillary wall. Concomitantly, there was an increased permeability of the glomerular capillary wall, shown by transient proteinuria (6). This may be related to neutralization of fixed negative charges by cationic proteins released from infiltrating inflammatory cells or to a direct effect of PAF. Evidence for the latter mechanism came from experiments demonstrating increased glomerular permeability in platelet- and neutrophil-depleted rabbits (11). The discovery of a dose-dependent accumulation of [^{125}I]fibrinogen in isolated, blood-free perfused rat kidney further suggested that PAF increases renal vascular permeability in the absence of circulating blood cells (45). Recent studies on isolated, perfused rat kidneys confirmed that PAF alters the sieving properties of the glomerular capillary wall and induces proteinuria in the absence of inflammatory cells by a mechanism independent of alterations of the glomerular electrostatic barrier (7,8). PAF induced proteinuria was evident 5 min after beginning the infusion, increased further during the infusion, and returned to basal values 20 min after ceasing PAF infusion (46). PAF induced proteinuria was prevented by pretreatment with a specific PAF receptor antagonist, but was not influenced by lipoxygenase or cyclooxygenase inhibitors (7). Dexamethasone also prevented the increase in glomerular permeability induced by PAF, although the mechanism remains unclear (47). In this same model it has been demonstrated that PAF alters the glomerular barrier size selectivity and increases the permeability to large neutral dextran molecules (46). As already discussed, in other vascular beds PAF induced permeability is thought to depend on increased intracellular calcium and endothelial cell shape changes (43). In fact, PAF induced changes in glomerular permselectivity properties are dependent on the Ca^{++} concentration in the extracellular medium (46). However, the glomerular filtration barrier is a complex structure, and actions on epithelial cells also may be relevant (44).

A ROLE FOR PAF IN EXPERIMENTAL PROLIFERATIVE, LEUKOCYTE-DEPENDENT GLOMERULONEPHRITIS

Several studies have indicated that PAF acts as an important mediator of immune glomerular injury when leukocytes and platelets are the final effectors of inflammation (11).

The first suggestion of the involvement of PAF in renal pathology was made in acute serum sickness induced in the rabbit by a single injection of bovine serum albumin. In this model, the formation of immune complexes in glomeruli is preceded by the interaction of IgE anti-bovine serum albumin antibodies with basophils (48), leading to degranulation and intravascular release of PAF (49). Platelets were specifically desensitized to the action of PAF and platelet-derived cationic proteins localized in the glomeruli, causing focal neutralization of fixed anionic charges (50). Furthermore, the administration of synthetic

PAF aggravated the histological picture and PAF receptor antagonists markedly decreased proteinuria and glomerular hypercellularity (51).

Our group first suggested a role for PAF in the chronic serum sickness of rats sensitized by ovalbumin (52). In this model (coincidentally with the appearance of immune deposits in the capillary wall and the abrupt onset of proteinuria) there was a specific desensitization of platelets to PAF (52). In more recent studies, we have demonstrated increased synthesis of PAF by glomeruli of nephritic rats (unpublished results). Glomerular PAF synthesis was maximal in the phase of moderate proteinuria, characterized by the mesangial localization of immune deposits, and decreased in the phase of maximal proteinuria, which coincided with the deposit of immune complexes in the capillary wall. In this model, PAF antagonists appear to be effective in decreasing proteinuria only if administered within a short time frame (unpublished results) before the full-blown nephrotic syndrome has developed, suggesting that PAF synthesis may be an early event leading to the recruitment of cells and mediators.

Lupus nephritis is a proliferative nephritis characterized by massive immune deposits in the mesangium and glomerular capillary wall that resembles the model of chronic serum sickness. In NZBxW lupus mice, urinary PAF excretion increased significantly with age, and correlated with proteinuria (53). Treatment with the PAF receptor antagonist L-659,989 delayed the onset of proteinuria and improved survival (53). In the MRL/Mpj-lpr/lpr (lpr) mice with systemic lupus erythematosus, synthesis of PAF, TxA_2 (thromboxane A_2) and PGE_2 (prostaglandin E_2) by renal microsomes was increased (54). Treatment with a PAF receptor antagonist, L-659,989, beginning before the development of proteinuria and maintained for four weeks improved renal function and reduced urinary protein excretion. Renal microsomal eicosanoid synthesis was reduced and renal microsomal PAF was normalized, indicating that in some way PAF also induces its own release *in vivo* (19,20). Although the immune complex deposition was not prevented, mesangial cell proliferation and interstitial fibrosis were reduced, suggesting that PAF blockade in this model interferes with the processes that follow the deposition of immune complexes, rather than with formation and deposition itself (54).

Nephrotoxic nephritis results from the injection of antiglomerular basement membrane antibodies. The degree of glomerular injury is species-specific, being more severe in the rabbit. PAF receptor antagonists have proved to be effective in decreasing the manifestations of the disease in rabbits, rats and mice, but the antagonists were always administered before disease induction (55–59). In this model, renal damage is complement- and leukocyte-dependent, but the mechanisms of proteinuria have not been completely elucidated. The onset of proteinuria is preceded by loss of anionic charges of the glomerular basement membrane and the infiltration of monocytes (60). Both phenomena may be PAF related. In this sense, glomerular PAF synthesis peaked three hours after induction of the disease and was reduced by complement and platelet depletion, but not by neutrophil depletion (57). Both glomerular TxB_2 and PGE_2 were increased (56). In rabbits, PAF receptor antagonists did not alter the glomerular binding of antibody (55), but prevented the development of crescentic nephritis, increased glomerular filtration rate, and reduced proteinuria (55,56). Therapy with a thromboxane synthase inhibitor was also beneficial, but less so than with PAF antagonists (56). In rats, an increased spontaneous release of PAF by nephritic glomeruli has been demonstrated (58). In our experience, the PAF receptor antagonists BN52021 and alprazolam reduced proteinuria in the heterologous phase of the disease (Fig. 1) (9). These results are consistent with those of others (58). In another model of complement- and leukocyte-dependent nephritis caused by implantation of cationic antigen in the glomeruli of preimmunized rats, PAF receptor antagonists lessened the proteinuria and the accumulation of inflammatory cells in glomeruli, but not the activation of complement (61). Interestingly, leukocyte depletion also prevented glomerular injury. Thus, in this model, PAF seems to act as a chemoattractant for neutrophils and macrophages, that are the ultimate cause of tissue damage. The PAF receptor antagonist also rendered high plasma PAF levels undetectable, suggesting that PAF was inducing its own synthesis.

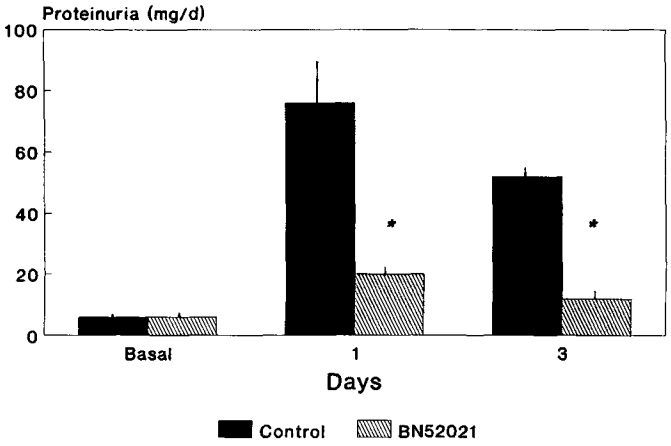


FIG. 1. PAF receptor antagonist BN52021 decreases proteinuria in the heterologous phase of rat nephrotoxic nephritis. Similar results were obtained with alprazolam; (*$p < 0.0025$); mean ± SEM.

PAF has been implicated in the pathogenesis of hyperacute kidney allograft rejection in the rabbit (62). In this model, preformed allotransplantation antibodies bind to the endothelium of glomerular and non-glomerular capillaries. PAF appeared rapidly in the venous effluent of the kidneys and was in close temporal relationship to the influx of platelets and neutrophils, with formation of intravascular thrombi (62). Both platelet and neutrophil-derived cationic proteins were located close to the anionic charges of the glomerular capillary wall, indicating the release of cell granules (62). PAF also possesses immunomodulatory effects that may affect graft survival (63); therapy with PAF receptor antagonists has resulted in improved survival of allografts (64).

PAF IN THE PATHOGENESIS OF NON-PROLIFERATIVE EXPERIMENTAL NEPHROTIC SYNDROME

The fact that PAF is capable of inducing proteinuria in the isolated perfused rat kidney model in the absence of inflammatory infiltrating cells led us to study the possible role of PAF in animal models of minimal change nephrotic syndrome. The experimental nephrosis induced

in rats by adriamycin (65) or aminonucleoside of puromycin (PAN) (66) is characterized by a virtual lack of inflammatory cells in the glomeruli and provides a useful model to study the participation of intrinsic glomerular cells in the genesis of proteinuria.

After receiving a single injection of adriamycin, rats developed severe proteinuria and degenerative lesions of epithelial glomerular cells (65). The toxic effect of adriamycin on the kidney requires only a few minutes of exposure to the drug (3); however, severe proteinuria persists and eventually leads to focal segmental glomerulosclerosis (67). We have hypothesized that adriamycin by itself, or through the release of some mediators from resident glomerular cells could damage epithelial glomerular cells (68–70). Several lines of evidence support the concept that PAF is one such mediator, released early in adriamycin-induced nephropathy.

The administration of PAF receptor antagonist BN 52021, begun the day before adriamycin injection and continued for three weeks, prevented the development of proteinuria (Fig. 2) as well as the biochemical manifestations of nephrotic syndrome (68). In treated rats, no alterations in epithelial glomerular cells were noted, while rats receiving vehicle displayed widespread effacement of podocytes, fusion of foot processes, and cytoplasmic vacuoles (68). Since the ginkgolide BN52021 also possesses antiprotease activities (22), we employed the synthetic specific PAF receptor antagonists, triazolam and the hetrazepine derivate, BN 52076, and obtained similar results (68). Therapy with PAF antagonists from day −1 to day +1 of adriamycin injection reduced proteinuria, although not to the extent observed with continued therapy (2). The administration of PAF receptor antagonists, once nephrotic syndrome was fully developed, had no effect on proteinuria. Together, these results suggest that PAF is an early mediator of proteinuria in this model of nephrosis. Cytokines or other lipid mediators, released in the cascade simultaneously or after PAF, could perpetuate renal damage (70).

We then evaluated the glomerular production of PAF and TNF_α, as both mediators have been shown to be closely related in several pathophysiological processes. Glomeruli obtained from adriamycin rats synthetized more PAF than those from healthy rats (70). The greatest production of PAF was on day 14, preceding maximal proteinuria and TNF_α production (both on day 21). Therapy with PAF receptor antagonists decreased glomerular PAF and TNF_α production (44). *In vitro* studies demonstrated that exposure of glomeruli and mesangial cells from healthy rats to adriamycin generated PAF production in a dose- and time-dependent fashion, peaking at 15 and 90 min (2; and unpublished data). Adriamycin also stimulates TNF_α synthesis by mesangial cells (44). A close relationship between PAF and TNF_α has been demonstrated in a variety of inflammatory responses (71).

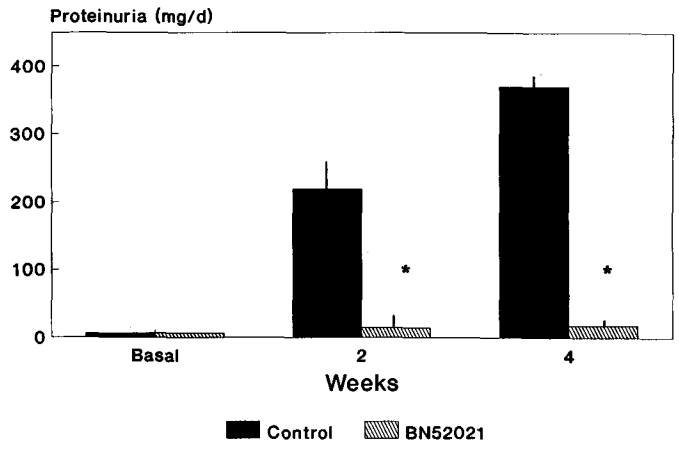


FIG. 2. PAF receptors antagonists prevent the development of proteinuria in adriamycin nephrosis; (*$p < 0.0005$); mean ± SEM.

The next question we addressed was whether PAF and TNF_α could induce epithelial cell damage. *In vitro* studies showed that both mediators were cytotoxic, cultured glomerular epithelial cells, as shown by a 6-hr ^{51}Cr release assay. PAF antagonists reduced TNF_α toxicity. Furthermore, adriamycin and PAN were also cytotoxic to epithelial cells, and there is preliminary evidence that this effect seems to be due to PAF and TNF_α (unpublished data).

A low protein diet is the only intervention, besides the use of PAF antagonists, that can prevent proteinuria in adriamycin nephropathy (72). Recent results from our laboratory suggest that a low protein diet may influence the glomerular production of PAF and TNF_α (73).

PAN nephrosis differs from adriamycin nephropathy in several aspects. Urinary protein excretion peaks earlier and then decreases only to rise again steadily in relation to the development of glomerulosclerosis (66). However, in both models epithelial cell damage is intense. In PAN nephrosis, PAF glomerular production peaked twice (days 1 and 15) before maximal proteinuria (day 11), and before the steady rise in proteinuria that follows, while TNF_α production peaked on day 2, and slowly declined until day 21. Treatment with the PAF receptor antagonist BN 52021 induced a striking decrease in proteinuria, a normalization of total serum proteins and serum cholesterol levels, and a diminution in TNF_α and PAF glomerular production (unpublished data).

Interstitial inflammation is associated with a variety of glomerular and non-glomerular renal diseases (74). The severity of interstitial damage correlates better with renal function than the severity of glomerular injury. However, the mechanisms involved are obscure. We recently characterized the interstitial cell infiltrates in adriamycin nephrosis as composed mainly of macrophages and T lymphocytes, that peaked on day 11, preceding maximal proteinuria (75). Previously, Eddy and Michael (76) had described a similar, but more severe, interstitial nephritis associated with puromycin nephrosis. In both models therapy with two structurally unrelated PAF antagonists decreased infiltration by leukocytes (75), suggesting a role for PAF in the interstitial nephritis associated with nephrosis.

By contrast, PAF receptor antagonists do not prevent proteinuria in passive Heymann nephritis and circulating PAF levels remain normal (77). Passive Heymann nephritis is an animal model of human membranous glomerulonephritis. The *in situ* formation of immune complexes on the subepithelial side of the glomerular basement membrane leads to epithelial cell damage and heavy proteinuria. There is no inflammatory cell recruitment in the glomerulus and the terminal sequences of complement are thought to be responsible for glomerular damage. The fact

that the pathological events take place in the subepithelial space may explain these findings. For this model we previously had reported the absence of an anaphylactic vasopermeability mechanism for immune complex deposition (78).

CONCLUSIONS

PAF has been shown to be released by a variety of renal and inflammatory cells. When administered systemically, PAF effects on the kidney depend on systemic hemodynamic changes. However, direct infusion of PAF into the renal artery induces changes in glomerular filtration and proteinuria both in the presence or absence of blood cells. The study of experimental models of glomerular injury has suggested a role for PAF in proliferative and nonproliferative, immune and toxic proteinuric nephritis. Increased glomerular PAF synthesis has been found to be an early event both in the absence or presence of infiltrating inflammatory cells. Prophylactic therapy with structurally different PAF receptor antagonists avoided or reduced proteinuria in experimental models of nephritis. On the other hand, therapy of established proteinuria has been disappointing so far. While the mechanisms of PAF induced proteinuria remains unknown, it may involve both loss of anionic charges and size selectivity of the glomerular filtration barrier, as well as epithelial cell damage and recruitment of other cells and mediators.

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The Effects of R-75,317 on Antiglomerular Basement Membrane Glomerulonephritis in Rats[1]

Masaaki Miyamoto[a], Hiroyuki Koike[a,*], Toshio Sada[a], Yasuteru Ijima[b], Junichiro Fukushige[a] and Norio Nakamura[c]

[a]Biological Research Laboratories, [b]New Lead Research Laboratories and [c]Fermentation Research Laboratories, Sankyo Co., Ltd., Tokyo 140, Japan

Platelet-activating factor (PAF) is a potent inflammatory mediator which is released by various inflammatory cells and produced by certain tissues, including the kidney. PAF has been shown to increase glomerular permeability to protein and to decrease glomerular filtration rate (GFR) by contracting mesangium. On the basis of these observations, it has been suspected that PAF may play a role as mediator of glomerular damage in glomerular nephritis. To examine this possibility, we studied the effects of a specific PAF antagonist, R-75,317, on the development of an experimental model of anti-glomerular basement membrane (anti-GBM) glomerulonephritis. Glomerulonephritis was initiated by injecting rabbit anti-rat GBM serum into rats. Proteinuria gradually developed after serum injection, plateaued at week 2, and remained at the high level of week 2 throughout the experimental period (6 wk). Chronic treatment with R-75,317 (10 mg/kg/day i.p.) tended to delay the onset of proteinuria and significantly accelerated the recovery phase. Creatinine clearance (Ccr) fell to 40% at week 3. R-75,317 treatment completely prevented this decline of Ccr. Histological changes in this model (glomerular hypertrophy, proliferation of mesangial matrix and interstitial fibrosis) were also ameliorated by the R-75,317 treatment. The results suggest that PAF may play a role in the development of glomerulonephritis and that PAF antagonists could be used in the treatment of human renal disease.

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Glomerular nephritis is the major cause of end stage renal diseases. Despite considerable advances in the understanding of the immunologic mechanisms of this disease, factors responsible for the initiation and progression of glomerular damage have still not been identified. Recent studies have indicated that platelet-activating factor (PAF) is a potent inflammatory mediator released by various inflammatory cells (1–3). PAF causes proteinuria and a decrease in glomerular filtration rate (GFR) when directly injected into the renal artery (4–6). Specific renal tissues, including mesangial, medullary and glomerular endothelial cells, produce PAF (7–9). It has therefore been suspected that PAF may play a role in glomerular nephritis.

The purpose of the present study was to examine the role of PAF in the development of glomerular nephritis by studying the effects of a specific PAF antagonist, R-75,317. We used a model of antiglomerular basement membrane (anti-GBM) nephritis induced in rats by injection of anti-rat GBM rabbit antiserum. R-75,317 was administered for 6 wk and its effects on urinary protein excretion, urinary *N*-acetyl-β-D-glucosaminidase (NAG) activity, creatinine clearance (Ccr), and on histological changes in nephritic kidneys were examined.

MATERIALS AND METHODS

Preparation of rabbit anti-rat GBM antiserum. Details of this model have been described elsewhere (10,11). Rat glomeruli were isolated by sieving techniques; isolated glomeruli were washed by centrifugation followed by repeated sonication. The isolated and sonicated glomeruli were incubated with trypsin to obtain GBM. GBM was emulsified in Freund's complete adjuvant (Defco Laboratory, Detroit, MI) and injected intradermally into rabbits. After five boosting immunizations, the rabbit anti-rat GBM anti-serum was collected.

PAF antagonist. The chemical structure of R-75,317, 3-{2-[*O*-[(2*R*)-3-(*N*-heptadecylcarbamoyloxy)-2-(3-isoxazoyloxy)propyl]phosphonoxy]ethyl} thiazolium (inner salt), is shown in Figure 1. The efficacy of this compound has been reported both *in vitro* and *in vivo* (12). R-75,317 is active after oral as well as parenteral administration. PAF-induced hypotension was inhibited by 70% in rats injected p.o. with 30 mg/kg of R-75,317 (12).

Intravenous injection of anti-GBM serum causes transient but severe hypotension in anesthetized rats. Pretreatment with 10 mg/kg R-75,317 i.p. completely prevented this hypotension (Fig. 2).

Experimental design. Female Wistar rats weighing between 170–180 g were divided into three groups of 10 animals each. The control group (group I) received 4 mL/kg of normal rabbit serum in the tail vein. The nephritic group (group II) received 4 mL/kg of anti-GBM serum in the tail vein. The PAF antagonist treated group (group III) received the same amount of anti-GBM serum as group II, but was also treated i.p. with 10 mg/kg/day of R-75,317 throughout the experiment.

Animals were studied on the day of injection of anti-GBM serum (week 0) and at 7, 14, 21, 28, 35 and 42 days (weeks 1, 2, 3, 4, 5 and 6) after the injection. Animals were housed in metabolic cages and urine was collected over 24-hr periods. Blood samples were taken at weeks 0, 3 and 6 for the determination of creatinine concentration.

[1]Based on a paper presented at the Third International Conference on Platelet-Activating Factor and Structurally Related Alkyl Ether Lipids, May 1989.

*To whom correspondence should be addressed at Biological Research Laboratories, Sankyo Co., Ltd., Shinagawa-ku, Tokyo 140, Japan.

Abbreviations: AA, arachidonic acid; Ccr, creatinine clearance; GBM, glomerular basement membrane; GFR, glomerular filtration rate; NAG, *N*-acetyl-β-D-glucosaminidase; PAF, platelet-activating factor, 1-*O*-alkyl-2-acetyl-*sn*-glycero-3-phosphocholine.

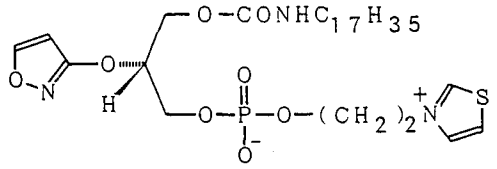


FIG. 1. Chemical structure of R-75,317.

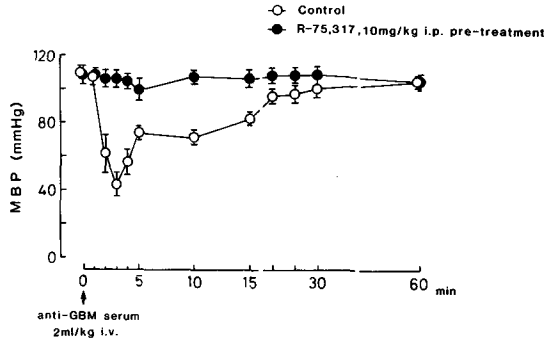


FIG. 2. Effects of R-75,317 on anti-GBM serum-induced hypotension in rats. The number of animals in each group was 5.

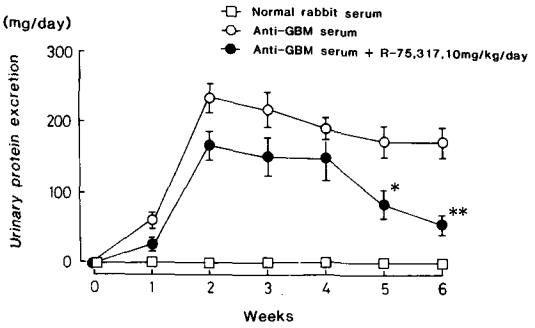


FIG. 3. Time course of urinary protein excretion. The number of animals in each group was 10. *p< 0.05, **p< 0.01, in comparison with the anti-GBM serum injected group (group II).

Histological study. All animals were sacrificed at week 6 to examine renal histology. For light microscopic examination, formalin-fixed sections were processed by routine methods and stained with hematoxylin and eosin or with periodic acid.

Analysis. Urinary protein concentration and urinary NAG activity were determined with a kit purchased from Wako, Osaka, Japan (Micro TP-test) and Shionogi, Osaka, Japan (NAG test), respectively. Plasma and urinary creatinine were measured by the enzyme colorimetric method (13); creatinine clearance was calculated by using standard formulae.

Statistical analysis. Results are expressed as mean ± SEM. Statistical analysis was by non-paired Student's t-test. Statistically significant differences were assumed to be $p < 0.05$.

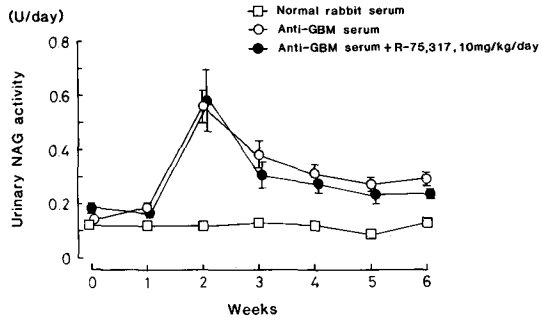


FIG. 4. Time course of urinary NAG activity. The number of animals in each group was 10.

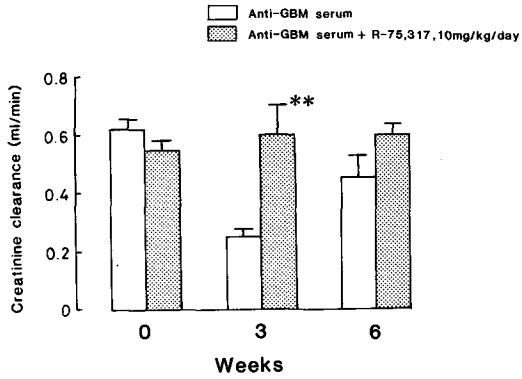


FIG. 5. Effects of R-75,317 on creatinine clearance in anti-GBM nephritic rats. The number of animals in each group was 10. **p<0.01, in comparison with the anti-GBM serum injected group (group II).

RESULTS

The time courses for urinary protein excretion in all groups are shown in Figure 3. Normal rabbit serum injection (group I) did not cause proteinuria at all. In group II, urinary protein excretion did not change on the day of anti-GBM serum injection, but gradually increased, reaching a peak at week 2 (230.4 ± 21.1 mg/24 hr) and maintained the week 2 level throughout the experimental period. Treatment with the PAF antagonist (group III) tended to suppress the onset of proteinuria and significantly accelerated the recovery phase.

The time course of urinary NAG activity after anti-GBM serum administration was similar to that of proteinuria (Fig. 4). Anti-GBM serum injection gradually increased urinary NAG activity in group II. R-75,317-treatment did not affect this enhanced activity.

Figure 5 shows the changes in Ccr in groups II and III. In group II, Ccr was reduced markedly at weeks 3 and 6 (0.62 ± 0.04, 0.25 ± 0.03, and 0.53 ± 0.08 mL/min for weeks 0, 3 and 6, respectively). Treatment with the PAF antagonist completely abolished this decline in Ccr.

Histologic changes in the kidneys were similar to those reported elsewhere for kidneys with anti-GBM nephritis (14–16). In the glomerular region, glomerular hypercellularity, mesangial expansion, thickening of GBM and

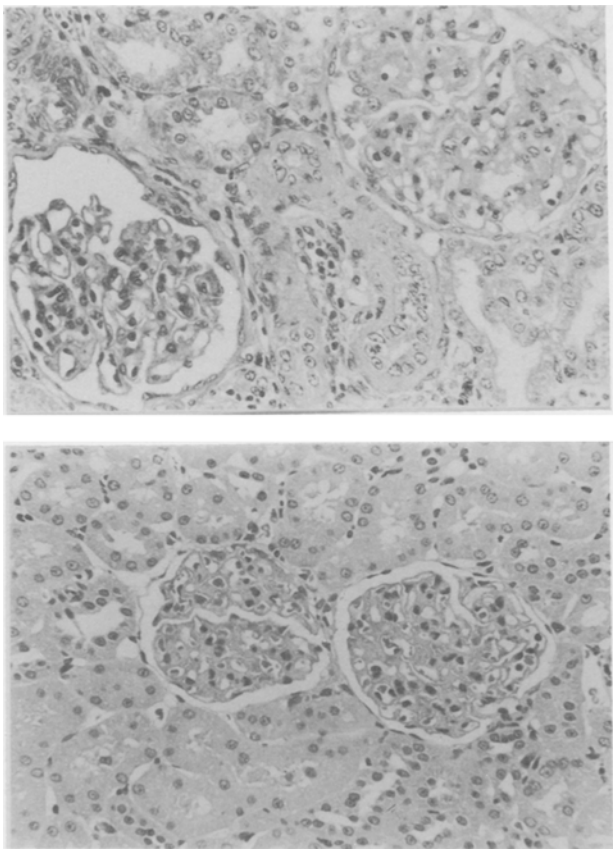


FIG. 6. ***Top.*** **Kidney section from nephritic rat 6 wk after injection with rabbit antiserum to rat GBM. There is glomerular hypercellularity, thickening of Bowman's capsule and tubular basement membrane and adhesion of glomeruli tuft to Bowman's capsule. Hematoxylin and eosin. Magnification × 100.** ***Bottom.*** **Kidney section from anti-GBM nephritic rat treated with R-75,317 for 6 wk. Glomerular and tubular changes are less pronounced than in anti-GBM animals without the PAF antagonist treatment. Hematoxylin and eosin. Magnification × 100.**

Bowman's capsule, and adhesion of glomeruli tuft to Bowman's capsule were obtained (Fig. 6, top). Thickened tubular basement membrane and interstitial fibrosis were observed in tubules. In the R-75,317 treated group, these histological changes were markedly improved (Fig. 6, bottom; Table 1).

DISCUSSION

Anti-GBM nephritis proceeds in two phases. First, the heterologous antibody binds to the glomerular basement membrane (GBM) within one hour after the injection of antiserum, and induces acute damage mediated mainly by complement and polymorphonuclear cells (17,18). In this heterologous phase, acute proteinuria and decrease in GFR are observed. The second phase (autologous phase) starts usually 7–10 days after the anti-GBM serum injection. In this phase, the host's immune responses to heterologous antibody bound to the GBM play an important role in deterioration of renal function and in bringing about morphological changes. Chronic proteinuria in the second phase is associated with enhanced glomerular permeability to protein.

The anti-GBM nephritic model in the present study has a different time course for urinary protein excretion. Proteinuria did not occur on the day of anti-GBM serum injection (week 0) but gradually developed thereafter, indicating that our model has a mild heterologous phase followed by a prominent autologous phase. The reason for the discrepancy between the results with our model and those of previous studies is not known. But it is quite possible that our anti-GBM serum has different activity or affinity from other anti-serum used by others since the GBM preparation is not pure and anti-GBM serum is polyclonal. The GBM-binding ability of our anti-serum might not have been potent enough to trigger the acute responses mentioned above. However, the injection of the anti-GBM serum did cause profound hypotension, an index of severe immuno-responses (Fig. 2). Another possibility is that the hypotension itself reduces proteinuria *via* the changes of intrarenal hemodynamics. Decrease of glomerular capillary pressure due to systemic hypotension is known to reduce glomerular filtration of macromolecules including protein. Therefore it is possible that reduction of glomerular capillary pressure induced by anti-GBM serum decreased the leakage of plasma protein. Treatment with the PAF antagonist partially, but significantly, enhanced the recovery phase of proteinuria. Proteinuria in this phase is associated with increased vascular permeability to proteins. Recent studies have indicated that PAF enhances vascular permeability (17) and causes proteinuria when directly injected into the renal artery (18,19). These findings suggest that PAF is involved in the proteinuria in this model through increased glomerular permeability.

NAG is a ubiquitous, lysosome-derived hydrolytic enzyme. Since proximal tubules are the major source of the enzyme in urine, urinary activity of NAG has been assumed to be a sensitive marker of tubular damage. The present results demonstrate that urinary NAG activity is increased in anti-GBM nephritic rats. This is in accord with histological findings that proximal tubules were injured in the nephritic kidneys. PAF may not be involved in NAG release from injured proximal tubules because the PAF antagonist did not affect the time course of urinary NAG activity.

The effects of R-75,317 on glomerular filtration rate (GFR) measured by Ccr were quite pronounced. Treatment with the PAF antagonist completely prevented the decrease of GFR in anti-GBM nephritic rats. Bertani *et al.* (20), using anti-GBM nephritic rabbits and L-652,731, another PAF antagonist, have found the PAF antagonist effective in preserving GFR. PAF has also been demonstrated to reduce GFR directly (5,6,19,21). These findings are in accord with the hypothesis that PAF plays an important role in the decrease of renal function in this model. However, other inflammatory mediators, namely thromboxane A_2 and leukotriene D_4, may also be responsible for the reduction of GFR in anti-GBM nephritis (22,23). Weisman *et al.* (24) have shown that PAF activates arachidonic acid (AA) metabolism in the kidney. The interrelationship between PAF and AA metabolites and their respective roles in nephritis induced by anti-GBM serum need to be determined.

TABLE 1

Effect of R-75,317 on Renal Morphology in Anti-GBM Nephritic Rats: Heterologous Phase[a]

	Anti-GBM serum	Anti-GBM serum + R-75,317 10 mg/kg/day
Glomerular hypercellularity	2.4 ± 0.5	1.9 ± 0.3
Mesangial expansion	2.2 ± 0.6	1.8 ± 0.4
Interstitial fibrosis	2.8 ± 0.8	1.8 ± 0.5

[a]Results were scored on a 0 to 4+ scale.

The histological changes of kidney observed in the present study are typically found in anti-GBM nephritis. The glomerular and tubular damages were moderately ameliorated by the R-75,317 treatment, which further supports the notion that PAF may play a role in the autologous phase of this model.

In summary, the present study has shown that R-75,317, a specific PAF antagonist, has preventive effects on the development of anti-GBM nephritis in rats. PAF may play an important role in this nephritic model. In order to clarify the role of PAF, the interrelationships between PAF and other inflammatory mediators and renal production of PAF in this model need to be examined.

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Nephrotoxicity of Cyclosporine: The Role of Platelet-Activating Factor and Thromboxane[1]

Oscar F. Pavão dos Santos[a], Mirian A. Boim[a], Elvino J.G. Barros[a], Eduardo Pirotzky[b], Pierre Braquet[b] and Nestor Schor[a,*]

[a]Nephrology Division, Escola Paulista de Medicina, São Paulo, SP, Brazil and [b]Institut Henri Beaufour, Les Ulis, France

Cyclosporine (CsA), an immunosuppressive agent, is potentially nephrotoxic. We had previously observed that acute administration of CsA to Munich-Wistar rats induced a decrease in single nephron glomerular filtration rate, due to a decline in glomerular plasma flow, and in the glomerular ultrafiltration coefficient. Moreover, these alterations were prevented when an antagonist of platelet-activating factor (PAF) was administered. In the present study we examined whether the protective effect of the PAF blocker in CsA nephrotoxicity could have been mediated by thromboxane (TxA_2). Our data show that the PAF effects were not mediated by TxA_2, since administration of dazmegrel, a thromboxane synthetase inhibitor, did not ameliorate the acute renal failure caused by CsA. Thus, PAF appears to be a direct mediator of acute CsA nephrotoxicity, while TxA_2 is not significantly involved in this process.
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Platelet-activating factor (PAF) is produced by the glomeruli and by various kidney cell types, including mesangial and medullary renal cells (1–3). PAF is also involved in inflammatory and vasoactive response (4); PAF infusion during maintenance of mean arterial pressure decreases renal plasma flow (RPF) and whole kidney glomerular filtration rates (GFR) (5). These alterations in superficial glomerular hemodynamics were also observed after cyclosporine (CsA) administration, which suggests that PAF may be generated during CsA treatment. Moreover, both PAF and CsA cause contraction of isolated mesangial cells (6,7), probably leading to a decrease in the glomerular ultrafiltration coefficient (K_f) (8).

BN 52021, a terpene isolated from *Gingko biloba*, significantly inhibits the immune and vascular effects of PAF (4). This antagonist was also found to inhibit the effects of both PAF and CsA on isolated mesangial cells (7), and to protect spontaneous hypertensive (9) and Munich-Wistar rats (10) from the nephrotoxicity induced by CsA. Thus, PAF may be one of the humoral factors modulating CsA nephrotoxicity. Moreover, PAF may participate directly in CsA-induced toxicity by its actions on glomerular hemodynamics, or indirectly by stimulating platelets and/or plasma and renal cells to release thromboxane (TxA_2). After chronic treatment with CsA, urinary thromboxane excretion increases, while GFR decreases (11). Thus, in order to evaluate a possible role of TxA_2 as a second mediator in CsA nephrotoxicity, we employed a specific TxA_2 blocker, dazmegrel (DAZ), during acute CsA administration.

MATERIALS AND METHODS

Materials. Cyclosporine was from Sandoz (Basel, Switzerland), dazmegrel from Pfizer (Sandwich, United Kingdom), and PAF from Institut Henri Beaufour (Les Ulis, France). The Munich-Wistar rats were provided by the Central Animal Facility of the Escola Paulista de Medicina, São Paulo, Brazil.

Methods. The studies were performed using male Munich-Wistar (MW) rats weighing 265–350 g. All animals were submitted to two periods of study: First, a control period (except for Group 4 which received DAZ in the first period); and second, acute drug administration which was as follows: Group 1 (n = 12) was treated i.v. with CsA, 50 mg/kg body wt, in bolus. Group 2 (n = 7) received a continuous infusion of PAF (0.012 ± 0.001 μg/kg body wt/min, i.v.) up to a maximum dose which did not cause a decrease in mean arterial pressure below the renal autoregulation level. Group 3 (n = 6) received DAZ (UK-38485, 5 mg/kg body wt, i.v., in bolus). The dose of dazmegrel chosen was that recommended by Pfizer Laboratories. Finally, Group 4 (n = 7) received the same dose of DAZ (as Group 3), in the first period, and then the animals were treated with CsA (as Group 1) in the second period. Each period lasted approximately 40 min and additional 30–45 min equilibration periods followed after surgery and following the first period.

General protocol. Rats were anesthetized with Inactin, 100 mg/kg body wt, i.p., and submitted to micropuncture studies as previously described (8). Throughout the study, all rats received continuous i.v. infusion of 10% inulin and 2% *p*-aminohippuric acid (PAH) solution and of isotonic rat serum to replace surgical losses (12). After 30–45 min equilibration, the initial study period began. Two samples of urine, and blood samples of about 60 μL each were collected for measuring hematocrit (Hct), protein concentration (C_A), inulin and *p*-aminohippuric acid (PAH) in plasma and urine. After the control period collections, the drugs were administered to the respective groups as described above, and a new 30–45 min equilibration period was allowed. Thereafter, the same procedure was followed in the second study period.

Micropuncture studies were performed in both experimental periods. Samples of fluid from surface proximal tubule convolutions were collected for determination of flow rate and inulin concentration, and for calculation of the single nephron glomerular filtration rate (SNGFR).

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*To whom correspondence should be addressed at Nephrology Division, Escola Paulista de Medicina, Rua Botucatu 740, 04023 São Paulo, SP, Brazil.

Abbreviations: AP, mean arterial pressure; C_A, afferent arteriolar protein concentration; CsA, cyclosporine; DAZ, dazmegrel; ΔP, transcapillary hydraulic pressure difference; FF, filtration fraction; GFR, glomerular filtration rate; Hct, hematocrit; K_f, glomerular ultrafiltration coefficient; MW, Munich-Wistar; PAF, platelet-activating factor; P_{GC}, glomerular capillary hydraulic pressure; P_T, tubular hydraulic pressure; Q_A, glomerular plasma flow rate; R_A, afferent arteriolar resistance; R_E, efferent arteriolar resistance; R_T, total arteriolar resistance; RPF, renal plasma flow; SNFF, single nephron filtration fraction; SNGFR, single nephron glomerular filtration rate; TxA_2, thromboxane.

Hydraulic pressures were measured in surface glomerular capillaries (P_{GC}), proximal tubules (P_T), efferent arterioles (P_{EA}), and peritubular capillaries (P_C) by a continuous recording servonull micropipette transducer system (IPM Inc., San Diego, CA). Protein concentration in femoral arterial and efferent arteriolar plasma was measured, as described previously (13), to estimate colloid osmotic pressure. Colloid osmotic pressure was calculated according to the equation of Deen *et al.* (14). The estimates of pre- and post-glomerular protein concentrations permit calculation of colloid osmotic pressure, single nephron filtration fraction (SNFF), initial glomerular capillary plasma flow rate (Q_A), and afferent (R_A), efferent (R_E) and total (R_T) arteriolar resistances, as well as of glomerular capillary ultrafiltration coefficient (K_f) using equations previously described (14).

Analytical procedures. Plasma and urinary inulin concentration were determined by the macroanthrone method of Fuhr *et al.* (15). The concentration of inulin in the tubular fluid was measured by the microfluorescence method (16). Protein concentration in efferent arteriolar and femoral arterial plasma was determined by the fluorimetric method (13). Plasma and urinary *p*-aminohippuric acid concentrations were measured according to Smith *et al.* (17).

Statistical analyses were by Student's t-test. Significance was defined as $p<0.05$. Data were presented as mean ($\bar{X} \pm SE$).

RESULTS

Body weight, kidney weight, hematocrit and plasma protein were similar in all groups (data not shown). Table 1 summarizes various whole kidney functions. CsA caused a decline in GFR from 0.96 ± 0.04 to 0.47 ± 0.07 mL/min ($p<0.05$), and in RPF from 2.91 ± 0.19 to 1.30 ± 0.23 mL/min ($p<0.05$). Similarly, PAF provoked a decline in GFR (1.09 ± 0.03 to 0.43 ± 0.10 mL/min; $p<0.05$) and in RPF (3.59 ± 0.17 to 1.78 ± 0.34 mL/min; $p<0.05$). DAZ alone did not change GFR, RPF or FF. Pretreatment with DAZ did not modify the global renal effects induced by CsA. In Group 4, GFR decreased from 1.01 ± 0.03 mL/min to 0.50 ± 0.08 mL/min ($p<0.05$) in the second period, and RPF decreased from 2.71 ± 0.15 mL/min to 1.35 ± 0.24 mL/min ($p<0.05$).

The glomerular hemodynamic studies are presented in Table 2. CsA caused a decline in SNGFR from 27.9 ± 3.4 to 14.0 ± 3.5 nL/min ($p<0.05$) and in Q_A from 101.0 ± 17.1 to 44.4 ± 13.4 nL/min ($p<0.05$). Since P_T (14 ± 3 mmHg) remained constant and P_{GC} increased (45 ± 1 to 55 ± 4 mmHg; $p<0.05$), the transcapillary hydraulic pressure difference (ΔP) rose significantly ($p<0.05$). Both R_A and R_E increased, but mainly R_E ($p<0.05$) as shown in Table 2. The K_f decreased by 70% after CsA administration (from 0.096 ± 0.003 to 0.031 ± 0.010 $nL \cdot s^{-1} \cdot mmHg^{-1}$, $p<0.05$). PAF induced a decline in SNGFR (31.7 ± 2.7 to 13.4 ± 1.7 nL/min; $p<0.05$) and in Q_A (96.7 ± 7.6 to 41.5 ± 5.7 nL/min; $p<0.05$), and caused an increase in both R_A and R_E. After PAF infusion there was a significant decrease in AP (99 ± 2 to 87 ± 1 mmHg; $p<0.05$).

As shown in Table 2, DAZ alone did not affect the superficial nephrons. Pretreatment with DAZ did not reduce the effects of CsA in SNGFR (33.8 ± 3.1 to 17.0 ± 3.3 nL/min; $p<0.05$), in Q_A (92.1 ± 6.0 to 55.3 ± 10.6 nL/min; $p<0.05$) or in K_f (0.081 ± 0.007 to 0.045 ± 0.010 $nL \cdot s^{-1} \cdot mmHg^{-1}$; $p<0.05$). However, DAZ normalized ΔP (32 ± 1 mmHg) and decreased total arteriolar resistance, although not significantly.

DISCUSSION

Cyclosporine, an immunosuppressive agent that is used in organ transplants can show nephrotoxic effects (18,19). On the other hand, the absence of morphological glomerular lesions (20) suggests that the acute toxic effects are caused by humoral factors (18). Impairment of renal function evoked by CsA is mainly due to a decrease in Q_A and K_f (8), effects which are also observed after PAF infusion (5). PAF and CsA induce contraction of mesangial

TABLE 1

Summary of Whole Kidney Function[a]

	AP (mmHg)	GFR (mL/min)	RPF (mL/min)	FF (%)
Group 1				
CONT[b]	108 ± 3	0.96 ± 0.04	2.91 ± 0.19	34 ± 2
CsA (n = 12)	108 ± 3	0.47 ± 0.07[c]	1.30 ± 0.23[c]	38 ± 2[c]
Group 2				
CONT	99 ± 2	1.09 ± 0.03	3.59 ± 0.17	30 ± 1
PAF (n = 7)	87 ± 1[c]	0.43 ± 0.10[c]	1.78 ± 0.34[c]	24 ± 2[c]
Group 3				
CONT	97 ± 3	1.05 ± 0.06	3.13 ± 0.37	36 ± 4
DAZ (n = 6)	96 ± 4	1.01 ± 0.05	3.00 ± 0.27	34 ± 2
Group 4				
DAZ	98 ± 4	1.01 ± 0.03	2.71 ± 0.15	38 ± 2
DAZ + CsA (n = 7)	101 ± 2	0.50 ± 0.08[c]	1.35 ± 0.24[c]	38 ± 2

[a]X ± SE; [b]CONT, control period; $p<0.05$: [c]*versus* first period.

TABLE 2

Glomerular Hemodynamics Data[a]

	SNGFR (nL/min)	Q_A (nL/min)	SNFF (%)	ΔP (mmHg)	R_A ($\times 10^{10}$ dyn·s·cm^{-5})	R_E ($\times 10^{10}$ dyn·s·cm^{-5})	K_f (nL·s^{-1}·mmHg^{-1})
Group 1							
CONT[b]	27.9 ± 3.4	101.0 ± 17.1	30 ± 3	30 ± 2	2.9 ± 0.4	1.5 ± 0.3	0.096 ± 0.030
CsA (n = 12)	14.0 ± 3.5[c]	44.4 ± 13.4[c]	34 ± 3	41 ± 3[c]	8.4 ± 3.8[c]	6.8 ± 2.0[c]	0.031 ± 0.010[c]
Group 2							
CONT	31.7 ± 2.7	96.7 ± 7.6	33 ± 2	32 ± 1	2.5 ± 0.3	1.7 ± 0.1	0.063 ± 0.006
PAF (n = 7)	13.4 ± 1.7[c]	41.5 ± 5.7[c]	33 ± 3	29 ± 2[c]	4.8 ± 0.4[c]	3.5 ± 0.5[c]	0.036 ± 0.006[c]
Group 3							
CONT	38.3 ± 3.5	148.5 ± 20.3	28 ± 3	33 ± 2	1.7 ± 0.2	1.1 ± 0.2	0.082 ± 0.021
DAZ (n = 6)	40.1 ± 7.2	138.4 ± 19.8	30 ± 3	31 ± 1	1.8 ± 0.2	1.1 ± 0.2	0.085 ± 0.026
Group 4							
DAZ	33.8 ± 3.1	92.1 ± 6.0	36 ± 1	32 ± 1	2.7 ± 0.3	1.7 ± 0.1	0.081 ± 0.007
DAZ + CsA (n = 7)	17.0 ± 3.3[c]	55.3 ± 10.6[c]	34 ± 3	32 ± 1[d]	5.2 ± 1.0[c]	3.3 ± 0.7[c]	0.045 ± 0.010[c]

[a]X ± SE; [b]CONT, control period; $p<0.05$: [c]*versus* first period, [d]DAZ + CsA *versus* CsA.

cells (7), which can lead to decreased K_f, suggesting that PAF is involved in CsA-induced nephrotoxicity.

PAF induces the release of secondary mediators from various cell types and organs. Weissman *et al.* (21) showed that isolated perfused rabbit kidney stimulated by PAF generated PGE_2 and TxB_2. The release of these mediators was greater when the kidneys had been previously subjected to ureteral ligation. Increased production of renal thromboxane under chronic CsA treatment has been demonstrated (11). These data suggest that an interrelationship may exist between PAF, CsA, and TxA_2 which results in the development of acute renal failure (ARF) induced by CsA.

During acute CsA treatment, substantial increases in R_A and R_E (mainly R_E), leading to a significant increase ($p<0.05$) in P_{GC} (Table 2), were observed. As P_T remained unaffected there was also an increase in mean ΔP. Reduction in SNGFR resulted from arteriolar vasoconstriction, and consequently from a substantial decrease in Q_A. Moreover, our data indicate that SNGFR was also decreased due to a significant decrease in K_f. Thus, in spite of an increased ΔP, the SNGFR was reduced resulting from a significant decrease in Q_A and in K_f (70%). In previous studies (8), the effect of CsA vehicle (cremophor) was not considered because no glomerular hemodynamic alterations were observed upon cremophor administration.

PAF infusion provoked a significant decrease in AP, but AP was kept at renal autoregulation levels. PAF induced a reduction in FF, as would be expected in a predominantly pre-glomerular vasoconstriction. However, after acute PAF infusion, microcirculation studies suggested proportional incremental increases in R_A and R_E. These results may reflect the higher increment in R_A of the juxtamedullary nephrons. Despite the proportional increases in both arteriolar resistances R_A and R_E, PAF induced a moderate decrease in P_{GC} leading to a decline in mean ΔP. Moreover, PAF also induced a significant reduction in K_f. The effect of PAF on K_f may be caused by direct action on PAF receptors in mesangial cells, since it is known that PAF contracts mesangial cells (7). Thus, the hemodynamic mechanism responsible for the decrease in SNGFR appears to involve intrarenal vasoconstriction with a decrease in Q_A and K_f. The data suggest that a similar hemodynamic situation exists after acute CsA infusion, and would indicate a potential role of PAF in CsA nephrotoxicity. However, since PAF may stimulate the release of TxA_2 from activated platelets, the aim of the present work was to determine whether this vasoconstrictor prostaglandin participates as a second mediator in CsA-induced ARF, by administering a specific thromboxane synthetase inhibitor, dazmegrel, prior to CsA administration.

In our experimental model, DAZ alone did not change total or superficial kidney function. These results are very similar to those observed with other blockers, such as prostaglandin synthesis inhibitor (22) and angiotensin I converting enzyme inhibitor (23). Thus, when euvolemic normal Munich-Wistar rats undergo a hormonal blocking treatment, other vasoactive systems are activated and renal function is maintained. Therefore, DAZ which has no direct effects on kidney function may be used as a tool to study the pathophysiology of TxA_2 in various renal disorders, including CsA nephrotoxicity.

We observed that pretreatment with DAZ did not prevent the decline in GFR and SNGFR after acute cyclosporine administration, despite the increase in ΔP (Group 4). However, the normalization of ΔP was insufficient to compensate for the reduction in Q_A and K_f. In contrast, Perico *et al.* (11) observed that the administration of DAZ after chronic treatment with CsA was accompanied by an increase in GFR and a reduction in urinary excretion of TxB_2. However, there is a difference between the study made by Perico *et al.* (11) and the present work. We evaluated the acute effects of CsA and dazmegrel, while the earlier authors studied a chronic situation. We observed differences between acute and chronic (short term) effects of CsA on glomerular hemodynamics (24). Moreover, in Perico's study, urinary TxB_2 rose gradually during the

first month, and it was significantly elevated after one month of CsA treatment.

In summary, our data suggest that PAF is the direct mediator of acute CsA nephrotoxicity, and that TxA_2 does not contribute to a significant extent to the changes observed in whole kidney and single nephron hemodynamics upon CsA administration.

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Effect of Platelet-Activating Factor Antagonist BN 52063 on the Nephrotoxicity of Cisplatin[1]

Oscar F. Pavão dos Santos[a], Mirian A. Boim[a], Elvino J.G. Barros[a], Eduardo Pirotzky[b], Pierre Braquet[b] and Nestor Schor[a,*]

[a]Nephrology Division, Escola Paulista de Medicina, São Paulo, SP, Brazil and [b]Institut Henri Beaufour, Les Ulis, France

Cisplatin (DDP) is an effective anticancer agent that has been successfully applied against various solid tumors. However, DDP commonly causes nephrotoxicity. We observed that DDP led to significant alterations in renal microcirculation when administered to Munich-Wistar rats, with a concomitant decrease in single nephron glomerular filtration rate due to reduction in glomerular plasma flow and transcapillary hydraulic pressure difference. BN 52063, a platelet-activating factor antagonist, caused a striking change in acute renal failure induced by DDP leading toward normalization of all parameters of renal function. The results suggest that BN 52063 could be used as a novel drug to control DDP nephrotoxicity. ***Lipids 26,*** **1324-1328 (1991).**

cis-Diamminedichloroplatinum (DDP) has been proven to be a valuable agent in oncologic therapy. It is used, in particular, in the treatment of testicular and ovarian malignancy (1). DDP has also been useful in the treatment of head, neck, bladder, cervical, prostate and lung cancers (2,3). However, DDP treatment can cause a number of side effects with nephrotoxicity being the most common and most serious (4).

Observed reduction in glomerular filtration rate (GFR) due to cisplatin results from a decrease in renal blood flow and an increase in total renal vascular resistance (5) suggesting that cisplatin-induced acute renal failure (ARF) may be related to renal hemodynamic alterations (6). Therefore, one aim of this study was to characterize the changes that occur in renal microcirculation in response to DDP treatment in rats.

Platelet-activating factor (PAF) is a lipid mediator involved in various renal disorders (7) that causes inflammatory and vasoactive responses in the kidney. It was also observed that PAF causes a decline in GFR and in renal plasma flow (RPF) which can be ascribed to arteriolar vasoconstriction and/or mesangial cell contraction (8,9).

We have previously shown that BN 52063, a terpene isolated from *Gingko biloba* which inhibits the immune and vascular effects of PAF, is able to minimize cyclosporine (CsA) nephrotoxicity by ameliorating vasoconstriction induced by CsA (9). Thus, a second purpose of this study was to monitor the effect of the PAF antagonist BN 52063 on renal microcirculation in DDP treated rats.

MATERIALS AND METHODS

Methods. The studies were done using 29 male Munich-Wistar rats weighing between 240 and 320 g. Experimental rats received cisplatin (Group 1, n = 9) in a single dose of 6 mg/kg body wt, i.p. (10,11) (Laborterápica Bristol, São Paulo, Brazil), and control rats (Group 2, n = 6) received 0.5 mL of 0.9% NaCl i.p. The third group (Group 3, n = 7) received cisplatin, in the same dose as Group 1, and BN 52063 (Institut Henri Beaufour, Les Ulis, France) at 50 mg/kg body wt, p.o. for 4 days. Rats in the fourth group (Group 4, n = 7) received BN 52063 daily in the same dose as Group 3, to study the effects of chronic BN 52063 administration on renal microcirculation. Clearance and micropuncture studies were performed after four days following the administration of DDP.

Rats were anesthetized with Inactin, 100 mg/kg body wt, i.p. (BYK, Gulden, Konstanz, Germany) and were prepared in a routine fashion for micropuncture studies (12). All rats received an i.v. infusion of 10% inulin and 2% *p*-aminohippuric acid (PAH) at a rate of 1.2 mL/min, and isotonic rat serum to replace surgical losses and to maintain the animals in euvolemic condition (13). After 45 to 60 min equilibration, the experimental study period began. Two urine samples and arterial blood samples were collected for determination of inulin and PAH concentrations. Hematocrit (Hct) and plasma (C_A) were also measured.

Micropuncture studies. Timed (1 to 3 min) samples of fluid were collected from surface proximal convolutions of at least three nephrons for determining flow rate and inulin concentration and for calculating single nephron glomerular filtration rates (SNGFR). Coincident with these tubule fluid collections, two or three samples of femoral arterial blood were obtained for determination of systemic arterial Hct, C_A, inulin and PAH concentration in the plasma.

Hydraulic pressure was measured in single capillaries within surface glomeruli by a continuous recording servonull micropipette-transducer system (IPM Inc., San Diego, CA). Direct measurements of hydraulic pressure in glomerular capillaries (P_{GC}), proximal tubules (P_T), afferent arterioles (P_{EA}) and peritubular capillaries (P_C) were recorded for each rat.

To obtain estimations of colloid osmotic pressure (π) of plasma entering and leaving glomerular capillaries, we measured protein concentration in femoral arterial (C_A) and efferent arteriolar (C_E) plasma (14). C_A was taken as the protein concentration in the afferent arteriole. These

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*To whom correspondence should be addressed at Nephrology Division, Escola Paulista de Medicina, Rua Botucatu 740, 04023 São Paulo, SP, Brazil.

Abbreviations: ARF, acute renal failure; BW, body weight; C_A, afferent arteriolar protein concentration; C_E, efferent arteriolar protein concentration; CsA, cyclosporine; DDP, *cis*-diamminedichloroplatinum; ΔP, transcapillary hydraulic pressure difference; FF, filtration fraction; GFR, glomerular filtration rate; Hct, hematocrit; K_f, glomerular ultrafiltration coefficient; KW, kidney weight; MAP, mean arterial pressure; PAF, platelet-activating factor; PAH, *p*-aminohippuric acid; P_{GC}, glomerular capillary hydraulic pressure; P_T, tubular hydraulic pressure; Π_A, afferent colloid osmotic pressure; Π_E, efferent colloid osmotic pressure; Q_A, glomerular plasma flow rate; R_A, afferent arteriolar resistance; R_E, efferent arteriolar resistance; R_T, total arteriolar resistance; RPF, renal plasma flow; SNGFR, single nephron glomerular filtration rate; SNFF, single nephron filtration fraction; TRVR, total renal vascular resistance; V', urinary volume.

TABLE 1

Initial (in) and Final (fn) Body Weight (BW), Kidney Weight (KW), Hematocrit (Hct) and Afferent Arteriolar Protein Concentration (C_A) for All Groups

	BWin (g)	BWfn (g)	KW (g)	Hct (%)	C_A (g/dL)
Vehicle	280[a]	291	1.16	48	5.3
(n = 6)	±7	8	0.03	1	0.2
DDP	293	267[b]	1.06	46	4.9
(n = 9)	8	7	0.03	1	0.2
BN + DDP	285	270	0.97	44	5.4
(n = 7)	9	10	0.06	2	0.2
BN	269	268	0.88[c]	46	5.3
(n = 7)	12	13	0.05	1	0.1

[a] $\bar{X}$ ± SE.

[b] $p<0.05$ *vs* BW_{in}.

[c] *vs* Vehicle.

estimations of pre- and post-glomerular plasma protein concentrations permit the calculation of colloid osmotic pressure, single nephron filtration fraction (SNFF), glomerular capillary plasma flow rate (Q_A), afferent (R_A), efferent (R_E) and total (R_T) arteriolar resistances, as well as of the glomerular capillary ultrafiltration coefficient (K_f) using equations previously described (15).

Analytical procedures. The concentration of inulin in the tubular fluid was measured by the microfluorescence method (16). The inulin concentration in plasma and urine was determined by the macroanthrone method (17). Protein concentration in efferent arteriolar and femoral arterial blood was determined by a fluorcolorimetric method (14). Plasma and urinary PAH concentrations were measured by the method of Smith *et al.* (18). Total renal vascular resistance was calculated as the MAP/RPF ratio. The filtration fraction was calculated as the quotient of GFR and RPF.

Data were analyzed by Student's unpaired and paired two-tailed test. Statistical significance was taken as $p<0.05$; all data are reported as X ± SE.

RESULTS

Table 1 shows that initial body weight, kidney weight, Hct and C_A were not different between the groups. However, the animals treated with DDP showed a significant weight loss after 4 days, from 293 ± 8 to 267 ± 7 g ($p<0.05$). Table 2 shows the date on whole kidney function. GFR decreased 66% ($p<0.05$) after cisplatin treatment when compared to the vehicle group. We similarly observed a reduction in RPF from 3.02 ± 0.34 to 0.86 ± 0.27 mL/min ($p<0.05$). Since mean arterial pressure was not different in both groups, *i.e.* in vehicle and DDP, calculated total renal vascular resistance (TRVR) increased from 20.5 ± 2.7 to 93.3 ± 12.5 $mmHg \cdot min \cdot mL^{-1}$, ($p<0.05$). As GFR and RPF decreased proportionally, filtration fraction (FF) remained unchanged. Urinary volume was significantly higher in DDP rats compared to controls, 12.4 ± 0.2 *vs* 3.3 ± 0.3 μL/min ($p<0.05$). Chronic BN 52063 administration did not change whole glomerular function compared to controls. Concomitant treatment with BN 52063 and DDP normalized GFR (0.33 ± 0.04 *vs* 1.03 ± 0.05 mL/min; $p<0.05$), RPF (0.86 ± 0.27 *vs* 3.49 ± 0.17 mL/min; $p<0.05$) and TRVR (93.3 ± 12.5 *vs* 28.3 ± 1.3 $mmHg \cdot min \cdot mL^{-1}$; $p<0.05$) when compared with results obtained with DDP alone. However, urinary volume remained unchanged (12.4 ± 0.2 *vs* 12.8 ± 3.4 μL/min).

Tables 3 and 4 summarize the data on superficial nephron function. Mean values for SNGFR and Q_A were significantly lower in cisplatin-treated rats than in vehicle rats, 20.1 ± 2.3 *vs* 34.2 ± 2.1 nL/min ($p<0.05$) and 61 ± 6 *vs* 106 ± 9 nL/min ($p<0.05$), respectively. SNFF did not change 33 ± 3 *vs* 34 ± 3%. Since mean P_{GC} decreased from 46 ± 1 to 41 ± 1 mmHg ($p<0.05$) and mean P_T remained unchanged (from 15 ± 1 to 17 ± 2 mmHg), the mean transcapillary hydraulic pressure difference (ΔP) decreased significantly from 31 ± 1 to 27 ± 1 mmHg ($p<0.05$). Cisplatin treatment induced an impressive increase in total arteriolar resistance (R_T), 7.0 ± 0.4 *vs* 3.8 ± 0.2 × 10^{10} $dyn \cdot s \cdot cm^{-5}$ ($p<0.05$) which resulted from the rise in both R_A (88%) and R_E (77%), but mainly in R_A as shown in Table 4. The mean glomerular ultrafiltration coefficient (K_f) was similar for the vehicle and the DDP group.

TABLE 2

Mean Arterial Pressure (MAP), Urinary Volume (V'), Glomerular Filtration Rate (GFR), Renal Plasma Flow (RPF) and Total Renal Vascular Resistance (TRVR) for All Groups

	MAP (mmHg)	V' (μL/min)	GFR (mL/min)	RPF (mL/min)	TRVR ($mmHg \cdot min \cdot mL^{-1}$)
Vehicle	103[a]	3.3	0.96	3.02	30.5
(n = 6)	±4	0.3	0.05	0.34	2.7
DDP	105	12.4[b]	0.33[b]	0.86[b]	93.3[b]
(n = 9)	2	0.2	0.04	0.27	12.5
BN + DDP	98	12.8[b]	1.03[c]	3.49[c]	28.3[c]
(n = 7)	3	3.4	0.05	0.17	1.3
BN	103	3.1	0.98	3.12	33.8
(n = 7)	1	0.4	0.05	0.18	2.3

[a] $\bar{X}$ ± SE.

[b] $p<0.05$ *vs* Vehicle.

[c] BN + DDP *vs* DDP.

TABLE 3

Single Nephron Glomerular Filtration Rate (SNGFR), Glomerular Plasma Flow (Q_A), Single Nephron Filtration Fraction (SNFF), Glomerular Capillary (P_{GC}) and Tubular (P_T) Hydraulic Pressure and Transcapillary Hydraulic Pressure Difference (ΔP) for All Groups

	SNGFR (nL/min)	Q_A (nL/min)	SNFF (%)	P_{GC} (mmHg)	P_T (mmHg)	ΔP (mmHg)
Vehicle	34.2[a]	106	34	46	15	31
(n = 6)	±2.1	9	1	1	1	1
DDP	20.1[b]	61[b]	33	41	14	27[b]
(n = 9)	2.3	6	3	1	2	1
BN + DDP	35.1[c]	120[c]	31	43	11[b]	32[c]
(n = 7)	2.1	12	3	1	1	1
BN	30.8	103	31	48	16	32
(n = 7)	2.3	9	2	1	1	1

[a]$\bar{X}$ ± SE.

[b]$p<0.05$ *vs* Vehicle.

[c]BN + DDP *vs* DDP.

TABLE 4

Afferent (R_A), Efferent (R_E) and Total (R_T) Arteriolar Resistances, Afferent (Π_A) and Efferent (Π_E) Colloid Osmotic Pressures, and Glomerular Ultrafiltration Coefficient (K_f) for All Groups

	R_A ($\times 10^{10}$ dyn·s·cm^{-5})	R_E ($\times 10^{10}$ dyn·s·cm^{-5})	R_T ($\times 10^{10}$ dyn·s·cm^{-5})	Π_A (mmHg)	Π_E (mmHg)	K_f (nL·s^{-1}·mmHg^{-1})
Vehicle	2.5[a]	1.3	3.8	16.8	31.2	0.0941
(n = 6)	±0.2	0.1	0.2	0.8	0.9	0.0102
DDP	4.7[b]	2.3[b]	7.0[b]	15.3	27.8[b]	0.0628
(n = 9)	0.5	0.3	0.4	0.7	0.8	0.0163
BN + DDP	2.2[c]	1.5[c]	3.7[c]	17.5	30.8[c]	0.0761
(n = 7)	0.2	0.2	0.5	0.8	1.1	0.0050
BN	2.4	1.6	4.0	16.8	29.1	0.0642
(n = 7)	0.2	0.2	0.4	0.6	0.7	0.0093

[a]$\bar{X}$ ± SE.

[b]$p<0.05$ *vs* Vehicle.

[c]BN + DDP *vs* DDP.

Reminiscent of what was observed for whole kidney function, chronic BN 52063 administration alone did not change the superficial renal microcirculation. However, the treatment with BN 52063 in the group receiving DDP induced a marked increase in SNGFR (20.1 ± 2.3 *vs* 35.1 ± 2.1 nL/min; $p<0.05$). The normalization of SNGFR resulted from increases in Q_A (61 ± 6 *vs* 120 ± 12 nL/min; $p<0.05$) and ΔP (27 ± 1 to 32 ± 1 mmHg; $p<0.05$) with decreases in both arteriolar resistance, especially R_A (4.7 ± 0.5 *vs* 2.2 ± × 10^{10} dyn·s·cm^{-5}; $p<0.05$). K_f remained unchanged (0.0628 ± 0.0163 *vs* 0.0761 ± 0.0050 nL/s·mmHg; $p>0.05$).

DISCUSSION

Cisplatin has been shown to have potent chemotherapeutic effects in a variety of human neoplasms, impeding the development of many solid tumors, including testicular, ovarian and lung cancers (1). Since the kidney is highly susceptible to toxic injury, it is not surprising that antineoplasic agents may cause nephrotoxicity. Thus, we employed an experimental protocol in which the renal function was evaluated after 4 days of cisplatin administration. Moreover, we studied the role of PAF in DDP nephrotoxicity, by using BN 52063, a PAF antagonist.

Vehicle administration did not cause any alteration in renal hemodynamics when compared to control Munich-Wistar rats. After 4 days of short-term BN 52063 treatment in control rats (Group 4), we did not observe any alterations in total or superficial kidney functions under euvolemic conditions. Thus, BN 52063 may be useful in the study of the pathophysiology of PAF in various renal disorders.

In fact, our data suggest a possibly important involvement of PAF in DDP nephrotoxicity, because normalization of renal function was achieved when a PAF antagonist was co-administered. However, despite the protective effect of BN on glomerular hemodynamics, we did not observe reversion of the polyuria induced by DDP. This suggests that the mechanism(s) involved in the urinary concentration may be independent of the vasoconstriction that causes ARF in DDP rats. Actually the polyuria induced by cisplatin have been suggested to occur by two distinct mechanisms. In the first phase (24 to 48 hr), polyuria results from a reduction in vasopressin release

or synthesis and a rise in prostaglandin production (19). In the later phase, the mechanism is probably dependent on the lack of urea addition to the fluid in the loop of Henle (20).

In the present study, cisplatin administration caused a substantial decrease in GFR and RPF with a marked increase in TRVR. The decrease in GFR has been attributed to the vasoconstriction of renal vessels with a decrease in renal plasma flow and in effective filtration pressure (5). To clarify the pathophysiological mechanism(s) of ARF induced by cisplatin, the glomerular hemodynamics were studied in Munich-Wistar rats. We observed a marked increase in both afferent and efferent arteriolar resistance, mainly in R_A. The increase in R_A was associated with a significant decrease in intraglomerular hydraulic pressure (P_{GC}). Since P_T remained unchanged, ΔP decreased significantly. The latter data pinpoint intratubular obstruction as a main factor in cisplatin-induced ARF. Similarly, it was noted that intratubular obstruction did not play an important role in the early phase of ARF (5).

Clinical (21,22) and experimental (5,23,24) studies suggest that cisplatin-induced ARF is in part caused by reduced renal blood flow. Actually, we also observed significant decline in mean Q_A as measured by observed arteriolar vasoconstriction. After volume expansion, Winston and Safirstein (5) observed that renal plasma flow in cisplatin-treated rats increased, but GFR and SNGFR remained below control euvolemic values, suggesting that a reduction in K_f contributes to the decreased GFR. In the present study we observed a ~35% decrease in K_f, although this reduction did not reach statistical significance.

Similar to the observations reported by others (5,10,23), the decline in SNGFR (41%) was disproportionately lower than that observed for GFR (66%). Various possibilities should be considered to explain this finding. Safirstein *et al.* (10) demonstrated that inulin leaks (~30%) out of the tubule fluid beyond the site of proximal tubule puncture. However, the magnitude of this leak is not enough to explain the discrepancy between SNGFR and GFR. Another possibility is that we have preferentially chosen and punctured slightly affected nephrons and not more severely altered nephrons (bias selection). However, we did not observe apparent differences in the surface morphology. A likely mechanism may involve a predominant effect of DDP on juxtamedullary nephrons, leading to a greater decrease in GFR as compared to SNGFR, thus accounting for the observed difference.

Several compounds have been reported to limit cisplatin nephrotoxicity. Sodium thiosulfate (11), superoxide dismutase (25) and atrial natriuretic factor (26) administration have been shown to have some partial beneficial effect on the experimental model of ARF induced by cisplatin. However, acute or chronic administration of captopril, an angiotensin I converting enzyme inhibitor or verapamil, a calcium channel blocker, did not change the evolution of cisplatin nephrotoxicity (20). In contrast, the present study showed that the treatment with a PAF antagonist (BN 52063) completely prevented the alterations induced by DDP in both global and superficial nephron functions. The increases in mean arteriolar resistances and the decrease in ΔP were abolished by simultaneous treatment with BN 52063. Moreover, the glomerular plasma flow rate remained unchanged and the SNGFR returned to normal levels. Thus, BN 52063 treatment induced a striking change in ARF caused by DDP; it normalized all parameters of renal function and improved morphological parameters and overall mortality (Pirotzky, E., unpublished observations).

In summary, our data have shown that the reduction in SNGFR caused by cisplatin treatment resulted from intrarenal vasoconstriction, mainly in the pre-glomerular arteriole (R_A), leading to a decrease in Q_A and ΔP. K_f was not substantially altered. BN 52063 was able to normalize renal function in DDP treated rats, suggesting that PAF may have a potential role in cisplatin-induced nephrotoxicity. Thus, the PAF antagonist BN 52063 offers itself as a novel drug that can prevent the deleterious effects of DDP on renal microcirculation.

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Effects of PAF Antagonists on Renal Vascular Escape and Tachyphylaxis in Perfused Rabbit Kidney[1]

M.G. Ferreira[a], P. Braquet[b] and M.C. Fonteles[a,*]

[a]Unidade de Pesquisas Clínicas, Centro de Ciencias da Saude, Universidade Federal do Ceará, Fortaleza, Brazil, and [b]Institut Henri Beaufour, F-92350, Le Plessis Robinson, France

Renal vascular escape is a physiological phenomenon of adaptation that occurs in vascular smooth muscle. It has been described in many preparations subjected to electrical stimulation or treated with vasoactive agents, such as noreprinephrine, angiotensin and vasopressin. We have recently demonstrated that a naturally occurring ginkgolide (BN 52021), which is a PAF antagonist, was able to block norepinephrine-induced escape in perfused rabbit kidney. In the present work other PAF antagonists, such as the ginkgolides BN 52022 and BN 52024, and the synthetic compounds 48740 RP and WEB 2086, were tested. Their effects on renal vascular escape, perfusion pressure and tachyphylaxis were evaluated. They all were shown to block the escape. Among the ginkgolides, BN 52024 is generally recognized as one of the weaker PAF antagonists. However, in spite of this, BN 52024 was able to significantly and simultaneously block renal vascular escape and tachyphylaxis in perfused rabbit kidney infused with norepinephrine.
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In 1964, Folkow *et al.* (1) demonstrated in anesthetized cats that electric stimulation of sympathetic nerves of the mesenteric vascular bed produced an initial vasoconstriction followed by a return of flow toward control levels, even during continued nerve stimulation. This phenomenon was called "autoregulatory escape" and later defined as the failure of the vascular smooth muscle to maintain its contraction when a constant stimulus is applied (2).

The occurrence of escape in vascular beds of different animals, induced by direct stimulation of sympathetic nerves or by infusion of vasoconstricting substances, has been reported. Sites of vascular escape described include the mesenteric bed (1,3,4–7), hepatic and portal beds (8–10), and renal artery (5,11,12). In all these experiments, escape was induced by neural stimulation. Adrenergic stimuli facilitated vascular escape at the mesenteric (13–16), hepatic (8,10,16) and renal (5,17,18) sites.

Escape occurs during constant pressure or constant flow (3–5) and is not altered by β-receptor blockade (19,10), atropine (6), reserpine (20), adenosine (21), aspirin (20), or endothelium-derived relaxing factor (EDRF)-blocking agents (22). Indomethacin partially blocks noradrenergic-induced escape (20), but not the escape produced by sympathetic stimulation (6).

Previous reports suggest that the mechanism of escape may involve either the release of an unknown vasodilator (15,17) or a cellular mechanism which results in the inability of the arteriolar smooth muscle to maintain its contraction (23). Identification in kidney of platelet activating factor (PAF) being a vasodilator (24), as well as the discovery of PAF antagonists (25), led us to propose that PAF may be involved in vascular escape. We have therefore studied the role of several PAF antagonists in this phenomenon.

MATERIAL AND METHODS

Rabbits of both sexes, each weighing 1–2 kg, were fasted for 24 hr. The animals had access to water and were kept in the laboratory in the morning of the experiment. They were anesthetized intraperitoneally with sodium pentobarbital (50 mg/kg). After an abdominal incision, the left kidney was isolated from surrounding tissues and the ureter and renal penduncle were carefully dissected. The renal vein was then severed proximally to the kidney and the renal artery immediately cannulated. Perfusion was initiated in a Petri dish containing Krebs-Henseleit solution, with the pH adjusted to 7.3–7.4. The kidney was flushed with oxygenated perfusate and, after cannulation of the ureter, the organ was connected to an open perfusion system.

This system consisted of a previously calibrated Gilson (Paris, France) Minipuls perfusion pump, a heat exchanger, an Imbracrios (Ribeirão Preto, Brazil) infusion pump, a membrane oxygenator (Travenol, Deerfield, IL) and a pressure device to monitor perfusion pressure. Several physiological parameters were measured including perfusate flow, urinary flow, resistance, and perfusion pressure. Perfusate flow was measured at 10-min intervals by collecting the perfusion effluent. Urinary flow was also determined. To measure renal vascular resistance, the ratios of the mean perfusion pressure (mmHg) and the perfusate flow rate were followed. To evaluate vascular escape, the kidney was submitted to noradrenergic vasoconstriction for 20 min; escape was present when at the end of the α-agonist infusion the perfusion pressure was less than the maximal effect. Renal vascular escape was calculated according to Fonteles *et al.* (17) and Henrich and Lutz (13), and was expressed as (Pmx-Pmn)/(Pmx-Pi) × 100, where Pmx and Pmn were, respectively, the maximal and minimal perfusion pressures obtained during norepinephrine (NOR) infusion, and Pi was the initial pressure measured just prior to α-agonist infusion.

The effects of several PAF antagonists were evaluated, including the naturally occurring ginkgolides BN 52022 and BN 52024 (2.27×10^{-5} M and 2.35×10^{-6} M,

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*To whom correspondence should be addressed at Centro de Ciencias da Saúde, Universidade Federal do Ceará, Departamento de Farmacologia, CEP 60425 - C. P. 657, Fortaleza, Ceará, Brazil.

Abbreviations: EDRF, endothelium-derived relaxing factor; NOR, norepinephrine; PAF, platelet-activating factor, 1-*O*-alkyl-2-acetyl-*sn*-glycero-3-phosphocholine.

respectively), the synthetic compound 48740 RP (10^{-5} M) and a triazolobenzodiazepine WEB 2086 (10^{-6} M). These drugs were added to the perfusion fluid at the beginning of the experiment and the response of the kidney vessels to the agents was observed during three bursts of 20-min NOR (1.3×10^{-6} M) infusion. All salts and chemicals used were reagent grade. The ginkgolides were donated by Institut Henri Beaufour. 48740 RP was a gift of Dr. Boris B. Vargaftig from Institut Pasteur, and WEB 2086 was donated by Boehringer-Ingelheim. Data were analyzed by paired and unpaired t-tests.

RESULTS

Control kidneys. Infusion of NOR (1.3×10^{-6} M) resulted in a sudden increase in perfusion pressure and resistance, which was followed by escape and tachyphylaxis as seen in Tables 1, 2 and 3.

The increase in resistance was due to α-agonist administration. Resistance dropped below control values during the wash-out periods. Urinary flow decreased from 0.093 ± 0.015 mL/g/min to 0.059 ± 0.008 mL/g/min during NOR infusion, while the perfusate flow rate remained constant during the experiment.

Kidneys treated with ginkgolides. When two specific PAF receptor antagonists, BN 52022 (2.27×10^{-6} M) and BN 52024 (2.35×10^{-6} M) were added to the perfusate, renovascular escape produced by NOR infusion was partially blocked (Table 3). The magnitutde of this blockage was greater in kidneys treated with BN 52024, which was also more effective in reversing tachyphylaxis (Fig. 1). BN 52022 caused throughout the set of experiments a drop in resistance which reached values below control levels (Table 2). Urine flow decreased slightly in kidneys perfused with BN 52022 during the last period of NOR infusion, but perfusate flow remained unchanged. The opposite occurred with kidneys treated with BN 52024 which showed increased urinary flow.

Kidneys infused with synthetic PAF antagonists. In kidneys treated with 48740 RP, noradrenergic vascular escape and tachyphylaxis were also blocked, although less efficiently (Table 3). The resistance values, however, were always above the averages registered for the other groups, even during the wash-out period (Table 2). The perfusate flow was constant and the urinary flow rate decreased only during NOR infusion. WEB 2086 (10^{-6} M) partially blocked vascular escape (Table 3) while other parameters (resistance and perfusion pressure) of kidney perfusion remained unaltered (Tables 1 and 2). The effects of several PAF antagonists on renal vascular escape are shown in Table 3.

DISCUSSION

The failure of the kidney vascular smooth muscle to maintain its contraction during prolonged NOR infusion at a constant flow rate characterizes renal vascular escape, as described previously (17,18,20). The mechanism of this phenomenon remains elusive, and some workers have suggested that release of a vasodilator, in response to constrictor stimuli, may be involved in this process (15,17,23).

Since PAF promotes hypotension (26,27) and is produced by kidney tissues under various conditions

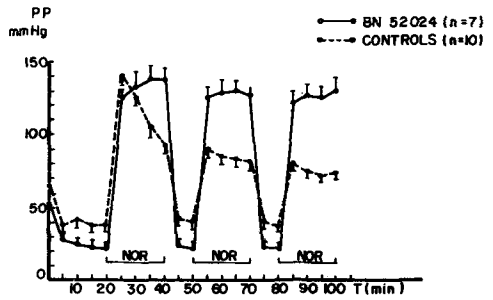


FIG. 1. Effects of repeated infusions of NOR (1.3×10^{-6}) on perfusion pressure of isolated rabbit kidney. Organs in this group were perfused with Krebs-Henseleit solution containing 2.35×10^{-6} M BN 52024. The control group was also plotted to demonstrate the complete reversal of tachyphylaxis.

(28,29), we hypothetized that PAF may be one such mediator.

Recently, we have demonstrated that the PAF antagonist ginkgolide B (BN 52021, 3.05×10^{-6} M) reduced vascular escape and blocked tachyphylaxis (30). BN 52021 is the most potent compound in displacing [^{3}H]PAF bound to its receptor and it has been considered to be the most potent PAF antagonist among the ginkgolides (31,32).

BN 52022 and BN 52024 share structural features which include the presence of a hydrophilic group close to lipophilic moiety, and this may well relate to their relative inability to displace bound [^{3}H]PAF (33). A recent study has compared the relative potencies of ginkgolides A, B and C (BN 52020, BN 52021 and BN 52022, respectively) in inhibiting PAF metabolism. These three compounds displayed in other systems the same order of potency as that described for platelet aggregation and PAF specific binding (32,34).

The fact that WEB 2086 and 48740 RP are also very active as escape blockers led us to believe that PAF is a mediator in this phenomenon.

In the present study, BN 52024 was used as a negative control because its PAF antagonistic effect is minimal. BN 52022 showed the ability to promote partial blockade of renovascular escape and tachyphylaxis. However, it was unexpected that BN 52024 would prove to be the most potent inhibitor of noradrenergic-induced escape and tachyphylaxis in rabbit kidney (Table 3).

Responses to NOR were always tachyphylatic in the present model (35). A high calcium concentration in the perfusate abolished NOR-promoted tachyphylaxis in rabbit kidney (36). Conversely, a low concentration of calcium and glucose in the infusate favors tachyphylaxis due to angiotensin II (37). Ginkgolides antagonize the rise in intracellular Ca^{2+} either by inhibiting calcium influx or calcium mobilization induced by PAF (38). It therefore seems unlikely that tachyphylaxis would be related to PAF antagonism and the effects observed here may be ascribed to a unique property of this compound. Escape could solely be dependent upon PAF receptor blockade. However, BN 52024 may possibly permeate the cell membrane and promote intracellular alterations which are linked to calcium mobilization, or it may act on a special PAF receptor.

TABLE 1

Effects of PAF Antagonists on Perfusion Pressure (mmHg) of Isolated Rabbit Kidneys Perfused with Krebs-Henseleit Solution and Infused with Norepinephrine (1.3×10^{-6} M)[a]

Time[b] (min)	Control (n = 10)	BN 52022 (n = 7)	BN 52024 (n = 7)	48740 RP (n = 7)	WEB 2086 (n = 7)
25	140.80 ± 8.04	124.85 ± 10.37	124.85 ± 11.67	102.57 ± 14.69	125.14 ± 12.07
30	126.80 ± 3.89	128.00 ± 10.37	132.28 ± 09.84	100.00 ± 13.97	126.85 ± 09.11
35	105.60 ± 5.09	120.00 ± 08.48	138.85 ± 06.46	098.28 ± 13.63	122.57 ± 08.96
40	093.20 ± 4.83	118.28 ± 08.37	137.42 ± 05.80	096.85 ± 13.22	122.85 ± 08.58
85	078.40 ± 3.53	092.57 ± 04.42	122.85 ± 04.73	078.57 ± 08.29	110.28 ± 08.83
90	073.60 ± 2.68	096.57 ± 04.41	128.28 ± 04.67	082.57 ± 09.34	102.00 ± 06.65
95	070.40 ± 2.39	092.00 ± 09.79	126.28 ± 05.31	082.28 ± 09.65	100.57 ± 06.86
100	071.40 ± 2.72	089.14 ± 03.02	130.00 ± 05.75	076.85 ± 10.18	100.57 ± 08.03

[a]Results are expressed as mean ± SEM.

[b]Time in min refers to the first and last periods of NOR infusion.

TABLE 2

Effects of PAF Antagonists on Resistance (mmHg/mL · g · min) of Isolated Rabbit Kidney Perfused with Krebs-Henseleit Solution and Infused with Norepinephrine (1.3×10^{-6} M)[a]

Period	Time (min)	Control (n = 10)	BN 52022 (n = 7)	BN 52024 (n = 7)	48740 RP (n = 7)	WEB 2086 (n = 7)
Control	10	55.44 ± 13.97	46.19 ± 10.32	19.09 ± 02.40	31.52 ± 04.56	14.81 ± 02.37
	20	51.16 ± 09.89	64.44 ± 12.40	16.52 ± 01.70	47.72 ± 05.60	16.79 ± 03.33
NOR[b]	30	145.02 ± 14.73	151.23 ± 18.93	91.04 ± 05.43	124.08 ± 20.22	80.35 ± 20.22
	40	101.60 ± 12.27	137.10 ± 17.68	105.60 ± 12.09	117.67 ± 17.83	74.00 ± 10.50
WO[c]	50	46.61 ± 10.98	41.58 ± 07.34	12.43 ± 01.70	40.39 ± 03.87	11.66 ± 01.98
NOR[b]	60	92.40 ± 11.03	37.32 ± 14.13	91.28 ± 06.34	106.10 ± 15.85	65.30 ± 10.20
	70	91.63 ± 09.86	22.48 ± 08.51	90.90 ± 06.50	110.95 ± 15.26	79.47 ± 07.62
WO[c]	80	39.21 ± 04.75	15.39 ± 05.82	12.96 ± 01.11	34.94 ± 03.10	10.89 ± 02.59
NOR[b]	90	81.84 ± 09.82	29.58 ± 11.20	83.54 ± 07.84	101.44 ± 13.76	79.67 ± 06.65
	100	78.10 ± 08.49	30.12 ± 11.40	89.92 ± 06.06	100.71 ± 14.74	73.41 ± 06.70

[a]Results are expressed as mean ± SEM.

[b]Norepinephrine.

[c]Wash-out.

TABLE 3

Effects of PAF Antagonists on Renal Vascular Escape in Perfused Rabbit Kidney[a]

Period[b]	Controls (n = 10)	BN 52022 (n = 7)	BN 52024 (n = 7)	48740 RP (n = 7)	WEB 2086 (n = 7)
P1	50.32 ± 4.61	35.69 ± 8.16[c]	9.70 ± 2.46[d]	30.18 ± 5.35[c]	33.18 ± 5.48[c]
P2	31.87 ± 3.81	16.72 ± 3.50	11.52 ± 1.87	21.28 ± 6.50	23.94 ± 2.52
P3	22.65 ± 4.22	14.62 ± 3.00	6.47 ± 1.68	20.47 ± 5.55	18.09 ± 3.09

[a]Results are expressed as mean ± SEM.

[b]P1, P2 and P3 are periods of 20 min during which NOR was infused at a rate of 1.3×10^{-6} M.

[c]$p < 0.01$.

[d]$p < 0.001$.

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Presence of Platelet-Activating Factor in Pyuria in Humans[1]

Ichiro Ikeda[a], Makoto Oda[a,*], Muneki Sakakura[a] and Kojiro Yasunaga[b]

[a]The Department of Internal Medicine, Saiseikai Izuo Hospital, Osaka 551 and [b]The First Department of Internal Medicine, Kansai Medical University, Moriguchi Osaka 570, Japan

The relationship between the occurrence of platelet-activating factor (PAF) and neutrophils in urine from patients with urinary tract infection was examined. PAF was detected in human pyuria, when leukocyte levels reached at least 300 cells/μL (n=45), but not in normal urine (n=12). The amount of PAF found in pyuria, measured by platelet aggregation assay, was 0.01 to 13.3 pmol/mL. A close correlation was seen between the amount of PAF present and the number of urinary leukocytes ($p < 0.01$, $r=0.70$). The leukocytes in pyuria consisted almost entirely of neutrophils (96 ± 4 %, mean ± S.D.). Our findings suggest that the occurrence of PAF is associated with the accumulation of neutrophils in urine.

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Platelet-activating factor (PAF) is generated by neutrophils, macrophages, monocytes, and endothelial cells, and is thought to play a role in the development of inflammation (1,2). However, the detailed mechanisms of PAF formation in these processes are not clear. Human neutrophils, which produce and are activated by PAF (3,4), are among the principal inflammatory cells. This suggests that the occurrence of PAF may be related to the accumulation of neutrophils in inflammatory lesions.

Sanchez-Crespo *et al.* (5) had found PAF at very low levels in the urine from normal volunteers. In the present study, the relationship between PAF and neutrophils in human urine was examined.

MATERIALS AND METHODS

1-*O*-Hexadecyl-2-acetyl-*sn*-glycero-3-phosphocholine (C_{16}PAF) and bovine serum albumin (BSA) were purchased from Sigma Chemical Co. (St. Louis, MO). CV-3988 (6) and CV-6209 (7) were gifts from Takeda Chemical Industries Ltd. (Osaka, Japan), and FR-900452 (8) was a gift from Fujisawa Pharmaceutical Co. (Osaka, Japan). Silica gel G plates (0.25 mm layer) were from Analtech (Newark, DE). Silicar (100–200 mesh type 60Å special) was obtained from Mallinckrodt Inc. (Paris, KY). Sources of other chemicals and other details are those described previously (9).

Fifty-seven samples of urine were obtained from 38 individuals as follows: 12 samples from 10 normal volunteers (6 male and 4 female; 51 ± 27 years of age, mean ± S.D.) and 45 samples from 28 patients with urinary tract infection (14 male and 14 female; 64 ± 20 years of age). The leukocytes in urine were counted using a blood cell counter. Pyuria is defined as urine containing more than or equal to 300 leukocytes/μL.

Total lipids were immediately extracted from 6 mL of fresh midstream urine by the method of Bligh and Dyer (10). Total lipids were applied to a silica gel column, and neutral lipids were eluted with chloroform. Phospholipids were recovered with methanol and suspended in 100 μL of 2.5 mg BSA/mL saline. For some samples, phospholipids were separated by preparative silica gel G thin-layer chromatography (TLC), using chloroform/methanol/water (65:35:6, v/v/v) as developing solvent. The silica gel was scraped from the TLC plates in 1 cm bands, and each fraction was extracted.

Washed rabbit platelets were prepared by the method of Pinckard *et al.* (11). The amount of PAF was quantified by measuring the ability of each sample to aggregate washed rabbit platelets using an aggregometer, as described previously (12). PAF levels are expressed as fmol/mL urine calculated from a calibration curve established for C_{16}PAF, which also is the major PAF species generated by human neutrophils (13). The minimum amount of PAF detectable by this method was 10 fmol/mL.

When the phospholipids from pyuria were diluted with BSA/saline and added to washed rabbit platelets, platelet aggregation decreased. By contrast, Nakayama *et al.* (14) reported that when similarly prepared samples from rat uterus were diluted with BSA/saline and added to washed rabbit platelets, platelet aggregation increased. This discrepancy was explained by the presence of endogenous inhibitors, such as lysophosphatidylcholine and sphingomyelin, in rat uterine samples. In our samples, the content of inhibitors was apparently insufficient to interfere with the platelet aggregation assay, even if endogenous inhibitors would have been present. The amount of PAF in urine was determined based on the aggregating activity of the phospholipid fraction obtained by column chromatography.

RESULTS AND DISCUSSION

When the phospholipid fraction from pyuria was reacted with washed rabbit platelets, immediate aggregation was observed. The aggregation curve was similar to that produced by authentic C_{16}PAF. The aggregation was completely inhibited upon preincubation with CV-3988, CV-6209, or FR-900452, which are specific antagonists of PAF (6–8) (Fig. 1).

The phospholipids from urine were also separated by thin-layer chromatography using chloroform/methanol/water (65:35:6, v/v/v) as solvent. Aggregation was induced by the fraction with the same migration rate (R_f) as that of C_{16}PAF, but not by the other fractions (Fig. 2). The results indicate that platelet-aggregating activity is associated with the PAF fraction isolated from the urine of human subjects with pyuria.

The amount of PAF in pyuria varied from 0.01 to 13.3 pmol/mL (n=45), but by our method PAF was not detected in normal urine (n=12). A close correlation was found between the amount of PAF and the number of

[1]Based on a paper presented at the Third International Conference on Platelet-Activating Factor and Structurally Related Alkyl Ether Lipids, Tokyo, Japan, May 1989.

*To whom correspondence should be addressed at the Department of Internal Medicine, Saiseikai Izuo Hospital, 3-4-5, Kitamura, Taisho-ku, Osaka 551, Japan.

Abbreviations: BSA, bovine serum albumin; C_{16}PAF, 1-*O*-hexadecyl-2-acetyl-*sn*-glycero-3-phosphocholine; PAF, platelet-activating factor; R_f, migration rate; TLC, thin-layer chromatography.

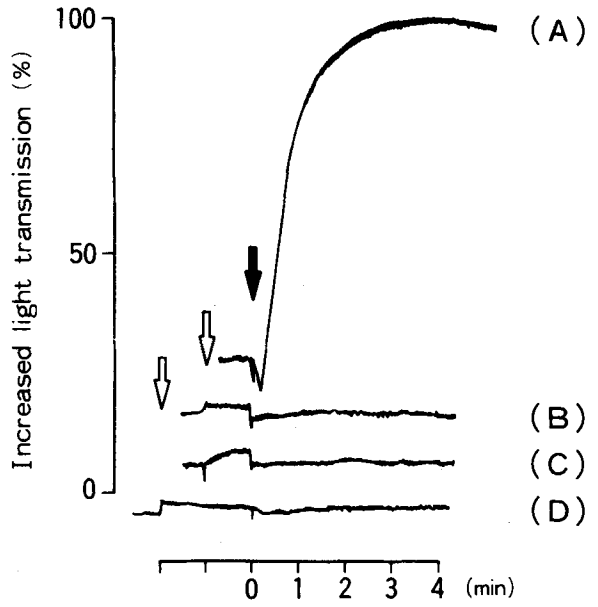


FIG. 1. Platelet aggregation by phospholipids extracted from urine of patients with pyuria. Phospholipids from 6 mL of urine were suspended in 100 μL of 2.5 mg BSA/saline solution. Washed rabbit platelets and 5 μL of the suspension were then allowed to react in an aggregometer (A). Washed rabbit platelets were preincubated with 50 nM CV-6209 for 1 min (B); 8 μM CV-3988 for 1 min (C); or 8 μM FR-900452 for 2 min (D); and the samples were then allowed to react with the platelets. The solid arrow indicates the time of sample addition; the outlined arrows indicate the time of CV-6209, CV-3988, or FR-900452 addition.

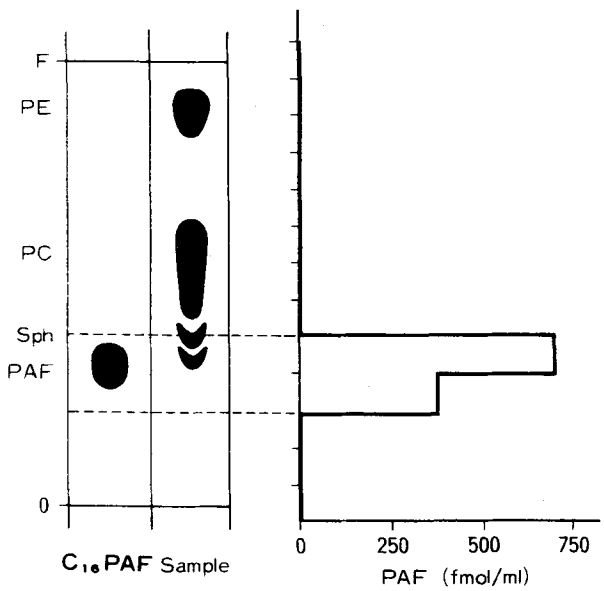


FIG. 2. The aggregating activity of the various fractions of phospholipids separated by TLC. The phospholipids from pyuria were separated by TLC using chloroform/methanol/water (65:35:6, v/v/v) as developing solvent. Fractions extracted from 1-cm silica gel bands were tested for their ability to promote platelet aggregation. Platelet aggregating activity was converted into amounts of C_{16}PAF. O, origin; F, front; PE, phosphatidylethanolamine; PC, phosphatidylcholine; Sph, sphingomyelin.

leukocytes in urine ($p < 0.01$, $r=0.70$) (Fig. 3). Light microscopic observation revealed that neutrophils were predominant among the leukocytes in pyuria (96 ± 4%, mean ± S.D., n=13). Hence, the presence of PAF appears to be related to the accumulation of neutrophils.

Sanchez-Crespo *et al.* (5) reported that urine from normal volunteers contained a platelet-activating component with characteristics similar to those of synthetic PAF. In contrast, our tests showed that normal urine which contained less than 300 leukocytes/μL contained no PAF. Reasons for the differences in the amounts of PAF observed in Sanchez-Crespo's study and ours may include differences in the initial volume of urine analyzed (about 1500 mL in their study and 6 mL in ours).

The observed relationship between the amount of PAF and the number of neutrophils present in urine appears to be reminiscent of what has been observed in scales from patients with psoriasis (15), and in pleural fluid from patients with pyothorax (16). There could be two explanations for this interrelationship between neutrophils and PAF. One is that PAF may be generated by neutrophils; the second is that PAF may be generated by other cells and that neutrophils may be accumulated in the inflammatory lesions. The source of PAF in pyuria is still unknown.

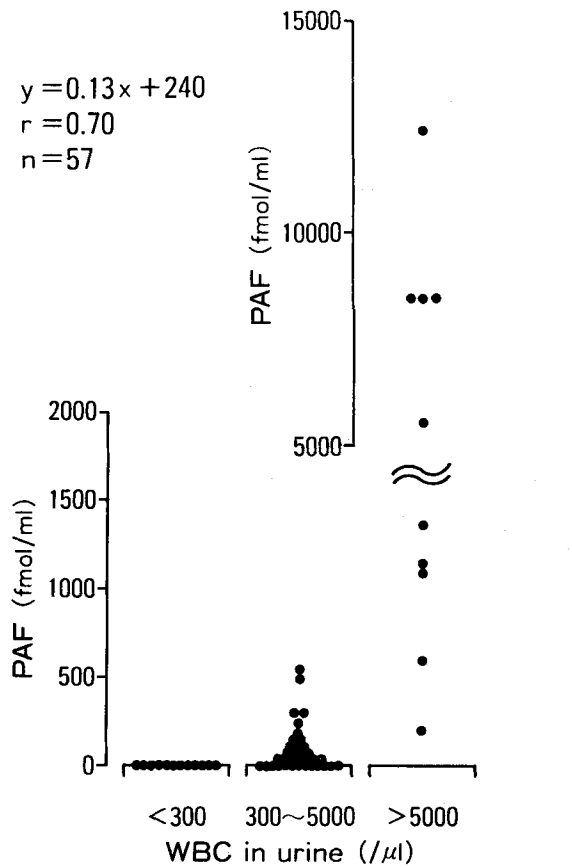


FIG. 3. Relationship between the amount of PAF and the number of leukocytes in urine. The amount of PAF was quantified by determining the ability of each sample to aggregate washed rabbit platelets in an aggregometer. The results are expressed as fmol/mL urine, calculated from a calibration curve established for C_{16}PAF. Pyuria was defined as urine containing more than or equal to 300 leukocytes/μL.

In conclusion, we found PAF in human pyuria and noted a close correlation between the amount of PAF and the number of neutrophils present. The occurrence of PAF appears to be associated with the accumulation of neutrophils in urine.

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REVIEW

Significance of Platelet-Activating Factor in Mesenteric Ischemia-Reperfusion[1]

János Filep[a,*], Pierre Braquet[b] and Tibor Mózes[c]
Departments of [a]Pathophysiology and [c]Traumatology, Semmelweis University Medical School, 1445 Budapest, Hungary, and [b]Institut Henri Beaufour, 92350 Le Plessis Robinson, France

Reperfusion of the ischemic mesenterium is frequently followed by acute circulatory collapse. This review focuses on the possible role of platelet-activating factor (PAF) in ischemia-induced damage. It provides evidence that (i) PAF concentrations are elevated in the mesenteric circulation following temporary ischemia; (ii) administration of exogenous PAF into the superior mesenteric vein mimics many events observed during reperfusion; and (iii) pretreatment of the experimental animals with specific PAF receptor antagonists prevent the circulatory collapse. These findings suggest that PAF may play an important role in the development of circulatory collapse caused by mesenteric ischemia-reperfusion.
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Reperfusion of an ischemic organ salvages some tissues that would have died if blood flow had not been restored. However, it results in prolonged but ultimately reversible dysfunction of the reperfused organ and often leads to acute circulatory collapse. Thus, reperfusion is a double-edged sword. During ischemia and reperfusion, various types of cells, including neutrophil granulocytes (1-3) and platelets (4), are activated. These cells are sources of numerous mediators, like free oxygen radicals (5,6), histamine (7), prostanoids (8-10), leukotrienes (9) and platelet-activating factor (PAF) (11). These mediators are thought to be responsible for the reperfusion injury and for the cardiovascular changes.

Since its identification in the early 1970's (12,13), PAF has emerged as a potential mediator of diverse pathological processes, such as airway hyperreactivity, shock, cardiac and systemic anaphylaxis, gastrointestinal mucosal damage and a variety of inflammatory conditions (11,14, 15). Evidence is accumulating which supports a role for PAF in ischemia-induced damage in various organs (16). PAF meets all of the major criteria required to define mediators of ischemia and shock: (i) it occurs in critical sites (i.e., in tissues or biological fluids of experimental animals) during the critical time; (ii) administration of exogenous PAF can mimic many events observed following ischemia; and (iii) PAF receptor antagonists protect against the reperfusion injury. This paper compiles available evidence on the possible role of PAF in the pathophysiology of mesenteric ischemia-reperfusion and analyzes the potential interactions of PAF and prostanoids.

REPERFUSION OF THE ISCHEMIC MESENTERIUM

Reperfusion of the ischemic mesenterium is frequently followed by an acute circulatory collapse (17). The hemodynamical changes may be reversible (i.e., correction of the impaired perfusion leads to restoration of systemic and organ functions) or irreversible (where uncorrectable changes have been produced during the ischemic state), depending on the duration of ischemia. One-hour occlusion of the superior mesenteric artery (SMA) in dogs resulted in reversible changes, while 2-hour occlusion evoked irreversible changes (18). Restoration of blood flow following 2-hour ischemia resulted in an immediate decrease in mean arterial blood pressure (MABP), and an increase in mean portal venous pressure (MPVP) and SMA blood flow (Fig. 1). The maximum reductions or elevations, respectively, were observed within 5-7 min. Then SMA blood flow returned to control levels, whereas MABP stabilized at low levels and MPVP remained elevated during the entire period of observation. Hematocrit values rose more than 30% during the first 5 min of reperfusion, and thereafter remained unchanged (19, 20). These changes were accompanied by a significant

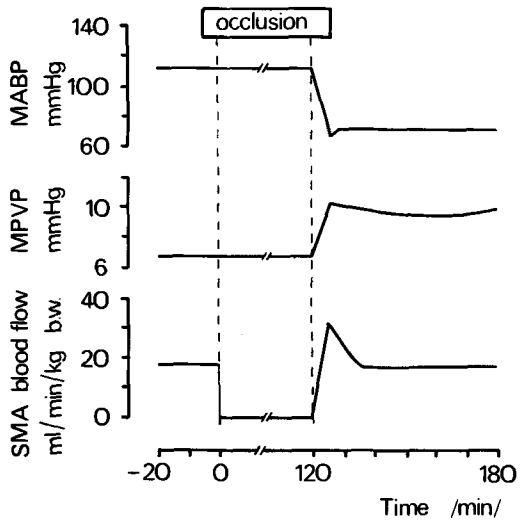


FIG. 1. Representative traces for the time course of changes in mean arterial blood pressure (MABP), mean portal venous pressure (MPVP) and superior mesenteric artery (SMA) blood flow in anesthetized dogs subjected to 2-hour occlusion of the superior mesenteric artery.

[1]Based on a paper presented at the Third International Conference on Platelet-Activating Factor and Structurally Related Alkyl Ether Lipids, Tokyo, Japan, May 1989.

*To whom correspondence should be addressed at the Department of Pharmacology, Faculty of Medicine, University of Sherbrooke, Sherbrooke, Quebec J1H 5N4, Canada.

Abbreviations: MABP, mean arterial blood pressure; MPVP, mean portal venous pressure; PAF, platelet-activating factor; SMA, superior mesenteric artery.

decrease in the number of circulating platelets and serum calcium levels (20). Serum potassium and uric acid levels were elevated at 5 min of reperfusion, while their levels returned to control values at 60 min of reperfusion (20). Concentrations of $PGF_{2\alpha}$, TXB_2 and 6-keto $PGF_{1\alpha}$ in the superior mesenteric vein were two- to six-fold higher than control levels after 5 min of reperfusion (18). Then plasma levels of these prostanoids tended to decrease, but their concentrations remained above their preocclusion values even after 60 min of reperfusion (18). Ischemia-reperfusion increased the mucosal permeability of terminal ileum of rats, released lysosomal enzymes from the mucosa to the lumen (21) and resulted in neutrophil invasion of the intestinal wall (3,22).

OCCURRENCE OF PAF DURING ISCHEMIA-REPERFUSION

To accept PAF as a mediator of ischemia-reperfusion, either it or its metabolite should be present during the disease process itself. Since PAF radioimmunoassay became available only very recently, the measurement of PAF was based on bioassays (aggregation of rabbit platelets or release of labeled serotonin from preloaded platelets). Measurement of PAF in blood is further complicated by the fact that it is rapidly hydrolyzed to biologically inactive lysoPAF (11,14).

To date, only a few reports document the presence of PAF in organs or in circulation during shock and ischemic states. Elevated plasma levels of PAF were found in *E. coli* endotoxemia (23) and in gram-negative bacterial sepsis (24). Enhanced PAF production has also been reported in glomeruli isolated from rats subjected to 60 min renal ischemia (25). Recently, we measured PAF in the mesenteric vein of the dog during reperfusion (19). PAF was extracted from blood, purified and quantitated by a bioassay system (aggregation of rabbit platelets). PAF concentrations in the mesenteric vein were found to be about thirteen-fold and four-fold higher than the preocclusion values at 5 and 60 min of reperfusion, respectively (Fig. 2). Similarly enhanced PAF release was reported during myocardial ischemia-reperfusion in rabbits (26). It is worth mentioning that low amounts of PAF were detected in the blood of healthy volunteers (27) and experimental animals (19,20,28). Whether these low concentrations of PAF represent its basal level or indicate its generation during blood collection and extraction procedure is not clear. The biological significance of PAF, if present in blood under control conditions, remains to be determined.

EFFECTS OF EXOGENOUS PAF

Systemic administration of exogenous PAF to experimental animals produced severe hypotension, shock and even death (29,30). The shock syndrome involved altered pulmonary (31) and coronary circulation (32), increased vasopermeability and hemoconcentration (33) and activation of various cells (11,14,15). PAF was also shown to increase formation of thromboxane A_2 (32,34,35) and leukotrienes (35,36), suggesting that its effects may be partly mediated by eicosanoids.

Local administration of PAF can mimic many events characteristic for ischemia-reperfusion in brain (37), heart (35) and intestine (21). Administration of PAF at a dose of 0.1 μg/kg body weight into the superior mesenteric vein of the dog evoked similar changes in MABP, MPVP (Fig. 3), hematocrit values and platelet count as those observed during reperfusion (19,20). On the contrary,

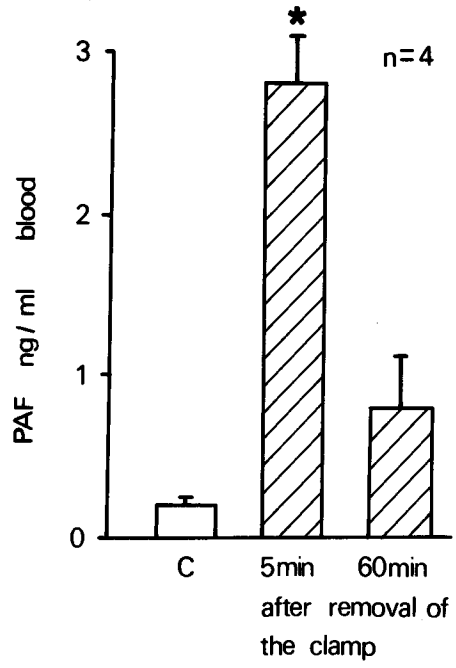


FIG. 2. PAF concentrations in the dog superior mesenteric vein during reperfusion. Blood was collected (a) 10 min before occlusion of the superior mesenteric artery (c, control); (b) at 5 min of reperfusion, when maximal hemodynamical changes were observed; and (c) at 60 min of reperfusion, when hemodynamical parameters had stabilized. PAF was extracted, purified and measured by using the rabbit platelet aggregation method. Identity of the PAF-bioactivity recovered from blood with authentic PAF was ascertained by thin-layer chromatography, high performance liquid chromatography and alkaline treatment (19,20).

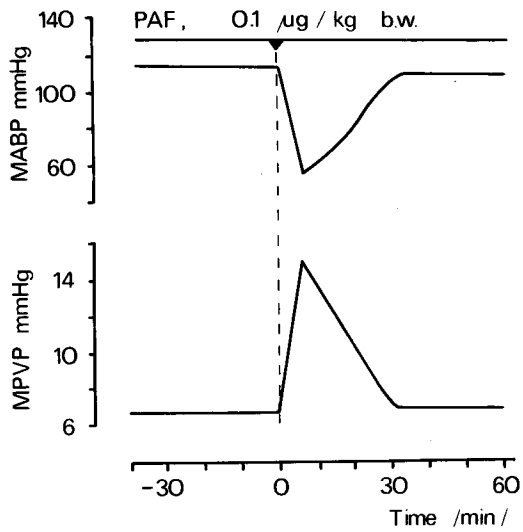


FIG. 3. Effects of exogenous PAF on mean arterial blood pressure (MABP) and mean portal venous pressure (MPVP) in anesthetized dogs. PAF, 0.1 μg/kg b.w. was injected into the superior mesenteric vein.

PAF did not effect serum uric acid, potassium and calcium levels (20). Since this dose of PAF was injected over a period of 1 min, and blood flow in the superior mesenteric vein was about 14–20 mL/min/kg body weight, peak concentrations of PAF in the mesenteric blood were calculated to be 5–7 ng/mL. These values are of the same order of magnitude as those measured at 5 min reperfusion (19,20) suggesting that the amount of PAF formed during reperfusion may be sufficient to evoke the observed changes.

PHARMACOLOGIC BLOCKADE OF PAF IN ISCHEMIA-REPERFUSION

Since the early 1980's, a group of specific PAF receptor antagonists have become available (38) that can be used to study the effects of PAF under various pathological conditions. There is accumulating evidence that the PAF receptor antagonists (e.g., BN 52021, kadsurenone, CV-6209) protected against reperfusion injury in brain (39–41), kidney (42) and heart (16,43,44). In mesenteric ischemia-reperfusion-induced shock, BN 52021, a competitive PAF receptor antagonist (45,46), prevented hypotension (19,20), hemoconcentration (19,20), activation of platelets (20), decrease in serum calcium concentration (20) and increases in serum potassium, uric acid (20), $PGF_{2\alpha}$, TXB_2 and 6-keto-$PGF_{1\alpha}$ levels (Filep *et al.*, unpublished observations). Furthermore, BN 52021 significantly reduced the increased intestinal permeability following ischemia (21) and attenuated the release of lysosomal enzymes from the mucosa to the lumen (21). Various PAF antagonists were found to exert a protective effect against mucosal damage similar to that observed with allopurinol, an inhibitor of xanthine oxidase in post-ischemic gastric lesion (22). However, BN 52021 had no effect on intestinal neutrophil accumulation following ischemia (21). Furthermore, it failed to modify the duration and the magnitude of reactive hyperemia in the mesenterium (20). This latter finding is consistent with the observation that BN 52021 has no effect on reactive hyperemia in gerbil brain (39).

SUMMARY AND CONCLUSIONS

Sufficient evidence has accumulated which suggest that PAF is an important mediator of reperfusion injury. Three major lines of observations support its role in mesenteric ischemia-reperfusion. PAF can be detected in mesenteric blood at concentrations sufficiently high enough to be responsible for many of the events observed during reperfusion. Secondly, exogenous PAF injected into the mesenteric circulation is able to produce acute circulatory collapse. Thirdly, pharmacological studies have shown that specific PAF receptor antagonists are capable of modifying the course of ischemia-reperfusion-induced shock. While any of these respective observations by themselves provide sufficient proof for a cause-effect relationship, the data taken together strongly suggest that PAF may play an important role in the early phase of ischemia-reperfusion. Figure 4 represents a simplified schematic diagram of the current understanding of PAF action during reperfusion. The cellular source(s) of the PAF released during ischemia-reperfusion is still unclear. Reperfusion injury is characterized by endothelial injury

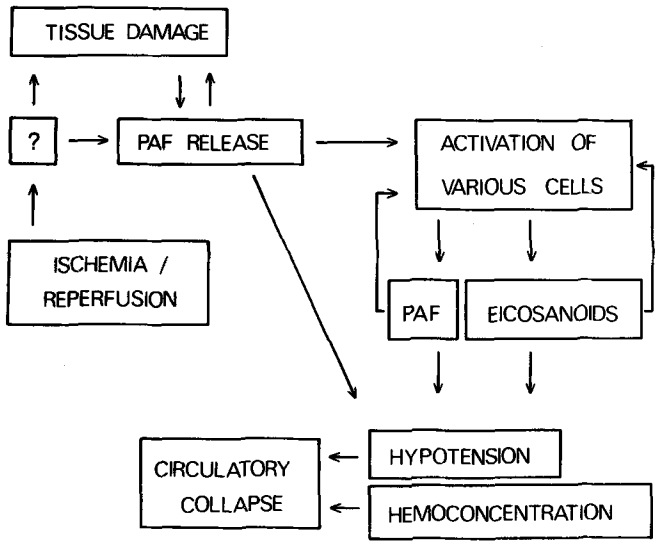


FIG. 4. Schematic presentation of the possible role of PAF in the early stage of mesenteric ischemia-reperfusion-induced shock. Reperfusion of the ischemic mesenterium leads to tissue damage and PAF release. The initial injury signal (questionmark) is still unknown. PAF released by either of the blood borne cells or by damaged tissues may cause further tissue destruction and activation of various cells. Stimulated cells, in turn, can release a wide range of vasoactive mediators, including PAF and eicosanoids. By activating the same and other cells, these lipid mediators can amplify the injury signal. Release of PAF and eicosanoids may lead to hypotension, hemoconcentration and to acute circulatory collapse.

(16) and ischemic tissues are infiltrated with different blood cells (3,16,22). All of these cells are capable of synthesizing PAF (11,14,15). Consequently, one or more of them may be responsible for the enhanced PAF concentrations in blood. Once released, PAF may exert a wide range of biological activities, including hypotension (29,30), hemoconcentration (33) and activation of various cells (11,14,15), which, in turn, release other vasoactive mediators, such as eicosanoids and oxygen radicals (6,32,34–36). Furthermore, PAF possesses the unique property of being able to induce its own synthesis *in vivo* (47,48), thus representing a self-generating, positive feedback system. However, one should keep in mind that none of the shock states are crucially dependent on any one class of lipid mediators. Indeed, PAF appears to play a minor (if any) role in reactive hyperemia (20,39), neutrophil infiltration (21) and tissue platelet accumulation (40). It remains to be established if the tissue damage is the cause or a consequence of PAF release. Although the signal that initiates reperfusion injury is not known, the data reviewed here suggest that PAF may serve as an early mediator to amplify the injury signal. As our understanding of the mediators and their complex interactions grows, the sequence of various events can be defined more precisely. The scheme may help to explain how the blockade of PAF can be an effective therapeutic modality in a complex multi-mediator system such as mesenteric ischemia-reperfusion-induced shock. Finally, more extensive experimental and clinical studies are needed to reveal whether the proposed role for PAF is correct and whether the PAF receptor antagonists developed so far have a therapeutic potential.

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REVIEW

Hypoxia, PAF, and Necrotizing Enterocolitis[1]

Michael S. Caplan[2], Xiao-Ming Sun and Wei Hsueh*
Departments of Pediatrics and Pathology, Children's Memorial Hospital, Northwestern University Medical School, Chicago, Illinois 60614

Necrotizing enterocolitis (NEC) is an important neonatal disease with a high mortality rate. The pathophysiology is unclear but epidemiologic studies suggest that hypoxia and infection are important risk factors. In this review we discuss the effect of hypoxia and platelet-activating factor (PAF) on intestinal blood flow and intestinal necrosis, and implicate PAF as an important mediator in hypoxia-induced intestinal injury. Finally we provide evidence that PAF may be important in neonatal NEC. ***Lipids 26,*** **1340–1343 (1991).**

Necrotizing enterocolitis (NEC) is a neonatal disease that affects 1–5% of all neonates admitted to the intensive care nursery, and up to 12% of premature infants weighing less than 1500 grams (1). Despite intensive management, the morbidity and mortality rates remain distressingly high. Extensive investigations have failed to clarify the etiology of NEC; however, many risk factors have been implicated. These include prematurity (2), formula feeding (3), bacterial infection (4), hypoxia (5), and intestinal ischemia (6). In addition, experimental animal models have been developed to investigate the pathogenesis of ischemic bowel disease. These experimental models have supported the epidemiologic evidence implicating hypoxia and infection as risk factors for NEC but have failed to define the etiology. In this review, we will discuss the role of hypoxia on bowel necrosis, describe the association of platelet-activating factor (PAF) and intestinal injury, and summarize the possible role of PAF as a mediator for hypoxia-induced intestinal injury. Finally we will review the association of PAF in human, neonatal NEC.

THE ROLE OF HYPOXIA IN BOWEL NECROSIS

Hypoxia is regarded as an important risk factor for neonatal NEC (7). In animal models, hypoxia and/or asphyxia result in decreased gastrointestinal blood flow (8) which presumably leads to intestinal injury. Using newborn piglets, Nowicki *et al.* (9) have shown that a reduction in pO_2 from 85 to 18 mm Hg resulted in marked reductions of gastrointestinal blood flow. Karna *et al.* (10) demonstrated significant reductions in intestinal perfusion following alterations in respiratory function. It is of interest, that hypoxia was more deleterious than asphyxia or acidosis on intestinal blood flow and O_2 consumption. Although these studies suggest that hypoxia results in intestinal vasoconstriction, they did not demonstrate the development of intestinal necrosis.

In other animal models, intestinal injury has been induced by hypoxia and/or asphyxia (11). In the newborn rat pup, repeated asphyxial insult resulted in severe intestinal injury (12). Similarly, in the neonatal piglet, asphyxia for 2 hr produced decreased intestinal blood flow with early changes of intestinal necrosis (13). In the neonatal dog, pure hypoxia (10% O_2 for 2 hr) caused intestinal injury when specimens were evaluated 24 hr later (14). Others have shown mild intestinal injury followed hypoxia alone without asphyxia or acidosis (15). Although these reports confirm that hypoxia can lead to intestinal ischemia and focal intestinal necrosis, they do not describe the mechanism of the hypoxia-induced bowel injury.

THE ROLE OF PAF IN ISCHEMIC BOWEL NECROSIS

Platelet-activating factor (PAF, PAF-acether) is an endogenous phospholipid mediator with potent biological effects. In various animal models, PAF infusions have resulted in shock, bronchoconstriction, capillary leakage, neutrophil aggregation and degranulation, platelet aggregation and degranulation, and pulmonary hypertension (16,17). We have shown that intravascular injection of PAF in the rat produces ischemic bowel necrosis morphologically identical to neonatal NEC (18). In addition, low dose PAF and low dose bacterial endotoxin (LPS) synergistically induce bowel necrosis that can be prevented by pretreatment with PAF receptor antagonists (19). Further, we have shown that LPS injection in the rat causes increased intestinal PAF production which leads to bowel necrosis (20). In this model, we have shown that leukotriene C_4 (21), norepinephrine (22), oxygen radicals (23), and activated complement fragments (24) all may play a role in PAF-induced intestinal injury. Finally, we have shown that tumor necrosis factor-alpha (TNF) induced intestinal injury that was predominantly mediated by PAF (25).

Other investigators have studied the importance of PAF in gastrointestinal blood flow and injury. In the isolated, perfused rat stomach, PAF induced a dose-dependent reduction in gastric vascular flow which was effectively reversed with PAF receptor blockade (26). Furthermore, intravenous PAF administration in the rat produced extensive hemorrhagic erosions in the gastric mucosa (27). PAF-induced gastric ulceration appears to involve lipid peroxidation (28), oxygen free radicals (29), and increased gastric vascular permeability (30). Finally, in endotoxin-induced gastrointestinal damage, PAF was shown to be an important mediator of hypotension, plasma leakage, and intestinal ulceration (31). Similar to our findings, LPS-induced gastrointestinal lesions were prevented by PAF receptor antagonists (31,32). These studies all point to the importance of PAF in gastrointestinal injury.

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[2]Current Address: Department of Pediatrics, Evanston Hospital, 2650 Ridge Avenue, Evanston, IL 60201.
*To whom correspondence should be addressed.

Abbreviations: ABG, arterial blood gas; BE, base excess; LPS, bacterial endotoxin (lipopolysaccharide); NEC, necrotizing enterocolitis; PAF, platelet-activating factor; TNF, tumor necrosis factor; WBC, white blood cells.

THE ROLE OF HYPOXIA AND PAF IN INTESTINAL NECROSIS

Although hypoxia can result in intestinal ischemia and necrosis, the pathophysiology is not well understood. It is unclear whether hypoxia causes intestinal vasoconstriction directly or whether this process is mediated by compounds released following hypoxic exposure. In the rat model, hypoxia resulted in decreased gastric prostaglandin production (33). Recent evidence suggests that hypoxia leads to increased endothelial cell permeability and capillary leak, which are changes present in ischemic bowel necrosis (34,35).

It has also been shown that hypoxia results in increased PAF concentrations in rat bronchoalveolar lavage (36). Furthermore, hypoxia-induced increase in PAF concentrations appears to result from increased activity of phospholipase A_2, a key enzyme involved in the formation of precursor for PAF synthesis (37). Finally, in the ischemia-reperfusion model in the mesenteric circulation of the dog, systemic plasma PAF levels are increased (38). These findings suggest that hypoxia may increase PAF locally and/or in the systemic circulation. Since the association of PAF-induced intestinal injury is well known, we studied the role of PAF in hypoxia-induced intestinal ischemia and necrosis.

As seen in Table 1, acute hypoxia was accomplished following exposure to 100% N_2 for two minutes (mean ABG: pH/pCO_2/pO_2/base excess: 7.27/57/18/−2); sustained, moderate hypoxia (10% O_2) for thirty minutes resulted in hypoxia and acidosis (39). Plasma PAF levels were markedly elevated following only two minutes of severe, acute hypoxia (100% N_2, PAF level: 13.8 ± 2.9 ng/mL *vs* control: 2.1 ± 0.8 ng/mL, $p<0.01$, Fig. 1). Even with challenge of moderate hypoxia (10% O_2), a significant elevation was observed within 30 min. (41.0 ± 11.7 ng/mL, $p<0.01$, Fig. 1). The observed elevation of PAF in this model is much more rapid than what was reported by Prevost *et al.* (36) who showed that in rats following four hours of hypoxia the bronchoalveolar lavage fluid contained increased PAF concentrations.

The intestinal perfusion, as measured by the Evans blue dye method, was abnormal in all animals (n = 14) treated with hypoxia for 30 min. The mean percent bowel perfused was 40 ± 7% for the hypoxic animals, and ranged from 0 to 95% (Fig. 2). In contrast, all control animals showed normal (100%) intestinal perfusion. Pretreatment with the PAF receptor antagonists, SRI 63-441 (40) and WEB 2086 (41) (data not shown) prior to the hypoxic exposure, completely prevented the hypoxia-induced intestinal hypoperfusion (Fig. 2). This observation strongly suggests that PAF is an important mediator of hypoxia-induced intestinal vasoconstriction and hypoperfusion. Although hypoxia leading to intestinal ischemia is well known, to our knowledge these data are the first to suggest PAF as the important mediator.

We have shown that following 30 min of hypoxia there were early changes of intestinal necrosis, in addition to intestinal hypoperfusion (39). Histological examination of intestine from animals subjected to hypoxia alone revealed 6 of 14 animals with mild or moderate intestinal injury (Fig. 3, top), and 3 of 14 with minimal intestinal necrosis (Table 2). In contrast, microscopic examination of specimens from animals treated with two structurally different PAF antagonists, SRI 63-441 and WEB 2086, showed complete prevention of hypoxia-induced bowel injury (Fig. 3, bottom): none of the rats showed any necrotic changes except for one animal with focal, minimal injury (see legend, Table 2 for definition of microscopic intestinal

TABLE 1

Effect of Hypoxia on Arterial Blood Gas (pH/pCO_2/pO_2/base excess)

Treatment	Time of exposure	Number of animals	ABG (mean)
Air		5	7.34/43/96/−2
100% N_2	2 min	7	7.27/57/18/−2
10% O_2	15 min	5	6.99/95/23/−12
10% O_2	30 min	10	6.80/101/40/−22

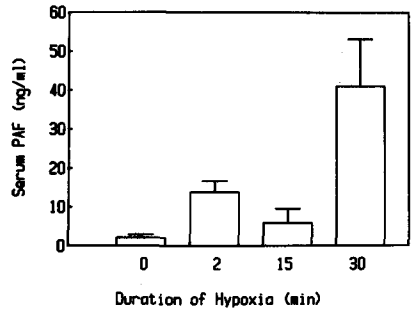


FIG. 1. The effect of hypoxia on serum PAF levels. The animals (male Sprague-Dawley rats, 60–90 g) were divided into four groups. The bars, from left to right, represent, rats breathing air (controls, n = 5); 100% N_2 for 2 min (severe hypoxia, n = 7); a mixture of 10% O_2, 15% CO_2, and 75% N_2 (moderate hypoxia) for 15 min (n = 5); the same gas mixture for 30 min (n = 10). Plasma PAF was extracted by the Folch method, purified by thin-layer chromatography, and quantitated with a rabbit platelet serotonin release assay. [^{3}H]PAF (10,000 cpm) was added at the start of each experiment to calculate extraction efficiency. Data are expressed as the mean ± SEM $P<0.05$ at 2 min, and $p<0.01$ at 30 min using analysis of variance with the Scheffe method to identify specific group differences.

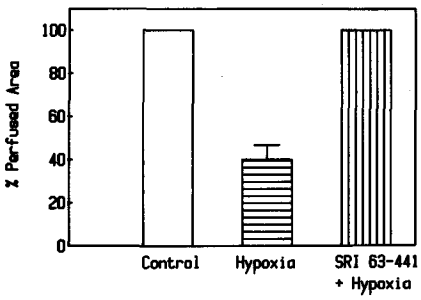


FIG. 2. Effect of hypoxia and PAF antagonist SRI 63-441 on intestinal perfusion. Intestinal perfusion was assessed after 30 min of hypoxia (n = 14) by the Evans blue dye method as described (39). Some animals were treated with the PAF receptor antagonist, SRI 63-441 (2.5 mg/kg i.v., Sandoz Research Institute, New Hanover, NJ), prior to hypoxic exposure (n = 5). The length of bowel not stained with Evans blue (hypoperfused) was measured and compared to the total intestinal length to calculate percent of perfused intestine (mean ± SEM). $P<0.01$ for hypoxia *vs* SRI pretreated groups.

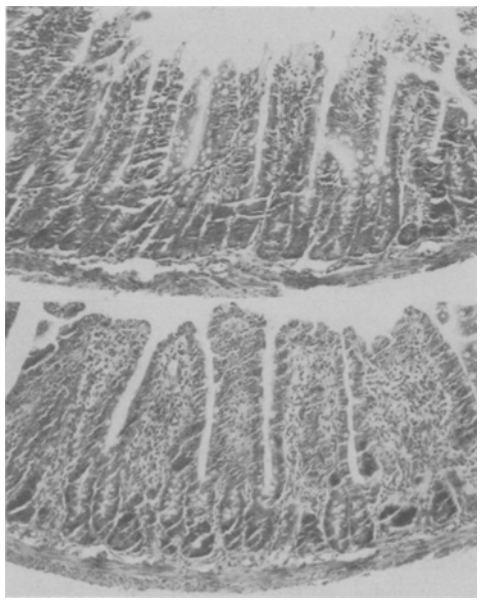


FIG. 3. Microscopic specimen from a rat. Top: treated with hypoxia (10% O_2) for 30 min. Bottom: pretreated with PAF receptor antagonist, SRI 63-441, 10 min prior to hypoxic exposure.

TABLE 2

Hypoxia-Induced Intestinal Injury and Its Prevention by PAF Antagonists

Treatment	Number of animals	Intestinal Injury[a]			
		None	Minimal	Mild	Moderate
Hypoxia 30 min	14	5	3	5	1
SRI 63-441 + Hypoxia	5	4	1	0	0
WEB 2086 + Hypoxia	5	5	0	0	0

[a]Intestinal injury was graded by microscopic examination. Sections were taken from the non-perfused areas of the small intestine; pathological changes of the most severe lesions are presented in the table (in animals pretreated with PAF antagonist in which no hypoperfused bowel was observed, random sections were taken for histological examination). Microscopic lesions were graded by one of the authors unaware of the codes. Minimal: Separation of the surface epithelial cells from lamina propria. Mild: Epithelial cell necrosis confined to the tips of villi. Moderate: Partial loss of intestinal villi. None of the animals showed necrosis beyond the mucosal layer. $P<0.05$ for antagonist *versus* hypoxia alone.

necrosis). Thus, PAF is probably an important mediator responsible for hypoxia-induced bowel injury. Interestingly, acidosis alone also increased PAF production, but failed to induce bowel necrosis (39). Thus it is probable that PAF and tissue hypoxia synergize in inducing tissue injury.

Acetylhydrolase is a PAF-specific enzyme that degrades PAF into biologically inert lysoPAF (42). This enzyme is found in most tissues, as well as in human serum (43). We found that serum acetylhydrolase activity was not significantly different between control and hypoxia-exposed rats at any time of blood sampling. However, intestinal acetylhydrolase activity increased following thirty minutes of hypoxia (3.8 ± 1.1 μmol/mL/min control *vs* 5.4 ± 0.6 μmol/mL/min hypoxia, $p<0.05$). Thus the acute rise in PAF must be a result of increased PAF production and not decreased degradation. This hypothesis is in accordance with the observation that hypoxia increases phospholipase A_2 activity, a key enzyme in the synthesis of PAF precursor (37).

In summary, severe hypoxia can rapidly (within two minutes) increase plasma PAF levels in the rat. In addition, following 30 min of moderate hypoxic challenge, intestinal hypoperfusion and mild ischemic bowel necrosis occurs, and can be prevented by PAF receptor antagonists. Although others have described the relationship of hypoxia inducing intestinal ischemia, hypoperfusion, and necrosis, these findings suggest that the changes are mediated by PAF.

PAF IN HUMAN NECROTIZING ENTEROCOLITIS

Although PAF is an important mediator of ischemic bowel necrosis in animal models, it is critical to investigate the role of PAF in human neonatal NEC. We have evaluated plasma PAF, plasma acetylhydrolase activity, and plasma TNF in 12 neonates with classic NEC and compared the levels to age-matched controls (44). Our findings showed that patients with NEC have higher plasma PAF (18.1 ± 3.6 ng/mL *vs* 3.1 ± 0.9 ng/mL control, $p<0.01$), lower acetylhydrolase activity (10.6 ± 0.7 nmol/mL/min *vs* 23.0 ± 1.4 nmol/mL/min control, $p<0.01$), and increased TNF (136 ± 75 U/mL *vs* 1.5 ± 0.8 U/mL control, $p<0.01$) compared to control infants. In these NEC patients, the individual plasma PAF concentrations did not correlate with plasma TNF levels. This pilot study shows that in human NEC, alterations in systemic PAF metabolism and TNF activity occur. The findings support the hypothesis derived from animal models, that PAF is a mediator in bowel necrosis. However, increased systemic PAF levels in neonates with NEC does not prove a cause and effect relationship. Further investigation of PAF in neonatal NEC is warranted. If follow-up studies are consistent with previous human and experimental reports describing the importance of PAF in ischemic bowel necrosis, then controlled trials of PAF receptor antagonists in this devastating disease would be indicated.

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Antagonism of Platelet-Activating Factor in Isolated Rat Colon: Possible Mechanism[1]

Akira Tokumura*, Nobuyuki Yube, Motonori Terao and Hiroaki Tsukatani
Faculty of Pharmaceutical Sciences, The University of Tokushima, Tokushima 770, Japan

The contractions of three different regions of rat colon in response to platelet-activating factor (PAF) were compared. The ascending colon was found to be the most responsive. The slow contraction of the ascending colon induced by PAF was dependent on external Ca^{2+}. CV-3988, a structural analog of PAF, slowly induced irreversible inhibition of PAF-induced contraction, whereas FR-900452, which is structurally unrelated to PAF, caused rapid reversible inhibition of PAF-induced contraction. No inhibitory effects of CV-3988 were observed when the strip was washed with Tyrode's solution containing 1% bovine serum albumin (BSA). The results suggest that PAF and CV-3988 penetrate slowly into the outer half of the lipid bilayer of plasma membranes of cells in isolated rat colon, and then rapidly diffuse laterally to associate firmly with specific binding sites.
Lipids 26, 1344-1346 (1991).

Platelet-activating factor (PAF, 1-*O*-alkyl-2-acetyl-*sn*-glycero-3-phosphocholine) has been implicated as a mediator of allergic and inflammatory processes (1,2). PAF has also been shown to cause contraction of smooth muscle of the gastrointestinal tract (3-6), lung (4,7-9) and uterus (10). However, the mechanism by which PAF induces smooth-muscle contraction is not fully understood. One problem in studies on this subject is the rapid tachyphylaxis of the isolated strips caused by PAF.

We previously developed a new method (11) for studies on isolated rat colon, the region most sensitive to PAF in the rat intestinal tract (5), by which tachyphylaxis was avoided. The binding of PAF to its putative sites seems to be very tight, because the contraction induced by this phospholipid agonist was not changed by repeated washings of the colon with Tyrode's solution (11). However, no contractions were observed when PAF bound to the specific binding sites was removed by treatment with bovine serum albumin (BSA). In the present study we further investigated the persistent contractile action of PAF on rat colon, using two different types of PAF antagonist.

MATERIALS AND METHODS

Materials. 1-*O*-Hexadecyl-2-acetyl-*sn*-glycero-3-phosphocholine (synthetic PAF) was purchased from Bachem Feinchemikalien AG (Bubendorf, Switzerland). CV-3988, *i.e. rac*-3-(*N*-octadecylcarbamoyloxy)-2-methoxypropyl-2-thiazolioethyl phosphate, was kindly provided by Dr. S. Tsushima Takeda Chemical Industries (Osaka, Japan). FR-900452, *i.e.* 1-methyl-3-(1-[5-methylthiomethyl-6-oxo-3-(2-oxo-3-cyclopenten-1-ylidine)-2-piperazinyl]ethyl)-2-indolinone, was a generous gift of Dr. M. Okamoto at Fujisawa Pharmaceutical Co. (Ibaraki, Japan). The other chemicals used in this study were as follows: acetylcholine hydrochloride (Daiichi Pharmaceutical Co., Tokyo, Japan), ethyleneglycol *bis*(β-aminoethylether)-*N,N,N',N'*-tetraacetic acid (EGTA; Nacalai Tesque, Kyoto, Japan), and fatty acid-free bovine serum albumin (BSA; Sigma Chemical Co., St. Louis, MO).

Measurement of contraction of rat colon. Male Wistar rats (200-300 g) were killed by a blow on the head, and colons were removed. Strips of colon (1.5-2 cm in length) were mounted in a 10-mL organ bath containing aerated Tyrode's solution (pH 7.8) of the following composition (mM): NaCl 136.9, KCl 2.7, $CaCl_2$ 2.5, $MgCl_2$ 1.0, $NaHCO_3$ 11.9, NaH_2PO_4 0.4, and glucose 5.5. After equilibration for 1 hr at 37°C, the colon was washed with Tyrode's solution containing 1% BSA, and remounted in Tyrode's solution with no BSA. After confirmation of constant responses of the strips to acetylcholine, they were exposed to PAF or PAF antagonist. The contractions produced by PAF were measured isotonically on a kymograph under 1-g load, and expressed as percentages of the maximal contraction produced by 100 μM acetylcholine. Results are shown as means ± S.E.M. Statistical analysis was performed by Student's t-test, and a value of $P<0.05$ was considered significant.

PAF was dissolved in saline with 0.1% BSA at various concentrations and a 0.1-mL aliquot of the PAF solution was added to a 10-mL organ bath. The final concentration of BSA was 0.001%. CV-3988 and FR-900452 were dissolved in ethanol. The final concentration of ethanol did not exceed 0.2%. Other chemicals were dissolved in saline.

RESULTS AND DISCUSSION

Regional differences in responses of rat colon to PAF. First we compared contractile responses to PAF of the ascending, transverse and descending regions of rat colon. The contraction of the ascending region in response to 0.3 nM PAF was 51.7 ± 2.4% (n = 14) of the maximal contraction induced by 100 μM acetylcholine. This response to PAF was significantly greater ($P<0.001$) than that of the transverse region (4.6 ± 1.4%, n = 7) or of the descending region (4.2 ± 1.25%, n = 8). Furthermore, the maximal contraction of the ascending region induced by PAF was 84.3 ± 1.7% (n = 20) of that induced by acetylcholine, whereas the maximal contractions of the transverse and descending regions induced by PAF were only 8.4 ± 0.8 (n = 7) and 12.5 ± 3.9% (n = 8), respectively, of those induced by acetylcholine. Therefore, the isolated ascending region of the colon was used in all subsequent studies.

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*To whom correspondence should be addressed at Faculty of Pharmaceutical Sciences, The University of Tokushima, Shomachi, Tokushima 770, Japan.

Abbreviations: BSA, bovine serum albumin; CV-3988, *rac*-3-(*N*-octadecylcarbamoyloxy)-2-methoxypropyl-2-thiazolioethyl phosphate; EGTA, ethyleneglycol *bis*(β-aminoethylether)-*N,N,N',N'*-tetraacetic acid; FR-900452, 1-methyl-3-(1-[5-methylthiomethyl-6-oxo-3-(2-oxo-3-cyclopenten-1-ylidine)-2-piperazinyl]ethyl)-2-indolinone; PAF, platelet-activating factor.

Resistance of PAF-induced contraction of rat colon to washing. Previously, we reported that PAF-induced contraction of isolated rat colon persisted even after repeated washing of the strip with Tyrode's solution (11). One explanation for this observation may be that a substantial portion of the added PAF was adsorbed to the Magnus vessel used, diffused quickly into the fresh Tyrode's solution after replacement of the bath solution, and then was bound to its specific binding sites on the rat colon in the Magnus vessel. We examined this possibility. For this purpose, a rat colon strip was contracted half-maximally with PAF, and then restored to the basal tone by addition of 3 mM EGTA (Fig. 1, A) or 10 μM FR-900452 (data not shown). The strip was then transferred to another Magnus vessel with 10 mL of fresh Tyrode's solution containing 3 mM EGTA (Fig. 1, B) or 10 μM FR-900452 (data not shown). The bath solution in this second Magnus vessel was then replaced by fresh Tyrode's solution, resulting in rapid contraction (second contraction). When the bath solution was replaced with fresh Tyrode's solution, the time for half-contraction was 0.8 ± 0.2 min (n = 8) which was significantly ($P<0.01$) shorter than that for the first PAF-induced half-contraction (4.5 ± 1.1 min, n = 8). This cycle of relaxation of PAF-treated colon induced by EGTA followed by restoration of contraction by washing could be repeated at least twice in the second Magnus vessel without any significant reduction in the extent of contraction (Fig. 1, B). The fourth contraction was completely abolished when 3 μM FR-900452 was used, indicating that the contraction was due to PAF, which probably remained in the plasma membranes of the cells in the colon.

Effect of BSA on the inhibitory action of PAF antagonists. After the colon had been treated with 10 μM FR-900452 for 15 min, no increase in tone of the colon was seen upon subsequent treatment with 0.3 nM PAF. When the colon was subsequently washed with Tyrode's solution containing 1% BSA, it showed no increase in tone; however, when the colon was washed with Tyrode's solution without BSA, it showed spontaneous contraction (Fig. 2, A). After washing with Tyrode's solution containing BSA, the colon responded normally to a second addition of PAF (Fig. 2, A). These results indicated that FR-900452, but not PAF, was removed by washing the strip with Tyrode's solution, and so PAF was able to maintain its stimulatory effect on the strips.

Similar experiments were performed with 10 μM CV-3988 instead of FR-900452. When a set of strips was pretreated with CV-3988 for 20 min and then challenged with 0.3 μM PAF, they showed no significant contraction (Fig. 2, B). When one strip was then washed with Tyrode's solution, no spontaneous contraction was observed, unlike the results when FR-900452 was used. In addition, the strip did not respond to a second addition of 0.3 nM PAF. When the other strip was washed with Tyrode's solution containing BSA, no spontaneous contraction was observed, but the strip was able to contract in response to a second addition of 0.3 nM PAF, as shown in Fig. 2, B. These results indicate that the inhibitory action of CV-3988 was resistant to washing with Tyrode's solution alone.

The present results suggest tight binding of PAF and CV-3988, but not of FR-900452, to PAF binding sites on rat colon. As previously reported (11), FR-900452-induced-relaxation of colon pre-contracted by PAF was as fast as either EGTA-induced relaxation of the colon, but unlike CV-3988-induced relaxation. This may mean that FR-900452 rapidly replaces PAF molecules bound to their specific binding sites, resulting in rapid cessation of stimulus contraction coupling and relaxation of rat colon.

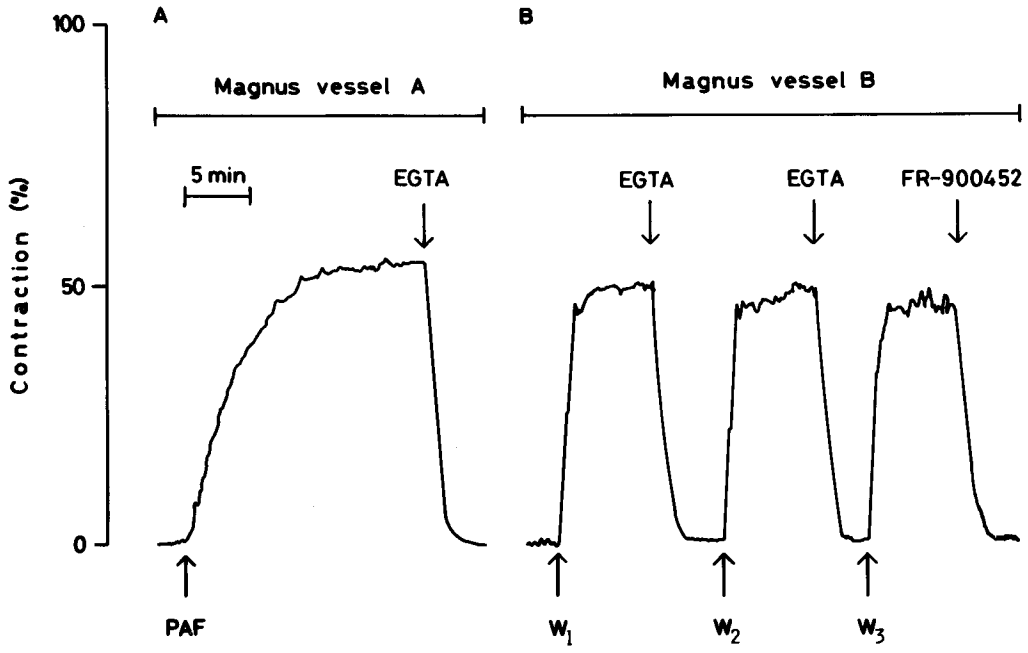


FIG. 1. Persistence of external Ca^{2+}-dependent contraction of rat colon induced by PAF after washing the colon with Tyrode's solution.

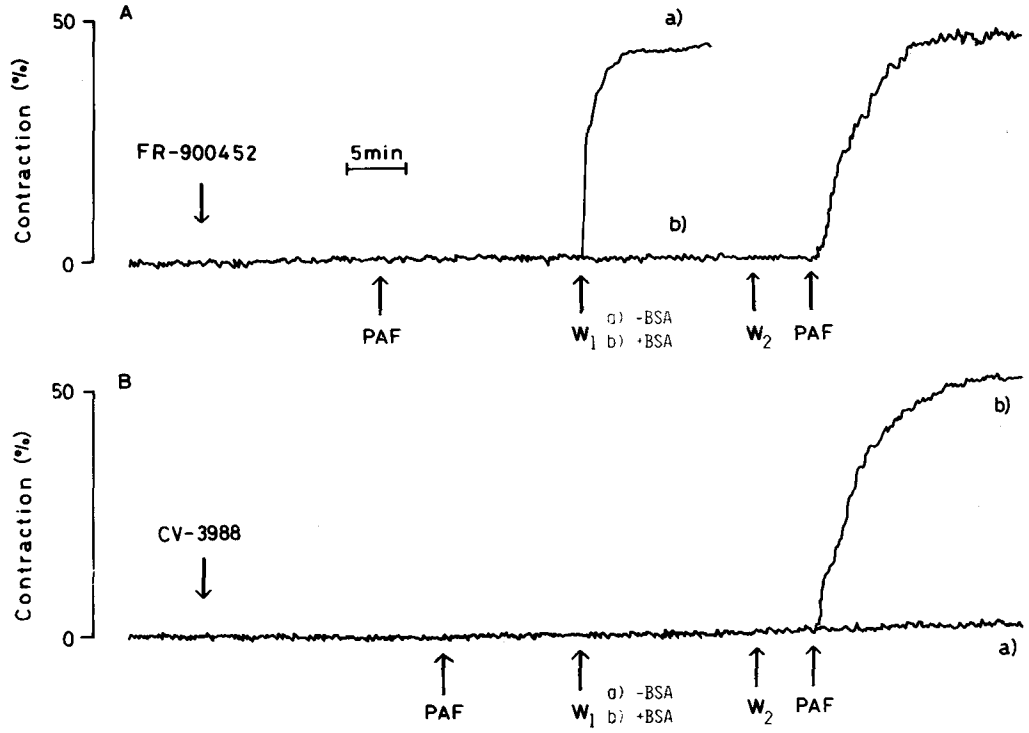


FIG. 2. Abolishment of the inhibitory action of FR-900452, but not of CV-3988, by washing rat colon with Tyrode's solution. A: Two strips of colon were pretreated with 10 μM FR-900452, and then challenged with 0.3 nM PAF. Strip a) was washed with Tyrode's solution (W_1). Strip b) was washed with Tyrode's solution containing 1% BSA (W_1), and then with Tyrode's solution with no BSA (W_2), and challenged with 0.3 nM PAF. B: Two strips of colon were pretreated with 10 μM CV-3988 and then challenged with 0.3 nM PAF. Strip a) was washed twice with Tyrode's solution (W_1 and W_2) and then rechallenged with 0.3 nM PAF. Strip b) was first washed with Tyrode's solution containing 1% BSA (W_1), and then with Tyrode's solution (W_2) to remove BSA. Strip b) was rechallenged with 0.3 nM PAF.

The binding sites for PAF are thought to be located near the interface between the plasma membrane and the extracellular fluid. Therefore, exogenously added PAF or its antagonists may be able to attach to binding sites either directly from the extracellular fluid (FR-900452) or after slow penetration into the outer half of the bilayer (PAF and CV-3988) followed by rapid lateral diffusion along the outer bilayer leaflet of the plasma membrane.

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Molecular Heterogeneity of Platelet-Activating Factor (PAF) in Rat Glandular Stomach Determined by Gas Chromatography/Mass Spectrometry. PAF Molecular Species Changes upon Water-Immersion Stress[1]

Junko Sugatani[a,*], Kazuyo Fujimura[b], Masao Miwa[c], Kiyoshi Satouchi[a] and Kunihiko Saito[a]
[a]Department of Medical Chemistry and [b]The Third Department of Internal Medicine, Kansai Medical School, Moriguchi, Osaka 570, and [c]Department of Biochemistry, School of Pharmaceutical Science, University of Shizuoka, Shizuoka 422, Japan

The molecular heterogeneity of 1-alkyl-2-acetyl-*sn*-glycero-3-phosphocholine (alkylacetyl-GPC) and 1-acyl-2-acetyl-*sn*-glycero-3-phosphocholine (acylacetyl-GPC) in normal rat glandular stomach was studied by gas chromatography/mass spectrometry (GC/MS) and tandem mass spectrometry. The percentage compositions of the molecular species of 1-alkyl-2-acetyl-GPC and 1-acyl-2-acetyl-GPC in the antrum were, respectively, 1-alkyl [16:0 (34%) and 18:0 (66%)]-2-acetyl-GPC and 1-acyl [16:0 (60%), 18:0 (14%) and 18:1 (26%)]-2-acetyl-GPC. The alkyl chain composition of 1-alkyl-2-acetyl-GPC was quite different from that of 1-alkyl-2-acyl-GPC in both the antrum and corpus, demonstrating a high degree of selectivity of alkyl chain utilization in PAF biosynthesis. The amount of 1-acyl-2-acetyl-GPC was much greater than that of 1-alkyl-2-acetyl-GPC. The molecular heterogeneity of 1-alkyl-2-acetyl-GPC and 1-acyl-2-acetyl-GPC in the corpus was similar to that in the antrum. Water-immersion stress affected not only the amount of 1-alkyl-2-acetyl-GPC and 1-acyl-2-acetyl-GPC, but also their molecular heterogeneity in the antrum and corpus. Whereas the amounts of 1-hexadecyl-2-acetyl-GPC and 1-acyl [16:0, 18:0 and 18:1]-2-acetyl-GPC decreased markedly (to less than one-fifth) in the antrum after such stress for 1 hr, the amount of 1-octadecyl-2-acetyl-GPC increased markedly (up to 4-fold) in the corpus and severe lesions were observed after stress for 7 hr. The changes may be associated with the pathogenicity of gastric ulcers.
***Lipids* 26, 1347–1353 (1991).**

Platelet-activating factor [PAF, 1-alkyl-2-acetyl-*sn*-glycero-3-phosphocholine (1)] is a chemical mediator with a wide spectrum of biological activities, *e.g.*, stimulation of platelets and neutrophils, and enhancement of vascular permeability and smooth muscle contraction (2). PAF is not only generated in a variety of cells upon chemical or immune stimulation associated with inflammation and anaphylaxis (2), but is also found in normal animal tissues [bovine brain (3), rat uterus (4) and fetal rabbit lung (5)]. In a previous study, we found a significant amount of PAF in normal stomach (especially in the antral mucosa), and observed an alteration in the level of PAF upon water-immersion stress, as determined by a bioassay based on the release of [^{3}H]serotonin from washed rabbit platelets (6). Over the last several years, data have been accumulating that suggest a molecular heterogeneity of PAF in cells (7–9) and tissues (3,4) which is dependent on the pathophysiological state. Evidence exists that the different molecular species of PAF homologs and analogs may exert different biological activities (1,10). In the present study, we determined the molecular species of rat gastric PAF homologs [1-alkyl-2-acetyl-*sn*-glycero-3-phosphocholine (alkylacetyl-GPC)] and analogs [1-acyl-2-acetyl-*sn*-glycero-3-phosphocholine (acylacetyl-GPC)], and compared the compositions of their alkyl and acyl chains with those of alkyl and acyl chains at the 1-position of 1-akyl-2-acyl-GPC and 1,2-diacyl-GPC in order to investigate the selectivity of alkyl and acyl chain utilization in PAF biosynthesis. The influence of water-immersion stress on the molecular heterogeneity of PAF was also elucidated by gas chromatography/mass spectrometry (GC/MS).

MATERIALS AND METHODS

Materials. Male Wistar rats were deprived of food, but not water, for 24 hr before the experiments. Animals were placed in a stress cage and immersed in water (23°C ± 1°C) to the level of xiphoid process, in accordance with the method of Takagi and Okabe (11). Control rats were placed in a cage under no restraint at 25°C ± 1°C. The rats were then sacrificed by decapitation under light diethyl ether anesthesia unless otherwise stated.

Chemicals. 1-Hexadecyl-GPC, 1-hexadecyl- and 1-octadecyl-2-acetyl-GPC and 1-hexadecanoyl-GPC were purchased from Bachem Feinchemikalien AG, Bubendorf, Switzerland. 1-Octadecenyl-2-acetyl-GPC was prepared from human polymorphonuclear leukocytes (12). 1-Octadec-*cis*-9′-enoyl- and 1-octadecanoyl-GPC were obtained from Serdary Research Laboratories Inc., Ontario, Canada. Perdeuterated acetic anhydride(d_6) was a product of E. Merck, Darmstadt, Germany. 1-Hexadecyl-2-perdeuteroacetyl-GPC (internal standard for 1-akyl-2-acetyl-GPC) and 1-hexadecanoyl-, 1-octadec-*cis*-9′-enoyl- and 1-octadecanoyl-2-acetyl-GPC were synthesized by acetylating 1-hexadecyl-GPC and 1-hexadecanoyl-, 1-octadec-*cis*-9′-enoyl- and 1-octadecanoyl-GPC with perdeuterated acetic anhy-

[1]Based on a paper presented at the Third International Conference on Platelet-Activating Factor and Structurally Related Alkyl Ether Lipids, Tokyo, Japan, May 1989.

*To whom correspondence should be addressed at Department of Medical Chemistry, Kansai Medical School, 1-Fumizonocho, Moriguchi, Osaka 570, Japan.

Abbreviations: GC/MS, gas chromatography/mass spectrometry; GPC, ***sn***-glycero-3-phosphocholine; PAF, platelet-activating factor; SIM, selected ion monitoring; *t*-BDMS, *tert*-butyldimethylsilyl; TLC, thin-layer chromotography; TNS, 6-*p*-toluidine-2-naphthalenesulfonic acid.

dride and acetic anhydride, respectively, in the presence of perchloric acid as a catalyst (13). 1-Heptadecanoyl-2-perdeuteroacetyl-GPC (internal standard for 1-acyl-2-acetyl-GPC) was synthesized as described previously (9). The *tert*-butyldimethylchlorosilane/imidazole reagent and all fatty acid methyl ester standards were obtained from Applied Science (State College, PA). Phospholipase C (EC 3.1.4.3) was partially purified from *Bacillus cereus* (14). Phospholipase A_2 (EC 3.1.1.4) from *Agkistrodon halys blomhoffi* was purified to homogeneity as reported previously (15).

Isolation of PAF, phosphatidylcholine and phosphatidylethanolamine from rat glandular stomach. The total lipids of antrum and corpus, respectively, were extracted by the method of Bligh and Dyer (16) at 4°C in the presence of 50 mM glycine-HCl buffer (pH 2.8) in a single-phase system to inactivate PAF acetylhydrolase, as described previously (6). The biological activity before and after the weak-acid treatment of the PAF extracted without acid treatment, as determined by rabbit platelet activation (17), was not altered, showing that only traces of the 1-alk-1′-enyl-type PAF, if any, were present. Lipid phosphorus was determined by the method of Bartlett (18). The lipids (*ca.* 2.5 mg phosphorus) were applied to a silic AR CC-7 column (8 × 90 mm). The neutral lipids were separated by elution with 40mL of chloroform. Phospholipids (*ca.* 500 μg phosphorus), eluted with 80 mL of methanol, were separated by thin-layer chromatography (TLC) on silica gel 60H plates (20 × 20 cm, 0.6 mm thick) using the solvent system chloroform/methanol/water (65:35:6, v/v/v). After spraying the plate with 1 mM 6-*p*-toluidine-2-naphthalenesulfonic acid (TNS) (19), the bands between lysophosphatidylcholine and sphingomyelin, and those of phosphatidylcholine and phosphatidylethanolamine, were localized under ultraviolet light, scraped off, and extracted. In order to separate acidic phospholipids, the phosphatidylcholine fraction (*ca.* 1 mg phosphorus) dissolved in chloroform was applied to a column of activated alumina 200 (8 × 16 mm) and eluted with 60 mL of chloroform/methanol (1:1, v/v).

Derivatization of PAF for mass spectrometry (MS) analysis. The isolated PAF fractions were digested with phospholipase C, and the resulting diradylglycerols were converted to *tert*-butyldimethylsilyl (*t*-BDMS) derivatives essentially as described previously (20). The *t*-BDMS derivatives were purified further by TLC (10 × 20 cm, 0.3 mm thick) using the solvent system hexane/diethyl ether (9:1, v/v).

Determination of 1-alkyl-2-acetyl-GPC and 1-acyl-2-acetyl-GPC by GC/MS coupled with selected ion monitoring (SIM). For measurement of 1-akyl-2-acetyl-GPC, the total lipids were mixed with 1-hexadecyl-2-perdeuteroacetyl-GPC ($4.2–4.8 \times 10^{-7}$ mol/mol total antrum phospholipds and 1.2×10^{-7} mol/mol total corpus phospholipids) as internal standard. For measurement of 1-acyl-2-acetyl-GPC, 1-heptadecanoyl-2-perdeuteroacetyl-GPC ($3.8–4.4 \times 10^{-6}$ mol/mol total antrum phospholipids and 2.4×10^{-7} mol/mol total corpus phospholipids) was used as internal standard.

A JEOL (Tokyo, Japan) JMS-DX300 instrument (ionization energy, 70 eV) with a JMA 3100 computer was coupled to a gas chromatograph equipped with a 1 m × 2 mm glass spiral packed with 1% OV-1 on Chromosorb W (80–100 mesh). The temperatures of the column, injection port, separator, inlet and ionization chamber were kept at 240, 290, 290, 290, and 270°C, respectively. The flow rate of helium was 55 mL/min. Ions $[M-57]^+$, produced by cleavage of the *tert*-butyl radical from the parent ion, and $[CH_3CO + 74]^+$ and $[RCO + 74]^+$, formed by rearrangements of the acetyl or acyl residue and dimethyl silanol, were selected for SIM. The abundance of each molecular species was calculated from the ratios of their peak areas to that of the internal standard.

Tandem mass spectrometry (MS/MS). MS/MS was performed on a JEOL HX110-HX110 tandem mass spectrometer equipped with a JMA-DA5000 computer. The instrument was used in the electron impact mode, and the sample was directly inserted into the ion source. The ionization energy, ionization current, ionization chamber temperature and accelerating voltage were 70 eV, 300 μA, 200°C and 10.0 kV, respectively. The selected $[M-57]^+$ ion was collided with helium.

Determination of the composition of ether-linked choline and ethanolamine glycerophospholipids. Choline and ethanolamine glycerophospholipids (358–587 μg phosphorus) were hydrolyzed with 2 mL of 0.1 N KOH in 90% methanol at room temperature for 60 min. The ether-linked type was recovered in the chloroform–soluble phase (32.2 and 28.7 μg phosphorus from choline glycerophospholipids of the antrum and corpus, and 154.7 and 113.6 μg phosphorus from ethanolamine glycerophospholipids of the antrum and corpus, respectively), and that derived from the diacyl type was recovered in the water-soluble phase (372.8 and 557.8 μg phosphorus from choline glycerophospholipids of the antrum and corpus, and 203.2 and 329.6 μg phosphorus from ethanolamine glycerophospholipids of the antrum and corpus, respectively) by the extraction method of Bligh and Dyer (16). Then the chloroform-soluble fraction was incubated in a mixture of 1 mL of chloroform, 2 mL of methanol and 0.8 mL of 0.5 N HCl at 37°C for 15 min. The products of alk-1′-enyl-GPC and alk-1′-enyl-*sn*-glycero-3-phosphoethanolamine (GPE) were recovered in the water-soluble phase (9.7 and 13.2 μg phosphorus from ether-linked choline lysoglycerophospholipids of the antrum and corpus, and 112.4 and 89.4 μg phosphorus from ether-linked ethanolamine lysoglycerophospholipids of the antrum and corpus, respectively), and alkyl-GPC and alkyl-GPE were recovered in the chloroform-soluble phase (20.9 and 13.8 μg phosphorus from ether-linked choline lysoglycerophospholipids of the antrum and corpus, and 31.2 and 20.7 μg phosphorus from ether-linked ethanolamine lysoglycerophospholipids of the antrum and corpus, respectively) in a two-phase system (16).

Analysis of the composition of acyl and alkyl chains at the 1-position of choline and ethanolamine glycerophospholipids. The composition of acyl and alkyl chains at the 1-position was determined by analysis of lysoglycerophospholipids obtained by phospholipase A_2 hydrolysis and of ether-linked lysoglycerophospholipids obtained by treatment with weak alkali and acid as described above, respectively. Choline and ethanolamine glycerophospholipids (6–7 μg phosphorus) were completely hydrolyzed with 50 μg purified phospholipase

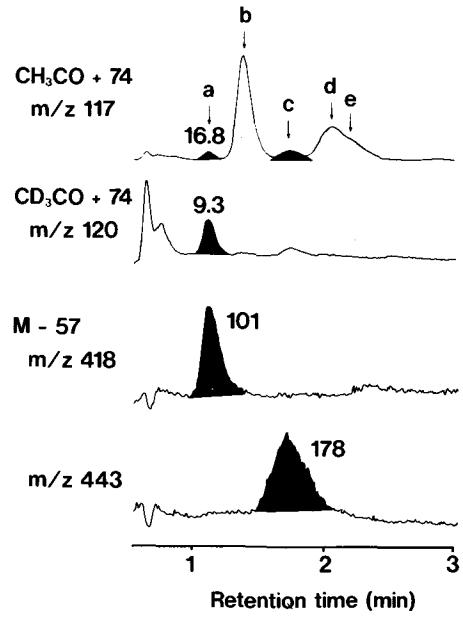


FIG. 1. Qualitative and quantitative analysis of 1-alkyl-2-acetyl-GPC in rat antrum by SIM analysis. Rats weighing 251 ± 31 g (157 animals), age 9–10 wk, were used (Experiment 1). The total antrum lipids were mixed with 1-hexadecyl-2-perdeuteroacetyl-GPC as internal standard. Peak areas are shown to the right of each of the peaks. Peaks of a, b, c, d and e correspond to those of *t*-BDMS derivatives of authentic 1-hexadecyl-2-acetyl-GPC and 1-hexadecanoyl-2-acetyl-GPC, 1-octadecyl-2-acetyl-GPC, 1-octadec-*cis*-9′-enoyl-2-acetyl-GPC and 1-octadecanoyl-2-acetyl-GPC, respectively. The result from one of 3 separate measurements is shown in the figure.

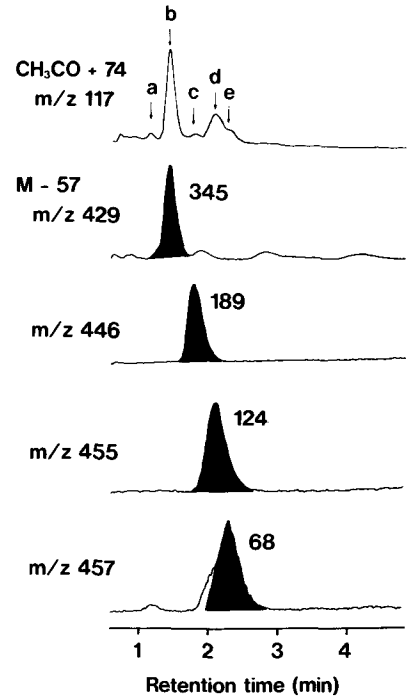


FIG. 2. Qualitative and quantitative analysis of 1-acyl-2-acetyl-GPC in rat antrum by SIM analysis. Rats weighing 223 ± 12 g (81 animals), age 9 wk, were used (Experiment 2). The total antrum lipids were mixed with both 1-hexadecyl-2-perdeuteroacetyl-GPC and 1-heptadecanoyl-2-perdeuteroacetyl-GPC as internal standards. Peak areas are shown to the right of each of the peaks. Peaks of a, b, c, d and e are as in Figure 1. The result from one of 3 separate measurements is shown in the figure.

A_2 (*Agkistrodon halys blomhoffi*) in a mixture of 3 mL of diethyl ether, 0.4 mL of 0.125 M Tris-HCl buffer (pH 8.2), 0.05 mL of 1.0 M NaCl and 10 mM $CaCl_2$ with vigorous shaking at room temperature for 16 hr. The lipid products were extracted and separated on a silica gel G plate in the solvent system chloroform/methanol/water (65:35:6, v/v/v). For analysis of the fatty acid composition of the purified lysoglycerophospholipids, the methyl esters derived by acid methanolysis were prepared (21). In order to determine the alkyl chain composition, the purified ether-linked lysoglycerophospholipids were mixed with 0.3 mL of acetic anhydride and 0.2 mL of glacial acetic acid at 150°C for 5 hr for conversion to 1-alkyl-2,3-diacetyl-glycerol (22). The fatty acid methyl esters and glyceryl ether diacetates were analyzed in a Shimadzu GC-7AG gas chromatograph (Kyoto, Japan) equipped with glass columns, packed 10% Silar 10C on Gas-Chrom Q (100-200 mesh) and 1% OV-1 on Chromosorb W (80–100 mesh) (both 2 m × 3 mm), respectively. The columns were operated at 185–215°C for analysis of fatty acid methyl esters and at 240°C for analysis of glyceryl ether diacetates. The peaks of each chromatogram were assigned on the basis of their retention times relative to those of standards.

RESULTS

Identification of 1-alkyl-2-acetyl-GPC and 1-acyl-2-acetyl-GPC in rat antrum. For GC/MS analysis, the *t*-BDMS derivative of the 'diglyceride' prepared enzymatically was used. The presence of both 1-alkyl-2-acetyl-GPC and 1-acyl-2-acetyl-GPG in rat antrum was demonstrated by SIM analysis (Figs. 1 and 2). Upon monitoring the ion at *m/z* 117, $[CH_3CO + 74]^+$, five peaks were detected with retention times of 1′07″, 1′24″, 1′44″, 2′04″ and 2′12″, which corresponded respectively to those of authentic 1-hexadecyl-2-acetyl-GPC, 1-hexadecanoyl-2-acetyl-GPC, 1-octadecyl-2-acetyl-GPC, 1-octadec-*cis*-9′-enoyl-2-acetyl-GPC and 1-octadecanoyl-2-acetyl-GPC. For determining the molecular species of 1-alkyl-2-acetyl-GPC, MS/MS analysis was performed. The $[M-57]^+$ ion was selected and collided with helium because this ion became more prominent than the $[M]^{+\cdot}$ ion in the electron ionization mass spectrum of the *t*-BDMS derivatives of the diglycerides (23). The collision-induced fragmentation of ions at *m/z* 415 and 443, due to $[M-57]^+$, yielded daughter ions at *m/z* 355 and 383, due to the characteristic $[R + 130]^+$ ion for the alkyl residue, and an ion at *m/z* 117, due to $[CH_3CO + 74]^+$ for the acetyl residue, for 1-hexadecyl-2-acetyl-3-*t*-BDMS-glycerol and 1-octadecyl-2-acetyl-3-*t*-BDMS-glycerol, respectively (Fig. 3). The collision-induced fragmentation of an ion at *m/z* 441 did not yield the

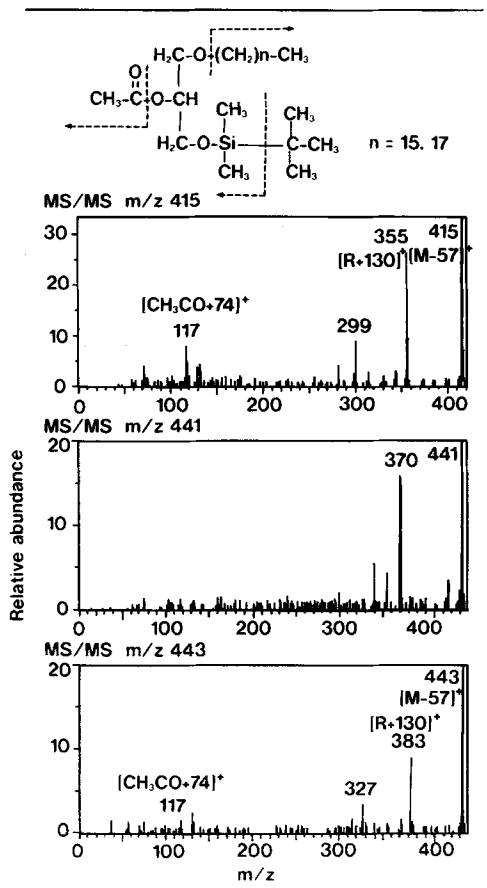


FIG. 3. MS/MS spectra of *t*-BDMS derivatives of antral 1-alkyl-2-acetyl-GPC in Experiment 2.

characteristic daughter ions for 1-octadecenyl-2-acetyl-3-*t*-BDMS-glycerol (Fig. 3). The observation indicates that 1-alkyl-2-acetyl-GPC was composed of 1-hexadecyl-2-acetyl-GPC and 1-octadecyl-2-acetyl-GPC. For the characterization of 1-acyl-2-acetyl-GPC, the ions at *m/z* 429, 455 and 457, due to $[M-57]^+$, and the ions at *m/z* 313, 339 and 341, due to $[RCO + 74]^+$, were selected. As shown in Figure 2, the peaks obtained by monitoring the ions at *m/z* 429, 455 and 457 were located at the same positions as those of authentic 1-hexadecanoyl-2-acetyl-GPC, 1-octadec-*cis*-9′-enoyl-2-acetyl-GPC, and 1-octadecanoyl-2-acetyl-GPC, respectively. Also, for the trace ions at *m/z* 313, 339 and 341, peaks were obtained at the same positions as those of 1-hexadecanoyl-2-acetyl-GPC, 1-octadec-*cis*-9′-enoyl-2-acetyl-GPC and 1-octadecanoyl-2-acetyl-GPC (data not shown).

Quantitation of 1-alkyl-2-acetyl-GPC and 1-acyl-2-acetyl-GPC in rat antrum. The amount of each molecular species was calculated from the ratios of their peak areas at $[M-57]^+$ relative to those of deuterated internal standards (1-hexadecyl-2-perdeuteroacetyl-GPC for measurement of 1-alkyl-2-acetyl-GPC and 1-heptadecanoyl-2-perdeuteroacetyl-GPC for measurement of 1-acyl-2-acetyl-GPC) using calibration curves (Figs. 1 and 2). In order to determine the amount of 1-hexadecyl-2-acetyl-GPC, the peak area at *m/z* $[CH_3CO + 74]^+$ relative to the internal standard was measured. The regression equations were $Y = 1.088X + 0.145$ and $Y = 0.583X + 0.121$ with correlation coefficients of $r = 1.000$ and 0.993 for 1-hexadecyl-2-acetyl-GPC and 1-octadecyl-2-acetyl-GPC, respectively, where X is the ratio by weight and Y is the ratio of peak areas. The regression equation for 1-hexadecanoyl-2-acetyl-GPC was $Y = 1.040X - 0.038$ with $r = 1.000$, which was coincident with the regression equation reported previously (14). Hence, for the calibration of 1-octadec-*cis*-9′-enoyl-2-acetyl-GPC and 1-octadecanoyl-2-acetyl-GPC, $Y = 0.871X - 0.044$ and $Y = 0.812X + 0.003$ were used as the regression equations, respectively, as reported previously (14). The results, presented as mol per mol total antrum phospholipids, are shown in Table 1. Thus, 1.9 ng of 1-alkyl [16:0 and 18:0]-2-acetyl-GPC and 15.6 ng of 1-acyl [16:0, 18:0 and 18:1]-2-acetyl-GPC were detected per rat antrum. The main species of 1-alkyl-2-acetyl-GPC was 1-octadecyl-2-acetyl-GPC, followed by 1-hexadecyl-2-acetyl-GPC. In contrast, the main species of 1-acyl-2-acetyl-GPC was 1-hexadecanoyl-2-acetyl-GPC, followed by 1-octadecenoyl-2-acetyl-GPC and 1-octadecanoyl-2-acetyl-GPC.

The composition of alkyl and acyl chains at the 1-position of choline and ethanolamine glycerophospholipids in perfused rat antrum and corpus. On analysis of choline and ethanolamine glycerophospholipids, rats were perfused with saline since it was necessary to remove phospholipids which might derive from blood cells and lipoproteins. As shown in Table 2, the composition of the diacyl, alkyl and alk-1′-enyl types of choline glycerophospholipids was quite different from that of ethanolamine glycerophospholipids in both the antrum and corpus. The amount of the alkylacyl type of both choline and ethanolamine glycerophospholipids was low (less than 5% of total phospholipids). The amount of the alkylacyl type in the antrum was about 2-fold greater than that in the corpus, which might be related to the localization of 1-alkyl-2-acetyl-GPC since the amount of PAF in the antrum was about 4-fold greater than that in the corpus (6). The alkyl chain distribution of choline glycerophospholipids in the antrum and corpus was 16:0, 18:0 and 18:1 with other components in less than trace amounts (Table 3). On the other hand, there was a significant difference in the fatty acid distribution between choline and ethanolamine glycerophospholipids. Whereas the predominant fatty acid in ethanolamine glycerophospholipids was 20:4 (28.9% in the antrum and 27.3% in the corpus), the predominant fatty acid in choline glycerophospholipids was 16:0 (25.8% in the antrum and 27.7% in the corpus) (data not shown). The acyl chain at the 1-position of diacyl type choline glycerophospholipids was composed mainly of 16:0 (36.3% in the antrum and 37.1% in the corpus) (Table 3), in contrast to the predominance of 18:0 (35.0% in the antrum and 28.8% in the corpus) in those of ethanolamine glycerophospholipids (data not shown).

Alteration in molecular species of 1-alkyl-2-acetyl-GPC and 1-acyl-2-acetyl-GPC associated with rat gastric ulcer induced by water-immersion stress. In a previous paper, we have reported that water-immersion stress for 1 hr and 3 hr, before the development of mucosal damage, produces a significant decrease in the gastric PAF level (39% of untreated controls), as determined by bioassay (6). The present study was done to confirm this observation physicochemically by GC/MS analysis. As shown

TABLE 1

Contents of Antral 1-Alkyl-2-acetyl-GPC and 1-Acyl-2-acetyl-GPC in Normal and Water-Immersion-Stressed Rats[a]

	Control		Water-immersion stress
	Experiment 1 9–10 wk old	Experiment 2 9 wk old	Experiment 3 9 wk old
	mol/mol total phospholipids		
1-Alkyl-2-acetyl-GPC			
1-Hexadecyl-2-acetyl-GPC	7.22×10^{-7} [b]	5.90×10^{-7} [b]	1.16×10^{-7} [b]
1-Octadecyl-2-acetyl-GPC	13.6×10^{-7}	11.3×10^{-7} [b]	7.00×10^{-7}
1-Octadecenyl-2-acetyl-GPC	not detected	not detected	not detected
1-Acyl-2-acetyl-GPC			
1-Hexadecanoyl-2-acetyl-GPC	not determined	8.20×10^{-6}	1.64×10^{-6}
1-Octadecanoyl-2-acetyl-GPC	not determined	1.94×10^{-6}	0.24×10^{-6}
1-Octadecenoyl-2-acetyl-GPC	not determined	3.60×10^{-6}	0.51×10^{-6}

[a]Rats used in experiments 1 and 2 are shown in Figures 1 and 2. In Experiment 3, rats weighing 233 + 17 g (189 animals), age 9 wk, were restrained in water maintained at 23°C ± 1°C for 1 hr. The antral lipid-extracts from 157, 81 and 189 animals were combined and analyzed in Experiments 1, 2 and 3, repsectively. The lipid-phosphorus contents were 484.4, 467.5 and 458.9 μg phosphorus/g antrum tissue in Experiments 1, 2 and 3, respectively.

[b]$[CH_3CO + 74]^+$ was selected for the quantitation.

TABLE 2

Composition of Choline and Ethanolamine Glycerophospholipids in Perfused Rat Antrum and Corpus[a]

Phospholipids	Antrum	Corpus
	mol%	
Ethanolamine glycerophospholipids[b]	26.8, 34.0	28.8, 31.8
Diacyl type[c]	56.8	74.4
Alkylacyl type[c]	9.4	4.8
Alk-1′-enylacyl type[c]	33.8	20.8
Choline glycerophospholipids[b]	44.9, 39.6	42.5, 40.5
Diacyl type[c]	92.1	95.1
Alkylacyl type[c]	5.4	2.5
Alk-1′-enylacyl type[c]	2.5	2.4

[a]Rats weighing 242 ± 17 g (means ± SD) (39 animals), age 9 wk, were anesthetized with sodium pentobarbital (50 mg/kg, i.p.) and perfused with saline warmed at 37°C *via* the ventral aorta at a rate of 42.5 mL per min for 90 sec. The total lipids were extracted in a single phase of chloroform/methanol/water (1:2:0.8, v/v/v) in order to avoid cleavage of the alk-1′-enyl linkage. The lipid-phosphorus contents were 586.1 ± 13.5 μg phosphorus/g corpus tissue and 455.7 ± 27.0 μg phosphorus/g antrum tissue.

[b]The phospholipid fraction (60 μg phosphorus) after silic AR CC-7 column chromatography, as described in Material and Methods, was separated into phospholipid subclasses on a silica gel G plate (3 × 20 cm, 0.25 mm thick) (Analtech, Newark, NJ) in the solvent system chloroform/methanol/acetic acid/water (50:25:8:4, by vol). After spraying the plate with TNS reagent, PAF, phosphatidylcholine and phosphatidylethanolamine were scraped-off and extracted. Data are from combined samples from 19 and 20 rats.

[c]Diacyl, alkyl, and alk-1′-enyl types were determined as alkali-labile, alkali-stable and acid-stable, and alkali-stable and acid-labile phospholipids, respectively. Data are from combined samples from 39 rats.

in Figures 4-1 and 4-2, an alteration in the ratio of each molecular species of 1-alkyl-2-acetyl-GPC and 1-acyl-2-acetyl-GPC was observed 1 hr after commencement of stress. The levels of 1-hexadecyl-2-acetyl-GPC and 1-acyl [16:0, 18:0 and 18:1]-2-acetyl-GPC in the antrum (Experiment 3) decreased to 12–20% of those in untreated controls 1 hr after stress began (Experiment 2) (Table 1). Interestingly, the degree of decrease in the level of 1-octadecyl-2-acetyl-GPC was insignificant (62% of untreated control), in contrast to a remarkable decrease in the level of 1-hexadecyl-2-acetyl-GPC. Stress for 7 hr resulted in severe mucosal damage mainly in the corpus. The previous study, based on bioassay, demonstrated that the PAF content 7 hr after the stress was still decreased in the antrum (49% of the level in untreated controls), but was restored to normal in the corpus (6). The present SIM analysis revealed a remarkable alteration in the composition and amount of molecular species of 1-alkyl-2-acetyl-GPC and 1-acyl-2-acetyl-GPC during the 7-hr period of stress, as shown in Figures 4-3 and 4-4. In the corpus after 7 hr of stress, the amounts of 1-hexadecyl-2-acetyl-GPC and 1-hexadecanoyl-2-acetyl-GPC decreased. Surprisingly, the amount of 1-octadecyl-2-acetyl-GPC increased 4-fold (1.01×10^{-6} mol/mol total phospholipids). The EC_{50} values of 1-hexadecyl-2-acetyl-GPC and 1-octadecyl-2-acetyl-GPC for rabbit platelet activation ([^{3}H] serotonin release) were 1.0×10^{-10} M and 2.7×10^{-10} M, respectively. Therefore, the discrepancy between the results obtained by the bioassay and those by GC/MS analysis can be considered to result from the different biological activities of each of the molecular species of PAF.

DISCUSSION

In the present study we elucidated the molecular heterogeneity of PAF in the rat glandular stomach by GC/MS analysis. It was observed that odd-numbered 1-alkyl-2-acetyl-GPC eluted at the same position as even-numbered 1-acyl-2acetyl-GPC upon simultaneous

TABLE 3

Composition of Acyl and Alkyl Chains at the 1-Position of Choline Glycerophospholipids in Perfused Rat Antrum and Corpus After Digestion with Phospholipase A_2 and Treatment with Weak Alkali and Acid [a]

Chain length and number of double bonds	Antrum		Corpus	
	Acyl[b]	Alkyl[c]	Acyl[b]	Alkyl[c]
	weight%			
14:0	0.8, 2.1		0.3, 1.5	
14:1	2.8, 4.8		1.8, 1.5	
16:0	37.6, 35.0	42.0	34.1, 40.0	44.3
16:1	2.0, 1.3		0.5, 1.1	
18:0	21.8, 22.9	18.2	17.6, 15.6	16.7
18:1	15.0, 14.8	33.0	15.1, 14.1	32.4
18:2	7.6, 4.4		11.3, 9.5	

[a]For details, see legend to Table 2 and Materials and Methods.

[b]Data are from combined samples from 19 and 20 rats.

[c]Data are from combined samples from 39 rats.

analysis of 1-alkyl-2-acetyl-GPC and 1-acyl-2-acetyl GPC. In the SIM analysis of fractions corresponding to 1-alkyl-2-acetyl-3-*t*-BDMS-glycerol purified by TLC using hexane/diethyl ether (9:1, v/v), the peaks at 1′24″, 2′04″ and 2′12″ almost disappeared whereas the peaks at 1′07″ and 1′44″ were mostly retained. The observation indicates that there are only trace components, if any, of odd-numbered alkyl and acyl chains. As a consequence of this finding, it was possible to determine 1-alkyl-2-acetyl-GPC and 1-acyl-2-acetyl-GPC simultaneously by SIM. The amount of 1-acyl [16:0, 18:0 and 18:1]-2-acetyl-GPC was about 8-fold greater than that of 1-alkyl [16:0 and 18:0]-2-acetyl-GPC (Table 1). A preponderance of 1-acyl-2-acetyl-GPC over 1-alkyl-2-acetyl-GPC is a common feature of tissues such as bovine brain (3) and rat uterus (4). However, this is not true in stimulated cells such as polymorphonuclear leukocytes, in which the amount of 1-alkyl-2-actyl-GPC is five times higher than that of 1-acyl-2-acetyl-GPC (24). There was a great degree of similarity in the alkyl chain distributions of 1-alkyl-2-acyl-GPC between the antrum and corpus (Table 3). Furthermore, the molecular heterogeneity of 1-alkyl-2-acetyl-GPC and 1-acyl-2-acetyl-GPC in the corpus was quite similar to that in the antrum (Fig. 4). These observations suggest that the cells producing PAF in the corpus might be the same as those in the antrum. The acyl chain distribution at the 1-position of 1-acyl-2-acetyl-GPC remained more or less the same as that at the 1-position of 1,2-diacyl-GPC (Tables 1 and 3). However, the alkyl chain composition of 1-alkyl-2-acetyl-GPC did not seem to be dictated by that of 1-alkyl-2-acyl-GPC (Tables 1 and 3). Whereas 1-hexadecyl-2-acyl-GPC was the major molecular species of 1-alkyl-2-acyl-GPC (42% in the antrum and 44% in the corpus), 1-octadecyl-2-acetyl-GPC (66% in the antrum and 59% in the corpus) was the major molecular species of 1-alkyl-2-acetyl-GPC. This suggests that enzymes in PAF biosynthesis may have an extraordinary selectivity of alkyl chain utilization or that substrates located in an extraordinary position of stomach tissues (cells) may be utilized for PAF biosynthesis.

In a previous study (6), we found that water-immersion stress affected the gastric PAF content based on bioassay. The present GC/MS analysis revealed that such stress was associated with both qualitative and quantitative alterations in the molecular species of

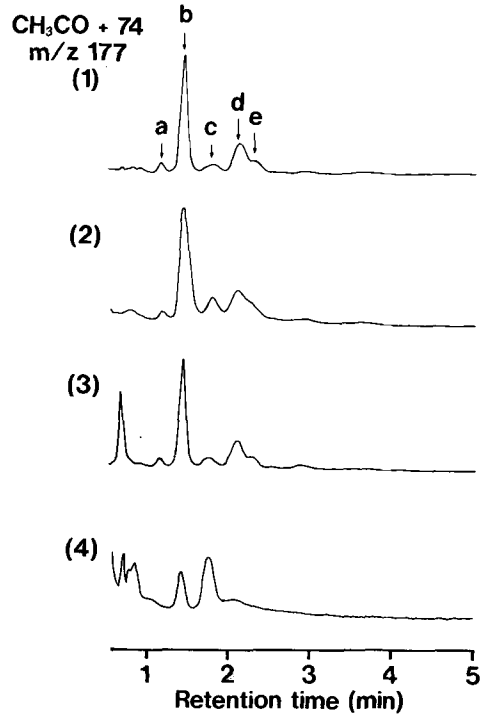


FIG. 4. SIM chromatograms of *t*-BDMS derivatives of gastric 1-alkyl-2-acetyl-GPC and 1-acyl-2-acetyl-GPC in control and water-immersion-stressed rats. 1. Control rat antrum (Experiment 1); 2. Antrum from 1-hr water-immersion-stressed rat (Experiment 3); 3. Control rat corpus (Experiment 4); 4. Corpus from 7-hr water-immersion-stressed rat (Experiment 5). In Experiments 4 and 5, rats weighing 225 ± 31 g (26 animals) and rats weighing 228 ± 14 g (22 animals) (age 9 wk) were used, respectively. The lipid-phosphorus contents were 702.5 and 722.8 μg phosphorus/g corpus tissue in Experiments 4 and 5, respectively. Peaks of a, b, c, d and e are as in Figure 1.

1-alkyl-2-acetyl-GPC and 1-acyl-2-acetyl-GPC in both the antrum and corpus. 1-Hexadecyl-2-acetyl-GPC and 1-acyl [16:0, 18:0 and 18:1]-2-acetyl-GPC showed a significant decrease before the development of mucosal damage in the glandular stomach upon application of water-immersion stress for 1 hr (Table 1 and Fig. 4). By contrast, 1-octadecyl-2-acetyl-GPC increased markedly in the corpus during a 7-hr period of stress, which resulted in severe mucosal damage to the corpus, characterized by hemorrhage and inflammatory infiltrates. The increased amount of 1-octadecyl-2-acetyl-GPC in the corpus may have been produced by stimulated resident cells or circulating blood cells. However, rat polymorphonuclear leukocytes and rat basophilic leukemia cells, either unstimulated or stimulated, have been reported to produce 1-hexadecyl-2-acetyl-GPC as the predominant molecular species of PAF (25). The cells producing endogenous 1-alkyl-2-acetyl-GPC and 1-acyl-2-acetyl-GPC in the rat glandular stomach and their biological significance still need to be clarified. However, our present observations may help in elucidating the biological function of gastric PAF. 1-Hexadecyl-2-acetyl-GPC and 1-octadecyl-2-acetyl-GPC may possibly play different roles in the stomach. The qualitative and quantitative alteration of specific molecular species of PAF might be associated with the pathogenesis of gastric ulceration. In order to clarify these aspects, it will be necessary to understand the intrinsic biological function of each molecular species of PAF homologs and analogs.

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The Effect of CV-3988 and CV-6209 on the Acute Gastric Erosions of Rats Due to Water-Immersion and Restraint Stress[1]

Makoto Nogami[a,*], Yoshio Hoshihara[a], Kunihiko Tsubura[a], Takashi Yamamoto[a], Masafumi Tabuchi[a], Terumasa Miyamoto[a] and Junji Shiga[b]

[a]Department of Medicine and Physical Therapy, and [b]Department of Pathology, Faculty of Medicine, University of Tokyo, 7-3-1, Hongo, Bunkyo-ku, Tokyo, 113, Japan

CV-3988 and CV-6209 inhibited gastric erosions in rats due to water-immersion and restraint stress in a dose-dependent manner. The above inhibitory effects of CV-3988 were observed in the presence of indomethacin, which may indicate that the inhibition is not prostaglandin dependent. The studies indicate that platelet-activating factor may be involved in the formation of erosions in rats under water-immersion and restraint stress. ***Lipids 26,*** **1354–1355 (1991).**

Platelet-activating factor (PAF) is a potent ulcerogenic agent (1). In the present investigation, the effect of the PAF antagonists CV-3988 and CV-6209 on acute gastric erosions due to water-immersion and restraint stress was studied in rats (2,3). The present study is an extension of our earlier work, in which the antagonist CV-3988 was used at relatively high concentrations (4,5).

CV-3988 (*rac*-3-(*N*-octadecylcarbamoyloxy)-2-methoxypropyl 2-thiazolioethyl phosphate) and CV-6209 (2-[*N*-acetyl-*N*-(2-methoxy-3-octadecylcarbamoyloxypropoxycarbonyl)aminomethyl]-1-ethylpyridinium chloride) were kindly provided by Takeda Pharmaceutical Company (Tokyo, Japan). Male Wistar rats (240–260 g for experiments 1 and 3), 190–210 g for experiment 2 were deprived of food for 24 hr. The drugs were then administered as follows: Experiment 1: CV-3988 was administered at doses of 0 mg, 10 mg, 20 mg, or 40 mg/kg body weight in saline (0.6 mL) by intravenous (i.v.) injection. Experiment 2: CV-3988 was administered i.v. in doses of 0 mg or 20 mg/kg body weight in saline (0.6 mL) and indomethacin (5 mg/kg in 0.6 mL of 8% $NaHCO_3$ adjusted to pH 9.0 by NaOH) were subcutaneously (s.c.) injected. As for the control, 0.6 mL of saline (i.v.) and the above solution used for indomethacin (s.c.) were administered. Experiment 3: CV-6209 was administered (i.v.) at doses of 0 mg, 0.1 mg, or 1 mg/kg in saline (0.6 mL).

After administration of the drugs, the rats were restrained in a cage and immersed in water at 22°C for 6 hr as described by Takagi and Okabe (6). Then the stomachs were fixed with 1% formalin and cut open by the great curvature. The lengths of linear hemorrhagic erosions were examined by one person unaware of the treatment, summed for each rat, and used as ulcer index.

The analysis of variances by F-test and Ryan's method were used for statistical analysis, $p < 0.05$ was regarded as statistically significant. Since the ulcer index varies between experiments, comparisons were made only within the same experiment and within the same group of rats.

[1]Based on a paper presented at the Third International Conference on Platelet-Activating Factor and Structurally Related Alkyl Ether Lipids, Tokyo, Japan, May 1989. The data on CV-3988 were originally reported in *Nippon Shokakibyo Gakkai Zasshi (Jap. J. Gastroenterol.) 85,* 2149–2154 (1988).

*To whom correspondence should be addressed.

RESULTS AND DISCUSSION

The effect of CV-3988 on erosions due to water-immersion and restraint stress is shown in Table 1. CV-3988 inhibited the erosions in a dose-dependent manner. As shown in Table 2, CV-3988 also inhibited the enhanced erosions due to indomethacin administration. The effect of CV-6209 on the erosions due to water-immersion and restraint stress is shown in Table 3. CV-6209 also inhibited the erosions in a dose-dependent manner.

TABLE 1

The Effect of CV-3988 on Erosions due to Water-Immersion and Restraint Stress in Rats[a]

CV-3988 (mg/kg)	n	Ulcer index (mm)
0	4	41.0 ± 8.9
10	5	26.3 ± 3.7
20	4	11.4 ± 5.1[b]
40	5	10.8 ± 4.0[b]

[a]Figures show means ± S.E.

[b]$P < 0.05$ as compared with 0 mg/kg correlation (coefficient r = −0.6737).

TABLE 2

The Effect of CV-3988 on the Erosions by Water-Immersion and Restraint Stress in Indomethacin-Treated Rats[a]

Treatment	Ulcer index (mm)
Vehicle + saline	15.3 ± 4.9
Indomethacin 5 mg/kg + saline	37.8 ± 2.5
Indomethacin 5 mg/kg + CV-3988 20 mg/kg	13.8 ± 3.7[b]

[a]Figures show means ± S.E. (n = 4 for each group).

[b]$P < 0.05$ as compared to indomethacin (5 mg/kg) and saline.

TABLE 3

The Effect of CV-6209 on the Erosions by Water-Immersion and Restraint Stress in Rats[a]

CV-6209 (mg/kg)	n	Ulcer Index (mm)
0	5	13.0 ± 0.5
0.1	4	8.3 ± 1.2[b]
1.0	5	3.5 ± 1.2[c]

[a]Figures show means ± S.E.

[b]$P < 0.05$ as compared with 0 mg/kg.

[c]$P < 0.01$ as compared with 0 mg/kg (correlation coefficient r = − 0.9267).

As shown here and reported earlier (4,5), CV-3988 significantly inhibited the erosions of rats caused by water-immersion and restraint stress. Since rather high doses were required for the inhibition, the possibility of a micellar effect of the drug could not be excluded (2). Micelles may injure cell membranes which, in turn, could possibly generate prostaglandins that could act as cytoprotective agents (7). However, the inhibitory effect of CV-3988 was also observed in the presence of indomethacin, which indicates that the inhibition is likely not to be mediated by prostaglandins (5,8). The more potent PAF antagonist CV-6209 also showed an inhibitory effect on the formation of erosions, but at much lower doses. At these drug concentrations, the inhibitory effect is observed well below the critical micelle concentration *in vivo*. Our data show that PAF might be involved in the formation of erosions in rats caused by water-immersion and restraint stress. Braquet *et al.* had earlier shown that BN 52021 and triazolam also reduced restraint-stress induced gastric damage (9). The data indicate that PAF may play a role in inducing gastric damage due to stress.

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Platelet-Activating Factor May Mediate Dexamethasone-Induced Gastric Damage in the Rat[1]

János Filep[a,*], Ferenc Hermán[b] and Pierre Braquet[c]
Departments of [a]Pathophysiology and [b]Pharmacodynamics, Semmelweis University Medical School, 1445 Budapest, Hungary and [c]Institut Henri Beaufour, 92350 Le Plessis Robinson, France

The possible role of platelet-activating factor (PAF) in dexamethasone-induced gastric mucosal damage was studied in rats. PAF was measured by a platelet aggregation assay. The identity of the PAF-like product recovered from gastric tissues was ascertained by thin-layer chromatography and high-pressure liquid chromatography. Low levels of PAF were detected in the normal rat stomach, while in dexamethasone-treated animals PAF levels were significantly higher. Pretreatment of the animals with BN 52021, a specific PAF receptor antagonist, significantly attenuated dexamethasone-induced mucosal injury. These findings suggest that PAF may be a mediator of mucosal damage induced by glucocorticoids.
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Platelet-activating factor (PAF) previously has been implicated as a mediator of allergic and inflammatory processes (1). Recent studies have identified PAF as the most potent endogenous ulcerogenic agent known to date (2–5). Further evidence suggesting a role for PAF in gastrointestinal damage comes from the observations that PAF predisposes the gastric mucosa to damage of topical irritants (6), and that PAF antagonists significantly reduce endotoxin-induced gastrointestinal damage (7–9) and prevent gastric lesions evoked by ethanol and stress (5,10).

Administration of glucocorticoids to experimental animals can result in acute gastric erosion formation (11,12). The mechanism of this damaging action of glucocorticoids is, however, far from being understood. Glucocorticoids have been reported to inhibit gastric mucus formation (13) and to affect epithelial cell turnover (14). Dexamethasone and prednisolone have been reported to be ineffective in blocking prostaglandin synthesis in rat stomach (12,15). The aim of the present experiments was to study the possible role of PAF in mediating gastric mucosal damage due to dexamethasone.

MATERIALS AND METHODS

Male Wistar rats (160–205 g) were used in all experiments. For six consecutive days (between 9:00 and 10:00 a.m. each day), the animals were given intraperitoneally 10 mg/kg of BN 52021 (ginkgolide B, Institut Henri Beaufour, Le Plessis Robinson, France), a specific PAF receptor antagonist (16,17) or its vehicle, and 30 min later they received a subcutaneous injection of dexamethasone sodium phosphate (Decadron, Merck, Sharp and Dohme-Chibret, Zürich, Switzerland) or vehicle. The animals were divided into four groups: (i) control (receiving vehicle only); (ii) 4 mg/kg dexamethasone; (iii) BN 52021; and (iv) BN 52021 plus dexamethasone. On day six, the animals were anesthetized with sodium pentobarbital 5 hr after the second injection. The stomach was immediately removed and examined. The severity of macroscopically visible damage was scored independently by two observers, who were unaware of the treatments, using a 0 to 3 scoring system (2,12). A score of 0 was assigned if the mucosa appeared to be normal; score 1, limited to diffuse superficial hyperemia; score 2, moderate hyperemia or focal hemorrhage; and score 3, multiple sites of bleeding or erosions. Scoring was completed within 2–3 min. Representative sections were taken, fixed in formalin, embedded into Durcupan ACM (Irvine, CA), and then 1 μm thick sections were made and stained with toluidine blue. The remaining glandular stomach was weighed, minced and homogenized in 10 mM morpholinoethansulfonic acid buffer containing 130 mM Na^+, 5.4 mM K^+, 1.8 mM Ca^{2+}, 112 mM Cl^- and 0.25% bovine serum albumin, pH 5.2. [^{3}H]PAF (220,000 dpm; 80 Ci/mmol, Amersham International, Amersham, U.K.) was added to monitor procedural losses. The lipids were extracted by the Bligh and Dyer method (18) and PAF was extracted on silica cartridges (Sep Pak, Waters Associates, Milford, MA) (19). PAF bioactivity was assayed by platelet aggregation as described (20). The identity of PAF bioactivity with authentic PAF was ascertained by thin-layer chromatography (TLC) and high-pressure liquid chromatography (HPLC) (20).

RESULTS AND DISCUSSION

As shown in Table 1, vehicle and BN 52021 treatment alone did not cause any gross or microscopic lesions. In contrast, all animals treated with dexamethasone developed extensive uniform hyperemia with multiple focal regions of petecchiae; in six out of nine animals mucosal erosions occurred in the glandular stomach. As evaluated histologically, dexamethasone treatment resulted in extravasation of erythrocytes, focal destruction of the surface epithelium, and necrosis extending down to one-fourth to one-third the depth of the mucosa.

Analysis of the PAF content showed that stomachs from rats treated with dexamethasone, at a dose which caused hemorrhagic mucosal damage, contained considerably higher amounts of PAF bioactivity than controls (Table 1). PAF was determined as a substance able to aggregate washed rabbit platelets in the presence of indomethacin and apyrase, an adenosine diphosphate (ADP) scavenger (20). Thus, it is unlikely that the aggregation observed was due to thromboxane A_2 or ADP. Furthermore, PAF bioactivity recovered from gastric extracts showed Rf values in TLC (0.24 ± 0.02, n=4) and

[1]Based on a paper presented at the Third International Conference on Platelet-Activating Factor and Structurally Related Alkyl Ether Lipids, Tokyo, Japan, May 1989.

*To whom correspondence should be addressed at: Department of Pharmacology, Faculty of Medicine, University of Sherbrooke, Sherbrooke, Quebec J1H 5N4 Canada.

Abbreviations: ADP, adenosine diphosphate; HPLC, high pressure liquid chromatography; PAF, platelet activating factor; TLC, thin-layer chromatography.

TABLE 1

Effects of Dexamethasone on Gastric Mucosa and PAF Content in the Rat Glandular Stomach, and Prevention by PAF Receptor Antagonist BN 52021

Treatment[a]	Gastric mucosal damage scores[b]	n	p[c]	PAF content ng/g tissue	n	p[c]
None	0.3 ± 0.2	9	—	0.14 ± 0.03	6	—
BN 52021, 10 mg/kg	0.4 ± 0.2	8	NS	0.12 ± 0.05	4	NS
Dexamethasone, 4 mg/kg	2.7 ± 0.2	9	0.001	5.31 ± 1.35	4	0.05
BN 52021, 10 mg/kg plus Dexamethasone, 4 mg/kg	0.7 ± 0.2	9	NS[d]	1.54 ± 0.60	4	0.05

Values are means ± SEM.

[a]The animals were given BN 52021, dexamethasone, or both drugs daily for six days. On day 6, the stomach was removed and examined. PAF was extracted, purified over silica cartridge and measured by a platelet aggregation assay (for details see Materials and Methods).

[b]Damage was scored on a 0 (normal) to 3 (severe, extensive hyperemia, multiple sites of bleeding/erosions) scale by two observers unaware of the treatments. The correlation between scores assigned by the two observers was r = 0.92 (n=35, p<0.001).

[c]Compared to untreated rats, [d]p<0.01 compared to dexamethasone treated animals (Dunn's multiple contrast test). NS, not significant; n, number of animals.

retention time (Rt) in HPLC (7.5 ± 0.2 min, n=4) identical to those of synthetic PAF (Rf 0.24 ± 0.02, n=3; Rt, 7.4 ± 0.2 min, n=4). Platelet aggregation induced by either synthetic PAF or by gastric extracts was completely abolished by pretreatment of the platelets with BN 52021, a specific PAF receptor blocker (16,17). Taken together, these findings strongly suggest that the material recovered from the rat stomach and assayed was indeed PAF.

In the present study, low amounts of PAF were detected in the stomach of untreated rats in five out of six cases. Although similar levels of PAF-like activity were detected in rat kidney (21) and uterus (22), it is still uncertain whether PAF is actually present *in situ*, or is generated by mechanical or chemical stimuli during the analytical procedure. The biological significance of endogenous PAF in the gastrointestinal area and the apparent contradiction that local injection of PAF can produce mucosal damage (2-5) remain to be clarified.

Pretreatment of the animals with BN 52021 (10 mg/kg) significantly reduced the severity of dexamethasone-induced gastric lesions (Table 1). In these animals no petecchiae or erosions were observed. BN 52021 also reduced the severity of hyperemia—in four out of nine rats, gastric mucosa appeared to be normal. Preliminary experiments showed that the protective effect of BN 52021 was dose-related. Pretreatment of the animals with 4 mg/kg of BN 52021 significantly reduced the number of erosions/petecchiae, but had little effect on hyperemia, while at a dose of 1 mg/kg, BN 52021 did not exert any protective effect (data not shown). PAF content of the stomach from BN 52021 plus dexamethasone-treated rats was significantly lower than PAF levels found in rats receiving dexamethasone alone (Table 1). Whether this difference mirrors a decrease in PAF formation and/or an increase in PAF metabolism secondary to its displacement from PAF receptors is not clear. The findings that BN 52021 is a competitive PAF receptor antagonist (16,17) and that it has a ten-fold lower affinity to PAF receptor than does PAF itself (17) seem to support the latter explanation. A further possibility might be that trace amounts of BN 52021 interacted in the platelet aggregation assay and that, consequently, PAF concentrations were underestimated.

The present data do not give any information on the source of PAF. However, it seems unlikely that PAF originated from neutrophil granulocytes since dexamethasone decreases gastric myeloperoxidase activity (12), a widely used index of neutrophil infiltration. On the other hand, dexamethasone treatment resulted in depletion and degranulation of mast cells in the gastric mucosa (23). Because these cells are capable of synthesizing PAF (1), it might be that mast cells are, at least partly, responsible for enhanced gastric PAF formation following dexamethasone treatment.

In conclusion, the present findings suggest that PAF may be an endogenous mediator of dexamethasone-induced gastric mucosal damage in the rat.

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Myoelectric Intestinal Disturbances in *Escherichia coli* Endotoxic Shock in Rats. Involvement of Platelet-Activating Factor[1]

Laurent Pons[a,*], Marié-Thèrese Droy-Lefaix[b], Pierre Braquet[b] and Lionel Buéno[a]
[a]Department of Pharmacology INRA, 31931 Toulouse Cédex, and [b]IPSEN/IHB Laboratories, 92350 Le Plessis-Robinson, France

Administration of BN 52021 (50 mg/kg *i.v.*), a specific antagonist of platelet-activating factor (PAF), significantly reduced the intestinal myoelectric disturbances induced by *E. coli* endotoxin injection (50 μg/kg *i.v.*) by 62%. Thus, PAF may be involved in the intestinal motor alterations observed in endotoxic shock. When given in combination with indomethacin (10 mg/kg *i.p.*), BN 52021 inhibited endotoxic shock intestinal disturbances. Indomethacin alone also reduced PAF induced (25 μg/kg *i.p.*) disruption of migrating myoelectric complexes. Endotoxins may act on intestinal motility *via* release of endogenous PAF and prostaglandins, the effects of PAF being mediated through the release of prostaglandins.
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Platelet-activating factor (PAF) is thought to be involved in the pathophysiology of endotoxic shock because it displays a variety of effects likely to occur in endotoxemia, such as extensive necrosis and vascular congestion in the stomach and in the small intestine (1). Whittle *et al.* (2) demonstrated increased gastrointestinal PAF synthesis in a rat model of endotoxic shock. In addition to comparing the effects of endotoxin and PAF on intestinal myoelectric activity in fasted rats, the present study was conducted to determine if the release of endogenous PAF contributes to the genesis of intestinal motor disturbances observed in endotoxic shock using BN 52021, a specific PAF antagonist. The role of released endogenous prostaglandins was also examined.

MATERIALS AND METHODS

Under halothane (Fluothane ND, Coopers Vétérinaire S.A., Meaux, France) anesthesia, male Wistar rats (280–320 g) were chronically implanted with nichrome electrodes in

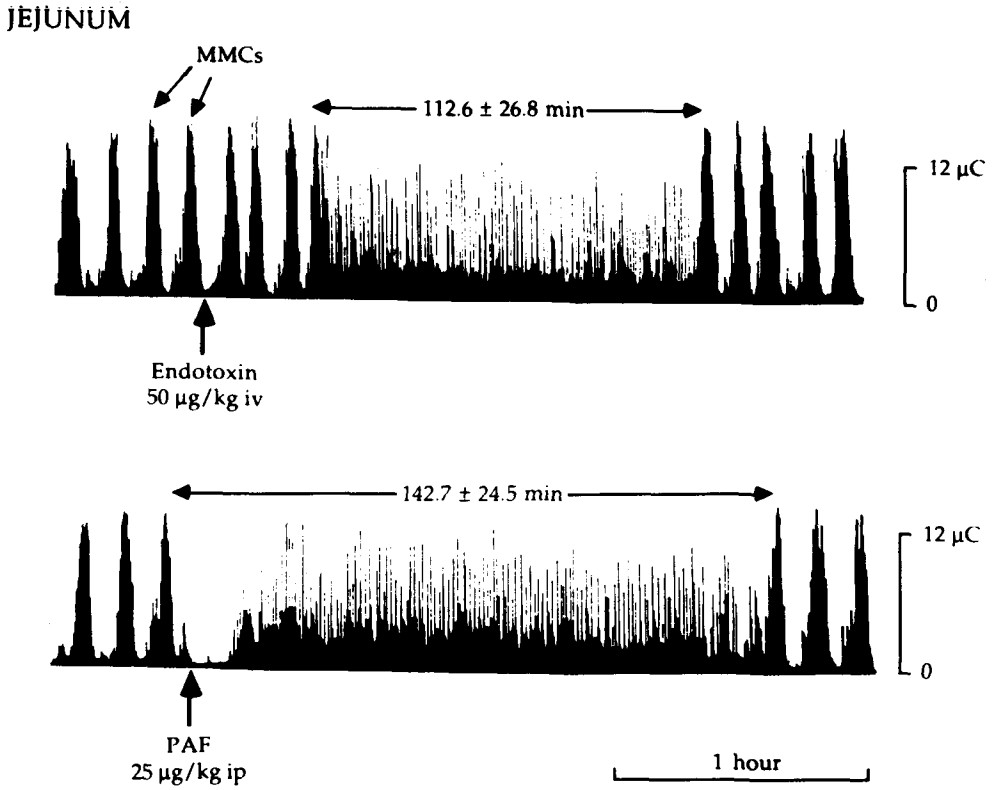


FIG. 1. Comparative effects of *E. coli* endotoxin (50 μg/kg *i.v.*) and PAF (25 μg/kg *i.p.*) administration on jejunal myoelectric activity (integrated records) in fasted rats.

[1]Based on a paper presented at the Third International Conference on Platelet-Activating Factor and Structurally Related Alkyl Ether Lipids, Tokyo, Japan, May 1989.

*To whom correspondence should be addressed at the Department of Pharmacology INRA, 180 chemin de Tournefeuille, B.P. 3, 31931 Toulouse Cédex, France.

Abbreviations: PAF, platelet-activating factor, 1-*O*-alkyl-2-acetyl-*sn*-glycero-3-phosphocholine; MMC, migrating myoelectric complex.

the duodeno-jejunum, using a previously described technique (3). After one week, the experiments were performed in animals that were fasted for at least 8 hr, but were given free access to water. In the first series of experiments animals received *E. coli* endotoxin (S.O111:B4, Sigma, La Verpillière, France) injected intravenously in 0.2 mL saline at a dose of 50 μg/kg. Alternatively, animals were submitted to intraperitoneal administration of 0.2 mL saline containing PAF (Sigma, La Verpillière, France) at a dose of 25 μg/kg. Control animals received 0.2 mL saline. In the second series of experiments, BN 52021, a specific PAF antagonist, was dissolved in 0.2mL saline and injected intravenously at a dose of 50 mg/kg, 5 min before PAF (25 μg/kg *i.p.*) or endotoxin (50 μg/kg *i.v.*). BN 52021 was a gift from IPSEN/IHB Laboratories (Le Plessis-Robinson, France). In the last series of experiments, similar administrations of endotoxin and PAF were performed 10 min after intraperitoneal administration of indomethacin (Interchim, Montluçon, France), a cyclooxygenase inhibitor, dissolved in 0.2 mL $NaHCO_3$ (5%) and injected intraperitoneally at a dose of 10 mg/kg.

Myoelectrical activity of the small intestine was recorded using an electroencephalograph (Reega VIII, Alvar, Paris). Identification of the intestinal motor pattern was facilitated by the summation of spiking activity every 20 seconds (4). Effects on intestinal motility were evaluated by measuring the time elapsed from the injection to the recovery of a normal fasted pattern, *i.e.*, migrating myoelectric complexes (MMC) on integrated electromyographic records. Values are means ± S.D. These values were compared using one- or two-way ANOVA, followed by Student's *t*-test for fasted values.

RESULTS AND DISCUSSION

The myoelectric activity of conscious, 8 hr-fasted rats is characterized by the occurrence of MMC having a mean period of 16.2 ± 5.8 min. Each MMC consisted of irregular spiking activity (phase II) lasting 4–6 min, followed by a short (4–5 min) period of intense activity called regular spiking activity, or phase III. These two periods of activity were separated by a quiescent (phase I) period (Fig. 1).

Animals were injected *i.v.* with 50 μg/kg *E. coli* endotoxin. This resulted in a disruption of the MMC pattern, which was replaced by a continous spiking activity for 112.6 ± 26.8 min (Fig. 1). Such a disruption appeared within 20 min after injection. PAF administered *i.p.* at a dose of 25 μg/kg induced a transient inhibition of the spiking activity immediately followed by a long-lasting

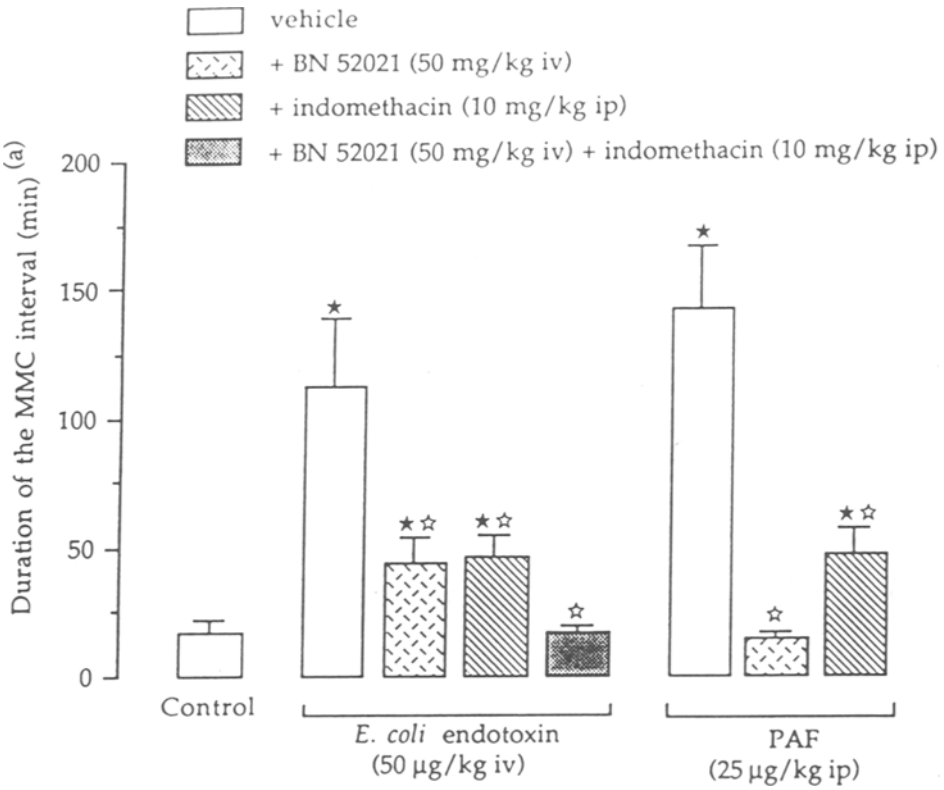


FIG. 2. Protective effects of BN 52021 (50 mg/kg *i.p.*), indomethacin (10 mg/kg *i.p.*) and a combination of both on endotoxin and PAF induced disruption of the MMC pattern in fasted rats. Values are means ± SD (n=6). A closed star and an open star indicate that values are significantly different from control and vehicle values, respectively. [a]Duration of the MMC interval was measured from the last phase III before to the first phase III after treatment at the jejunal level.

disruption of the MMC pattern for 142.7 ± 24.5 min (Fig. 1).

BN 52021, injected *i.v.* at a dose of 50 mg/kg 5 min prior to *i.p.* administration of PAF, abolished the effects of PAF on the intestinal myoelectric activity (Fig. 2). When BN 52021 was injected 5 min before endotoxin, the period of endotoxin-induced continous spiking activity persisted, but its duration was significantly reduced from 112.6 ± 26.8 min to 43.3 ± 10.7 min (Fig. 2). Injected 10 min before endotoxin, indomethacin (10 mg/kg *i.p.*) also significantly shortened the MMC disruption (Fig. 2). The same dose (10 mg/kg) also reduced the PAF induced intestinal myoelectric alterations (Fig. 2). A combination of indomethacin (10 mg/kg *i.p.*) and BN 52021 (50 mg/kg *i.v.*) injected 10 and 5 min before endotoxin, respectively, abolished the effects of endotoxin on the intestinal MMC pattern (Fig. 2).

Because of the similar effects of PAF to those of endotoxin, PAF is suspected to play a role in endotoxic shock. Our findings indicate that *i.v.* administration of endotoxin abolishes the normal pattern of intestinal myoelectric activity. The present work demonstrates that PAF is also able to induce a disruption of the MMC pattern. Furthermore, BN 52021 has been shown to affect endotoxin-induced lethality (5) or to prevent the hypotension induced upon PAF administration (6). In our model of endotoxic shock, BN 52021 appeared to be able to reduce endotoxin-induced alterations of the MMC pattern, consistent with an involvement of PAF in *E. coli* endotoxin-induced intestinal motor disturbances. In our experiments, PAF and endotoxin induced alterations are reduced by prior administration of indomethacin, a cyclooxygenase inhibitor, suggesting that prostaglandins are involved in endotoxin-induced motility alterations, but are also implicated in the action of PAF on the intestinal motor pattern. Prostaglandins appear to be involved in at least two levels, because indomethacin reduced PAF induced motor alterations, and a combination of indomethacin and BN 52021 abolished the effects of endotoxin.

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REVIEW

Platelet-Activating Factor and Granulocyte-Mediated Oxidative Stress. Strategy for *in vivo* Oxyradical Visualization[1]

Makoto Suematsu and **Masaharu Tsuchiya***
Department of Internal Medicine, School of Medicine, Keio University, Tokyo 160, Japan

Platelet-activating factor (PAF) and leukotriene B_4 are potent lipid mediators of endothelium-granulocyte interaction which results in granulocyte-dependent increased microvascular permeability. The specific mechanism by which PAF induces granulocyte-mediated endothelial injury has not been fully investigated. Digital imaging photonic intensified microscopy has revealed that PAF effectively induces granulocyte-mediated oxidative stress on microvascular beds. The method has made it possible to visualize luminol-dependent photonic burst released from PAF-treated microvascular beds in the rat mesentery. The photonic activities clearly corresponded to the localization of sticking granulocytes in post-capillary venules. By contrast, no significant chemilumigenic response could be detected in the leukotriene B_4-induced activation of endothelium-granulocyte interaction *in vivo*. The present findings suggest that oxygen radicals are not prerequisites for granulocyte adhesion in the microcirculation and provide evidence for a dissociation of *in vivo* granulocyte function between leukotactic and oxidative activation in leukotriene B_4-induced microvascular changes.
***Lipids 26,* 1362–1368 (1991).**

Platelet-activating factor (PAF) is formed by many different cells and is believed to contribute to allergic and inflammatory processes (1,2). PAF shows a broad spectrum of pharmacological effects including platelet aggregation, granulocyte activation, bronchoconstriction, and increased microvascular permeability. The effect of PAF on hyperpermeability in microvascular beds is thought to be mediated by both granulocyte-independent and granulocyte-dependent mechanisms (3). In the latter mechanism, granulocytes are thought to adhere to the venular endothelial surface and in an unknown way induce macromolecular leakage. Björk and coworkers (3,4) first reported that pretreatment with anti-neutrophil sera reduced PAF-induced microvascular hyperpermeability in hamsters, which suggested the involvement of permeability-altering mediators derived from sticking granulocytes.

PAF is known to stimulate secretagogue activation of granulocyte *in vitro*, including the release of lysosomal enzymes, active oxygen metabolites, and leukotrienes (5). Among these mediators, active oxygen metabolites have attracted considerable interest as a tissue breakdown factor during acute inflammatory processes. Superoxide anion (O_2^-) is the primary product derived from NADPH oxidase (6) from which hydrogen peroxide (H_2O_2), hydroxyl radicals (•OH) and hypochlorous anions (ClO^-) are thought to be subsequently generated (7). Although there is a great deal of information on the mechanism by which granulocytes release active oxidants, whether oxidative activation of granulocytes actually occurs during inflammatory processes *in vivo* has not been fully established. Most observations that have suggested participation of active oxidants in tissue injury are based upon the preventive effects of antioxidants such as superoxide dismutase (SOD), catalase, or dimethyl sulfoxide (DMSO). It has been difficult to obtain direct evidence for oxidative stress that occurs during endothelium-granulocyte interaction in the disturbed microcirculation. Because oxygen radicals are highly reactive and short-lived compounds which react with the nearest target molecule, the cell membrane of endothelial cells is believed to be the primary site of granulocyte-mediated oxidative metabolite interaction (8). However, it has been technically impossible to evaluate spatial and temporal alterations of oxidant generation even *in vitro*.

This report is intended to summarize current methodology to *visualize* oxidative stress *in vitro* and *in vivo*, which may help in clarifying the topographic relationship between oxidant-generating sources and damaged sites. As an application, we discuss the role of PAF in granulocyte-mediated oxidative stress in microcirculatory disturbances.

CURRENT APPROACHES FOR OXYRADICAL VISUALIZATION

Chemical probes. Chemical probes intended to obtain spatial information on oxidative stress in biological systems require certain properties. An electron microscopic technique and use of cerium derivatives ($CeCl_3$) has first revealed the location of H_2O_2 in granulocytes stimulated by zymosan particles or soluble stimuli such as phorbol myristate acetate (PMA) or bacterial chemotactic peptide (FMLP). Karnovsky and coworkers (9) reported the spatial difference of H_2O_2 generation between PMA- and zymosan-treated cells. When granulocytes were stimulated by zymosan particles, H_2O_2-generating sites were observed to distribute in the contact surface between particles and the phagosome-forming membrane of granulocytes. By contrast, granulocytes stimulated by soluble stimuli produced a diffuse pattern of cerium deposits surrounding the entire membrane. Through this technique, the

[1] Based on a paper presented at the Third International Conference on Platelet-Activating Factor and Structurally Related Alkyl Ether Lipids, Tokyo, Japan, May 1989.

*To whom correspondence should be addressed at Department of Internal Medicine, School of Medicine, Keio University, 35 Shinanomachi, Shinjuku-ku, Tokyo 160, Japan.

Abbreviations: AO, acridine orange; ClO^-, hypochlorous anion; DCF, dichlorofluorescein; DCFH, dichlorofluorescin; DMSO, dimethyl sulfoxide; FMLP, *N*-formylmethionylleucylphenylalanine; LTB_4, leukotriene B_4; NBT, nitroblue tetrazolium; O_2^-, superoxide anion; PAF, platelet-activating factor; PMA, phorbol myristate acetate; SOD, superoxide dismutase.

oxyradical distribution during intercellular reaction between phagocytes and parenchymal cells can be investigated. Vissers *et al.* (10) clearly demonstrated that oxyradical burst could actually occur in the non-phagocytosable contact surface between granulocytes and the glomerular basement membrane *in vitro*. The electron microscopic approach helps to shed light on the topographic relationship between the oxidant-generating effector and target cells. However, it can not adequately trace the time history of oxidative stress.

Oxyradical-sensitive chemical probes may be useful tools for the analysis of the spatial distribution and temporal alteration of oxidant generation *in vitro* and *in vivo*. Table 1 lists previous reports which have provided quantification of oxidant generation from a single phagocyte through a microscope. The goal of these investigations is to clarify the spatial distribution of oxidant release and the heterogeneity of oxidative reactivity among cells. DiGregorio *et al.* (11) applied nitroblue tetrazolium (NBT), a chemical probe which is oxidized by O_2^- to form insoluble blue deposits (formazan), at the site of O_2^- generation. To determine the O_2^- generation, they quantified the optical turbidity of cells which was caused by formazan deposits by microscopic videodensitometry.

Another probe to visualize oxidant activities is dichlorofluorescin (DCFH), the usefulness of which was first established by Cathcart *et al.* (12). This method is based upon the original work of Keston and Brandt (13), in which DCFH is oxidized to the fluorescent, dichlorofluorescein (DCF) by H_2O_2 and peroxidase (13,14). Bass *et al.* (15) quantified intracellular oxidizing species in individual granulocytes using the ester-type precursor, DCFH diacetate. This reagent enters living cells to be catalyzed into DCFH by esterase. The technique has been applied to determine inorganic and organic hydroperoxide formation in renal epithelial cells (16) and hepatocytes (17). Using the fluorochrome, we have recently investigated the intralobular heterogeneity of carbon tetrachloride-induced hydroperoxide formation in perfused rat liver (18). The DCF-associated fluorescence was predominantly enhanced in the pericentral area, and was attenuated by SKF-525A, a potent inhibitor of cytochrome P-450. In order to use this technique for quantitative observation of oxidative stress *in vivo*, technical difficulties in fluorescence quantification such as a correction for photobleaching attenuation need to be solved (19).

Digital imaging photonic microscopy and chemiluminescence imaging. Besides the use of $CeCl_3$ and DCFH, the third strategy for oxidant visualization is the chemiluminescence method. Compared with the fluorescence method, this technique has several advantageous properties which prove useful for evaluation of oxidative stress *in vivo*: (i) the absence of a photobleaching effect; (ii) no need for excitation illumination, resulting in attenuation of cellular damages; (iii) availability of different chemilumigenic probes which makes it possible to pinpoint the active oxidants that are involved in different experimental models. However, the chemilumigenic response induced by the release of oxidants is generally too weak to be observed as a visible image even in the presence of chemiluminescence sensitizers. A special device for the low-level chemiluminescence visualization and an analytical evaluation is therefore required.

The objective of the conventional chemiluminescence study is to count oxidant-associated scintillating photonic activity by a photomultiplier. Since Tsuchiya *et al.* (20) first developed a two-dimensional photon-counting system equipped with a computer-assisted digital image processor, recent work with this system was aimed at the collection of quantitative information about the spatial distribution of chemiluminescence within a single cell. Figure 1 shows a system diagram of the ultrasensitive photonic image intensifier (C-2400-20, Hamamatsu Photonics, Hamamatsu, Japan). When an optical image from a microscope is focused on the bialkali photocathode in the front portion of camera head, photoelectrons are emitted in proportion to the intensity of the microscopic image. These photoelectrons are amplified in a 2-stage microchannel plate by a million-fold and transmitted to a phosphor screen. The scintillating photonic images on the screen are visualized by a low-lag vidicon and then digitally processed.

One major problem is the specificity of chemilumigenic probes. For example, the probe luminol is widely used to determine oxyradical generation from phagocytes. Using ultrasensitive photonic image intensified microscopy and a computer-assisted image processing device, we investigated the mechanism of luminol-dependent photoemission derived from single human neutrophils (21). This technology has revealed spatial differences of respiratory burst, heterogeneity of oxidative activation among neutrophils, and the crucial role of extracellular release of myeloperoxidase in this type of chemilumigenic reaction. When neutrophils were stimulated by opsonized zymosan, luminol-dependent photonic burst clearly corresponded to the distribution of cells in the microscopic field and each

TABLE 1

Chemical Probes for Measurement of Oxidative Activation in Single Cells

Probe	Specificity	Analyzing device	References
Nitroblue tetrazolium	O_2^-	videodensitometry	(11)
Dichlorofluorescin diacetate	H_2O_2 or hydroperoxides	fluorescence intensitometry or fluorescence-associated cell sorting	(12,16,17) (15)
Luminol	myeloperoxidase-H_2O_2-Cl^- system	single photon counting and imaging	(21,23,27)

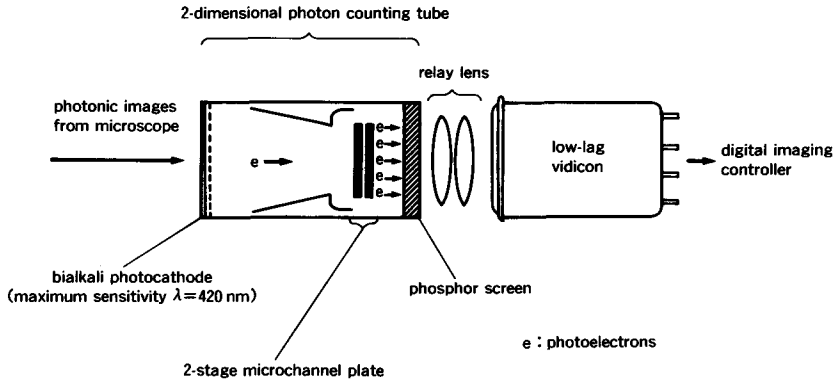


FIG. 1. System diagram of an ultrasensitive photonic image intensifier. The maximum response of bialkali photocathode to generate photoelectrons is ranged at approximately 420 nm, nearly corresponding to the maximum photoemission band in luminol-dependent chemiluminescence.

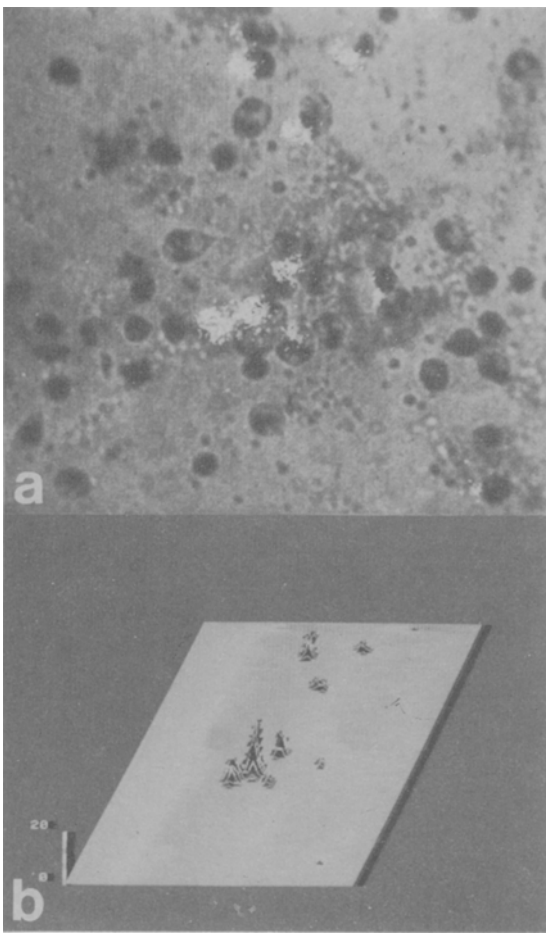


FIG. 2. Imaging of luminol-dependent chemiluminescence generated from zymosan-stimulated neutrophils. (a) Accumulated photonic frame superimposed upon the cellular image. (b) Three-dimensional display of photonic intensity. (From ref. 21, with permission.)

chemilumigenic site was observed in a restricted region close to each active cell (Fig. 2). These photonic activities were completely attenuated by sodium azide or catalase, and were enhanced by adding exogeneous myeloperoxidase (22). It is therefore likely that extracellular and/or pericellular release of myeloperoxidase-associated oxidants such as ClO^- is involved in the luminol-dependent photoemission. To further elucidate the role of azulophilic degranulation and the extracellular release of myeloperoxidase, luminol-dependent chemiluminescence from neutrophils stimulated by PMA and calcium ionophore A 23187 was visually investigated (23). Neutrophils stimulated by PMA alone produced only a negligible level of photonic activities in the presence of luminol. The addition of calcium ionophore induced explosive changes of photonic burst which was closely correlated with the cellular distribution in the image. The photonic activities gradually spilled from the cells into the extracellular space. These findings suggest that degranulation and myeloperoxidase release is a prerequisite in luminol-dependent photoemission from stimulated neutrophils. This type of chemiluminescence was not detectable in neutrophils of patients with chronic granulomatous disease (complete cytochrome b558 deficiency) or myeloperoxidase deficiency (24). Taken together with these data, luminol was proposed to be a useful chemilumigenic probe to detect active oxidants derived from the H_2O_2-myeloperoxidase-chloride system.

IN VIVO OXYRADICAL VISUALIZATION DURING PAF-INDUCED MICROVASCULAR DAMAGE

Intravital microscopy is an indispensable technique to evaluate the dynamics of endothelium-granulocyte interaction *in vivo* (25). By using an intravital microscope equipped with an ultrasensitive photonic intensifier system, we observed spatial distribution and temporal changes of oxyradical-dependent chemilumigenic sites in damaged microvascular beds. Male Wistar rats were anesthetized and the ileocecal portion of the mesentery was carefully exposed and mounted on a plastic stage. The stage bottom was made of a non-fluorescent thin cover glass according to our methods described previously (21). The

preparation was continuously superfused (0.5 mL/min) with Krebs-Ringer bicarbonate-buffered solution. The dynamics of the mesenteric microcirculation was observed and visualized through an inverted microscope incorporated with a digital imaging photonic intensified system (C-2400-20 / ARGUS, Hamamatsu Photonics). After completing the preparation procedure, the luminol-containing Krebs-Ringer solution (final concentration 1.0 μg/mL) was superfused. After a control period of 10 min, the solution of vasoactive lipid mediators (PAF-acether or leukotriene B_4) was added to the superfusion buffer. The luminol-dependent chemiluminescence activity detected by the C-2400-20 through the intravital microscope was observed in a light-excluding chamber at 37°C. Real-time scintillating photonic activities were digitally accumulated for 180 sec and stored in a video frame memory of the image processor. Microvascular images were also recorded by using a transmission light source of the microscope immediately after the 180-sec photon-counting period. The stored photonic image was superimposed upon the vascular image to identify oxyradical-generating sites in the microvasculature. Temporal alterations of photonic activities were automatically calculated in preselected area of each microscopic field containing a 100 μm segment of a post-capillary venule (40 μm in diameter).

The photonic intensity derived from the microvascular segment is thought to depend on both the oxyradical-generating activity of individual granulocytes and the population of sticking cells. We therefore determined the number of sticking granulocytes in the preselected venular segment using acridine orange-associated fluorographic analysis (26). The acridine orange solution (0.1%, 0.2mL) was administered intravenously and the intravital fluorescence image in the microvascular segment was visualized by a silicon intensified target camera (C-2400-08, Hamamatsu Photonics).

Figure 3 indicates a representative study of endothelium-granulocyte interaction induced by leukotriene B_4 (100 nM superfusion for 5 min). The same concentration of PAF-acether also induced significant activation of granulocyte sticking in post-capillary venules (Fig. 4). These findings reflect the powerful stimulating effect of these reagents on granulocyte adherence as reported previously (3,4).

By contrast, the intravital photonic responses showed quite different pictures among the two groups. Figure 5

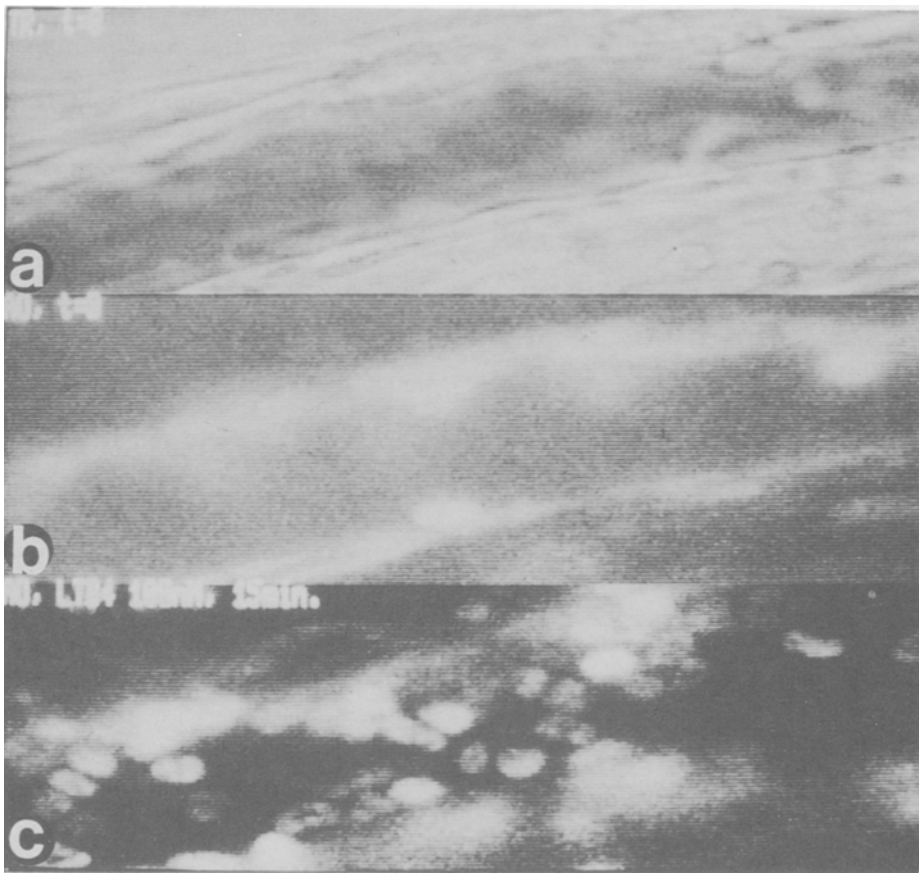


FIG. 3. Leukotriene B_4-induced granulocyte adherence in rat mesenteric microcirculation. Leukotriene B_4 (LTB_4) was superfused at 100 nM for 15 min. After the intravenous injection of acridine orange (AO) solution, rolling and sticking granulocytes were observed as spheroid-shaped fluorogenic cells in post-capillary venules. (a) A representative image of a venular segment visualized by transillumination (TR). (b) AO-associated epifluorescence image of the same venular segment before LTB_4 superfusion. (c) Granulocyte adherence induced by the LTB_4 superfusion for 15 min.

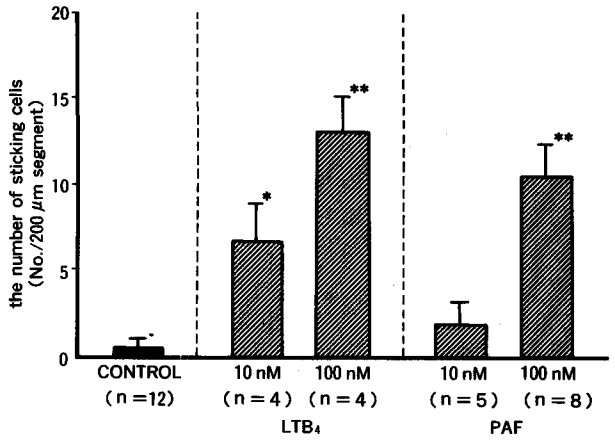


FIG. 4. *In vivo* morphometric analysis of the number of sticking granulocytes in rat mesenteric venules (200 μm segment, 40 μm in diameter) after 15 min superfusion of leukotriene B_4 or PAF-acether (PAF). Acridine orange-positive spheroid cells adhering to the endothelium and remaining in the same position for 1 min were considered to be 'sticking cells'.

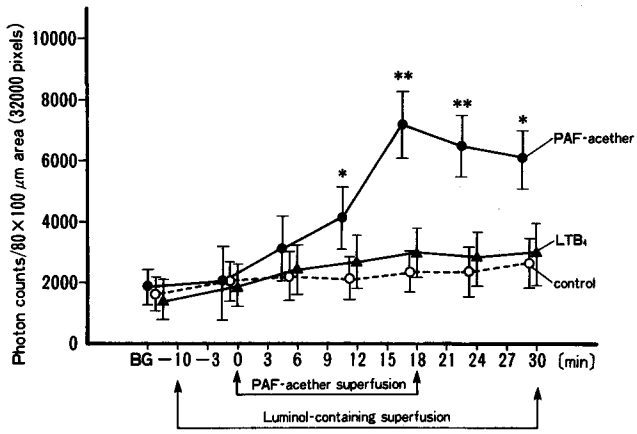


FIG. 6. Effects of PAF-acether and leukotriene B_4 (LTB_4) on luminol-dependent chemiluminescence activities derived from the preselected microscopic field (80 μm × 100 μm) containing a 100-μm venular segment. BG denotes background photonic activities before experiments in a light-excluding chamber. The photon-accumulating period is 180 sec, common in all experiments. Mean ± S.D. of 6 experiments. *$p<0.05$ and **$p<0.01$ as compared to the control.

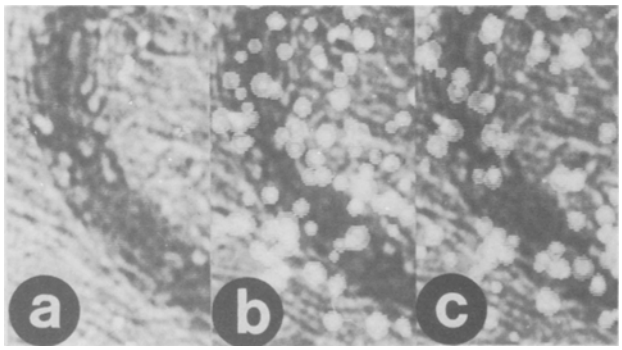


FIG. 5. Temporal and spatial alterations of luminol-dependent photoemission during endothelium-granulocyte interaction in a post-capillary venule treated with 100 nM PAF-acether. Parts a, b and c represent photonic images superimposed on microvascular frames in 3–6 min (a), 15–18 min (b) and 21–24 min (c) after starting the PAF-acether superfusion. (From ref. 27 with permission.)

represents temporal and spatial alterations of luminol-dependent photoemission derived from the venular segment treated with PAF-acether (100 nM). Six minutes after the start of PAF-acether superfusion, remarkable changes in granulocyte adherence were observed, but no significant elevation of chemiluminescence was detectable. However, the photonic activities gradually increased and their spatial distribution clearly corresponded to the localization of intravascular sticking cells (27). The photonic intensitometric analysis by the digital image processor indicated that approximately 15–18 min were required to obtain the maximum photonic counts. It was concluded that oxyradical-dependent photoemission in the luminol-perfused and PAF-treated microvascular beds reflects active oxidants derived from sticking and migrating granulocytes for the following reasons: (i) Luminol-dependent photoemission from PAF-stimulated granulocytes *in vitro* was attenuated by chlorpromazine, which is known to inhibit O_2^- generation by competing with oxygen for NADPH oxidase (28). Catalase (1000 units/mL) or sodium azide (1.0 mM) completely attenuated the photoemission, suggesting participation of the H_2O_2-myeloperoxidase-chloride system in this type of photoemission (27). (ii) The sites of photonic burst clearly corresponded to the location of sticking granulocytes in post-capillary venules. (iii) Luminol-dependent photoemission *in vivo* from sticking cells was also suppressed by the topical application of chlorpromazine or sodium azide (27). These results suggest that PAF is a powerful lipid mediator of endothelium-granulocyte interaction ensued by granulocyte-mediated oxidative stress *in vivo*.

By contrast, leukotriene B_4 did not cause any significant photonic activation at a concentration of 100 nM which is high enough to induce granulocyte adherence in post-capillary venules (Fig. 6). Although the luminol-dependent photoemission does not directly reflect O_2^- production in sticking granulocytes, our results may imply that secretagogue activation of the H_2O_2-myeloperoxidase-chloride system in granulocytes is not apparent in leukotriene B_4-induced leukotactic activation *in vivo*. The present findings raise the possibility that oxidative activation of granulocytes may not be a prerequisite for endothelium-granulocyte interaction in disturbed microvascular beds. The independence of granulocyte diapedesis from oxidant production is supported by the fact that NADPH oxidase-deficient granulocytes in patients with chronic granulomatous disease are accumulated in pus at the sites of infection (29). This idea is also consistent with the previous report by Harlan *et al.* (30) which demonstrated that granulocytes mediate the rapid nonlytic disruption of the endothelial monolayer integrity by an oxygen radical-independent mechanism.

ROLES OF PAF AND LEUKOTRIENE B_4 IN GRANULOCYTE-MEDIATED OXIDATIVE STRESS DURING MICROVASCULAR DAMAGE

Leukotaxis-inducible doses of leukotriene B_4 did not cause any significant activation of the photoemission from microvascular beds. Most inflammatory mediators with

chemotactic properties *in vitro* are known to increase microvascular permeability *in vivo* through a process entirely or partly dependent on granulocytes. Although the exact mechanism by which granulocytes damage the microvascular integrity remains unknown, recent studies have suggested the involvement of oxygen radicals in endothelial damages (31,32). Leukotriene B_4 is a potent chemotactic agent for granulocytes, and in high concentrations it can induce an oxyradical burst (33). It has also been reported that O_2^- and H_2O_2 may play a role in leukotriene B_4-induced granulocyte chemotaxis *in vitro* (34). However, there have been few reports suggesting that leukotriene B_4 may stimulate oxidant generation in adherent granulocytes *in vivo*. Rosengren *et al.* (35) demonstrated that SOD and/or catalase does not influence the leukotriene B_4-induced microvascular permeability and granulocyte sticking in the hamster cheek pouch microcirculation. This suggests that oxygen radicals may be not crucially involved in endothelium-granulocyte interaction and the resultant microvascular permeability changes. Our results showing the failure of leukotriene B_4 to produce photonic activities in sticking granulocytes *in vivo* provide evidence for a functional dissociation between leukotactic and secretagogue activation in leukotriene B_4-stimulated granulocytes.

The mechanism by which PAF-acether causes an increase in microvascular permeability seems different from that involved in leukotriene B_4-induced hyperpermeability. As shown in experiments with animals treated with a specific anti-neutrophil serum (36), the leukotriene B_4-induced plasma leakage is almost exclusively granulocyte-dependent. By contrast, PAF has been reported to cause a direct vasoactive effect on the microvascular endothelium. Humphrey *et al.* (37) have demonstrated that picomolar levels of PAF-acether induce an early, transiently increased microvascular permeability and that PAF-induced vasoactivity occurs in the absence of granulocyte, platelet or mast cell stimulation and during H_1 histamine blockade. With increasing amounts of PAF-acether, early granulocyte infiltration followed by relatively prolonged activation of vascular permeability has been documented (38). According to Kubes *et al.* (39), PAF-induced intestinal microvascular hyperpermeability was *partially* attenuated by pretreatment with a monoclonal antibody (IB_4) against the leukocyte adhesive glycoprotein complex. It should be noted that SOD attenuates the PAF-induced granulocyte adherence on the venular endothelium without changing microvascular hemodynamics, suggesting the involvement of O_2^- which may be generated at the interface between the venular endothelium and adherent granulocytes in PAF-induced granulocyte diapedesis (40). Taken together with these previous reports, the present study demonstrates that active oxidants from the H_2O_2-myeloperoxidase-halide system (*e.g.* ClO^-) may be possible candidates for PAF-induced and granulocyte-dependent microvascular hyperpermeability.

Our recent data showed that the PAF-induced granulocyte adherence and photonic burst are attenuated by pretreatment with AA-861, a specific 5-lipoxygenase inhibitor (41). This suggests involvement of leukotrienes in granulocyte-mediated oxidative stress in PAF-treated microvascular beds, and is supported by *in vitro* experiments showing the preventive effect of 5-lipoxygenase inhibitor on PAF-induced granulocyte chemotaxis through cultured endothelial cell monolayers (42). As shown previously, leukotriene B_4 *alone* did not show any significant photoemission at the sites of endothelium-granulocyte interaction. The potent stimulatory effect of PAF on the luminol-dependent photoemission from sticking granulocytes *in vivo* may be due to the concurrent action of PAF and endogenous leukotriene B_4, which is subsequently released from sticking granulocytes. Since H_2O_2 is known to stimulate PAF generation in umbilical venous endothelium *in vitro* (43), the exogenous PAF-induced granulocyte-mediated oxidative stress may stimulate the endothelium-dependent endogenous PAF generation at the sites of granulocyte adherence. Further *in vitro* investigations using cultured endothelium and granulocytes will be required to confirm this scenario. However, the reciprocal activation between venular endothelium and granulocytes *via* lipid mediators (PAF *plus* leukotriene B_4) may play an important role in secretagogue activation of sticking granulocytes in the PAF-treated microvascular beds.

In conclusion, the present findings which were obtained by digital imaging photonic intensified microscopy support the following concepts: (i) Oxygen radicals are not a prerequisite for endothelium-granulocyte interaction, and (ii) PAF is a potent lipid mediator of granulocyte-mediated oxidative stress upon venular endothelial integrity. Since our methods using luminol-dependent chemiluminescence can evaluate myeloperoxidase-mediated oxidants, it seems difficult to determine the direct activity of O_2^- produced by other oxidant-generating sources such as xanthine oxidase in endothelial cells. To determine and visualize intravital O_2^- generation, development of better chemilumigenic probes with O_2^--specific reactivity (44), high photonic efficiency, and low-level background photoemission are needed. Although the development of such an ideal chemilumigenic probe will be difficult, it would provide further insights into the cellular and subcellular mechanisms through which oxygen radicals exert their cytotoxic effects on tissues during microcirculatory disturbances.

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REVIEW

Involvement of Platelet-Activating Factor (PAF) in Septic Shock and Priming as Indicated by the Effect of Hetrazepinoic PAF Antagonists[1]

Hubert O. Heuer*
Department of Pharmacology, Boehringer Ingelheim KG, W-6507 Ingelheim/Rhein, Germany

Pharmacological data obtained with hetrazepinoic platelet-activating factor (PAF) antagonists, such as apafant (WEB 2086) and bepafant (WEB 2170), indicate a role for PAF in septic shock and in the priming process. The effect of PAF antagonists in different models of shock states favors a role for PAF in endotoxin associated lethality, activation of inflammatory blood cells with release of mediators, cardiovascular failure and increased vascular permeability, and in the development of shock organs and organ failure. The priming process (*e.g.*, by endotoxin or tumor necrosis factor) towards an increased susceptibility towards minute amounts of PAF has to be taken into account when considering the pathophysiological significance of PAF under *in vivo* conditions and in septic shock.
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There is substantial evidence that platelet-activating factor (PAF) is one of the main mediators of shock states, including endotoxin-induced shock. PAF can induce most of the pathophysiological features associated with endotoxin shock, such as pulmonary hypertension and edema, cardiac failure, alteration of the microcirculation resulting in an increase in vascular permeability and tissue damage, activation of immune cells with associated mediator release, and systemic hypotension (for a review, see ref. 1). Figure 1 summarizes the pathophysiological derangements which can be mimicked by exogenously introduced PAF (1). Increased levels of PAF-like activity have been detected in blood from endotoxin-treated animals (2,3) and in septic patients (4–6). Tumor necrosis factor (TNF) can mimic most of the pathophysiologic features of endotoxin (7,8), and TNF has been shown to release PAF from endothelial and other cells (9).

Selective and potent PAF antagonists can be used to demonstrate a causative role of PAF in septicemia and septic shock. Partial effectiveness in animal models of septicemia suggests a contributory role of PAF in the pathophysiology, including interaction with other mediators.

The purpose of this synopsis is to summarize and evaluate the data available so far for hetrazepinoic PAF antagonists, exemplified by apafant (WEB 2086), bepafant (WEB 2170) and other hetrazepines (reviewed in refs. 10 and 11) in animal models of septic shock.

[1]Based on a paper presented at the Third International Conference on Platelet-Activating Factor and Structurally Related Alkyl Ether Lipids, Tokyo, Japan, May 1989.

*Present address: Boehringer Ingelheim Pharmaceuticals, Inc., Dept. of Pharmacology, 900 Ridgebury Rd, Ridgefield, CT 06877-0368.

Abbreviations: ARDS, adult respiratory distress syndrome; PAF, paf-acether, platelet-activating factor, 1-*O*-alkyl-2-acetyl-*sn*-glycero-3-phosphocholine; TNF, tumor necrosis factor.

LETHAL EFFECT OF ENDOTOXIN AND/OR TUMOR NECROSIS FACTOR (TNF)

The role of PAF in the lethal effect of endotoxin, as indicated by a protective effect of selective PAF antagonists, including hetrazepines, remains somewhat controversial. Variable results may have been obtained because different animal species were used in different studies, and the routes of endotoxin administration varied. The kinetics, potency and bioavailability of PAF antagonists also differ. The lethal effect of endotoxin from *E. coli* in rats is inhibited dose-dependently by oral and intravenous treatment with WEB 2086 (12). Also, post-treatment with WEB 2086, starting at 2 hr after administration of endotoxin from *Salmonella enteritidis*, significantly improves survival as compared to vehicle treatment (13).

Animal susceptibility to endotoxin may be different even within the same species depending upon the age of the animals, and the dose and route of endotoxin administration. Although Balb/C mice seem to be sensitive to the lethal effect of endotoxin (14), younger Balb/C-mice do not have a high mortality rate when 10 mg/kg *i.v.* endotoxin alone is given (15). In addition, the lethal effect of endotoxin when given alone takes longer to manifest itself in younger mice (15). Oral WEB 2170 protected Balb/C mice from the lethal effect of the endotoxin even when a high dose (10 mg/kg *i.v.*) is used (15).

Experimental protocols which would produce clear-cut and reproducible high mortality rates 8–24 hr after administration of endotoxin, possibly at low doses, are desirable. Three different approaches have been chosen in previous studies (15,16). Human recombinant TNF (0.04 mg/kg *i.v.*) synergizes with endotoxin to increase its lethal effect (15,16). Rothstein and Schreiber (17) found that when both endotoxin and TNF are used, cytotoxicity, hemorrhagic necrosis in the skin, and lethal shock increase. Oral WEB 2170 inhibited the lethal effect of a combination of endotoxin and TNF in a dose-dependent manner, which suggests that PAF plays a role in this model (Fig. 2; refs 15,16).

Propranolol (0.1–0.5 mg/kg *i.v.*) administered 8 hr after endotoxin increases the toxic (lethal) potency of the endotoxin (15,16). The failure of co-administered propranolol to potentiate the lethal effect of endotoxin suggests that during the time interval between the administration of endotoxin and of propranolol, endotoxin may prime the generation of shock mediators such as thromboxanes, cytokines, leukotrienes, peptides, and PAF. Propranolol synergizes with a number of mediators (18). The ability of the selective PAF antagonist WEB 2170 to antagonize the propranolol-precipitated lethal effect of endotoxin (15,16) indicates that PAF may play a key role in the release of mediators and their interaction *in vivo*. Furthermore, the ability of propranolol to enhance the lethal and

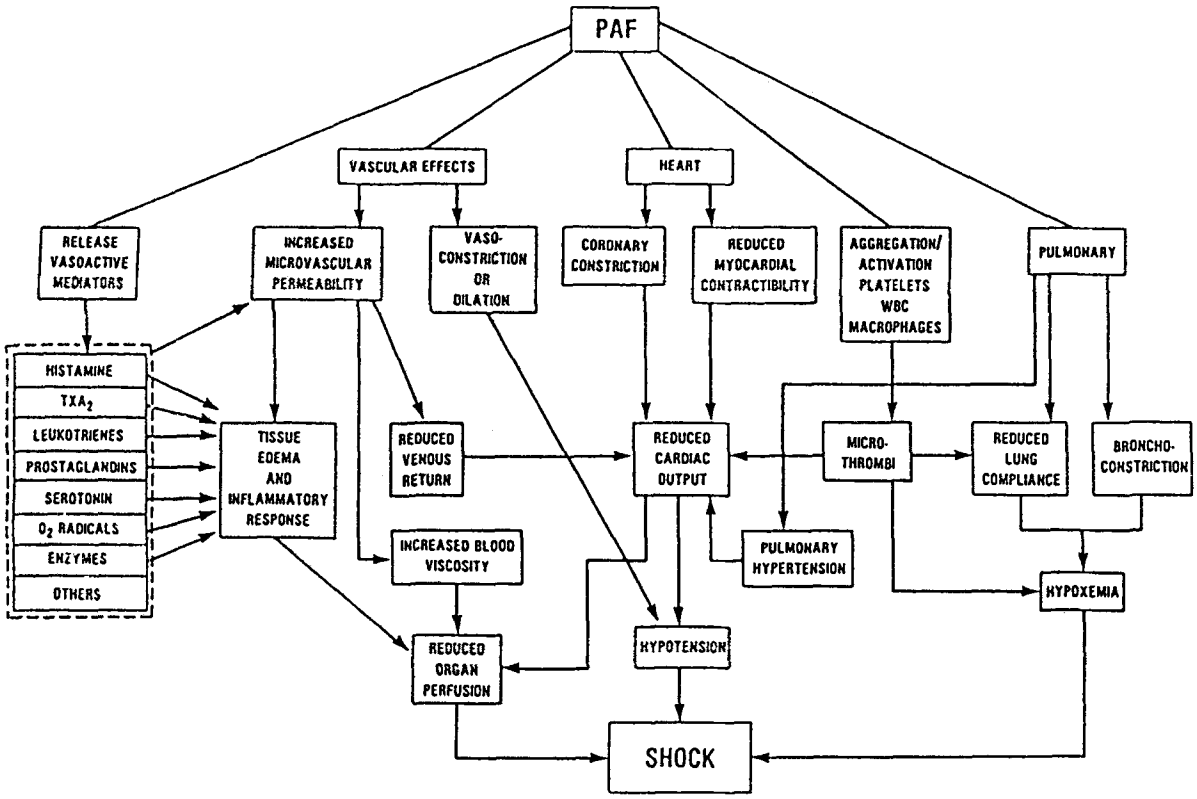


FIG. 1. Sequence of events and pathophysiology of PAF induced shock (adapted from ref. 1).

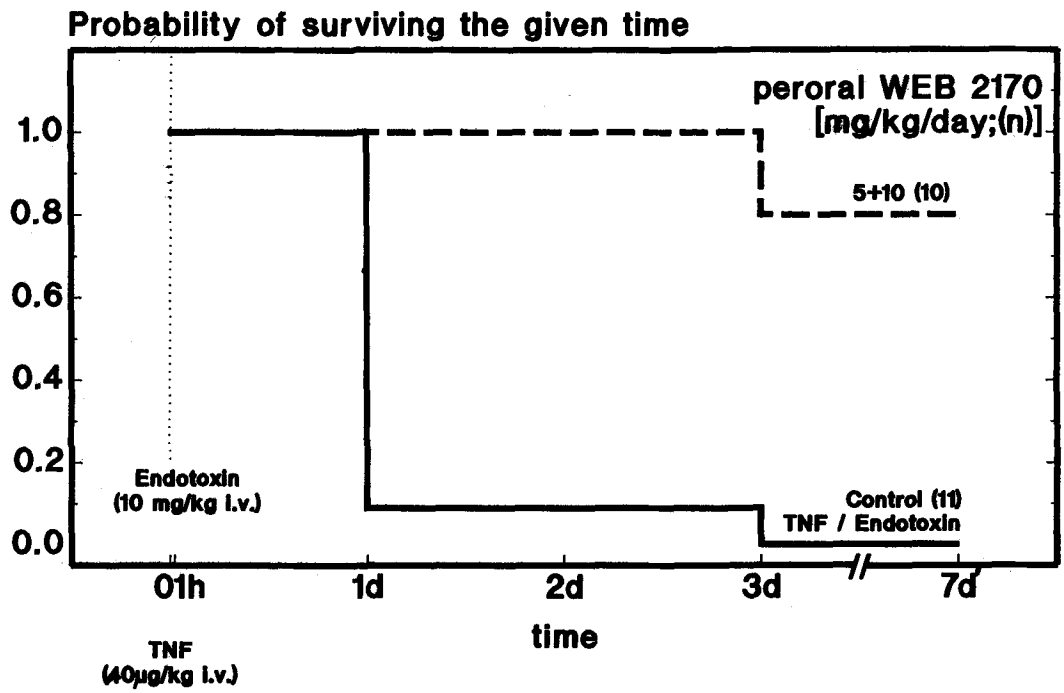


FIG. 2. Inhibition by oral WEB 2170 of the lethal effect of a combination of endotoxin (*E. coli* 0111:B_4, 10 mg/kg *i.v.*) and TNF (0.04 mg/kg *i.v.*). WEB 2170 was given in the morning and 8 hr later; 1 hr time interval between endotoxin and TNF; observation period, seven days; n is indicated in parentheses.

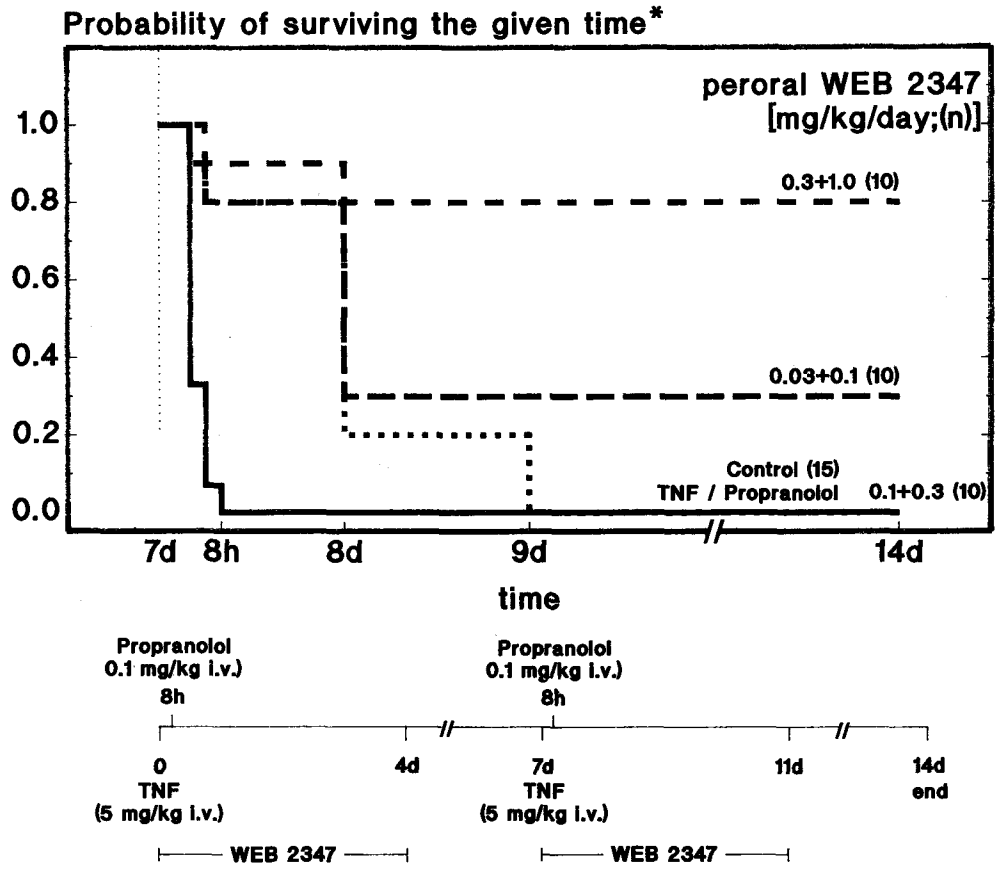


FIG. 3. Inhibition by oral WEB 2347 of the lethal effect of repeated human recombinant TNF/propranolol in Balb/C-mice; n indicated in parentheses.

toxic effects of exogenously introduced PAF (10,19) can be extended to endogenously released PAF *in vivo*; for example, the PAF antagonist, WEB 2170, blocks propranolol potentiated anaphylactic death in mice (16).

Although a single treatment with TNF and subsequent propranolol administration fails to have lethal effect on Balb/C-mice, it primes the mice for enhanced sensitivity to a second challenge with TNF (combined with subsequent administration of propranolol) one week later (15). This resembles a Sanarelli-Shwartzman-like phenomenon, which is induced here by TNF. The selective and very potent PAF antagonist [WEB 2347 (11)] protects Balb/C-mice from the lethal effect of TNF in the Sanarelli-Shwartzman-like phenomenon, which suggests a key role of PAF in this process (Fig. 3; ref. 15).

CARDIOVASCULAR EFFECTS OF ENDOTOXIN

The hypotensive action of endotoxin (from *E. Coli* 0111:B_4) in the rat is prevented by oral or intravenous treatment with WEB 2086, WEB 2170, or STY 2108 (12,20,21). In addition, therapeutic intervention at the nadir of endotoxin-induced hypotension (6 min after endotoxin) reverses endotoxin-induced hypotension in a dose-dependent fashion (12,21). In contrast to other pharmacological agents, PAF antagonists only increase blood pressure when the blood pressure is lowered by PAF or endotoxin. These results are in agreement with previously reported results for other PAF antagonists (2).

Tables 1 summarizes the ED_{50} values for prevention of endotoxin-induced hypotension by WEB 2086, WEB 2170, or STY 2108 (*p.o.*, *i.v.*) administered before endotoxin in comparison with those of the same drugs given after administration of endotoxin (12,21). The doses of PAF antagonists required to inhibit endotoxin-induced hypotension correlated with the doses needed to antagonize PAF induced hypotension in the rat (Table 2), which supports a specific involvement of PAF in this process.

In contrast, in endotoxin-induced shock, when WEB 2086 at the tested dose is given to pigs, the systemic hypotension occurring after infusion of endotoxin is not lessened (22). However, the pulmonary hypertension that occurred in the pig after infusion of endotoxin is significantly inhibited by treatment with WEB 2086 (22).

Perfusion of the hindlimb and systemic blood pressure in the rat are reduced by intravenous injection of 30 mg/kg *i.v.* endotoxin from *E. coli* 0111.B_4. Perfusion in the presence of WEB 2086 reverses the decreases in hindlimb perfusion and systemic blood pressure (23).

Endotoxin-induced microvascular leakage with edema formation is considered one of the earliest and most prominent symptoms in the development of adult respiratory distress syndrome (ARDS). WEB 2086 infused at 20 mg/kg/hr inhibits the endotoxin-induced lymph flow and

TABLE 1

ED_{50} Values of Hetrazepinoic PAF Antagonists Given Orally or Intravenously to Antagonize Hypotension Induced by Endotoxin from *E. coli*.

	Antagonism of endotoxin-induced hypotension[a]		
	Prophylactic[b] ED_{50} (mg/kg)		Curative[c]
	i.v.	*p.o.*	ED_{50} (mg/kg *i.v.*)
WEB 2170	0.099 (...-...)[d]	0.32 (0.001-0.6)	0.026 (0.001-0.084)
STY 2108	0.004 (...-...)[d]	3.51 (1.9-8.77)	0.085 (0.055-0.19)
WEB 2086	0.41 (0.052-1.17)	6.25 (2.42-...)[d]	0.34 (0.063-1.62)

[a]The ED_{50} values were calculated on the basis of the BP levels at the last measurement time point (20 min; in the case of a prophylactic effect) or 6 min after the "curative" administration of the PAF antagonist. ED_{50} values in comparison with WEB 2086; 95% confidence limits in parentheses.

[b]ED_{50} calculated 20 min after ET.

[c]ED_{50} calculated 6 min after curative administration of PAF antagonist.

[d]Confidence limit(s) could not be determined.

TABLE 2

ED_{50} Values of Hetrazepinoic PAF Antagonists Given Orally or Intravenously to Antagonize Hypotension Induced by PAF

	Antagonism of PAF induced hypotension[a]	
	i.v. Route ED_{50} = mg/kg *i.v.*[b]	Oral route ED_{50} = mg/kg *p.o.*
WEB 2086	0.061 (0.01-0.4)[b]	9.23 (7.89-10.8)
WEB 2170	0.016 (0.01-0.026)	0.26 (0.19-0.35)
STY 2108	0.011 (0.008-0.014)	0.52 (0.31-0.81)

[a]Inhibition of PAF induced [30 ng/(kg × min)] hypotension in the rat by hetrazepines given orally or intravenously; ED_{50}, effective dose of hetrazepine that antagonizes PAF induced hypotension by 50%.

[b]95% Confidence limits.

lymph protein clearance in conscious sheep (24). By contrast, WEB 2086 has little effect on early pulmonary hypertension and alterations in lung mechanics (24). In a recent study, Chang *et al.* (13) found that WEB 2086 inhibits lung vascular leakage not only when given before endotoxin but also when given 30 min after endotoxin. In the same study, post-treatment with WEB 2086, starting at 90 min after endotoxin, markedly attenuated the vascular lung leakage measured 6 hr after endotoxin (13).

In hypoxic pulmonary vasoconstriction in the rat, perfusion with WEB 2086 attenuated the pulmonary vasoconstrictor response in isolated and ventilated lung in a concentration dependent manner (25). The inhibition of the hypoxic pulmonary response is complete at the highest concentration (25). However, the involvement of PAF has not been fully clarified, as the result could not be reproduced in the pig (26).

In a traumatic shock model using anesthetized rats, WEB 2086 maintained a higher mean arterial blood pressure in traumatized rats than in rats receiving only physiologic saline (27). In addition, WEB 2086 increased the overall survival time of traumatized rats (27). Other indicators of traumatic shock, such as accumulation of lysosomal hydrolase, cathepsin D, and free amino-nitrogen compounds and increase of myocardial depressant factor were also inhibited by WEB 2086 (27).

The hetrazepine related benzodiazepine receptor antagonist and weak PAF antagonist, Ro 15-1788, had beneficial effects in a rat model of hemorrhagic shock (28). The specific PAF antagonist WEB 2086 attenuated hemorrhagic shock (survival and blood pressure drop) in another model of the rat (R. Reichl, personal communication).

ACTIVATION OF BLOOD CELLS AND ORGAN FAILURE

Activation of blood cells, including platelets and leukocytes, with mediator release may particularly contribute to the development of lung injury (ARDS). When conscious guinea pigs are exposed to an endotoxin aerosol, pretreatment with the hetrazepine brotizolam inhibits the endotoxin-induced pulmonary platelet recruitment dose-dependently (29). Also in the pig, WEB 2086 retards the sharp decline in peripheral white blood cell count occurring after the start of an infusion of endotoxin (22). In addition, the endotoxin associated deterioration in gas exchange and inspiratory pressure are also partially prevented by WEB 2086 (22).

Endotoxin-induced gastrointestinal ulceration is at least partially mediated *via* PAF, because it is blocked by PAF antagonists and PAF is released from the jejunum of endotoxin treated rats (30).

PRIMING OF PAF EFFECTS BY ENDOTOXIN/TNF

When PAF, endotoxin or TNF are given alone, high doses are necessary to induce any changes of transit velocity-index (TV-index) or mortality because mice are relatively resistant to PAF (31), TNF (7) and endotoxin (14).

Treatment with endotoxin (*S. typhosa*) primes mice for enhanced sensitivity towards PAF, resulting not only in decreased gastrointestinal transit velocity, but also in increased mortality (32). The same priming has been shown between tumor necrosis factor (TNF), which mimics pathophysiologic features of endotoxin, and PAF (33-35). The priming effect by endotoxin or TNF towards PAF occurs at doses at which PAF, endotoxin or TNF given alone do not significantly change lethality or TV-index (34,35). Furthermore, the priming effect of endotoxin/TNF towards PAF with respect to decreased gastrointestinal transit velocity and increased lethality is maximal when the time interval between administration of endotoxin/TNF and PAF is about 1-2 hr. WEB 2086 at 0.01-1 mg/kg *i.p.* prevents (in a dose-dependent manner) the enhanced sensitivity to PAF induced by endotoxin/TNF (34,35).

There may be several explanations for the need of a time interval between treatment with TNF or endotoxin and subsequent PAF challenge for maximum decreases in TV-index and survival rate. TNF or endotoxin may stimulate the synthesis of different mediators, such as PAF (9), leukotrienes, prostaglandins (36) and thromboxanes, dur-

ing the time period between administrations of TNF or endotoxin and PAF. These preformed mediators may be released upon PAF challenge and synergize with PAF to decrease TV-index and increase mortality. Thus both endotoxin and TNF prime effects of PAF *in vivo*. This is in accordance with the ability of TNF to prime PAF induced superoxide generation by human neutrophils *in vitro* (37). Interestingly, a similar time interval for the maximum priming response has been observed *in vitro* (37). TNF (38), like endotoxin (30), may induce intestinal damage which increases the susceptibility to PAF. Furthermore, TNF may be a mediatory of endotoxin shock (7) because the same synergism to PAF is also observed after treatment with endotoxin (32); the synergism is maximal when the time delay between endotoxin and PAF administration is about 1–2 hr, a "window phenomenon of dramatically enhanced sensitivity to PAF following a lag-time after pretreatment with endotoxin (32).

These results are in agreement with those reported by Salzer and McCall (39), namely that priming with endotoxin for 60–120 min leads to explosive enhancement of tissue injury caused by a triggering dose of PAF.

CONCLUSIONS

In summary, based on different models of shock states, the action of specific PAF antagonists of the hetrazepine series suggest a role for PAF in endotoxin-induced lethality, activation of blood cells with release of enzymes and mediators, cardiovascular derangements, edema formation, vascular permeability changes in the lung, development of shock organs, as well as in organ failure.

Since the pathophysiology of sepsis and septic shock is very complex, other mediators and mechanisms apart from PAF may have to be taken into account. In addition, contradictory results have been obtained in similar standardized animal models; the same PAF antagonists, given as a single therapeutic agent, have failed to show beneficial effects in some models, while in other models they have shown protective effects. Particular pathophysiological significance may have the ability of endotoxin and/or TNF to prime effects of PAF *in vivo*. This priming occurs at concentrations of the respective mediators at which they will not induce any pathophysiological change when given alone.

In conclusion, PAF may be a key mediator in endotoxin- and TNF-induced shock and in the Sanarelli-Shwartzman-like phenomenon. The effect of selective PAF antagonists in human sepsis and in TNF associated shock-like syndromes still needs to be examined further. When used in addition to the standard therapeutic treatments, PAF antagonists may provide a new therapeutic approach to the still severe and potentially fatal diseases of sepsis and septic shock.

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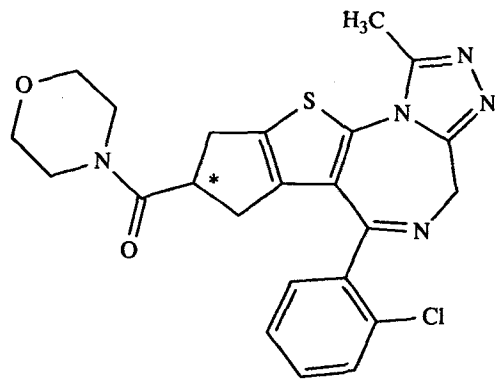
Effect of the Hetrazepinoic Platelet-Activating Factor Antagonist Bepafant (WEB 2170) in Models of Active and Passive Anaphylaxis in Mice and Guinea Pigs[1]

H.O. Heuer
Department of Pharmacology, Boehringer Ingelheim KG, W-6507 Ingelheim/Rhein, Germany

The selective hetrazepinoic platelet-activating factor (PAF) antagonist WEB 2170 (Bepafant) was used to study the pathophysiological role of PAF in several models of anaphylaxis in mice and guinea pigs. In actively sensitized mice, the PAF antagonist WEB 2170 (1.0–10 mg/kg *p.o.*) protected mice from anaphylactic death in a dose-dependent manner when the anaphylactic response was potentiated by the beta-receptor antagonist propranolol. When active anaphylaxis in guinea pigs was induced intravenously by 100 mg/kg ovalbumin (OA) in the presence of small doses of the antihistamine mepyramine, additional treatment with oral or intravenous WEB 2170 protected the guinea pigs from anaphylactic death. Also, the remaining anaphylactic bronchoconstriction and blood pressure changes (including anaphylactic hypotension) were attenuated. When guinea pigs were passively sensitized with a heterologous antibody *via* the tracheal route and then challenged by ovalbumin (100 mg/kg *i.v.*) 24 hr after sensitization in the presence of 0.003 mg/kg *i.v.* mepyramine, additional treatment with tracheal WEB 2170 at 0.1–1 mg/kg protected the guinea pigs dose-dependently not only from anaphylactic death but also from a further decrease of respiratory flow and changes of blood pressure. Increased levels of PAF-like activity (20–50 ng PAF/whole lung) were detected in lungs removed from antigen-challenged animals. The results suggest a causative role for PAF in active and passive anaphylaxis. *Lipids 26,* 1374–1380 (1991).

Bronchoconstriction during anaphylactic shock in the guinea pig continues to be used as a model in the search of antiasthmatic drugs, although guinea pig anaphylaxis is mediated to a greater extent *via* histamine, particularly in its early phase (1), than it is in human asthma.

The results that have been obtained with platelet-activating factor (PAF) antagonists in models of *in vivo* anaphylaxis vary widely. Most investigators observed at least some protective effect by PAF antagonists during passive anaphylaxis in the guinea pig (2–6). On the other hand, in active anaphylaxis inhibition by PAF antagonists has been observed only in some instances (1,6–8). These results have to be considered in light of other findings where even the same PAF antagonists have failed to antagonize active anaphylaxis under differing experimental conditions, particularly without antihistamine supplementation (5,6,9).



WEB 2170

FIG. 1. Chemical structure of Bepafant (WEB 2170).

The purpose of the present study was to reinvestigate the role of PAF in several models of active and passive anaphylaxis and to define the conditions under which a protective effect by a PAF antagonist can be demonstrated. Since booster sensitization has been suggested to make active anaphylaxis refractory to treatment with PAF antagonists, the action of Bepafant in two series of boosted guinea pig anaphylaxis experiments was investigated. The selective hetrazepinoic PAF antagonist WEB 2170 (INN: Bepafant; Fig. 1) was used as a tool to investigate the role of PAF in *in vivo* anaphylaxis. Bepafant (WEB 2170) is one of the most potent antagonists of PAF *in vivo* (10).

MATERIALS AND METHODS

Five different series of experiments on active and passive anaphylaxis in mice and guinea pigs were carried out.

Active sensitization of mice. Fasting male mice (Chbi: NMRI, SPF; weight range 20–25 g at first sensitization) were given, on days 1, 3 and 5, injections *i.p.* of an ovalbumin (OA)/$Al(OH)_3$ suspension containing 40 μg/kg OA. OA (5 times re-crystallized, maximum purity; Serva, Heidelberg, Germany) and the aluminum hydroxide [2 g $Al(OH)_3$/10 mL] were suspended in physiological saline. The OA/$Al(OH)_3$ suspension was administered in a volume of 0.1 mL per animal *i.p.* per day [*i.e.,* 40 μg/kg OA and 100 mg $Al(OH)_3$ per mouse]. Because the mice failed to show any significant anaphylactic reaction upon antigen challenge 4 weeks after the first sensitization, the mice were sensitized again on days 30, 32 and 35 according to a protocol similar to that described above. Antigen

[1]Based on a paper presented at the Third International Conference on Platelet-Activating Factor and Structurally Related Alkyl Ether Lipids, Tokyo, Japan, May 1989.

Address correspondence at Department of Pharmacology, Boehringer Ingelheim Pharmaceuticals, Inc., 900 Ridgebury Road, P.O. Box 368, Ridgefield, CT 06877-0368.

Abbreviations: AUC, area under the curve; MAP, mean arterial blood pressure; OA, ovalbumin; PAF, platelet-activating factor, 1-*O*-alkyl-2-acetyl-*sn*-glycero-3-phosphocholine; TLC, thin-layer chromatography.

challenge with 100 mg/kg OA *i.v.* was carried out 115 days (mice then weighed 40–50 g) after the first day of sensitization in the presence of 0.1 mg/kg *i.v.* of propranolol.

Active sensitization of guinea pigs. Three groups of guinea pigs (Pirbright white Kisslegg, Ivanovas strain, weight range 250–350 g at first sensitization) were actively sensitized following three different protocols.

In the first group of anaphylaxis, female guinea pigs were sensitized using a modification of the method described by Andersson (11). Ovalbumin (OA) (40 μg/kg) and 100 mg $Al(OH)_3$ per guinea pig suspended in physiological saline (0.5 mL) were injected into each guinea pig *i.p.* Four weeks after this first sensitization, a booster sensitization followed using the same antigen-mixture as before. The animals were pretreated with cyclophosphamide (30 mg/kg *i.p.*) 2 days prior to this booster sensitization. Antigen challenge (100 mg/kg OA *i.v.*) was carried out approximately 30 weeks after the second sensitization in the presence of the antihistamine mepyramine (50 μg/kg *i.v.*). The weight of the guinea pigs at that time ranged from 750–970 g.

In the second group of active anaphylaxis, male guinea pigs of the same strain and weight as above were sensitized again using a modification of the method described for the first group. In the second group the booster sensitization after five weeks was not preceded by cyclophosphamide treatment. Antigen challenge with 100 mg/kg *i.v.* followed about 15 weeks after the first (10 weeks after the second) sensitization in the presence of a small dose of mepyramine. In the third group of active anaphylaxis a dose of 100 mg OA/guinea pig was injected *i.p.* and 100 mg OA/guinea pig *s.c.* (one site). Antigen challenge with 100 mg/kg *i.v.* was carried out approximately five weeks after sensitization in the presence of 15 μg/kg mepyramine *i.v.*

Passive sensitization of guinea pigs. Male albino guinea pigs (Pirbright white Kisslegg, Ivanovas strain, weight range 290–420 g) were passively sensitized by intratracheal instillation of 0.4 mL/kg of an heterologous anti-ovalbumin antibody (from rabbit) about 24 hr before antigen challenge (100 mg/kg OA *i.v.*) in the presence of 0.003 mg/kg of mepyramine *i.v.*

Measurement of respiratory flow and mean arterial blood pressure (MAP). The fasting guinea pigs were anaesthetized with urethane (1.5 g/kg *i.p.*) before insertion of an intra-arterial (carotid artery) and an intravenous (jugular vein) catheter and a tracheal cannula. Spontaneously breathing guinea pigs were used. The respiratory flow was monitored using a Fleisch pneumotachograph [Hugo Sachs Elektronik (HSE), Freiburg/Breisgau, Germany] attached to the intratracheal cannula and connected *via* a differential pressure transducer (MKS recorder) directly to a Servogor recorder, model 220 or SE 130 (HSE).

In the case of passive anaphylaxis, the spontaneously breathing guinea pigs were positioned in a body plethysmograph (HSE) and connected *via* a tracheal cannula to the outside, allowing the guinea pigs to breath air from outside the sealed plethysmography box. Respiratory flow was indirectly monitored by following the changes in the thorax volume, which, in turn, resulted in changes in the air flow measured with the pneumotachograph (HSE) mounted to the wall of the plethysmograph box.

The blood pressure (carotid artery) was recorded continuously with a pressure transducer (Statham P 23Db, HSE) connected to an amplifier (Burster) and a Servogor recorder 220 or SE 130 (HSE).

Experimental protocols. Animals were pretreated either intravenously (first group of active anaphylaxis) or *via* the intratracheal route (passive anaphylaxis) 10 min, or orally 60 min, before antigen challenge (ovalbumin at 100 mg/kg *i.v.*) with the PAF antagonist WEB 2170, *i.e.* (8-(R,S)-6-(2-chlorophenyl)-8,9-dihydro-1-methyl-8-[(4-morpholinyl]-carbonyl)-4H,7H-cyclopenta(4,5)thieno(3,2-f)(1,2,4)triazolo-(4,3-a)(1,4)diazepine. When intravenous or tracheal pretreatment was used, WEB 2170 was dissolved in 0.1 N HCl and further diluted with distilled water. For oral treatment WEB 2170 was dissolved in 0.5% Methocel (Tylose®) and further diluted with the same vehicle.

In the case of anaphylaxis in the guinea pig, the antihistamine mepyramine (Bayer, Leverkusen, Germany) was administered 5 min before antigen challenge by the intravenous route to protect the guinea pigs from immediate death upon antigen challenge. The indicated dose of mepyramine was individually titrated for each group of guinea pigs in such a way that immediate death was avoided, but a severe anaphylactic reaction was attained. Respiratory flow and mean arterial blood pressure were monitored after antigen challenge over a time period of 30 min.

Evaluation and statistical analysis. The results are expressed as the mean ± SEM. In mice, the experiments were evaluated by probit analysis, and the dose of WEB 2170 which protected 50% of the mice from anaphylactic death was calculated.

In the active anaphylaxis group of guinea pigs, the area under the curve (AUC) of peak respiratory flow during the 30-min evaluation period was referenced to AUC before antigen challenge.

In the group of passive anaphylaxis of guinea pigs, a representative baseline value was determined before each OA challenge. Respiratory flow and mean arterial blood pressure were evaluated at time points 2, 5, 10, 15, 20, 25 and 30 min after antigen challenge. A decrease in respiratory rate below 15/min marked the onset of gasping, and this respiratory flow was defined as zero.

An analysis of variance for respiratory flow and MAP was calculated. A Dunnett-Test comparing the mean values of the treated animals with the controls at any time point of measurement was applied.

Determination of PAF-like activity. For determination of PAF-like activity, lungs from control guinea pigs were removed immediately after death from active anaphylaxis, washed with saline, and homogenized in 0.25 M hydrochloric acid in methanol. After extraction according to Bligh and Dyer (12), with intermittent centrifugation to precipitate protein, this extract was further purified by thin-layer chromatography (TLC). TLC plates (Si 60 on alufoil, Merck, Darmstadt, Germany) were developed in chloroform/methanol/water (65:35:6, v/v/v). The band comigrating with synthetic C_{16}PAF was scraped off, extracted according to Bligh and Dyer (12), and further processed for measuring PAF-like activity using the aggregation assay described by Heuer *et al.* (13).

RESULTS

Active anaphylaxis in mice. In actively sensitized mice, the PAF antagonist WEB 2170 (1.0–10 mg/kg *p.o.*)

protected mice from anaphylactic death in a dose-dependent manner when the anaphylactic response was potentiated by the beta-adrenoreceptor antagonist propranolol. PD_{50} (dose of WEB 2170 which protects 50% of the mice from anaphylactic death) was determined as 3.3 (1.7–6.8) mg/kg *p.o.*

Active anaphylaxis in guinea pig. Preliminary tests in all three groups of active anaphylaxis failed to show a protective effect by WEB 2170 in the absence of an antihistamine. By contrast, control animals which had been treated with mepyramine (dose individually adjusted for each group of guinea pigs) were protected from immediate death. However, the guinea pigs still exhibited a very severe anaphylactic reaction, as shown by a decrease in respiratory flow (Figs. 2 and 3) and by an initial increase of MAP followed by a subsequent decline. The guinea pigs still died during the 30-min evaluation period.

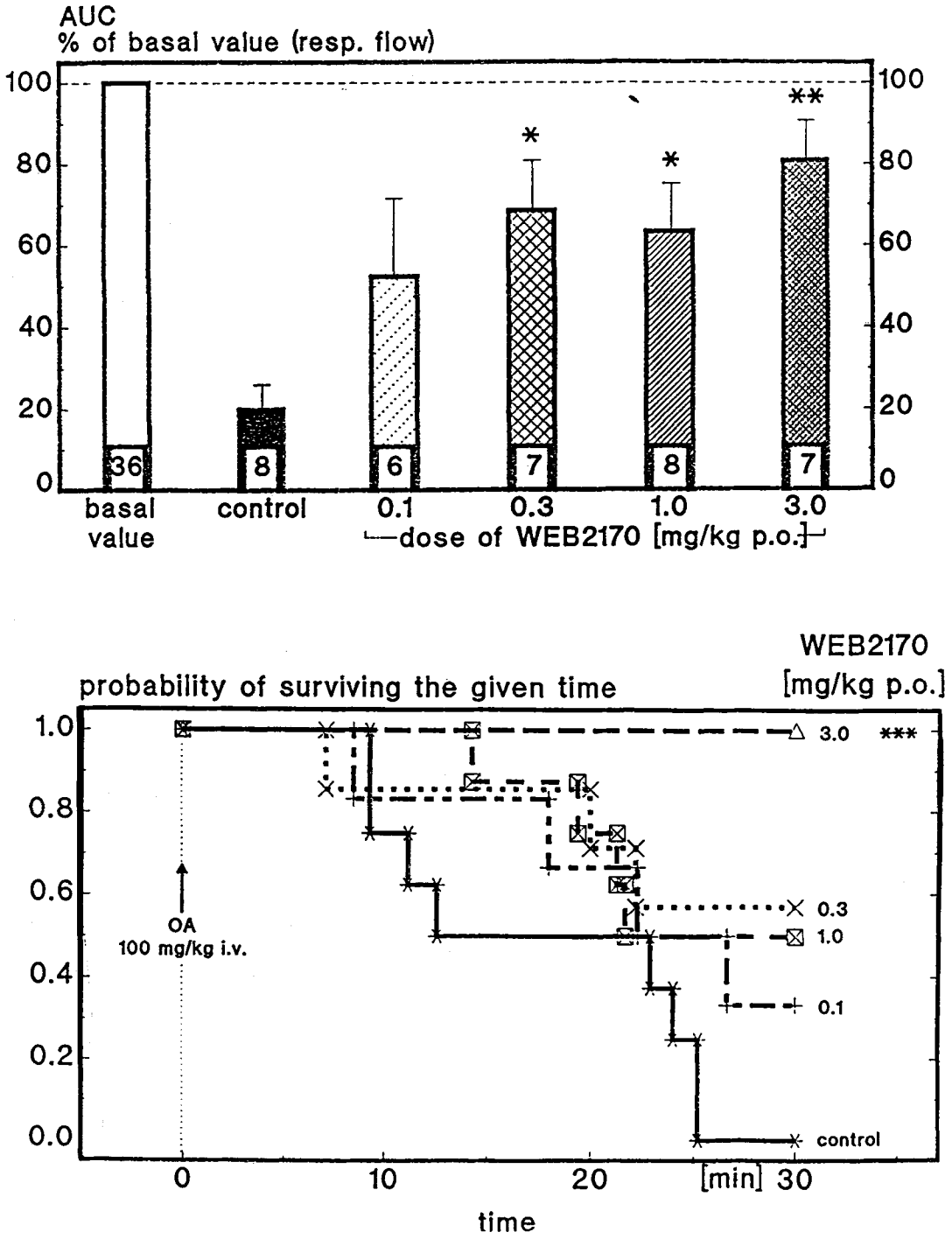


FIG. 2. Effect of oral WEB 2170 on anaphylaxis-associated decrease of respiratory flow (top, AUC, area under curve) and on mortality (bottom) during a 30-min observation period. Actively sensitized guinea pigs (second group) were challenged with 100 mg/kg *i.v.* ovalbumin in the presence of 10 μg/kg *i.v.* mepyramine; *p < 0.05, **p < 0.01, ***p < 0.001; n is indicated at the bottom of the bars; mean ± SEM.

When the guinea pigs were treated with WEB 2170 in addition to a small dose of mepyramine, the animals were protected from anaphylactic death (Figs. 2 and 3). The antigen-induced changes in respiratory flow (Figs. 2 and 3) and blood pressure were also attenuated significantly. In the first group, only one dose of WEB 2170 (5 mg/kg *i.v.*) was tested. In the latter two groups, oral Bepafant (WEB 2170) dose-dependently inhibited the remaining anaphylactic reaction in terms of lethality, decrease of respiratory flow, and blood pressure change (Figs. 2 and 3). With respect to the last group of active anaphylaxis, even 0.04 mg/kg *p.o.* WEB 2170 provided a very significant protection against antigen-induced bronchoconstriction (Fig. 3) and circulatory changes with protection being essentially complete at the highest dose (1 mg/kg *p.o.*).

Passive anaphylaxis in the guinea pig. Guinea pigs

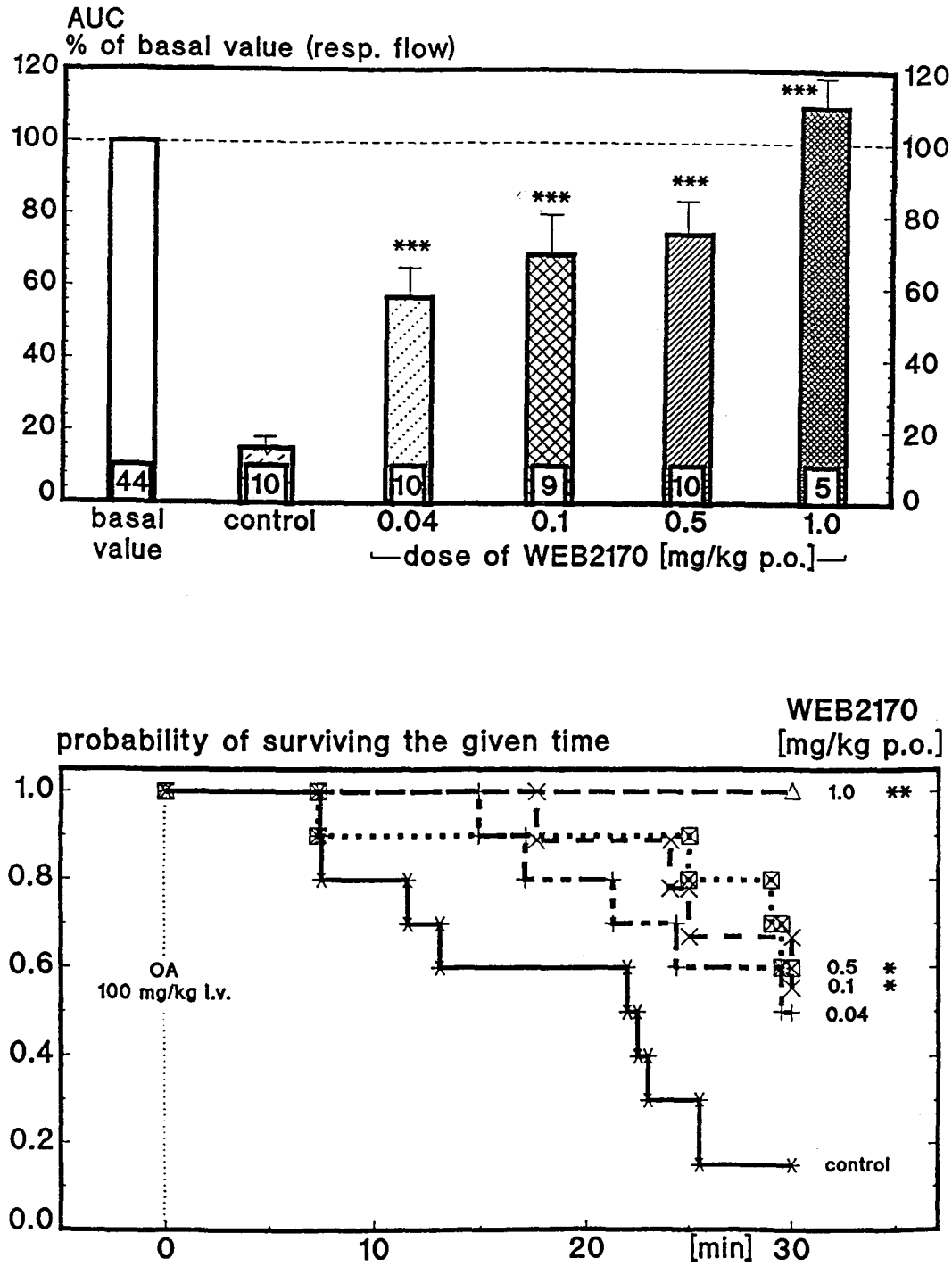


FIG. 3. Effect of oral WEB 2170 on anaphylaxis-associated decrease of respiratory flow (top) and mortality (bottom) during a 30-min observation period. Actively sensitized guinea pigs (third group) were challenged with 100 mg/kg *i.v.* ovalbumin in the presence of 15 μg/kg *i.v.* mepyramine, *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$ indicates significant difference from control; n is indicated at the bottom of the bars; mean ± SEM.

passively sensitized *via* the tracheal route were dose-dependently protected by tracheal WEB 2170 (0.1–1 mg/kg) not only from anaphylactic death, but also from residual decrease in respiratory flow and changes in MAP (Fig. 4).

PAF-like activity in lungs from guinea pigs in anaphylaxis. Increased levels of PAF-like activity (20–50 ng PAF/whole lung) were detected in lungs removed from antigen-challenged animals (third group of active anaphylaxis) (Fig. 5). This PAF-like activity co-migrated with synthetic PAF and was completely suppressed by WEB 2086. Lungs from non-sensitized, non-challenged guinea pigs contained about 1–2 ng PAF/whole lung.

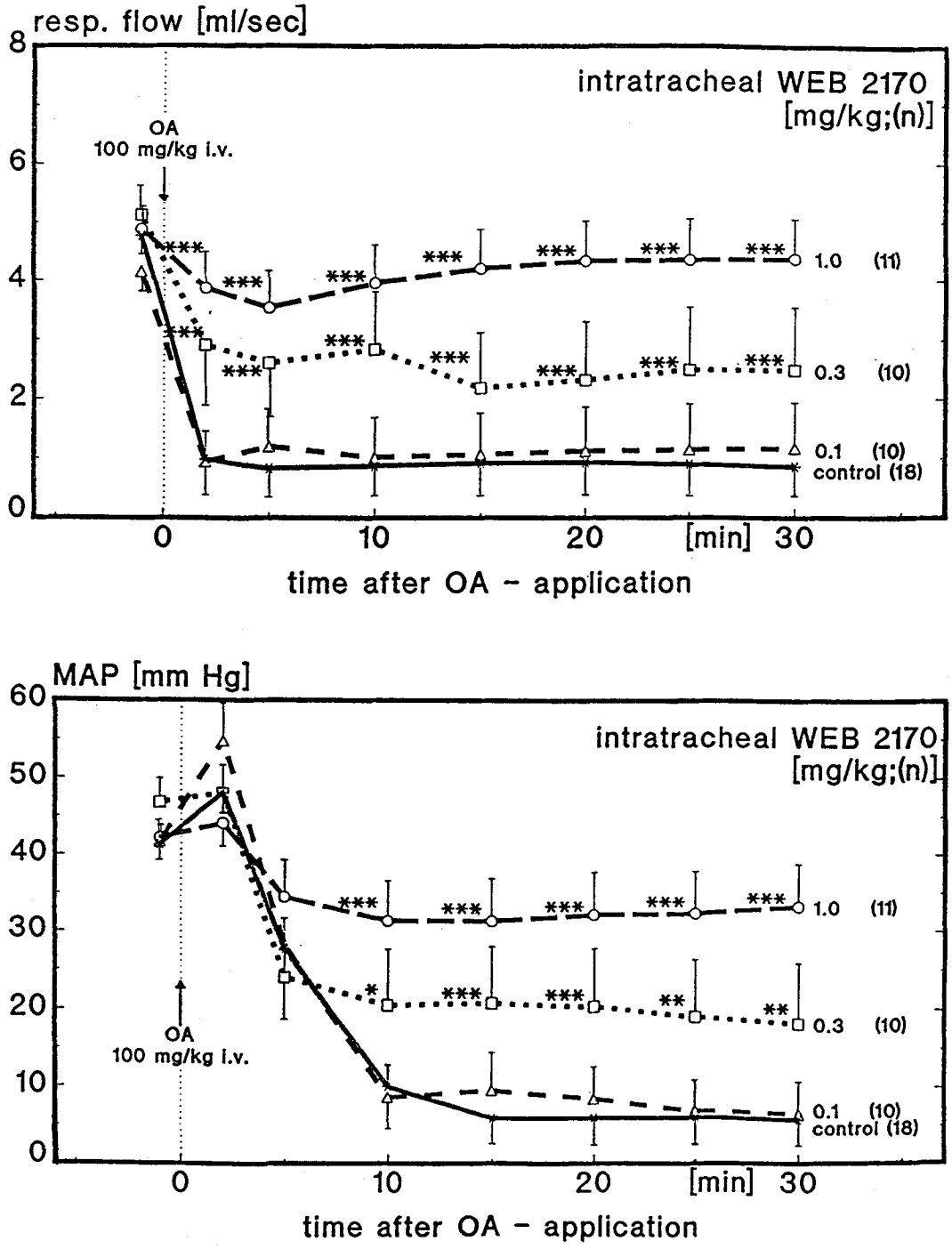


FIG. 4. Effect of tracheal WEB 2170 on anaphylaxis-associated decrease of respiratory flow (top) and blood pressure changes (bottom) induced by 100 mg/kg *i.v.* ovalbumin in the presence of 3 μg/kg *i.v.* mepyramine. Guinea pigs were passively sensitized by the tracheal route; n is given in parentheses; mean ± SEM; *p < 0.05, **p < 0.01, ***p < 0.001 indicates significant difference from control.

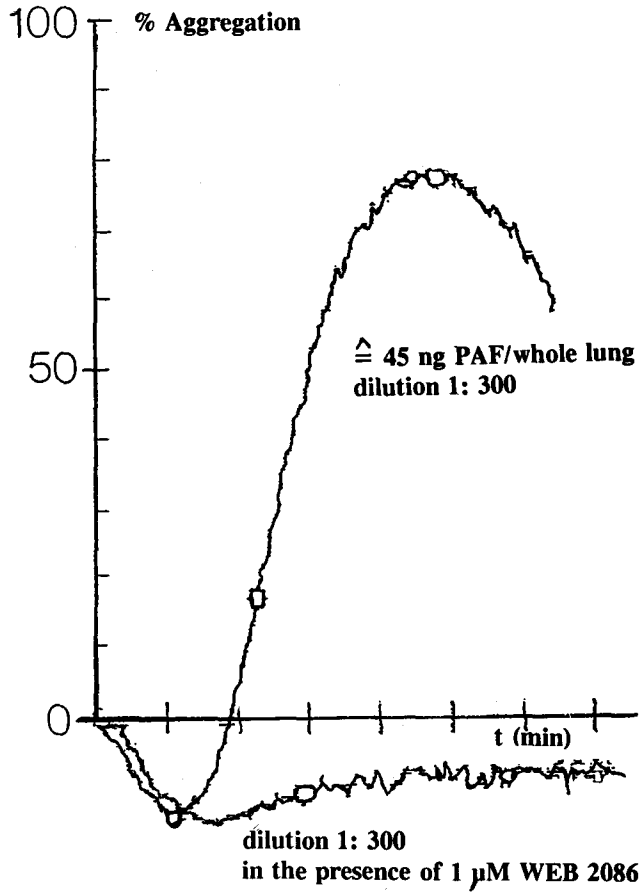


FIG. 5. PAF-like activity in the lung from active anaphylaxis in the guinea pig (third group). PAF-like activity co-migrated with synthetic PAF and could be completely suppressed by the selective PAF antagonist WEB 2086 (superimposed lower tracing). Control lungs from non-sensitized, non-challenged animals contained 1-2 ng PAF/lung.

DISCUSSION

The ability of WEB 2170 to protect actively sensitized mice from propranolol-potentiated anaphylactic death is the first report that a PAF antagonist can overcome an anaphylactic reaction when potentiated with propranolol. It is in contrast to results reported for guinea pig anaphylaxis (2). However, the finding is in agreement with the ability of the beta-antagonist propranolol to potentiate PAF effects in mice and guinea pigs (14,15).

The failure of WEB 2170 (given alone) to protect guinea pigs from anaphylaxis is in agreement with findings on actively sensitized guinea pigs (1,5,6) and actively sensitized rabbits (9) in which other PAF antagonists given alone neither attenuated anaphylactic bronchoconstriction nor circulatory anaphylaxis nor changes in blood cell count.

The characteristic of the protocols followed in the present study is the high antigen-load used for challenge. Higher doses of antigen during guinea pig anaphylaxis have been shown to reduce the relative contribution of histamine as a predominating mediator (16), and may favor a more prominent role of other mediators (such as PAF and leukotrienes) beside histamine, during guinea pig anaphylaxis.

Thus the presence of an antihistamine negates the early histamine-predominated phase of anaphylaxis, thereby revealing the subsequent PAF-predominated phase (see ref. 1). Discrepancies with other literature data (5,9) where PAF antagonists did not show a protective effect in active anaphylaxis may be due to the specific conditions used, such as a small dose of antihistamine in combination with a high challenging dose of ovalbumin, as we used here. Another reason for the failure of PAF antagonists to block active anaphylaxis has been suggested recently, namely the modification of the anti-anaphylactic action of PAF antagonists by a booster sensitization (17,18). By contrast, this report shows that even in boosted guinea pigs (first two series of active anaphylaxis) the hetrazepine Bepafant attenuated the anaphylactic reaction. This is in accordance with results obtained with WEB 2347 (19) which showed that in as often as five-times boosted guinea pigs anaphylaxis was inhibited dose-dependently (19). Thus, the booster would shift the already PAF-dependent anaphylaxis to an even more pronounced PAF-dependent process as indicated by the higher dose of PAF antagonists needed in boosted guinea pigs as compared to simply sensitized guinea pigs (third series of active anaphylaxis). Thus, the booster could modify the lung response to mediators, including PAF, and generate increased reactivity not only by activation of the lipoxygenase pathway but also *via* PAF.

The important role of PAF in passive anaphylaxis was confirmed in this study by use of tracheal Bepafant (WEB 2170). In addition, this is the first report describing guinea pigs to be passively sensitized by the tracheal route in an attempt to restrict the anaphylactic reaction to the lung. However, upon intravenous challenge with antigen, systemic anaphylactic reaction still occurred as documented by blood pressure changes. Tracheal Bepafant (WEB 2170) also antagonized the blood pressure changes, suggesting good systemic uptake of Bepafant from the lung and a role for PAF in passive anaphylaxis after intrapulmonary sensitization.

The present results suggest a causative role of PAF in active and passive anaphylaxis, because PAF cannot only mimic anaphylaxis but anaphylaxis also can be inhibited by the selective PAF antagonist Bepafant (WEB 2170). Furthermore, increased PAF-like activity can be detected in the lungs from antigen-challenged animals.

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Platelet-Activating Factor Type Activity in Plasma from Patients with Septicemia and Other Diseases[1]

Hubert O. Heuer[a,*], Harald Darius[b], Helmut F. Lohmann[c], Juergen Meyer[b], Manuela Schierenberg[b] and Norbert Treese[b]

[a]Department of Pharmacology, Boehringer Ingelheim KG, 6507 Ingelheim, [b]University Clinic of Johannes Gutenberg University Mainz, 6500 Mainz, [c]Human Pharmacology Center, Boehringer Ingelheim KG, 6507 Ingelheim, Germany

The purpose of the present study was to determine whether increased levels of platelet-activating factor (PAF) type activity can be detected in plasma from patients with septicemia and other diseases. A level of PAF below 0.5 ng/mL of plasma was considered normal. We found that plasma from a patient with adverse anaphylactoidic reaction to intravenous analgetics contained 2.1 ng PAF/mL. In seven patients with septicemia, including urosepsis, endocarditis and peritonitis, and with positive blood culture, increased plasma PAF levels (1–20 ng PAF/mL) were observed. Other patients with clinical indications of septicemia had negative blood cultures and/or increased levels of C-reactive protein (CRP). Yet, in the plasma from these patients, no increased PAF levels were detected under the assay conditions used. Two patients with allergic asthma, requiring treatment with steroids, had no measurable plasma PAF. In the plasma from a patient with idiopathic thrombocytopenic purpura (ITP) only an "endogenous" inhibitor of PAF induced platelet aggregation was initially observed. In spite of this, the patient responded to treatment with the PAF antagonist WEB 2086 with a dramatic increase in platelet count (Lohmann *et al., Lancet ii,* 1147, 1988). Thereafter, also increased PAF levels (3.3 ng PAF/mL) were detected in plasma, although some "endogenous" inhibitor of PAF was still present. In conclusion, increased PAF levels in plasma from patients support a role of PAF in certain human disease states, such as in anaphylactoid reaction, sepsis and septic shock. The type, relevance and specificity of endogenous inhibitors of PAF deserve further study.

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Increased levels of platelet-activating factor (PAF) type activity have been detected in blood from endotoxin treated animals (1,2). So far, there are only few reports on an increased level or turnover of PAF in human sepsis (3,4). This may be due in part to technical limitations in the measurement of PAF in human tissue and blood. Different approaches are conceivable to detect changes in turnover rates or levels of PAF under pathophysiological conditions in tissues and fluids, including blood, ascites, saliva, lavage fluid or urine, such as the measurement of lysoPAF or of PAF acetylhydrolase. However, any of these approaches has certain disadvantages, and the direct measurement of PAF may most likely reflect any relationship that may exist between PAF levels and various disease states. The purpose of the present study was to determine whether a modified aggregation assay for PAF type activity (5) may be suited for detecting and differentiating increased PAF-like activity in human plasma samples from patients with septicemia and other diseases.

MATERIALS AND METHODS

Blood samples from volunteers and patients were prepared for analysis of PAF-like activity by mixing 1 vol of whole blood with one volume of a modified ACD buffer (citric acid, 25 g; sodium citrate, 15 g; dextrose, 10 g; distilled water to a total volume of 1 L, pH 3.5). After centrifugation, the lipids of ACD plasma (pH 3.5) were extracted according to Bligh and Dyer (6), with intermittent centrifugation to precipitate protein. The chloroform phase of the Bligh and Dyer extract was taken to dryness and reconstituted in 0.8 mL of the aggregation buffer (see below).

Washed rabbit platelets were prepared according to a modification of described procedures (7–9). The washing solution/aggregation buffer had the following compositions (per 1 L distilled water): KCl (195 mg/195 mg), $MgCl_2 \times 6\ H_2O$ (212.5 mg/212.5 mg), NaCl (8 g/8 g), $NaHCO_3$ (1.015 g/–), Tris (–/1.21 g), $CaCl_2 \times 2\ H_2O$ (–/126.4 mg), EGTA 0.2 M; 76.07 g/L (1 mL/–), anhydrous glucose (1.0 g/1.0 g), bovine serum albumin (BSA) (fatty acid (FA) free) (2.5 g/2.5 g), HCl (pH 6.5/pH 7.4).

PAF-like activity was identified and quantitated by an aggregation assay using washed rabbit platelets under conditions described (5,10). PAF aggregatory activity was identified based on inhibition by the selective PAF antagonist WEB 2086, and in some cases was also confirmed by comigration in thin-layer chromatography (TLC) of PAF-like activity with synthetic PAF. Reproducibility and sensitivity of the PAF aggregation assay were improved by making some adjustments (5,10). Concentration response curves for PAF were well reproducible and independent of whether PAF was added to the assay system before (BAP) or after (AAP) addition of platelets. Aggregation was started according to the BAP procedure by addition of 50 μL of platelet suspension to 200 μL of PAF containing 1.0 aggregation buffer. The selective PAF antagonist WEB 2086 inhibited PAF induced aggregation in a specific and concentration-dependent manner with inhibition being complete above 1×10^{-7} M final concentration of WEB 2086 (IC_{50} 28 nM; PAF, 100 pg/mL). In the presence of subthreshold concentrations of cooperative aggregation stimuli, such as ADP or adrenaline, the concentration response curves for PAF were shifted to the left. Increase of pH (*e.g.*, pH 8.0) resulted in increased sensitivity for PAF, whereas a decrease in pH (pH 7.2) shifted the

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*To whom correspondence should be addressed at Boehringer Ingelheim Pharmaceuticals, Inc., Dept. of Pharmacology, 900 Ridgebury Road, P.O. Box 368, Ridgefield, CT 06877-0368.

Abbreviations: AAP, after addition of platelets; ARDS, adult respiratory distress syndrome; BAP, before addition of platelets; CRP, C-reactive protein; DIC, disseminated intravascular coagulation; ET, endotoxin; ITP, idiopathic thrombocypenic purpura; PAF, platelet-activating factor; SFA, septic fever attack; TLC, thin-layer chromatography.

concentration response curve to the right. Addition of fibrinogen to the aggregation buffer (0.01–0.06%) shifted the concentration response curves for PAF to the left (*e.g.*, final concentration, 0.02%). Consideration of these factors and appropriate choice of ion concentrations led to a sensitive PAF assay with a lower detection limit of about 1 pg/mL.

RESULTS

Under the conditions used for the extraction and assay of PAF in blood, no detection of PAF or a level of PAF below 0.5 ng/mL of plasma was considered normal.

Plasma from a patient who had collapsed due to adverse anaphylactoid reaction upon intravenous injection of a metamizol containing mixture of analgetics, exhibited a PAF level of 2.1 ng/mL (see Fig. 1). In seven plasma samples from patients with septicemia, including urosepsis, endocarditis and peritonitis, and with positive blood culture, increased levels of PAF (1–20 ng PAF/mL) were detected (see Fig. 2 and Table 1). In contrast, other patients with clinical indicators or suspicion of septicemia had negative blood cultures and/or increased levels of C-reactive protein (CRP); however, no increased levels of PAF were measured under the conditions used (see Tables 2 and 3). Two patients with allergic asthma, who required treatment with steroids, did not show measurable PAF in their plasma.

In plasma from a patient with idiopathic thrombocytopenic purpura (ITP), initially no PAF was detected. Instead an "endogenous" inhibitor of PAF induced platelet aggregation was identified. However, the patient responded to treatment with the PAF antagonist WEB 2086 with a dramatic increase in platelet count (Fig. 3). Thereafter and upon treatment with WEB 2086, also increased levels of PAF (3.3 ng PAF/mL) appeared in plasma (Fig. 4A). Upon purification by TLC, a yet higher level of PAF was measured in the same sample (Fig. 4B) suggesting the presence of some type of endogenous PAF inhibitor in ITP plasma.

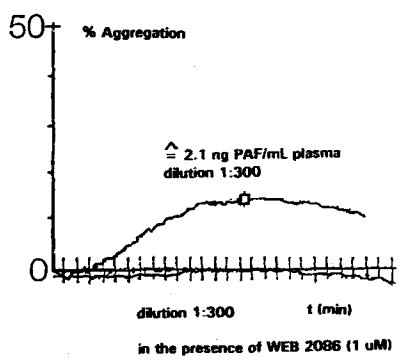


FIG. 1. PAF-like activity in plasma from a patient who collapsed upon anaphylactoid reaction after i.v. injection of a metamizol containing analgetic mixture. PAF-like activity is completely suppressed in the presence of the selective PAF antagonist WEB 2086 (superimposed tracing below).

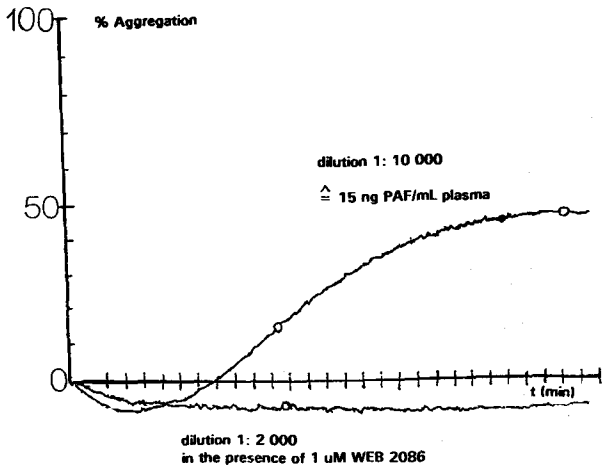


FIG. 2. Increased PAF-like activity in the plasma from a patient with acute myeloid leukemia and septicemia (severe peritonitis with necrosis of the bowel) from *Pseudomonas aeruginosa*. PAF-like activity comigrated with synthetic PAF in TLC and was completely suppressed in the presence of the selective PAF-antagonist WEB 2086 (superimposed tracing below). The patient died later in septic shock.

TABLE 1

PAF Levels in Plasma from Patients with Septicemia and Positive Blood Culture

Patient	Clinic (disease, symptoms, course)	Bacteria (species identified)	PAF (ng/mL plasma)
1	Acute myeloid leukemia; septicemia from peritonitis with necrosis of bowel; patient died in septic shock	*Ps. aeruginosa*	15.0
2	Endocarditis; SFA; dyspnea; thrombocytopenia	β-Hemolytic streptococci	2.46
3	Pulmonary edema; cardiac failure; septic fever	Propionibacterium spec. 697 (blood); tracheal secretion: *Staph. aureus*, *E. coli* group	1.3
4	Pyesis from left foot and SFA (after leg amputation, no PAF detected)	β-Hemolytic streptococci	1.12

TABLE 2

Patients with High C-Reactive Protein (CRP), Negative Blood Culture and No Increased/Detectable PAF in Plasma

Patient	Age	Sex	Clinic (disease, symptoms, course)	CRP (mg/mL)
1	51	female	After abscess surgery suspicion of meningoencephalitis and septicemia;	7.0
			performation of ulcus duodeni; CRP after surgery	4.3
2	63	male	Anti-GBM glomerulonephritis; tuberculosis; suspicion of pneumonia;	20.8
			no improvement after nine days	16.6
3	75	male	Cerebral stroke; suspicion of pneumonia and urinary tract infection	4.7

TABLE 3

Patients with High CRP and No Increased/Detectable Level of PAF

Patient	Age	Sex	Clinic (disease symptoms, course)	Bacteria	CRP (mg/mL)
1	59	female	Thrombocytopenia; DIC undetermined origin;	gram-positive rod-shaped bacillus	5.1
			improvement 8 days later		0.8
2	69	male	Septicemia after abdominal surgery; nine days later SFA and DIC; exitus	n.i.[a]	10.4 16.2
3	55	male	Septicemia after Billroth II surgery and bowel resection; fulminant hepatitis B	n.i.[a]	32.7
4	35	male	Metastatic thyroid carcinoma; pneumonia; nephrolithiasis	n.d.[b]	39.5
5	52	male	Septicemia	*Staph. aureus*	13.5
6	28	female	Caesarean section; DIC; ARDS; hyperfibrinolysis; 2 days later exitus	n.d.[b]	21.7
7	44	female	Acute myeoid leukemia; signs of septicemia and shock	n.d.[b]	23.7

[a]n.i., not identified. [b]n.d., not determined.

DISCUSSION

The bioassay used here to measure PAF type activity is highly sensitive and specific through use of the selective PAF antagonist WEB 2086. Under the modified conditions (pH 8.0, 0.02% fibrinogen, and cooperative stimuli), a detection limit of as low as 1 pg PAF/mL can be achieved, particularly when the Mg^{2+} and Ca^{2+} ion concentrations of the aggregation buffer (pH 8.0) are carefully adjusted (7,11,12).

Measurement of increased levels of PAF-like activity in plasma samples from patients in certain disease states illustrates that the bioassay can detect and differentiate PAF-like activity in plasma samples from patients. The level of PAF-like activity observed in this study is in the range found in the literature (3,13). The increased level of PAF-like activity found during an anaphylactoidic reaction after intravenous administration of analgesics is also in line with reports by others (14,15).

The increased level of PAF-like activity in septicemic patients (particularly those whose blood cultured gram-negative bacteria) is in agreement with previous studies in children (3) as well as studies in rats after infusion of endotoxin (1,2).

Our inability to find PAF-like activity in the plasma of asthmatic patients is consistent with earlier reports (16). On the other hand, changes in PAF metabolism in human allergies and asthma are indicated by the increased levels of lysoPAF found in plasma (16), by changes in PAF turnover, and/or by PAF occurrence in lung and other target sites of allergies (17–20). Furthermore, treatment with steroids may suppress PAF formation in asthmatics.

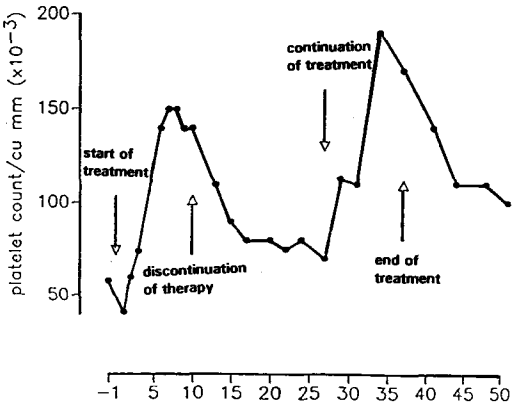


FIG. 3. Effect of repeated treatment with the PAF antagonist WEB 2086 (4 × 20 mg/day) on platelet count in a patient with idiopathic thrombocytopenic purpura (ITP; Morbus Werlhof).

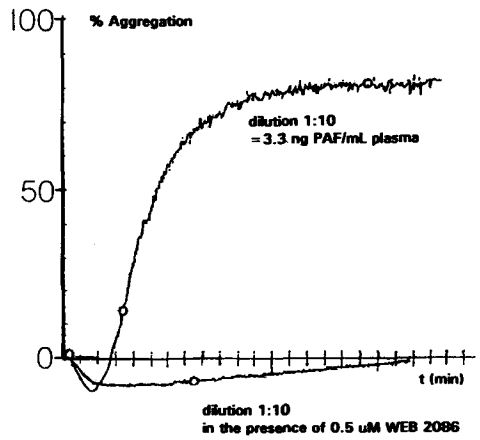


FIG. 4A. PAF-like activity in the plasma from a patient with idiopathic thrombocytopenic purpura (ITP) which came up after the second treatment cycle with WEB 2086 (see Fig. 3). Identification of platelet aggregating activity as PAF-like activity by WEB 2086 (superimposed tracing below).

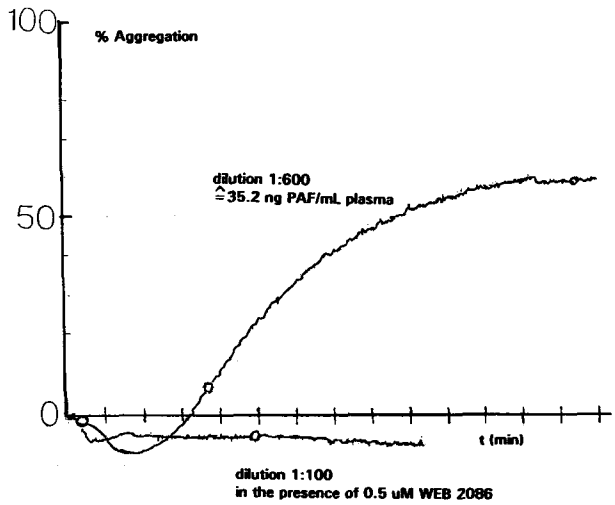


FIG. 4B. PAF-like activity in the plasma sample used above (see Fig. 4A) after purification by TLC. Increased PAF-like activity (35.2 ng PAF/mL plasma) compared to unpurified sample (3.3 ng PAF/mL plasma) suggests the presence of an endogenous inhibitor of PAF. PAF-like activity comigrated with synthetic PAF and was completely blocked by WEB 2086 (superimposed tracing below).

The functional bioassay using washed rabbit platelets allows the detection and quantification of specific and nonspecific inhibitors of PAF induced platelet aggregation, if in the apparent absence of platelet aggregating activity, added PAF is also inhibited. The amount of inhibitor present can then be estimated by serial dilution of the sample until the inhibitor disappears.

Different endogenous inhibitors of PAF, including certain phospholipids and C-reactive protein (CRP), have been described in the literature (21–27).

In some samples from patients suspected of septicemia, an endogenous inhibitor of PAF induced platelet aggregation was detected. In other samples where no PAF-like activity was identified, increased levels of CRP were found. It is entirely possible that PAF-like activity may be bound, and inactivated, by CRP and lipoproteins (25,27,28).

The dramatic increase in platelet count in a patient with idiopathic thrombocytopenic purpura (ITP) upon treatment with the selective PAF antagonist WEB 2086 (29) in the apparent absence of PAF and in the presence of an endogenous PAF inhibitor indicates that the apparent absence of PAF, as judged by the assay used, should not necessarily preclude the presence of PAF. In fact, the presence of an endogenous inhibitor of PAF may actually be indicative of a PAF-mediated process.

In conclusion, the very sensitive and selective aggregation assay for PAF-like activity as used here is suited to detect and differentiate PAF-like activity in plasma from patients. The results can provide additional support for a role of PAF in human disease, such as sepsis, anaphylaxis, and immunological disorders.

Various points should be taken into account when interpreting the data measured. For example, compartmentalization of PAF-mediated processes at the organ level (*e.g.*, in asthma) or binding and inactivation by lipoproteins or CRP may affect the level of unbound PAF in plasma, as suggested by the results presented here. Furthermore, the level of PAF relative to its metabolites, such as lysoPAF, the involvement of enzymes (*e.g.*, acetylhydrolase), and the presence of endogenous inhibitors (CRP, certain phospholipids) have to be considered. In addition, the level of PAF in relation to other inflammatory mediators, their interaction and synergism with PAF, and the sequence of their formation and release may have impact on the physiological and pathophysiological role of PAF.

Due to this complexity, the use of selective and potent PAF antagonists may be a more useful and convincing approach to pinpointing a causative role of PAF in human disease.

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Differential Effect of a PAF Antagonist CV-3988 on Active and Passive Anaphylactic Shock in Various Mouse Strains[1]

Akinori Arimura* and **Minoru Harada**
Shionogi Research Laboratories, Shionogi & Co., Ltd., Osaka 553, Japan

To define the role of platelet-activating factor (PAF) in anaphylactic shock in the mouse, the suppressive effect of CV-3988, a PAF antagonist, on active and passive anaphylactic shock was studied. Various mouse strains treated or not treated with *Bordetella pertussis (B. pertussis)* were used. We found that the effect of CV-3988 on anaphylactic shock in the mice that were actively sensitized with bovine serum albumin plus *B. pertussis* differed markedly according to mouse strain. CV-3988 suppressed the anaphylactic shock in C3H/He and CBA/JN mice at a low dose of 3 mg/kg, whereas antagonists to other mediators such as histamine, serotonin, thromboxane A_2 and leukotrienes did not show a suppressive effect. This suggests that PAF plays a major role in anaphylactic shock in these strains. On the other hand, CV-3988 did not suppress active anaphylactic shock in cataract Shionogi (CTS), NOD and DS strains even at a high dose of 30 mg/kg, which could be interpreted to suggest that PAF is not active in these strains. However, this possibility was ruled out based on the similar results obtained in passive anaphylactic shock and PAF-induced shock in these mice. Passive anaphylactic shock in CTS mice mediated by IgG_1 antibody was markedly suppressed by CV-3988 but not at all by antagonists to other mediators. Furthermore, the suppressive action of CV-3988 against passive anaphylactic shock, and PAF-induced shock was greatly attenuated when the mice were pretreated with *B. pertussis*. From these results, the conclusion can be drawn that PAF is the main mediator of active and passive anaphylactic shock in the mouse in general, even though the effect of CV-3988 differs depending on the mouse strain and on whether or not *B. pertussis* treatment is used.
***Lipids 26,* 1386-1390 (1991).**

We have previously reported that the cataract Shionogi (CTS) mouse is T lymphocytopenic and that its *in vitro* and *in vivo* immune responses to T cell-dependent antigens are markedly impaired (1,2). For instance, the *in vivo* production of antibodies to bovine serum albumin (BSA) is minimal when the mouse is immunized with dead *Bordetella pertussis (B. pertussis)* organisms used as an adjuvant. Unexpectedly, however, lethal active anaphylactic shock was elicited in almost all mice 7 or more days after the immunization. These results suggest that CTS mice are very susceptible to the effector phase of active anaphylactic shock. This is supported by the finding that this strain is also highly susceptible to passive anaphylactic shock mediated by allogeneic IgE and IgG_1 antibodies (3). There are several possible explanations for the high susceptibility of CTS mice. Firstly, mast cells and other cell types may easily be sensitized by the antibodies. Secondly, a larger amount of mediators may be released from mast cells, or other cells, by antigen-antibody interactions in this strain than in others. Thirdly, CTS mice may be highly susceptible to the chemical mediators released. Before gaining some insight into the mechanism of the potent anaphylactic shock in CTS mice, we needed to identify the main mediator responsible for anaphylactic shock in the mouse. Using CV-3988 as a PAF antagonist, Terashita *et al.* (4) showed that PAF plays an important role in active anaphylactic shock in ICR mice. However, it is still unclear whether this is true for all mouse strains and all types of anaphylactic shock.

To examine whether or not the suppressive effect of CV-3988 against anaphylactic shock can be generally observed in the mouse, a systematic study was undertaken with various mouse strains which were sensitized by active and passive means with or without *B. pertussis* treatment.

MATERIALS AND METHODS

Animals. CTS, NOD, C3H/He, BALB/c, C57BL/6J, DS/Shi and BDF1 mice were bred under specific pathogen-free conditions at Shionogi Aburabi Laboratories (Shiga, Japan). CBA/JN and DBA/2 mice were purchased from Charles River Japan Inc. (Kanagawa, Japan), and Jcl:ICR mice were obtained from Clea Japan Inc. (Tokyo, Japan). Both sexes of NOD mice, and females of the other strains were used. Unless otherwise described, tests were done using mice 7 to 10 wk old. WS rats at 8 wk of age, which were bred at Shionogi Aburabi Laboratories, were used as recipients for passive cutaneous anaphylaxis (PCA) mediated by mouse IgE antibody.

Antigen and antibody. Ascitic fluids of BDF_1 mice containing IgE or IgG_1 monoclonal antibody to benzylpenicilloyl (BPO) haptens (5) were used. The antibody titers of both ascitic fluids were 1:1024 when assayed by means of 1-day PCA in rats (for IgE antibody) and 1-hr PCA in DS/Shi mice (for IgG_1 antibody). As a polyvalent eliciting antigen, BPO-GSA, the conjugate of BPO haptens with guinea pig serum albumin (GSA, Sigma Chemical Co., St. Louis, MO), was prepared according to the method of Levine and Ovary (6). In addition, to obtain anti-ovalbumin (OVA) serum, BALB/c mice were hyperimmunized by three intraperitoneal injections at 1-wk intervals of 1 mg of OVA (Seikagaku Kogyo, Tokyo, Japan) emulsified with complete Freund's adjuvant (CFA, Difco, Detroit, MI), and bled 1 wk after the last sensitization. The IgE and IgG_1 titers of the pooled serum were both

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*To whom correspondence should be addressed at Shionogi Research Laboratories, Shionogi & Co., Ltd., 5-12-4 Sagisu, Fukushima-ku, Osaka 553, Japan.

Abbreviations: *B. pertussis, Bordetella pertussis*; BPO, benzylpenicilloyl; BSA, bovine serum albumin; CFA, complete Freund's adjuvant; GSA, guinea pig serum albumin; OVA, ovalbumin; PAF, 1-*O*-alkyl-2-acetyl-*sn*-glycero-3-phosphocholine; PCA, passive cutaneous anaphylaxis.

1:1024. Anti-BPO ascites and anti-OVA serum were stored frozen at −20°C.

Active anaphylactic shock. Mice were sensitized by i.p. injection of 0.2 mL of saline containing 1 mg of bovine serum albumin (BSA, Nakarai Chemicals, Kyoto, Japan) and 2×10^9 cells of dead *B. pertussis* organisms (Nacarai Tesque, Tokyo, Japan). Shock was elicited by an intravenous challenge with 1 mg (0.2 mL in saline) of the homologous antigens 1 wk after sensitization in CBA/JN, C3H/He, DBA/2, BDF_1, BALB/c, NOD and Jcl:ICR mice and two weeks after sensitization in C57BL/6J, DS/Shi and CTS mice.

Passive anaphylactic shock. NOD and CTS mice were passively sensitized by an intravenous injection of 0.2 mL of ascitic fluid of BDF_1 mice containing anti-BPO IgE or IgG_1 monoclonal antibody or by an intravenous injection of 0.2 mL of anti-OVA hyperimmune serum, as these two strains are most susceptible to anaphylactic shock (3). To elevate susceptibility of mice to chemical mediators, some animals were injected intraperitoneally with *B. pertussis* (2×10^9 cells, unless otherwise stated) one week before the sensitization. IgG_1- and IgE-mediated anaphylactic reactions were elicited by an intravenous injection of BPO·GSA (1 mg) 3 to 5 hr and 1 day after sensitization, respectively. Anaphylaxis mediated by anti-OVA serum was caused by an injection of OVA (1 mg) after 3- to 5-day sensitization. The lethality was estimated at 60 min after antigen challenge in both active and passive shock tests, though lethal shock usually occurred within 30 min.

Platelet-activating factor (PAF)-induced shock. PAF (1-*O*-alkyl-2-acetyl-*sn*-glycero-3-phosphocholine, Sigma, 2 mg/mL in chloroform) was diluted with 0.9% saline solution to the concentrations needed. PAF was injected through the tail vein at 3.13 to 200 μg/kg (injection volume: 0.1 mL/10 g body weight). Intensity of PAF-induced shock was expressed in terms of the incidence of lethal shock within 1 hr after PAF injection, although death occurred usually within 30 min after the injection.

Drugs. CV-3988 [(RS)-2-methoxy-3-(octadecylcarbamoyloxy)-propyl-2-(3-thiazolioethylphosphate)], S-145 Na [sodium dl-(5Z)-7-(3-endo-(phenylsulfonyl)amino)bicyclo-[2.2.1]hept-2-exo-yl)heptenoic acid] (7) and FPL-55712 (sodium 7-[3(4-acetyl-3-hydroxy-2-propylphenoxy)-2-hydroxypropoxy]-4-oxo-8-propyl-4H-1-benzopyran-2-carboxylate were synthesized in our laboratories. Cyproheptadine (Sigma) was purchased. All drugs were dissolved in 0.9% saline and administered intravenously 5 min before the challenge with antigen or PAF.

RESULTS

Effect of various antagonists on active anaphylactic shock. To determine the dominant mediator in active anaphylactic shock, we examined the effects of various antagonists on lethality in C3H/He mice. Lethal shock occurred in almost all of the control mice within 30 min after antigen challenge, although it was somewhat delayed in a few cases. As shown in Table 1, cyproheptadine (histamine and serotonin antagonist), S-145 Na (thromboxane A_2 antagonist) and FPL-55712 (leukotriene antagonist) did not suppress the shock at all. In contrast, CV-3988 (PAF antagonist) protected mice from death caused by active anaphylactic shock at doses ranging from 1 to 10 mg/kg, when injected intravenously 5 min before the antigen challenge. These results are consistent with the previous report (4), and suggest that PAF plays a primary role in C3H/He mice. To see if this could be confirmed in general, the effect of CV-3988 was tested in other strains.

TABLE 1

Effect of Various Antagonists on Active Anaphylactic Shock in C3H/He Mice[a]

Drugs	Dose (mg/kg)	Lethality (death/total mice)
Control		27/31
CV-3988	1	5/12
	3	5/24
	10	0/4
Cyproheptadine	20	5/5
S-145 Na	10	5/5
FPL-55712	10	5/5

[a]Drugs were given i.v. 5 min before the antigen challenge.

As illustrated in Figure 1, the suppressive effect of CV-3988 differed according to mouse strain. Suppression was potent in C3H/He and CBA/JN mice and moderate in BALB/c, DBA/2, C57BL/6J, BDF_1 and Jcl:ICR mice. In CTS, NOD and DS mice, no suppression was observed even at a high dose of 30 mg/kg.

Effect of various antagonists on passive anaphylactic shock. As reported previously, CTS as well as NOD mice were much more susceptible to passive anaphylactic shock (3). Briefly, when the mice were sensitized with IgG_1 monoclonal antibody, lethal shock was easily produced in CTS and NOD strains regardless of the presence or absence of *B. pertussis* pretreatment. In some strains such as NON, C57BL/6J and DS/Shi, lethal shock was produced only when the mice had been treated with *B. pertussis*. In contrast, IgE antibody was less effective in sensitizing mice for anaphylactic shock than IgG_1 antibody. Lethal anaphylactic shock mediated by IgE antibody was observed in CTS, NOD, C57BL/6J and DS mice treated with *B. pertussis*. Based on these findings, the effects of

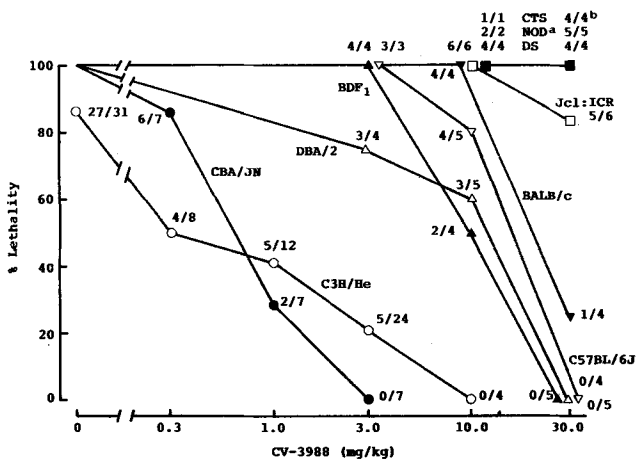


FIG. 1. Effect of CV-3988 on active anaphylactic shock in various mouse strains immunized with BSA and *B. pertussis*. CV-3988 was given i.v. 5 min before the antigen challenge. [a]Females aged 15 wk. [b]Incidence of lethal shock.

several drugs were examined against passive anaphylactic shock in *B. pertussis*-treated and untreated CTS and NOD mice.

Table 2 shows the drug effect against passive anaphylactic shock in untreated CTS mice sensitized with IgG_1 monoclonal antibody. Unlike active anaphylactic shock, this type of passive anaphylactic shock was markedly suppressed by the PAF antagonist CV-3988, but not by the antagonist to other mediators. Table 3 demonstrates the difference in the suppressive effect of CV-3988 between *B. pertussis*-treated and untreated mice sensitized with anti-OVA serum or anti-BPO ascites. CV-3988 suppressed the anaphylactic shock mediated by anti-OVA hyperimmune serum or anti-BPO IgG_1 antibody in the untreated NOD and CTS mice at lower dosages ranging from 3 to 10 mg/kg, but did not suppress shock in the *B. pertussis*-treated mice even at a high dose of 30 mg/kg. Because *B. pertussis* treatment is necessary to provoke lethal shock as far as IgE antibody is concerned, the suppressive effect of CV-3988 against IgE-mediated passive anaphylactic shock was compared between NOD mice treated with a low dose (2×10^8 cells) and a high dose of *B. pertussis* (2×10^9 cells). The suppressive effect was more pronounced in the mice treated with a low dose of *B. pertussis* than in those treated with a high dose. From these results, one could speculate that the suppressive effect of CV-3988 against passive anaphylactic shock is alleviated by *B. pertussis* treatment in general, even though PAF might play a crucial role.

Effect of B. pertussis *treatment on the antagonistic action of CV-3988 against PAF-induced shock in DS mice.* To support the aforementioned speculation, the effect of CV-3988 against PAF-induced shock between untreated and *B. pertussis*-treated DS mice was compared. In view of the observation that the minimal dose of PAF to produce 100% lethality in this strain was 25 μg/kg, the effect of CV-3988 was assessed against two doses of PAF, *i.e.,* 50 and 100 μg/kg. As shown in Table 4, protection of mice by CV-3988 from PAF-induced lethal shock decreased, accompanied by an increase in PAF dose, and was greatly reduced in the *B. pertussis*-treated animals.

Comparison of susceptibility to PAF-induced shock in untreated and B. pertussis*-treated mice of various strains.* The above described results that *B. pertussis* affects the suppressive action of CV-3988 against passive anaphylactic shock and PAF-induced shock suggest that differences between strains in the suppressive effect of CV-3988 against active anaphylactic shock depends on how much it is influenced by *B. pertussis.* To examine this possibility, we compared the PAF-induced shock in untreated and *B. pertussis*-treated mice of various strains. As demonstrated in Table 5, the susceptibility to PAF-induced shock in the untreated animals differed according to strain. However, the susceptibility was enhanced by *B. pertussis* in all the strains tested, although differing in intensity. The enhancement was minimal in C3H/He and CBA/JN strains as compared with the other strains. This may explain the potent suppressive action of CV-3988 against active anaphylactic shock in these strains. CTS mice were more susceptible than the other strains regardless of *B. pertussis* treatment.

TABLE 2

Effect of Various Antagonists on IgG_1-Mediated Passive Anaphylactic Shock in the Untreated Male CTS Mice[a]

Drugs (death/total mice)	Dose (mg/kg)	Lethality (death/total mice)
Control		7/7
CV-3988	10	3/10
	30	0/5
Cyproheptadine	20	5/5
S-145 Na	10	5/5
FPL-55712	10	5/5

[a]Drugs were given i.v. 5 min before the antigen challenge.

DISCUSSION

Our results show that the effect of CV-3988 against active anaphylactic shock differed widely according to mouse strain. The suppressive effect was potent in C3H/He and CBA/JN strains, moderate in BALB/c,

TABLE 3

Effect of CV-3988 on Passive Anaphylactic Shock in Untreated and *B. pertussis*-Treated NOD and CTS Mice[a]

Strain	Sex	Age (W)	Antibody	*B. pertussis* treatment	CV-3988 (mg/kg) Cont.	1	3	10	30
NOD	F	18–20	anti-OVA serum	−	6/6[b]	2/2	0/4		
				+ (2×10^9)[c]	5/5				4/5
NOD	M	13–14	anti-BPO·IgG_1[d]	−	4/4	3/3	3/4	1/4	
				+ (2×10^9)	4/4			3/4	
NOD	M	20	anti-BPO·IgE[d]	+ (2×10^8)	3/4		2/2	2/4	1/5
				+ (2×10^9)	4/5			2/2	0/3
CTS	F	10–14	anti-BPO·IgG_1[d]	−	6/6		5/5	0/4	0/5
				+ (2×10^9)	5/5				5/5

[a]CV-3988 was given i.v. 5 min before the antigen challenge. *B. pertussis* was given i.p. 1 wk before the PAF injection.

[b]Lethality (death/total mice).

[c]Cells/animal.

[d]Monoclonal antibody.

TABLE 4

Effect of CV-3988 on PAF-Induced Shock in Untreated and *B. pertussis*-Treated DS Mice[a]

PAF (μg/kg)	*B. pertussis* treatment	CV-3988 (mg/kg)				
		Cont.	1	3	10	30
50	−	23/24[b]	3/6	0/5	0/5	
	+	4/4			4/4	1/6
100	−	5/5		3/5	1/5	
	+	2/2				4/4

[a]CV-3988 was given i.v. 5 min before the i.v. injection of PAF at doses of 50 and 100 μg/kg. *B. pertussis* was given i.p. at a dose of 2×10^9 cells 1 wk before the PAF injection.

[b]Lethality (death/total mice).

TABLE 5

Strain Difference in the Susceptibility to PAF-Induced Shock in the Animals Untreated or Treated with *B. pertussis*[a]

Strain	*B. pertussis* treatment	Incidence of PAF-induced lethal shock at the dose (μg/kg) of						
		100	50	25	12.5	6.3	3.2	1.6
CBA/JN	−		3/3[b]*	2/9	0/7			
	+			8/8*	8/13	0/5		
C3H/He	−			4/4*	9/15	0/13		
	+				9/9*	11/15	0/8	
C57BL/6J	−	6/7*	4/7	3/7	0/7			
	+				6/6*	2/7	0/5	
BDF_1	−	0/14	0/3	0/11				
	+			16/19*	13/20	0/9		
BALB/c	−	0/9						
	+			9/9*	6/10	0/9		
Jcl:ICR	−	3/3*	3/4*	2/4	0/2			
	+				4/4*	2/5	0/4	
DS	−			12/12*	5/13	1/12		
	+				10/10*	13/14*	0/6	
NOD[c]	−		2/2*	0/1	0/1			
	+				3/3*	1/3	0/2	
CTS	−			3/3*	10/10*	7/16	3/11	0/4
	+		1/1*	1/1*	4/4*	3/4*	3/3*	0/3

*Lethality over 75%.

[a]*B. pertussis* was given i.p. 1 wk before the PAF injection.

[b]Lethality (death/total mice).

[c]Females aged 13 to 23 wk.

DBA/2 and BDF_1 strains and absent in CTS, NOD, C57BL/6J and DS strains. For the lack of suppression in CTS and some other strains, two explanations are possible. One is that mediators other than PAF are responsible. The other is that the action of CV-3988 can be greatly influenced by *B. pertussis* used as an adjuvant, even though PAF might be the main mediator for mouse anaphylactic shock in general. The first possibility can be disregarded on the basis of the analogy of the results with passive anaphylactic shock in CTS mice, where high dosages of antagonists to histamine, serotonin, thromboxane A_2 and leukotrienes sufficient to produce complete antagonism did not protect the mice from lethal shock. Involvement of histamine and serotonin can also be ruled out based on the finding that i.v. injection of a high dose (40 mg/kg) of exogenous histamine and serotonin failed to cause lethal shock in untreated mice (data not shown). By contrast, the second possibility is plausible in view of the observation that in these strains the suppressive effect of CV-3988 was markedly attenuated by *B. pertussis* treatment not only against passive anaphylactic shock, but also against PAF-induced shock. Actually, active anaphylactic shock in DS mice, which had been actively sensitized for 4 wk after an immunizing injection using CFA in place of *B. pertussis*, was dramatically suppressed by CV-3988 (data not shown).

It is known that *B. pertussis* and its components enhance the susceptibility to various chemical mediators

such as histamine, serotonin, methacholine and bradykinin and some immunological and physical stimuli in mice and rats (8), probably through the beta-adrenergic blockage (9–11). The present study demonstrated that the susceptibility to PAF was also enhanced by *B. pertussis* in all the mouse strains tested, probably due to its beta-blocking activity in view of the facts that plasma epinephrine and norepinephrine levels are markedly elevated by the injection of PAF in rats (12) and that PAF-induced shock is potentiated by propranolol, a beta-adrenergic blocker, and by adrenalectomy in mice (13,14). However, it should be noted that the *B. pertussis*-induced enhancement of the PAF-induced shock differed markedly according to strain, the enhancement being minimal in C3H/He and CBA/JN strains as compared to the others. This may explain why the potent suppression by CV-3988 was observed against active anaphylactic shock in these two strains even though they had been injected with *B. pertussis* as an adjuvant.

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Effect of a Selective PAF Antagonist SM-10661 ((±)-*cis*-3,5-Dimethyl-2-(3-pyridyl)thiazolidin-4-one HCl) on Experimental Disseminated Intravascular Coagulation (DIC)[1]

Noriaki Imanishi*, Yoshihiro Komuro and Shigeaki Morooka
Research Laboratories, Sumitomo Pharmaceuticals Co., Ltd., Osaka, 554 Japan

Intravenous infusion of endotoxin (0.25 mg/kg/hr for 4 hr) was shown to induce disseminated intravascular coagulation (DIC) in rats, which resulted in hypofibrinogenemia, prolongation of prothrombin (PT) and partial thromboplastin time (PTT), thrombocytopenia, and elevated levels of fibrinogen/fibrin degradation products (FDP). Oral administration (100 mg/kg) of the selective PAF antagonist, SM-10661 ((±)-*cis*-3,5-dimethyl-2-(3-pyridyl)thiazolidin-4-one HCl), counteracted the changes caused by the endotoxin. Intravenous infusion of SM-10661 (6mg/kg bolus 2 min before endotoxin infusion + 6 mg/kg/hr for 4 hr infusion) also counteracted DIC. When suboptimal doses of gabexate mesilate, a synthetic protease inhibitor (3 mg/kg i.p.), and SM-10661 (2 mg/kg bolus + 2 mg/kg/hr for 4 hr infusion) were administered concomitantly, hematological parameters improved. The results suggest that PAF may play a role in the pathogenesis of DIC, and that together with the results already reported for other PAF antagonists, SM-10661 may be useful in the treatment of DIC.
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Disseminated intravascular coagulation (DIC) is a pathological syndrome in which the formation of fibrin thrombi, the consumption of specific plasma proteins, the loss of platelets, and the activation of fibrinolytic systems occur. Clinically, these effects may manifest themselves as diffuse hemorrhage and, less frequently, as thrombosis (1).

The most common cause of DIC is infection (2–4). It is usually induced by endotoxin from gram negative bacteria (5–7). The principal goals in the treatment of DIC are to decrease the symptoms of the disease and to prevent the development and formation of fibrin thrombi.

The pathophysiology of DIC induced by endotoxin is complicated. Endotoxin activates platelets, leukocytes, blood coagulation-fibrinolytic systems and complement systems. The possible involvement of various chemical mediators such as reactive oxygen radicals, prostaglandins and thromboxane A_2 has been suggested (8–11).

It has been shown that injection of endotoxin increases the level of platelet-activating factor (PAF) in rats (12). *In vitro* studies have indicated that endotoxin-activated endothelial cells and monocytes produced PAF *via* the secretion of interleukin 1 and/or tumor necrosis factor (13–16). Therefore, PAF is thought to play a pathophysiological role in DIC caused by septic shock.

We previously developed an orally active PAF antagonist SM-10661 (17,18). We now have studied the effect of SM-10661 on endotoxin-induced DIC and the possibility of involvement of PAF in the pathogenesis of DIC.

MATERIALS AND METHODS

Materials. SM-10661 ((±)-*cis*-3,5-dimethyl-2-(3-pyridyl)-thiazolidin-4-one HCl) was synthesized by Sumitomo Pharmaceuticals Co. Ltd. (Osaka, Japan). Endotoxin (*S. typhimurium*) was purchased from Sigma Chemical Co. (St. Louis, MO). The fibrin/fibrinogen degradation products (FDP) test and gabexate mesilate were obtained from Teikoku Hormone Mfg. Co. (Tokyo, Japan), and Ono Pharmaceutical Co. Ltd. (Osaka, Japan), respectively. SM-10661 was dissolved in saline and adjusted to pH 5–6 by 1 N NaOH.

Effects of drugs on experimental DIC. The rat DIC model was developed according to Yoshikawa *et al.* (19), with slight modifications. Male Wistar rats, 7 to 8 wk old, were anesthetized with sodium pentobarbital (50 mg/kg i.p.), and right and left jugular veins were cannulated for injection of endotoxin and drugs (SM-10661 or saline). Gabexate mesilate was administered intraperitoneally before the start of endotoxin infusion. Endotoxin (0.25 mg/kg/hr) was infused at a flow rate of 0.6 mL/hr for 4 hr. SM-10661 was given both orally and by infusion to test its effect on DIC. In the first treatment, SM-10661 (5 mL/kg) was given orally 1 hr before the start of the endotoxin infusion. In the second treatment, 0.1 mL of SM-10661 was given in a bolus, and subsequently an additional 0.35 mL was infused. The control group was given saline instead of SM-10661, and the normal group was given saline instead of endotoxin and SM-10661. Blood samples (3 mL each) were taken from the abdominal artery into a plastic syringe containing citrate anticoagulant (final concentration 0.36%) 4 hr after the start of endotoxin infusion. Platelets were counted using an automatic counter (Sysmex PL Taiyodensi, Kobe, Japan). FDP was determined by the latex aggregation test and is reported in μg/mL of an equivalent of human fibrinogen base (20). Fibrinogen was determined according to the method of Ellis and Stransky (21); prothrombin time (PT) and partial thromboplastin time (PTT) were measured according to the methods of Quick *et al.* (22) and Langdell *et al.* (23), respectively, using a Blood Coagulation Analyzer (Sysmex PL Taiyodensi, Kobe, Japan).

Statistical analysis. The values in the text and figures represent the mean ± SEM. Student's t-test was used

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*To whom correspondence should be addressed at Research Laboratories, Sumitomo Pharmaceuticals Co., Ltd., 1-98, Kasugade-Naka 3-Chome, Konohana-ku, Osaka, 554 Japan.

Abbreviations: DIC, disseminated intravascular coagulation; FDP, fibrin/fibrinogen degradation products; PAF, platelet activating factor, 1-*O*-alkyl-*sn*-glycero-3-phosphocholine; PT, prothrombin time; PTT, partial thromboplastin time.

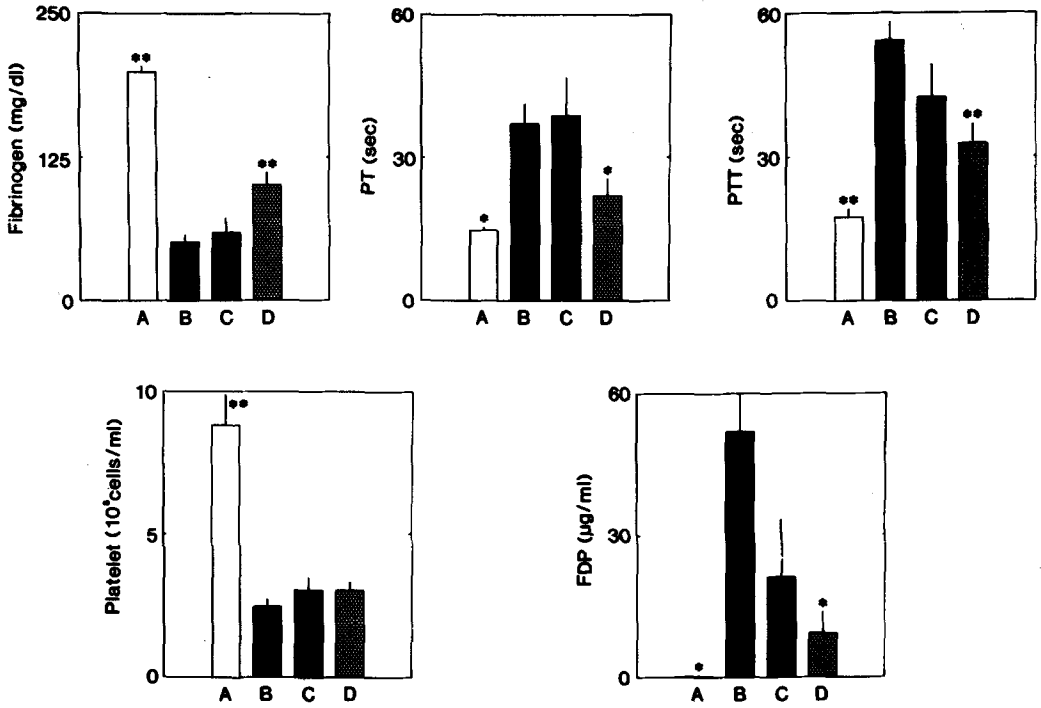


FIG. 1. Effect of orally administered SM-10661 on endotoxin-induced changes of the hematological parameters in rats. (A) Vehicle was administered and saline was infused intravenously for 4 hr (normal group) (n = 5). (B) Vehicle was administered and endotoxin (0.25 mg/kg/hr) was infused intravenously for 4 hr (control group) (n = 19). (C) SM-10661 (30 mg/kg) was administered and endotoxin was infused intravenously for 4 hr (n = 8). (D) SM-10661 (100 mg/kg) was administered and endotoxin was infused intravenously for 4 hr (n = 12). Statistical significance: *P<0.05, **P<0.01 as compared with the control group B.

for the statistical analysis. A value of less than 0.05 was considered significant.

RESULTS

A 4-hr sustained infusion of endotoxin (0.25 mg/kg/hr for 4 hr) caused DIC symptoms in rats (Fig. 1). Fibrinogen decreased from 199 ± 5 to 51 ± 6 mg/dL; PT and PTT were prolonged from 16.7 ± 0.5 sec and 26.1 ± 1.7 sec to 37.2 ± 4.1 sec and 54.2 ± 0.7 sec, respectively. Platelet counts decreased from 8.82 ±0.06×10^8 cells/mL to 2.47 ± 0.26 × 10^8 cells/mL, and FDP increased from 0.17 ± 0.17 μg/mL to 52.0 ± 13.7 μg/mL.

Orally administered SM-10661 partially inhibited endotoxin-induced DIC (Fig. 1). When 100 mg/kg of SM-10661 was administered, fibrinogen (101 ± 11 mg/dL), PT (22.0 ± 1.5 sec), PTT (32.8 ± 1.9 sec) and FDP (9.1 ± 1.5 μg/mL) were improved significantly, but platelet counts were not significantly affected (3.03 ± 1.28 × 10^8 cells/mL).

The effect of SM-10661 intravenous infusion on hematological parameters was examined in another series of experiments. A sustained 4-hr infusion of endotoxin caused DIC symptoms in rats (Fig. 2). Treatment with SM-10661 (6 mg/kg bolus + 24 mg/kg/4 hr infusion) improved fibrinogen (139 ± 8 mg/dL), PT (17.1 ± 0.4 sec), PTT (22.8 ± 0.7 sec), platelet counts (9.2 ± 8.1 × 10^8 cells/mL) and FDP (5.1 ± 1.0 μg/mL).

The effect of post-treatment with SM-10661 intravenous infusion on the hematological parameters was examined (Fig. 2). SM-10661 (6 mg/kg) was given in a bolus 2 hr after the start of endotoxin infusion, and subsequently 24 mg/kg was infused in addition to endotoxin. This treatment also improved fibrinogen (130 ± 10 mg/dL), PT (17.6 ± 0.3 sec) and FDP (9.2 ± 2.1 μg/mL), but did not significantly affect PTT and platelet.

The effect of intraperitoneally administered gabexate mesilate, a potent synthetic protease inhibitor, on hematological parameters is presented in Figure 3. Treatment with 10 mg/kg to 30 mg/kg gabexate mesilate improved the endotoxin-induced changes. The effects of gabexate mesilate were saturable.

To investigate whether co-administration of SM-10661 and gabexate mesilate is more effective than administration of each drug, both SM-10661 (2 mg/kg bolus + 6 mg/kg/4 hr infusion) and gabexate mesilate (3 mg/kg i.p.) were used (Fig. 3). Treatment with both drugs improved the parameters significantly in spite of the ineffectiveness of each drug at the same dose; fibrinogen (119 ± 11 mg/dL), PTT (23.2 ± 1.2 sec), platelet counts (5.85 ± 1.34 × 10^8 cells/mL) and FDP (3.4 ± 1.1 μg/mL). These results demonstrate that the co-administration of SM-10661 and gabexate mesilate had additive effects.

SM-10661 (final concentration; 10^{-9} to 10^{-3} M) did not prolong blood coagulation time *in vitro* (data not shown).

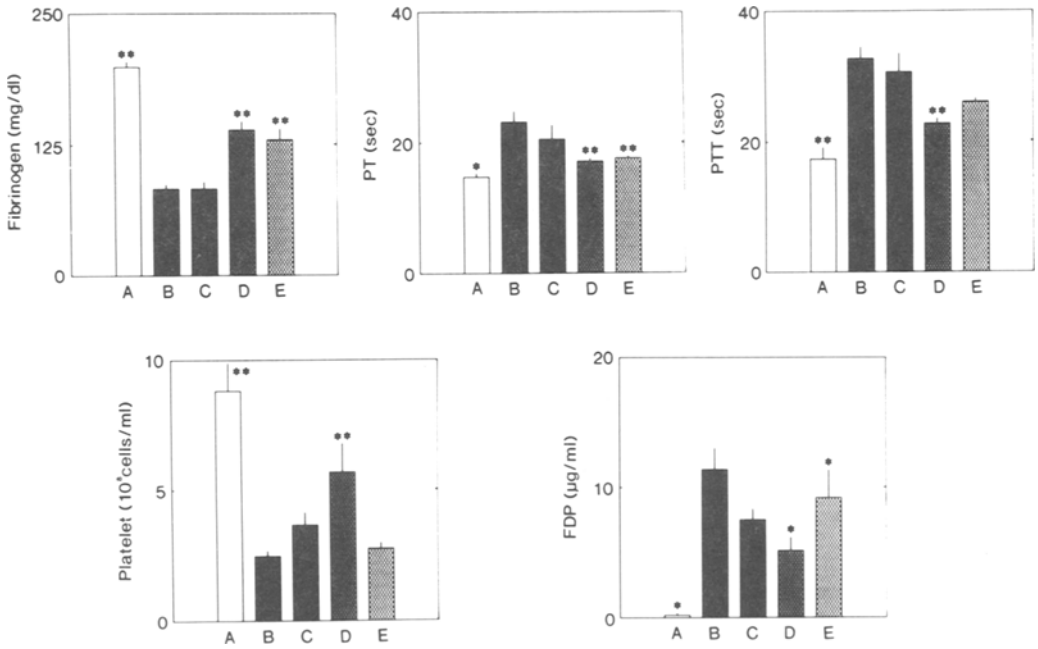


FIG. 2. Effect of intravenously administered of SM-10661 on endotoxin-induced changes of the hematological parameters in rats. SM-10661 or saline was administered in bolus and by infusion. (A) Saline was infused for 4 hr (normal group) (n = 5). (B) Saline was administered and endotoxin (0.25 mg/kg/hr) was infused for 4 hr (control group) (n = 52). (C) SM-10661 (2 mg/kg) was administered before the start of endotoxin infusion and infused (8 mg/kg) for 4 hr (n = 20). (D) SM-10661 (6 mg/kg) was administered before the start of endotoxin infusion and infused (24 mg/kg) for 4 hr (n = 19). (E) SM-10661 (6 mg/kg) was administered 2 hr after the start of endotoxin infusion and infused (24 mg/kg) for 2 hr (n = 15). Statistical significance: *P<0.05, **P<0.01 as compared with the control group B.

DISCUSSION

The intravenous administration of endotoxin evokes serious responses associated with systemic hypotension, shock, pulmonary hypertension, thrombocytopenia, leukopenia and increased vascular permeability. Similar responses were recently reported to be produced by PAF. Treatment by certain PAF antagonists, CV-3988, Kadsurenone and BN-52021 reduced these symptoms (12,24,25). Therefore, PAF is thought to be involved in the conditions induced by endotoxin. However, it is not clear whether PAF is involved in intravascular coagulation caused by endotoxin. Recently, Imura *et al.* (26) reported that CV-3988 prevented changes in all hematological parameters induced by endotoxin. Furthermore, in human patients with sepsis or DIC, high amounts of PAF have been described in blood or urine (27–29). These results suggest that PAF participates in the pathogenesis of endotoxin-induced DIC.

In the present studies, orally administered SM-10661 (100 mg/kg) reduced endotoxin-induced changes in the hematological parameters examined except for platelet counts. Intravenously administered SM-10661 affected these parameters, and thrombocytopenia was also improved. When SM-10661 was administered orally, anti-PAF activity in blood peaked at about 1–2 hr (17,18). Different effects on platelet counts may have resulted when SM-10661 was administered p.o. and i.v. because anti-PAF activity 1–2 hr after the onset of endotoxin infusion is necessary for thrombocytopenia inhibition. SM-10661, given in a bolus (6 mg/kg) 2 hr after the onset of endotoxin infusion and infused (24 mg/kg) with endotoxin for 2 hr, also improved the changes in hematological parameters except for platelet counts and PTT. Anti-PAF activity soon after the administration of endotoxin may therefore be important for the prevention of thrombocytopenia.

It is reported that the intravenous administration of endotoxin causes a transient neutropenia (30). The decrease of neutrophils in the blood is considered to be the result of adhesion of the cells to capillary vessels (31). Accumulation of neutrophils in the microvasculature may play an important role in endothelial damage because neutrophils produce a variety of vasoactive substances such as lysosomal enzymes and active oxygen metabolites (32-34). This injury induces blood coagulation, and is thought to be related to the etiology of DIC. Recent studies have indicated that endotoxin induces secretion of interleukin-1 and tumor necrosis factor in leukocytes (13,14) and that these cytokines activate the adhesion of leukocytes to cultured endothelial cells (35,36). This interaction is inhibited by various PAF antagonists (37,38), which suggests that PAF is involved.

In the present experiment, thrombocytopenia induced by endotoxin infusion may not be a direct effect of PAF because PAF has not been found to induce platelet aggregation in rats (39). However PAF does induce platelet aggregation in humans (40), and therefore PAF antagonists may be more effective in endotoxin-induced DIC in humans than in rats. Further investigation is needed using guinea pig or rabbit whose platelets are sensitive to PAF (40).

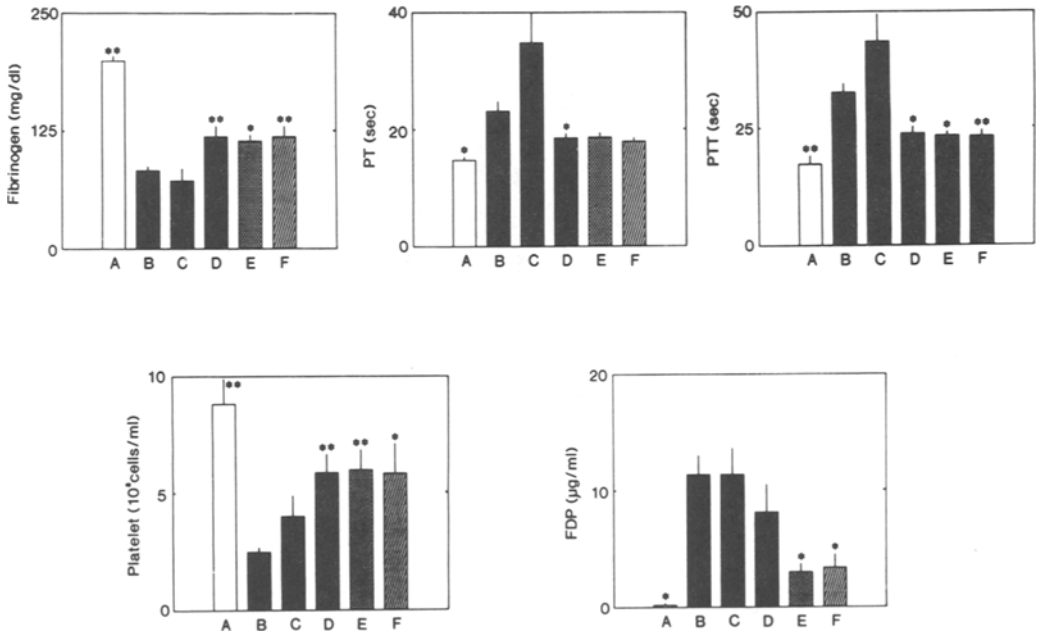


FIG. 3. Effects of intraperitoneally administered gabexate mesilate and intravenously administered SM-10661 on endotoxin-induced changes of the hematological parameters in rats. Gabexate mesilate was administered before the start of endotoxin infusion. (A) Saline was infused for 4 hr (normal group) (n = 5). (B) Saline was administered and endotoxin (0.25 mg/kg/hr) was infused for 4 hr (control group) (n = 52). (C) Gabexate mesilate (3 mg/kg) was administered before the start of endotoxin infusion (n = 11). (D) Gabexate mesilate (10 mg/kg) was administered before the start of endotoxin infusion (n = 9). (E) Gabexate mesilate (30 mg/kg) was administered before the start of endotoxin infusion (n = 6). (F) Gabexate mesilate (3 mg/kg) and SM-10661 (2 mg/kg bolus) were administered before the start of endotoxin infusion and SM-10661 (6 mg/kg) was infused for 4 hr (n = 9). Statistical significance: *P<0.05, **P<0.01 as compared with the control group B.

Several drugs which interfere with the activation of the coagulation system, or which remove fibrin (thrombosis or end-organ failure), are presently used for the treatment of DIC (41). These include synthetic inhibitors of coagulation enzymes such as heparin (42), gabexate mesilate (FOY) (43) and FUT-175 (44) and in some cases, thrombolytic agents (45).

The preventive effects of gabexate mesilate on the changes in hematological parameters were saturable with a bell-shaped dose response curve. Yoshikawa *et al.* (43) reported that an optimal dose of gabexate mesilate had to be administered to obtain the best results. Gabexate mesilate possesses not only anti-thrombin activity but also anti-plasmin activity. The requirement of the optimal dose is thought to be due to its anti-plasmin activity. The choice of optimal drug doses for clinical use may not always be easy. We demonstrated that coadministration of suboptimal doses of both SM-10661 and gabexate mesilate has additive effects. It is reported that protease inhibitors inhibited PAF production stimulated by phagocytosis in macrophages and induced with interleukin-1 in neutrophils or with tumor necrosis factor in vascular endothelial cells (46,47). Such additive effects may be due to the inhibitory effect of gabexate mesilate on PAF production. Therefore SM-10661, in combination with other protease inhibitor, may be useful for the treatment of DIC.

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We are grateful to Junko Nakahara and Naoko Takeda for technical and secretarial assistance.

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Effect of PAF-Acether Antagonists on Active Anaphylactic Mouse Paw Edema[1]

Claudia Zuany Amorim[a], Maria das Graças Muller de Oliveira Henriques[a], Vivian Baseches Weg[a], Renato Sérgio Balão Cordeiro[a,*] and B. Boris Vargaftig[b]

[a]Fundacao Oswaldo Cruz, IOC, Departamento de Fisiologia e Farmacodinâmica, CEP 20040, Rio de Janeiro, Brazil and [b]Unité de Pharmacologie Cellulaire, Unité Associee Institut Pasteur-INSERM U.285, 75015 Paris, France

A new model of active anaphylactic reaction in mice was developed. The edematogenic reaction appeared 5 min after the intraplantar injection of ovalbumin, peaked at 30 min after the antigenic challenge, and decreased thereafter. Using the non-steroidal, anti-inflammatory agents indomethacin and aspirin, we found that cyclooxygenase products do not participate in the reaction. In contrast, vasoactive amines appear to be involved, because meclizine and methysergide reduced the edema. Dexamethasone, BW755C, LY 171883 and WEB 2170 effectively interfered with the edematogenic reaction, which suggests that lipid mediators such as leukotrienes and PAF play a role in the active anaphylactic response.
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Platelet-activating factor (PAF, PAF-acether, 1-*O*-alkyl-2-acetyl-*sn*-glycero-3-phosphocholine) (1) is a mediator of allergic reactions which is involved in acute inflammatory processes (2,3). PAF is released by different cell types, including platelets, mast cells, endothelial cells, monocytes, macrophages, neutrophils and eosinophils (4). In addition, PAF promotes the activation of several inflammatory cells after immune and non-immune stimulation. Evidence suggests that PAF plays an important role in anaphylaxis. Its intravenous administration mimics different characteristics of systemic anaphylaxis, such as a drop in the number of circulating platelets and neutrophils, circulatory collapse with reduced coronary blood flow, and bronchoconstriction (5). Recent studies have also demonstrated the importance of PAF in shock, following the intravenous administration of human IgG aggregates to rats (6), and in mouse anaphylaxis, using the specific antagonist CV 3988 (7,8). Evans *et al.* (9), however, excluded a role for PAF in the increased vascular permeability induced by ovalbumin in actively sensitized guinea pigs. Furthermore, Yoshida *et al.* (10) argued that PAF does not participate in pulmonary anaphylactic dysfunction of guinea pigs or in passive cutaneous anaphylaxis of rats.

Since the role of PAF in mouse anaphylaxis is not fully understood, the focus of the present study was to investigate PAF participation in paw edema induced by intraplantar injection of ovalbumin in sensitized mice.

MATERIALS AND METHODS

Animals. Male Swiss Webster mice raised at the Oswaldo Cruz Foundation (Rio de Janeiro, Brazil) and weighing 25 to 30 g were used.

Sensitization. Animals were sensitized by the subcutaneous administration of 0.2 mL of 0.9% NaCl (saline) containing 10 μg ovalbumin and 1 mg aluminium hydroxide. The animals received two injections of the antigen separated by a 15-day interval (11).

Induction of paw edema. Seven days after the second antigen injection, the animals were challenged with 10 μg ovalbumin in 50 μL saline; the mixture was injected intraplantarly into one of the hind paws. The contralateral paw was injected with saline alone. Non-sensitized animals were injected with 2.5 μg of PAF or ovalbumin diluted with 50 μL saline. An equal volume of saline was injected into the contralateral paw. Animals injected with PAF were given intraperitoneally the PAF antagonist WEB 2170 (2, 4 or 8 mg/kg). The volume of the paw up to the tibiotarsal articulation was determined by plethysmography (12) 30 min after the challenge with ovalbumin or PAF; the results are expressed as increase in volume (μL). The control values obtained for the paws injected with saline alone were subtracted from the volume of the paws injected with the stimuli.

Treatments. Animals were treated intraperitoneally with the anti-inflammatory drugs indomethacin (0.25–0.5 mg/kg), aspirin (100–200 mg/kg), dexamethasone (0.25–2.5 mg/kg), and prednisolone (10–20 mg/kg), with the mixed cyclooxygenase and lipoxygenase inhibitor BW 755C (10–20 mg/kg), with the antihistaminic agents meclizine (12.5–25 mg/kg) and cimetidine (12.5–25 mg/kg), with the antiserotonin agent methysergide (1–5 mg/kg), with the anti-PAF agent WEB 2170 (2–16 mg/kg), and with the leukotriene D_4 antagonist LY 171883 (15–30 mg/kg). All drugs were administered 1 hr before the challenge, except that dexamethasone and prednisolone were administered 12 hr and 1 hr before the challenge, respectively, and meclizine was adminisitered 30 min before the challenge.

Drugs. Ovalbumin, indomethacin and methysergide were purchased from Sigma (St. Louis, MO). Aspirin was from Aspergic Egic Jouille (Paris, France). Dexamethasone was from Merck-Sharp and Dohme (Rio de Janeiro, Brazil) and prednisolone (Meticortem) was from Schering (Rio de Janeiro, Brazil). BW 755C was kindly provided by Dr. S. Moncada, Wellcome Research Laboratories (Beckenham, United Kingdom). Meclizine was from Pfizer (São Paulo, Brazil). Cimetidine was from CEME (Rio de Janeiro, Brazil). WEB 2170 (6-(2-chlorophenyl)-8,9-dihydro-1-methyl-8-(4-morpholinylcarbonyl)-4H, 7H-cyclopenta(4,5)thieno(3,2-f)(1,2,4)-triazolo-(4,3-a)(1,4)di-

[1]Based on a paper presented at the Third International Conference on Platelet-Activating Factor and Structurally Related Alkyl Ether Lipids, Tokyo, Japan, May 1989.

*To whom correspondence should be addressed at Fundação Oswaldo Cruz, IOC, Departmento de Fisiologia e Farmacodinâmica, Avenida Brasil, 4365, Caixa Postal 926, CEP 20040, Rio de Janeiro, Brazil.

Abbreviations: LTD_4, leukotriene D_4; PAF, platelet-activating factor.

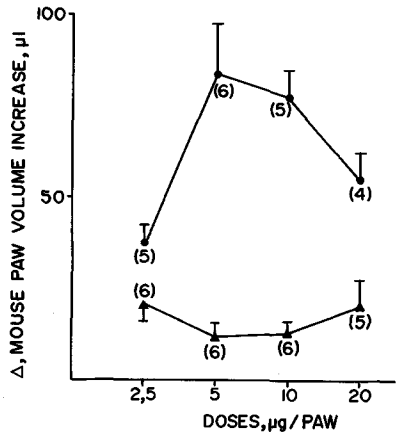


FIG. 1. Increase in mouse paw volume (µL) measured 30 min after intraplantar injection of ovalbumin (2.5–20 µg/paw); ●, sensitized animals; ▲, non-sensitized animals. Vertical bars indicate the mean ± SEM. Numerals in parentheses refer to the number of animals used.

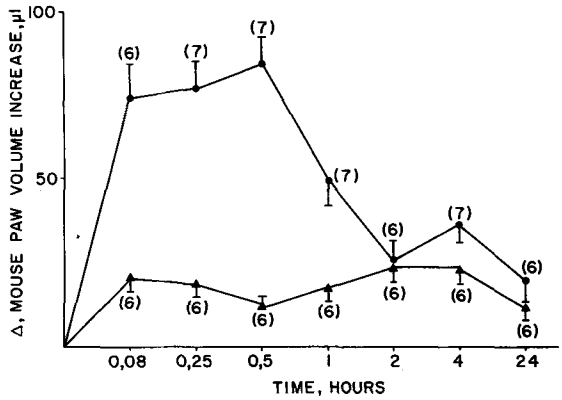


FIG. 2. Analysis of the time course of ovalbumin-induced edema in the mouse paw. The time is expressed in hr (0.08–24 hr). Each point represents the mean ± SEM of at least 5 animals; ●, sensitized animals; ▲, non-sensitized animals. The number of animals used is given in parentheses.

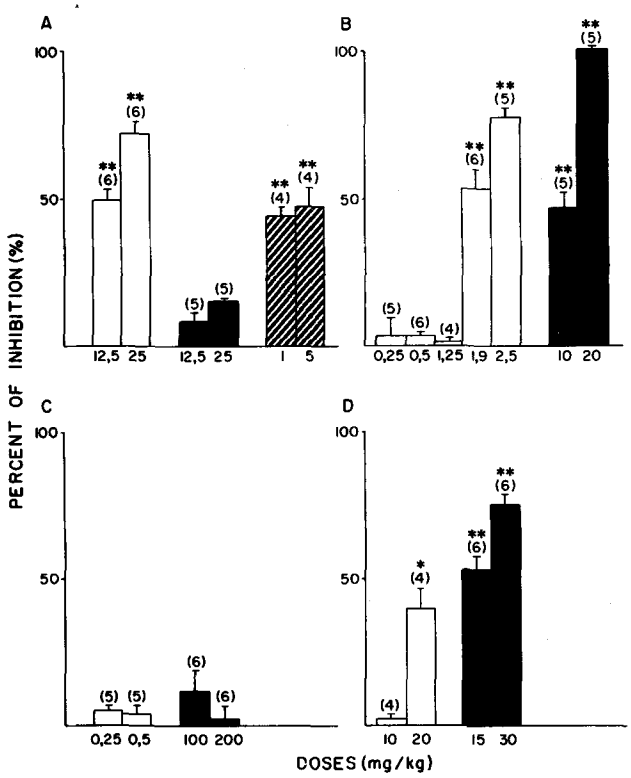


FIG. 3. Percent inhibition of ovalbumin-induced mouse paw edema by intraperitoneal pretreatment with synthesis and receptor antagonists. A, meclizine (12.5–25.0 mg/kg), open bar; cimetidine (12.5–25.0 mg/kg), solid bar; and methysergide (1–5 mg/kg), hatched bar. B, dexamethasone (0.25–2.5 mg/kg), open bar; prednisolone (10–20 mg/kg), solid bar. C, indomethacin (0.25–0.5 mg/kg), open bar; aspirin (100–200 mg/kg), solid bar. D, BW 755C (10–20 mg/kg), open bar; LY 171883 (15–30 mg/kg), solid bar. Vertical bars represent the mean ± SEM. The number of animals is given in parentheses; *, p< 0.01; **, p< 0.001 compared to control. Control groups consisted of at least six animals.

azepine) was kindly donated by Boehringer Ingelheim (Ingelheim, Germany) and the leukotriene D_4 antagonist LY 171883 (1-[2-hydroxy-3-propyl-4[4-(1H-tetrazol-5-yl)butoxy]pheny]ethanone) was kindly donated by Eli Lilly (Indianapolis, IN).

Statistical analysis. Results are reported as means ± standard error of the mean (SEM). The statistical significance of the differences between groups was calculated by analysis of variance by the method of Newman-Keuls.

RESULTS

Animals challenged with ovalbumin (2.5, 5, 10 or 20 µg) developed paw edema 21 days after sensitization (Fig.1). The maximal response, 80 µL, occurred with 5 and 10 µg ovalbumin; therefore 10 µg was used in further experiments. Analysis of the time course of this reaction (Fig. 2) showed an intense edematogenic reaction as early as 5 min after challenge, which reached a maximum at 30 min, and declined thereafter. In contrast, intraplantar injection of up to 20 µg ovalbumin failed to trigger edema in non-sensitized animals (Fig. 1).

Intraperitoneal administration of the H1 antihistaminic agent meclizine (12.5 or 25 mg/kg) and H2 antihistaminic agent cimetidine (12.5 or 25 mg/kg) to sensitized animals showed that only the H1 receptor inhibitor, meclizine, was effective in reducing the edema in a dose-dependent manner, with a maximum inhibition of 80% ($p < 0.001$) for 25 mg/kg (Fig. 3A). In addition, the figure shows that the anti-serotonin agent methysergide (1 or 5 mg/kg) caused a statistically significant inhibition (45%) of the response ($p < 0.001$) (Fig. 3A).

The cyclooxygenase inhibitors indomethacin (0.25 or 0.5 mg/kg) and aspirin (100 or 200 mg/kg), and the mixed cyclooxygenase and lipoxygenase inhibitor BW 755C (10 or 20 mg/kg) were used to examine the possible involve-

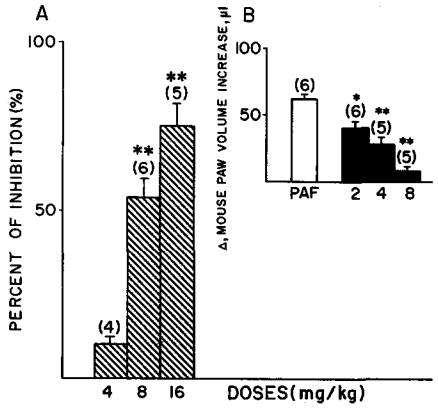


FIG. 4. A, Inhibition of ovalbumin-induced edema by WEB 2170, 4–16 mg/kg (hatched bar); B, Inhibition of PAF induced edema by saline (open bar) and WEB 2170, 2–8 mg/kg, (solid bar) in non-sensitized animals. The results are expressed as mean ± SEM. The number of animals is given in parentheses; *, p< 0.01; **, p< 0.001 compared to control. Control groups consisted of at least 6 animals.

ment of prostaglandins and leukotrienes. Indomethacin and aspirin did not interfere with edema, while BW 755C reduced it by 40% ($p < 0.01$) at 20 mg/kg (Fig. 3C, 3D). Dexamethasone was effective only at doses above 1.25 mg/kg (60% for 1.9 mg/kg and 80% for 2.5 mg/kg, $p < 0.001$). Prednisolone (10 and 20 mg/kg), reduced paw edema dose-dependently, with full suppression at 20 mg/kg ($p < 0.001$) (Fig. 3B). The intraperitoneal treatment of non-sensitized animals with the anti-PAF agent WEB 2170 (2, 4 and 8 mg/kg) inhibited dose-dependently ($p < 0.001$) the paw edema induced by the intraplantar injection of 2.5 μg PAF (Fig. 4A). Figure 4B shows that WEB 2170 8 and 16 mg/kg, administered i.p. caused a potent inhibition of ovalbumin-induced paw edema in sensitized mice ($p < 0.001$) at both doses. Finally, the LTD_4 inhibitor LY 171883 at a dose of 15 mg/kg reduced the increase of mouse paw edema by 55%, and reduced it by 75% at a dose of 30 mg/kg ($p < 0.001$) (Fig. 3D).

DISCUSSION

Administration of ovalbumin into the paw of sensitized mice triggered an edema which peaked within 30 min. The response started to decrease after intraplantar injection of 20 μg ovalbumin, probably because the antigen leaked into the circulation, triggering systemic shock. Shock was distinctly evident when antigen doses higher than 20 μg were injected, causing death of animals (data not shown). In contrast, control non-sensitized animals failed to respond to ovalbumin.

The use of specific antagonists indicated the participation of histamine *via* type H1 receptors, and of serotonin as reported by Kaneta *et al.* (13) in passive paw anaphylaxis of the mouse.

The fact that this reaction was not inhibited by the non-steroidal, anti-inflammatory agents indomethacin and aspirin, whereas both the cyclooxygenase and lipoxygenase mixed inhibitor BW 755C and the leukotriene D_4 antagonist compound LY 171883 interfered with edema, suggests that the cyclooxygenase metabolites of arachidonate are not involved in this type of anaphylactic model, in contrast to the lipoxygenase metabolites. These results differ from those observed in passive anaphylaxis of the rat by Ohuchi *et al.* (14) who found that leukotrienes do not participate in the reaction, but histamine and serotonin do.

The mechanism of action of steroidal anti-inflammatory drugs may involve the synthesis of phospholipase A_2 inhibitors, such as the lipocortins (15). The present results show that the glucocorticoid prednisolone suppresses the inflammatory response, whereas dexamethasone at doses that inhibit carrageenin-induced paw edema (0.25 and 0.5 mg/kg) (16) failed to interfere with the reaction and required doses as high as 1.9 mg/kg to be effective. Similar results were reported by Kaneta *et al.* (17) who showed that dexamethasone does not protect mice from passive paw anaphylaxis at doses of 0.1 mg/kg administered once a day for two days. On the basis of the model used by our group (edema by antigen *versus* edema by carrageenin), the different dose requirements for glucocorticosteroids confirm that these anti-inflammatory agents interfere with multiple targets and/or the synthesis of inhibitory proteins (18). In our study the specific antagonist WEB 2170 (19) was used. WEB 2170 has been recently found effective in inhibiting PAF-induced paw edema and pleurisy by Henriques *et al.* (20). Our present results confirm the previous data and show that the PAF antagonist WEB 2170 suppresses paw edema induced by PAF and by ovalbumin in sensitized animals, which suggests the participation of PAF in active anaphylaxis in mice.

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Pooling of Blood in Postischemic Shock Is Modulated by Platelet-Activating Factor (PAF)[1]

V.F. Sagach*[,a], A.V. Dmitrieva[a] and P. Braquet[b]
[a]A.A. Bogomolets Institute of Physiology, Kiev, USSR and [b]Institut Henri Beaufour, Le Plessis-Robinson, France

In experiments on dogs, *i.v.* administration of platelet-activating factor (PAF) (500 ng/kg) was shown to induce hypotension which, apart from decreased myocardial contractility, was characterized by blood pooling in veins (82.6 ± 6.8 mL/kg). This was accompanied by restriction of venous return to the heart and reduction of cardiac output (CO). During postischemic shock the cardio- and hemodynamic disturbances were similar to those induced by *i.v.* administration of PAF. In the postischemic shock model, preliminary blockage of PAF receptors with the PAF receptor antagonist BN 52021 (6 mg/kg, *i.v.*) significantly decreased the amount of blood pooled in shock from 38.7 ± 5 to 18.3 ± 2 mL/kg ($p<0.01$). Simultaneously, the reduction of CO and blood pressure, induced by reperfusion of the continuously ischemized tissues of a rear limb, was less significant in pretreated *vs.* the nontreated group. The data suggest that PAF may be involved in postischemic blood pooling and that PAF antagonists could be used to correct postischemic cardio- and hemodynamic disturbances.
***Lipids* 26, 1400–1403 (1991).**

In recent years, the development of a number of shock-related cardio- and hemodynamic disturbances has been attributed to lipid mediators, and particularly to platelet-activating factor (PAF). The administration of PAF to animals has been shown to produce systemic hypotension, inhibition of myocardial contractility, and reduction of cardiac output (CO). These disturbances are characteristic for shock (1,2). There is evidence for a protective effect by different PAF receptor antagonists on the cardiovascular system in endotoxic and anaphylactic shock. Terashita *et al.* (3) reported the protective effect of PAF antagonists in traumatic shock, while Sagach and Dmitrieva (4) noted similar effects in tourniquet shock. During traumatic shock, an increase of PAF content was found in peritoneal fluid (5). However, the precise mechanisms of the involvement of PAF in the shock-related development of cardio- and hemodynamic disturbances are still unclear.

The purpose of the present investigation was to clarify whether PAF is involved in postischemic blood pooling and in the development of hemodynamic disturbances.

MATERIALS AND METHODS

Twenty mongrel dogs were premedicated with Calipsol and then anesthetized with chloralose/urethane mixture (5 and 50 mg/kg, *i.v.*). Three series of experiments were carried out. In group 1 (n=13) postischemic shock was produced. Group 2 (n=5) was treated with PAF antagonist BN 52021 (6 mg/kg, *i.v.*) prior to reperfusion of the ischemic rear limb. PAF (500 ng/kg, *i.v.*) was administered in Group 3 (n=7).

Postischemic shock was modeled as follows. The left femoral artery and vein were ligated, and the left rear limb was ischemized using tourniquet for 3.5 hr. Then the ischemic rear limb was reperfused for 3 hr.

The right and left external jugular veins, main carotid arteries, subclavian arteries and right femoral artery and vein were exposed. A thermodilution CO catheter was positioned in the descending aorta through the left subclavian artery. The second catheter was placed in the left jugular vein for administration of cold solution. The left ventricle was catheterized through the left main carotid artery. The right and left external jugular veins were used for catheterization of the pulmonary artery and right atrium.

A special system for autoperfusion of coronary vessels was mounted. The right subclavian artery was catheterized, then the flow probe, offset to the pressure transducer, and a stiff metal catheter were connected in sequence. The metal catheter was placed in the aorta semi-lunar valves through the right main carotid artery, then turned around until it touched the opening of the left coronary vessels under pressure control. The amount of blood flowing from the subclavian artery to one of the left coronary artery branches was measured using an electromagnetic flow probe.

The vessels in the nonischemic rear limb were monitored by resistography, using pumps which perfused a constant volume of blood. The blood flowed from the femoral vein into a graduate burette, then was returned to the body by pumping through the proximal portion of the same vein.

The following hemodynamic parameters were measured: mean blood pressure, venous and pulmonary pressure, cardiac output (using the thermodilution method), coronary flow, left ventricular pressure and its first derivative (dP/dt), myocardial contractility (6), and total vascular, pulmonary and coronary resistance. In addition, we measured femoral mean filling pressure (7), compliance of venous vessels, and coefficient of capillary filtration (8). The amount of blood pooled was measured by means of a graduate burette in the resistography system. All indices were registered using a polycardiograph (Mingograph-82, Siemens-Elema, Berlin Germany).

C_{16}PAF (Novabiochem, Switzerland) was dissolved in dimethylsulfoxide (1 mg/mL) and diluted to the required concentration prior to injection. The PAF receptor antagonist BN 52021 (Institut Henri Beaufour, Plessis-Robinson, France) (6 mg/kg) was administered *i.v.* 15 min prior to reperfusion of the ischemic rear limb.

All data presented in the text and Figures are expressed as mean ± SEM of n observations. The differences within and between groups were analyzed using Student's *t*-test.

[1]Based on a paper presented at the Third International Conference on Platelet-Activating Factor and Structurally Related Alkyl Ether Lipids, Tokyo, Japan, May 1989.

*To whom correspondence should be addressed at A.A. Bogomolets Institute of Physiology, Bogomolets St. 4, 252601 Kiev-24, USSR.

Abbreviations: CO, cardiac output; CVP, central venous pressure; LVEDP, left-ventricular end-diastolic pressure; MBP, mean blood pressure; PAF, platelet-activating factor; TVR, total vascular resistance.

A value of 0.05 or less was considered statistically significant.

RESULTS

The reperfusion of a continuously ischemic rear limb was accompanied by a gradual decline in mean blood pressure (MBP), which reached 52% of the initial level, or 68 ± 6.1 mm Hg ($p<0.001$), 3 hr after initiation of reperfusion. The development of hypotension was characterized by a drop of CO from 1864 ± 106 to 1003 ± 98 mL/min ($p<0.001$), which occurred without significant changes in total vascular resistance (TVR). The decrease in CO was associated with a reduction in myocardial contractility. Thus, the index of myocardial contractility decreased from 51.1 ± 3.2 to 33.3 ± 8 s^{-1} ($p<0.05$) 3 hr after initiation of the reperfusion. These changes were accompanied by a decrease in the circumflex coronary artery blood flow from 59.2 ± 5.8 to 37.4 ± 4.3 mL/min ($p<0.05$) without significant variation in coronary resistance.

Another major cause of reduced CO and subsequent hypotension was the restriction of venous blood return to the heart as exemplified by the decrease in central venous pressure from 2.2 ± 2 to −2.5 ± 1.1 mm Hg ($p<0.05$) and in left-ventricular end-diastolic pressure (LVEDP) to reach negative values. The decrease in venous return was associated with blood pooling in the periphery of the vascular bed, which resulted from the increase in compliance of venous vessels from 0.12 ± 0.01 to 0.56 ± 0.08 mL/mm Hg ($p<0.001$). The amount of blood pooled in the nonischemic rear limb was 38.7 ± 10 mL/kg. Decreased resistance and compliance of peripheral vessels could be attributed to the substantial fall in filling pressure of the rear limb's vascular bed, which decreased from 60.4 ± 3.3 to 13.2 ± 3.4 mm Hg at the end of the observation period (Fig. 1).

A similar pattern of cardio- and hemodynamic changes was noted following *i.v.* administration of PAF (500 ng/kg) (Fig. 2). Development of marked hypotension with a fall in MBP from 106 ± 6 to 47.4 ± 3.9 mm Hg ($p<0.001$) was accompanied by a sharp reduction in CO from 2251 ± 191

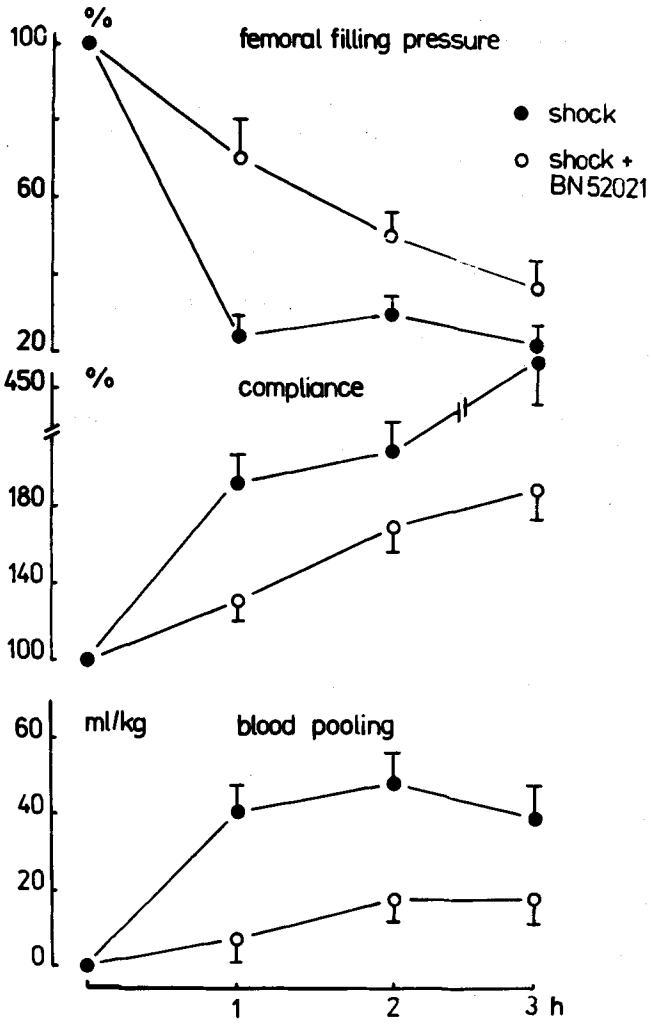


FIG. 1. Effect of the PAF antagonist BN 52021 on circulatory changes in the femoral vascular bed in postischemic shock reaction. The ordinates depict femoral filling pressure, compliance of venous vessels, and the amount of blood pooled; the abscissae depict the time of reperfusion. The data are expressed as means ± SEM.

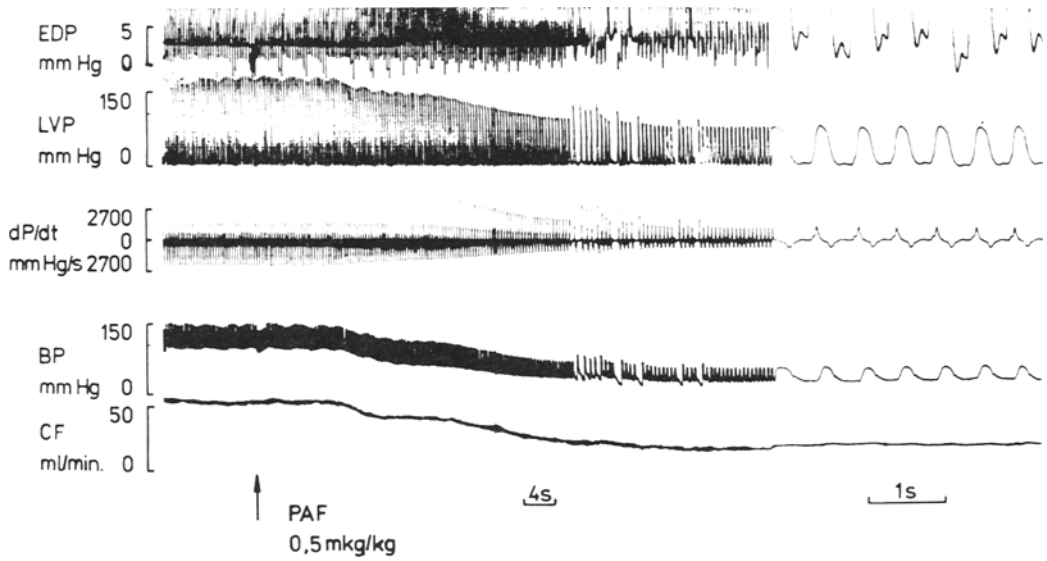


FIG. 2. Cardio- and hemodynamic changes in dog after *i.v.* PAF administration (500 ng/kg). Abbreviations: EDP, end-diastolic pressure; LVP, left-ventricular pressure; BP, arterial blood pressure; CF, coronary blood flow.

to 1181 ± 145 mL/min ($p<0.001$), and a decrease of the index of contractility from 55.8 ± 1.9 to 41.1 ± 2.7 s^{-1} ($p<0.001$). These animals showed marked signs of restriction of venous blood return to the heart due to blood pooling at the periphery of the vascular bed. The amount of blood pooled reached 86 ± 9.2 mL/kg and was accompanied by an increase of compliance of venous vessels from 0.27 ± 0.02 to 0.4 ± 0.037 mL/mm Hg ($p<0.01$) (Fig. 3). Apart from this, a significant increase in capillary permeability occurred: The coefficient of capillary filtration increased from 0.038 ± 0.005 to 0.055 ± 0.001 mL·mm $Hg^{-1}·g^{-1}·min^{-1}$ ($p<0.01$). The femoral filling pressure fell from 24.8 ± 3.8 to 11.7 ± 1.9 mm Hg ($p<0.02$); and central venous pressure (CVP) became negative.

Administration of BN 52021 prior to reperfusion of the ischemic rear limb significantly diminished both changes in cardiac function and in the phenomenon of blood pooling. In BN 52021 pretreated animals, the decrease in MBP was less marked, associated with a less significant reduction in CO, which fell from 1578 ± 118 to 1184 ± 131 mL/min ($p<0.05$). Inhibition of myocardial contractility was less pronounced in BN 52021 pretreated dogs. Thus, 3 hr after initiation of reperfusion, the left ventricular dP/dt was 37% lower than the initial value, while the inhibition in the nontreated group corresponded to 49%. In contrast to nontreated animals, the index of contractility changed from 52.8 ± 5.9 to 47.2 ± 5 s^{-1}. The decrease in CVR and LVEDP was only half of that of the nontreated group. These changes, indicative of restrictions in venous return and blood pooling, were less pronounced in BN52021 pretreated animals.

The compliance of venous vessels increased from 0.21 ± 0.02 to 0.4 ± 0.035 mL/mm Hg ($p<0.01$) *vs.* more than 400% in nontreated animals (Fig. 1). The amount of blood pooled was 18.3 ± 2.0 mL/kg.

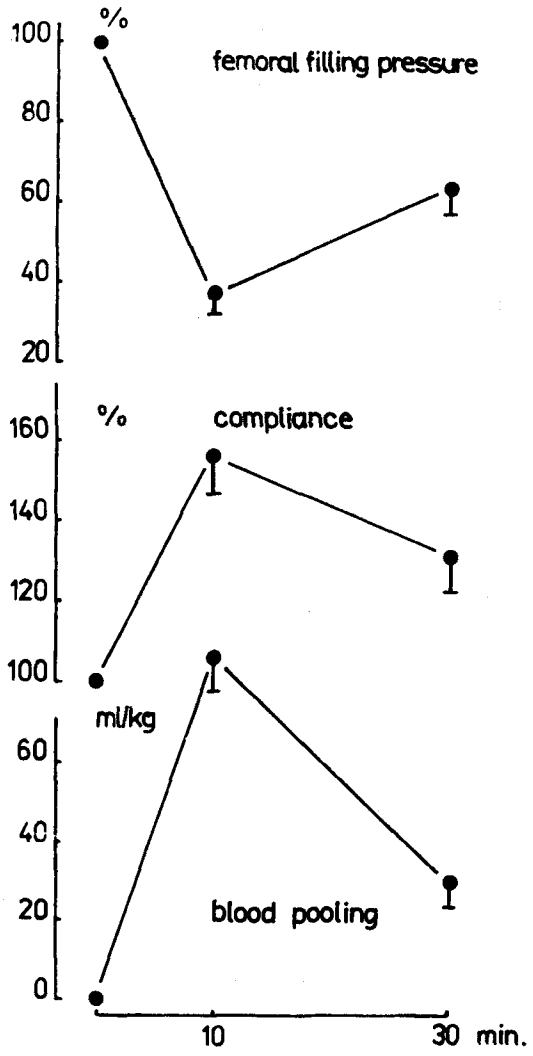


FIG. 3. Hemodynamic changes in the femoral vascular bed after PAF administration (500 ng/kg, *i.v.*). Coordinates and other details are as in Figure 1.

DISCUSSION

We have shown that hypotension, which develops following reperfusion of ischemic rear limb tissue, was associated with substantial blood pooling at the periphery of the vascular bed with subsequent restriction of venous return to the heart and decreased CO. PAF recently has been shown to participate in the development of lesions that occur in different organs and tissues in ischemia and subsequent reperfusion (9,10). PAF has been implicated as an important mediator in a variety of shock states, including anaphylactic (2,11), endotoxic (12), and traumatic shock (3,5). The capacity of PAF receptor antagonists to diminish hypotension and to preserve cardiac pumping and contractility functions suggest PAF involvement in shock reactions (1-3,12).

Our data show that blockage of PAF receptors by BN 52021 significantly decreased blood pooling, which developed due to increased compliance of peripheral venous vessels and due to increased capillary permeability, and thus reduced restrictions of venous return to the heart.

These effects of PAF on the cardiovascular system, namely its capacity to increase compliance of the veins, capillary permeability and pooling of blood in venous vessels, and the restriction imposed on venous return, have not been observed previously (1,13,14). Our results show that *i.v.* administration of PAF, apart from its known effects (systemic hypotension, decrease of coronary flow and myocardial contractility, pulmonary hypertension), may increase venous compliance, capillary permeability (*e.g.*, that of femoral vessels) and blood pooling. The latter is accompanied by the signs of restricted venous return to the heart (decrease of CVP, LVEDP, intravascular filling pressure) with subsequent reduction in CO. In a special series of experiments with stabilized venous flow to the heart, we showed that during *i.v.* administration of PAF the restriction of venous return to the heart was the major cause of reduced CO and hypotension (15). It is unclear whether this is a direct effect of PAF on the vessels, or whether it occurs *via* mediators capable of producing similar effects (16) that are released upon PAF stimulation.

In conclusion, it appears that blood pooling and subsequent hemodynamic disturbances in postischemic shock may be induced by PAF, and that PAF antagonists, such as BN52021, may be useful in attenuating these disturbances.

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Induction of Platelet-Activating Factor in Mice by Intravenous Administration of a Neutral Fraction of Bakers' Yeast Mannan[1]

Takeshi Mikami[a], Ken Fukushi[a], Miki Ishitani[a], Kouki Ishitani[a], Shigeo Suzuki[b] and Masuko Suzuki[a,*]
[a]Department of Microbiology and [b]Second Department of Hygienic Chemistry, Tohoku College of Pharmacy, Sendai 981, Japan

A neutral subfraction of mannan of bakers' yeast (WNM) was found to show a lethal effect in mice when administered intravenously. Symptoms caused by intravenous (i.v.) administration of WNM resembled those resulting from the administration of platelet-activating factor (PAF). CV-3988 and ONO-6240, selective PAF antagonists, prevented hypotension and death caused by the administration of WNM or PAF. A β-adrenoceptor agonist was shown to prevent death caused by WNM, whereas propranolol increased the lethal activity of WNM. Intravenous administration of WNM into mice produced PAF in gall bladder fluid which was determined by platelet aggregation assay. The findings indicate that WNM is able to induce PAF in mice and that the resultant PAF may participate in the WNM-induced lethal activity observed in mice.
***Lipids 26,* 1404–1407 (1991).**

A neutral subfraction of mannan of bakers' yeast (WNM) has been shown to cause various biological effects in mammalian systems such as antitumor effects against several transplantable tumors in mice (1–3), *Limulus* amoebocyte lysate-gelation activity (4), pyrogenicity in rabbits (5), and a mitogenic effect on murine splenic B-lymphocytes (6).

In the course of our studies of the biological effects of mannan, we found that WNM showed strong lethal activity in mice when it was administered intravenously (i.v.) (7), and that the acidic fraction of mannan from the same yeast containing phosphate, hexosamine, and chemically phosphorylated WNM was practically ineffective. We also demonstrated that the pretreatment of mice with D-mannose, D-galactose and dexamethasone (an anti-inflammatory steroid) protected the mice from death.

It is now well recognized that PAF shows a potent systemic hypotensive effect when administered either i.v. or orally (8,9). Myers *et al.* (10) reported that i.v. administration of PAF caused lethal shock in mice, and this could be prevented by pretreatment with cortisone acetate.

In the present paper, we report on the lethal effect of WNM in mice which we studied by using PAF agonists and antagonists.

[1]Based on a paper presented at the Third International Conference on Platelet-Activating Factor and Structurally Related Alkyl Ether Lipids, Tokyo, Japan, May 1989.

*To whom correspondence should be addressed at Department of Microbiology, Tohoku College of Pharmacy, 4-4-1 Komatsushima, Aoba-Ku, Sendai 981, Japan.

Abbreviations: CV-3988, R,S-2-methoxy-3-(octadecylcarbamoyloxy)-propyl 2-(3-thiazolio)ethyl phosphate; DEAE, diethylaminoethyl; i.p., intraperitoneally; i.v., intravenous; LPS, lipopolysaccharides; ONO-6240, 1-*O*-hexadecyl-2R,S-*O*-ethyl-3-*O*-(7-thiazolipoheptyl)-glyceryl methanesulfonate; PAF, platelet-activating factor, 1-*O*-alkyl-2-acetyl-*sn*-glycero-3-phosphocholine; WNM, a neutral subfraction of mannan of bakers' yeast (*Saccharomyces cerevisiae* wild type strain).

MATERIALS AND METHODS

Materials. C_{16} PAF (1-*O*-hexadecyl-2-acetyl-*sn*-glycero-3-phosphocholine) was purchased from BACHEM Feinchemikalien AG (Bubendorf, Switzerland). CV-3988, namely R,S-2-methoxy-3-(octadecylcarbamoyloxy)propyl 2-(3-thiazolio)ethyl phosphate, was provided by Takeda Chemical Industries, Co., Ltd. (Osaka, Japan). ONO-6240, namely 1-*O*-hexadecyl-2R,S-*O*-ethyl-3-*O*-(7-thiazolipoheptyl)glyceryl methanesulfonate was provided by ONO Pharmaceutical Co. (Osaka, Japan). Salbutamol, an agonist of β-adrenoceptors, and propranolol, an antagonist of β-adrenoceptors, were purchased from Sigma Chemical Co. (St. Louis, MO), and Nakarai Tesque, Inc. (Kyoto, Japan), respectively.

Preparation of the neutral fraction of mannan. The neutral mannan of bakers' yeast of a wild type strain of *Saccharomyces cerevisiae* (WNM) was prepared in accordance with the description by Suzuki *et al.* (3). Commercially available bakers' yeast was extracted with water at 140°C for 3 hr. After cooling, the extract was centrifuged and the residue was washed with water. The supernatant was concentrated *in vacuo*. The concentrated solution was poured into ethanol. The precipitate formed was collected and dissolved in water. Fehling solution then was added until the precipitation was completed. The mannan copper complex was washed several times with water, suspended in water, and concentrated HCl was added to dissolve the complex. The resultant solution was poured into ethanol, and the precipitate formed was collected by centrifugation. The crude polysaccharides were dissolved in water and passed through a column of Amberlite IR-120. The eluted solution was concentrated and precipitated with ethanol. The crude mannan fraction was dissolved in water, applied on a column of diethylaminoethyl (DEAE)-Sephadex (acetate type), and washed thoroughly with water. The eluate was concentrated and dialyzed against distilled water.

The results of the chemical analyses of WNM showed that the carbohydrate, phosphate, and nitrogen contents were 98.0, 0.0, and <0.3%, respectively. A *Limulus* amoebocyte lysate gelation assay showed that WNM was not contaminated with any bacterial lipopolysaccharides (LPS) (4).

Animals. ddY Strain male mice, 5 to 8 wk old, were obtained from Shizuoka Agricultural Cooperative Association for Laboratory Animals (Hamamatsu, Japan).

Assay of the effect of several drugs on the lethality of WNM in mice. The lethal effect of WNM was determined as described in a previous report (7). CV-3988 and ONO-6240 were administered i.v. to ddY mice, eight mice per group, at a dose of 10 mg/kg, 30 min before challenge with a lethal dose of WNM or at the time of WNM administration. The influence of β-adrenoceptors on lethality was measured as described by Criscuoli and Subissi (11). Salbutamol was used as an agonist and propranolol was used as an antagonist of β-adrenoceptors. Salbutamol and propranolol were administered intraperitoneally (i.p.) 30

min before WNM at doses of 1 mg/kg and 10 mg/kg, respectively. In all assays, mortality was expressed as the total number of dead mice from duplicate experiments, and percent of survival was calculated.

Assay of the effect of WNM and PAF on blood pressure of mice. Two groups of ddY strain mice, 10 mice per group, were given 10 mg/kg of CV-3988 or ONO-6240. Thirty minutes after CV-3988 treatment, or immediately after the administration of ONO-6240, 5 mg/kg of WNM was injected i.v. Blood pressure was measured until 30 min after the injection of WNM using the electric Rat-Mouse Manometer-Tachometer TK-350 (Unicom Co., Tokyo, Japan).

Detection of PAF in gall-bladder fluid from WNM-treated mice. The methods used were adopted from Doebber *et al.* (12). Mice were injected with 10 mg/kg of WNM i.v., and 10 min after injection, gall-bladder fluid was collected under anesthetized conditions. The lipid fraction in the bile was isolated according to the method of Bligh and Dyer (13) and was applied on a column of SEP-PAK Silica Cartridges (Waters Associates, Milford, MA) and eluted with chloroform, acetone, acetone/methanol (1:1, v/v), chloroform/methanol (7:3, v/v), and chloroform/methanol/water (10:20:8, v/v/v), subsequently. The finally eluted fraction was evaporated to dryness, and was designated as PAF specimen.

Bioassay of PAF by platelet aggregation. Rabbit blood, obtained by arterial puncture, was collected in a 3.8% trisodium citrate/citric acid buffer and processed as described by Benveniste *et al.* (14). The blood was centrifuged at room temperature for 10 min at 500 × *g* to obtain the platelet-rich fraction. Platelet aggregation by PAF was measured by a modified method described by Camussi *et al.* (15), using a platelet aggregation analyzer AA-100 (SYSMEX, Koube, Japan). A suspension of 0.25 mL containing 5 × 10^8 platelets/mL in HEPES-Tyrode buffer was added to a siliconized cuvette containing a Teflon stirbar revolving at 1000 rpm. After a 5-min delay to allow warming of cells to 37°C, 25 μL of aggregant was added and the resultant changes in light transmission were recorded as ΔT. Pretreatment of platelets with PAF inhibitor was also done with CV-3988, 25 ng, 30 min before treatment of samples.

RESULTS

Effects of selective PAF antagonists and β-adrenoceptor reagents on lethality of WNM in mice. As shown in Figure 1, CV-3988 (10 mg/kg) and ONO-6240 (10 mg/kg) lowered the lethality of WNM. CV-3988 increased the survival rate of mice from 25.0 to 77.7% and ONO-6240 from 25.0 to 100.0%. Following the administration of WNM at a dose of 20 mg/kg and propranolol at a dose of 20 mg/kg i.v., survival decreased. However, death of mice due to 50 mg/kg of WNM was completely prevented by salbutamol at a dose of 1 mg/kg, indicating that this β-adrenoceptor agonist is a very potent inhibitor of the WNM-induced lethal effect.

Inhibitory effects of PAF antagonists on hypotension induced by WNM. In order to test whether PAF was one of the substances causing hypotension in mice, the animals were pretreated with either of the two PAF antagonists. For this purpose, 10 mg/kg of CV-3988 or 10 mg/kg of ONO-6240 was administered 30 min before or simultaneously with WNM, and blood pressure changes

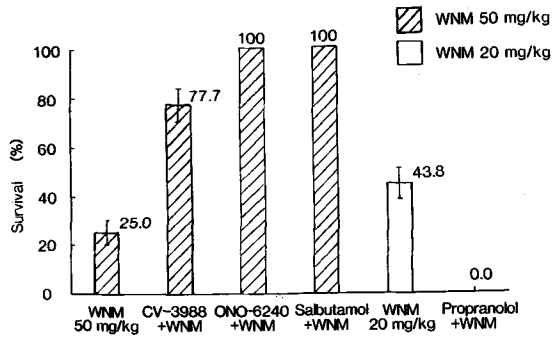


FIG. 1. Effect of several drugs on the lethal activity of WNM in mice. WNM (50 mg/kg): 50 mg/kg of WNM-treated group; CV-3988 + WNM, 10 mg/kg of CV-3988 was pretreated i.v. 30 min before 50 mg/kg of WNM administration; ONO-6240 + WNM, 10 mg/kg of ONO-6240 was administered i.v. at the same time with 50 mg/kg of WNM; Salbutamol + WNM, 1 mg/kg of salbutamol was pretreated i.p. 30 min before 50 mg/kg of WNM administration. WNM (20 mg/kg): 20 mg/kg of WNM-treated group; Propranolol + WNM, 10 mg/kg of propranolol was pretreated i.p. 30 min before 20 mg/kg of WNM administration. Figure shows mean survival percentage ± the standard error.

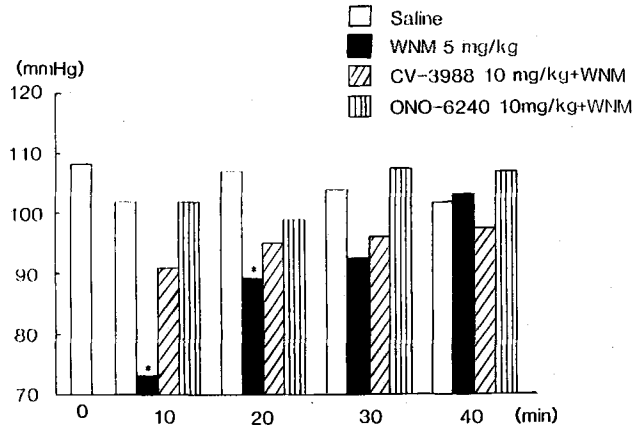


FIG. 2. Effect of PAF inhibitors on hypotension induced by WNM. Diagonal-lined bar, CV-39888 (10 mg/kg) pretreated i.v. 30 min before 5 mg/kg of WNM administration; vertical-lined bar, ONO-6240 (10 mg/kg) administered i.v. at the same time with 5 mg/kg of WNM; solid bar, WNM (5 mg/kg) in saline; open bar, saline control. * Indicates statistical significance between the experimental group and control group (P<0.05). Figure represents mean blood pressure of each sample.

in groups of mice given WNM alone or WNM plus PAF antagonists were measured. As shown in Figure 2, groups of PAF antagonist-treated mice showed no hypotension after receiving an i.v. injection of WNM at 50 mg/kg. The antagonists alone also showed no hypotensive effect.

Amount of PAF from gall-bladder fluid in WNM-treated mice. The results of this study indicate that i.v. administration of WNM induced *in vivo* PAF formation which caused various biological effects, including lethality. Therefore, we made an attempt to detect the PAF induced by WNM in gall-bladder fluid. The amount of PAF produced by administration of 10 mg/kg of WNM (0.13 pg/mouse) was estimated by measuring platelet aggregation and by comparing the aggregatory effect with that observed at various PAF concentrations (Fig. 3). Pretreatment with CV-3988 at a dose of 25 ng completely inhibited aggregation of platelets by gall-bladder fluid after the administration of WNM (data not shown).

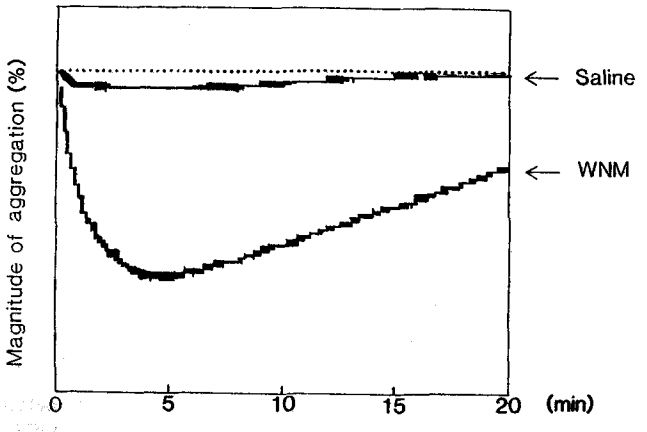


FIG. 3. Aggregation of rabbit platelets by gall-bladder fluid from WNM- or saline-treated mice. Platelet aggregation was assayed with a Sysmex AA-100 on 5 × 10^8 cells using 25 μL of sample at 37°C (20 min).

DISCUSSION

It has been reported (16–18) that cells of the yeasts *Candida albicans* and *Saccharomyces cerevisiae*, and an alkali-soluble extract of the mannan fraction from a cell wall preparation of *Candida albicans*, can elicit aggregation of rabbit platelets causing fibrin-platelet clots.

We previously also reported that WNM can act as a polyclonal B-cell mitogen, and that the effect was not due to contamination with bacterial lipopolysaccharides (LPS). The gelation activity of WNM was preserved upon treatment with 100 mM NaOH at 30°C for 48 hr, while bacterial LPS are not stable under these conditions. The results clearly demonstrated that *Limulus* amoebocyte lysate gelation activity of mannan was not due to contamination by bacterial LPS (4,6).

The present study revealed that the lethal effect of WNM is caused mainly by PAF produced by WNM. However, in a previous study (5) it was noted that the number of circulating platelets in mice treated with WNM did not change when compared with untreated-mice, and that administration of anti-platelet aggregating agents (*e.g.*, ticlopidine, indomethacin) did not inhibit the lethal effect of WNM. Mouse platelets cannot be aggregated by PAF (19) because these platelets lack PAF receptors (20). Therefore, WNM-induced lethality in mice must be mediated by some other mechanism.

Criscuoli and Subissi (11) reported that i.v. injection of PAF caused death in mice without causing platelet aggregation, and propranolol potentiated the effect of PAF (LD_{20}) in a dose-dependent fashion. On the other hand, salbutamol completely protected mice from PAF-induced death (LD_{80}). High doses of indomethacin, aspirin, benoxaprofen and FPL 55712 failed to counteract the lethal effect of PAF. Therefore, the authors concluded that manipulation of β-adrenoceptors affects PAF-induced death in mice, while 5-lipoxygenase and cyclooxygenase products derived from arachidonate do not appear to contribute to the lethal effect observed.

Biological and immunological effects of yeast mannan, containing a high-mannose type sugar chain, have been reported by several investigators (21,22). The existence of lectin-like proteins which bind with yeast mannan with high affinity has been demonstrated by Kawasaki *et al.* (23,24). One could further speculate that this mannan-binding protein serves as a carrier for the transport of glycoproteins by interacting with the oligomannoside-type carbohydrate chains. This transport is thought to occur, in the course of biosynthesis, from the rough endoplasmic reticulum to the Golgi.

In addition, IgE, an important allergic factor, has been shown to have high-mannose type sugar chains at its Fc portion which can react with the surface of mast cells (25). The structure of WNM, consisting of a linear chain with an α (1→ 6) linkage and side chains with α (1→ 2) and α (1→ 3) linkages, closely resembles the structure of high-mannose type sugar chains that are located on the cell surface of myeloid cells (26). The lethal effect of WNM in mice may thus involve the high-mannose type sugar chains that exist in mammalian glycoproteins.

Based on the results of our study, it appears reasonable to conclude that the factor that causes the lethal effect of WNM in mice is PAF. The observation points at the potential biological significance of high-mannose type carbohydrate chains located on various glycoproteins which may serve as the interacting sites.

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We are grateful to Takeda Chemical Industries Co., Ltd., and Ono Pharmaceutical Co., Ltd., Osaka, Japan, for kindly providing the PAF antagonists, CV-3988 and ONO-6240, respectively. We wish to thank Dr. W. J. Baumann for a critical reading and valuable advice for the manuscript.

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Involvement of Platelet-Activating Factor in Zymosan-Induced Rat Pleurisy[1]

Yohsuke Imai, Masahiko Hayashi and Sachiko Oh-ishi*
Department of Pharmacology, School of Pharmaceutical Sciences, Kitasato University, Tokyo 108, Japan

The role of platelet-activating factor (PAF) in inflammatory reactions was studied in zymosan-induced rat pleurisy. Pleurisy was induced by injection of a 2% zymosan suspension into the pleural cavity of rats. The time course of pleural exudate accumulation, the exudation rate, and exudate leukocyte numbers were followed then for 96 hr. Peak pleural exudate accumulation was about 3 mL at 24 hr, whereas the exudation rate increased biphasically with peaks at 0.5 hr and 5 hr. The migration of leukocytes into the pleural cavity increased with time up to 48 hr. The polymorphonuclear leukocytes were the dominant white cells in the exudate between 5 and 16 hr, but mononuclear leukocytes started to outnumber them around 24 hr. Pretreatment with cyproheptadine (5 mg/kg), an inhibitor of both histamine and serotonin, significantly suppressed pleural fluid accumulation and the exudation rate at 0.5 hr. The PAF antagonist CV-6209 (1 mg/kg) significantly suppressed pleural fluid accumulation and the exudation rate at both 0.5 and 5 hr. At either time point, the parameters were not suppressed by indomethacin. We detected PAF activity in the high-performance liquid chromatography (HPLC) fraction (with a retention time corresponding to that of authentic PAF) of the exudates at 0.5 hr, 5 hr, and 16 hr using an aggregation bioassay with washed rabbit platelets. The results suggest that in zymosan-induced rat pleurisy, histamine and/or serotonin are the main mediators of exudation at 0.5 hr and that PAF may be partly responsible for exudation at 0.5 hr and later at 5 hr to 16 hr.
***Lipids 26,* 1408–1411 (1991).**

Platelet-activating factor (PAF) has been reported to exhibit a variety of biological effects including pro-inflammatory activities (1). PAF is synthesized by various types of cells, such as neutrophils (2), mononuclear leukocytes (3), and endothelial cells (4), as well as by lung (5) and amniotic (6) tissues. We previously suggested that PAF is a mediator for inflammatory exudation, based on our findings that a PAF-antagonist suppressed the exudation, and that PAF, in addition to histamine and PGI_2, was detected in the exudate when rat pleurisy was induced by phorbol myristate acetate (PMA) (7–9). When injected into the rat pleural cavity, PAF caused a vascular permeability increase and leukocyte accumulation (10,11). The factor also caused significant vascular permeability increases in rat skin and potentiated vascular permeability increases induced by other autacoids, such as histamine, bradykinin, and PGI_2 (8). In the present study, we further examined the role of PAF in the development of inflammatory exudation using zymosan-induced rat pleurisy.

[1]Based on a paper presented at the Third International Conference on Platelet-Activating Factor and Structurally Related Alkyl Ether Lipids, Tokyo, Japan, May 1989.

*To whom correspondence should be addressed at Department of Pharmacology, School of Pharmaceutical Sciences, Kitasato University, 5-9-1 Shirokane, Minatoku, Tokyo 108, Japan.

Abbreviations: BSA, bovine serum albumin; HPLC, high-performance liquid chromatography; PAF, platelet-activating factor, 1-*O*-alkyl-2-*O*-acetyl-*sn*-glycero-3-phosphocholine; PMA, phorbol myristate acetate; UV, ultraviolet.

MATERIALS AND METHODS

Animals. Eight-week-old male Sprague-Dawley rats (Shizuoka Laboratory Animal Center, Hamamatsu, Japan) were used in this study. Male Japanese white rabbits (2–3 kg, Doken, Ibaragi, Japan) were used to prepare washed platelets.

Materials. 1-*O*-Hexadecyl- and 1-*O*-octadecyl-2-*O*-acetyl-*sn*-glycero-3-phosphocholine (C_{16}PAF, C_{18}PAF, Bachem Feinchemikalien, Bubendorf, Switzerland) were gifts from Funakoshi Pharmaceutical Co. (Tokyo, Japan). CV-6209 (2-[*N*-acetyl-*N*-(2-methoxy-3-octadecyl-carbamoyloxypropoxycarbonyl)aminomethyl]-1-ethyl-pyridinium chloride) and CV-3988 (*rac*-3-(*N*-n-octadecylcarbamoyloxy)-2-methoxypropyl 2-thiazolioethyl phosphate) were kindly supplied by Takeda Chemical Industries, Ltd. (Osaka, Japan). Zymosan A (Sigma, St. Louis, MO), indomethacin (Sigma), cyproheptadine (Nihon Merck Banyu Co., Tokyo, Japan), [^{3}H]C_{16}PAF (1-*O*-[^{3}H]hexadecyl-2-*O*-acetyl-*sn*-glycero-3-phosphocholine, 40 Ci/mmol)(New England Nuclear, Boston, MA), and SEP-PAK silica cartridges (Waters, Milford, MA) were obtained commercially.

The zymosan particles were washed with saline for 3 hr and autoclaved. Indomethacin and cyproheptadine were each prepared as emulsions in 0.5% sodium carboxy methyl cellulose solution at 5 mg/mL and injected intraperitoneally (i.p.) into the rats. CV-6209 was dissolved in saline (1 mg/mL) and intravenously (i.v.) injected.

Induction of rat pleurisy. Male Sprague-Dawley rats were intrapleurally injected with 0.1 mL of 2% zymosan suspension in saline under light diethyl ether anaesthesia and sacrificed by exsanguination at selected time points after injection in the same manner as for phorbol myristate acetate (PMA)-induced pleurisy (8). To assess the exudation rate, pontamine sky blue (PSB; Tokyo Kasei, Tokyo, Japan) solution (50 mg/kg) was injected intravenously 20 min before sacrifice (12). Either indomethacin or cyproheptadine was injected i.p. into rats at a dose of 5 mg/kg, 30 min before the zymosan injection. CV-6209 was injected i.v. at a dose of 1 mg/kg, 5 min before the inciter. The pleural exudate volume, the exudation rate, and the number and type of leukocytes in the exudate were measured as previously described (12).

PAF extraction and purification. PAF was extracted from the exudate and purified as described previously (7). The pleural exudates from 2–5 rats were pooled, immediately acidified to pH 3.0 in an ice bath, and centrifuged at 1,600 × *g* for 10 min at 4°C to separate the supernatant from the cell fraction. To determine recoveries, [^{3}H]C_{16}PAF was added as internal standard to the samples before extraction. The supernatants of the exudate and the cell pellets were extracted with methanol containing 2% acetic acid according to Bligh and Dyer (13). The lower layer was loaded on a SEP-PAK silica

cartridge, and the cartridge was washed sequentially with 10 mL aliquots of chloroform, acetone, acetone/methanol (1:1, v/v), and chloroform/methanol (7:3, v/v). PAF and choline-containing phospholipids were eluted with 11.4 mL of chloroform/methanol/water (1:2:0.8, v/v/v) and re-extracted by the Bligh and Dyer method (13). The combined chloroform layers were evaporated to dryness under reduced pressure. The residue was dissolved in 50 μL of isopropanol/hexane (1:2, v/v) for high-performance liquid chromatography (HPLC) separation. HPLC was performed with a Shimazu LC-3A system fitted with a μ-bondasphere silica column (Waters; 3.5 × 150 mm) and connected to a ultraviolet (UV) detector (Uvidec-V, Japan Spectroscopic Co., Tokyo, Japan). The fractions corresponding to the retention times of authentic C_{16}PAF and C_{18}PAF were eluted with isopropanol/hexane/water (110:100:20, v/v/v) at the flow rate of 0.4 mL/min, and the eluates were evaporated to dryness under reduced pressure. The residue was resuspended in Tyrode's solution containing 0.25% bovine serum albumin (BSA) and 1.3 mM Ca^{2+}, and used for the determination of PAF levels by the washed platelet aggregation method.

Washed rabbit platelet aggregation for PAF assay. Washed platelets were prepared from citrated rabbit blood as described previously (7). The number of platelets was adjusted to 10^6 cells/μL in Tyrode's solution containing 0.25% BSA and 1.3 mM Ca^{2+} (counted by Sysmex platelet counter PL-110, Tokyo, Japan), and aggregation was monitored with an aggregometer (Rikadenki RAM-31, Tokyo, Japan). The amount of PAF was calculated from the standard curve of authentic C_{16}PAF and corrected for losses in the extraction procedure. PAF in the sample was identified by the following criteria: i) inhibition of aggregation by CV-3988, and ii) suppression of the activity by treatment with phospholipase A_2, as previously reported (7).

Statistics. Statistical data evaluation was by Student's t-test. Differences with $p<0.05$ were considered significant.

RESULTS

Time course of zymosan-induced rat pleurisy. Figure 1 shows the time course of rat pleurisy induced by intrapleural injection of zymosan. The exudation volume, the exudation rate assessed by dye leakage during 20 min, and the leukocyte number in the exudate were examined up to 96 hr. The pleural exudate volume increased with time and peaked at 24 hr. The maximal volume was about 3 mL, and the volume then gradually decreased, but still amounted to about 0.13 mL even at 96 hr (Fig. 1). The exudation rate increased biphasically with peaks at 0.5 hr and 5 hr after the injection of zymosan, as shown in Figure 1. The first peak was transient but sharp, and corresponded to the initial rapid increase in exudate volume. The second peak at 5 hr was rather broad and corresponded to the gradual increase in exudate volume. Leukocyte migration into the pleural cavity occurred gradually and the total number increased until 48 hr, and slowly decreased thereafter up to 96 hr (Fig. 1). The total number of leukocytes in the exudate at 96 hr was still significantly higher than that of resident cells.

Classification of migrated leukocytes in the pleural exudate was done by microscopic examination of methanol-fixed specimens with Giemsa stain, as shown in Figure 2.

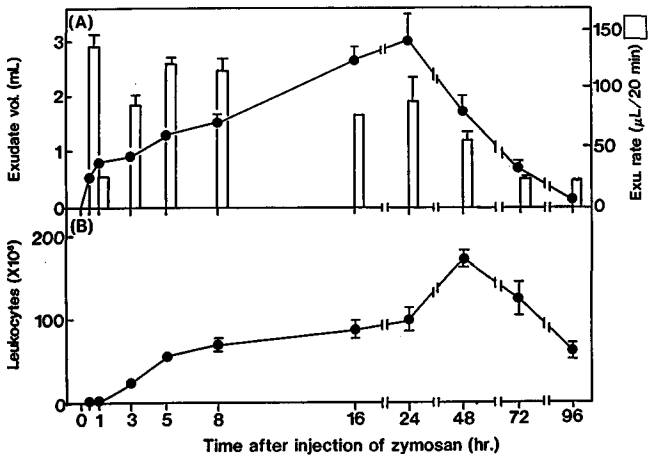


FIG. 1. Time course of zymosan-induced rat pleurisy. Rat pleurisy was induced by injection of 2% zymosan into the pleural cavity. The parameters indicated in panels (A) and (B) were determined at the indicated times after zymosan injection: (A) Pleural exudate volume (closed circles); exudation rate: rate of plasma exudation during 20 min before sacrifice (open column). (B) Infiltrated leukocytes counted in the exudate. Each value indicates the mean with standard error. The number of rats used were 4-16 for each group.

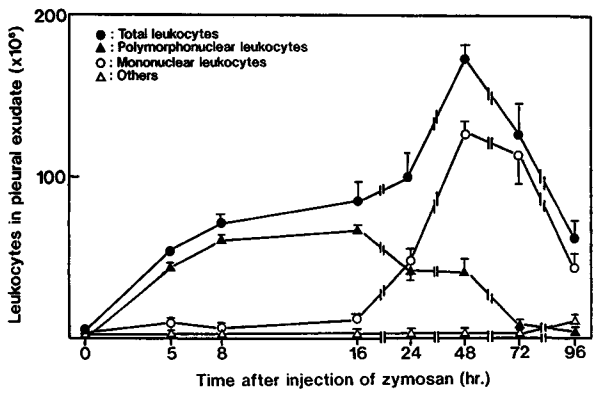


FIG. 2. Time course of leukocyte migration into the pleural cavity in zymosan-induced rat pleurisy. Classification of leukocytes was done on Giemsa-stained specimens of the exudate collected at the indicated times. The samples are the same as those in Figure 1. The type of leukocytes is shown by the following symbols: ●, total leukocytes; ○, polymorphonuclear leukocytes; ▲, mononuclear leukocytes; △, eosinophils and mast cells. Each value indicates the mean with standard error.

By this examination we could differentiate mast cells, eosinophils, polymorphonuclear leukocytes, and mononuclear cells. The latter cells included monocytes/macrophages and lymphocytes. Examination of a film sample of the exudate showed that polymorphonuclear leukocytes predominated up to 16 hr, and then decreased to half of their maximal number by 24 hr. After 16 hr, mononuclear cells started to increase in number and became predominant up to 96 hr. The number of mast cells and eosinophils did not change throughout the observation period until after 72 hr. The total number of eosinophils in the exudate at 96 hr was significantly higher than that at 72 hr.

Effect of selected inhibitors on rat zymosan pleurisy. As shown in Figure 3, the effects of indomethacin (5 mg/kg), CV-6209 (1 mg/kg), and cyproheptadine (5 mg/kg)

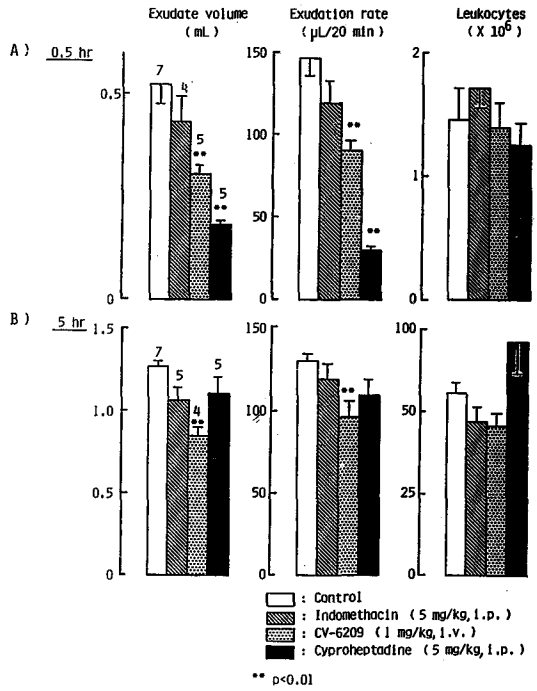


FIG. 3. Effect of various inhibitors on the rat zymosan pleurisy at 0.5 hr (A) and 5 hr (B). Cyproheptadine (5 mg/kg) and indomethacin (5 mg/kg) were injected intraperitoneally 30 min prior to the zymosan injection. CV-6209 (1 mg/kg) was injected into the tail vein 5 min prior to the zymosan injection. The data represent means with standard errors, with the number of rats examined indicated by the numerals above the columns. ** Values are significantly different at $p<0.01$ from those of the corresponding controls.

on the pleurisy were examined at 0.5 hr and 5 hr when the exudation rate peaked. Pretreatment with cyproheptadine, a dual inhibitor of histamine and serotonin, or CV-6209, a PAF antagonist, lowered the pleural exudate volume and the exudation rate significantly, but did not affect the leukocyte number of 0.5 hr pleurisy. Indomethacin had no effect. At 5 hr, only CV-6209 was significantly effective in reducing exudate volume and exudation rate.

Detection of PAF activity in the exudate of zymosan-induced rat pleurisy. Each supernatant and cellular fraction prepared from exudates pooled from 2–5 rats was extracted separately, and purified as described in Materials and Methods. PAF activity was detected and quantified in the pleural exudate of 0.5 hr, 5 hr, and 16 hr, as shown in Table 1. The HPLC fraction corresponding to authentic C_{16}PAF caused platelet aggregation, but no detectable activity was found in other fractions. The activity was suppressed by CV-3988 or treatment with phospholipase A_2 (data not shown), as indicated previously (7), suggesting that the activity in the exudate was due to intrinsic C_{16}PAF.

DISCUSSION

Rat pleurisy was induced by intrapleural injection of zymosan particles, which is known to activate an alternative pathway of the complement system (14). The time course of the accumulation of the pleural exudate of the

TABLE 1

PAF Activity in HPLC-Separated Fractions of Exudate of Rat Pleurisy Induced by Zymosan

Time after injection	Number of experiments[a]	ng/rat (C_{16}PAF equivalent)	
		Supernatant	Cell fraction
0.5 hr	5	0.662 ± 0.10	0.469 ± 0.16
5 hr	5	1.191 ± 0.20	3.068 ± 1.00
16 hr	5	0.590 ± 0.10	3.265 ± 0.94

[a]Each experiment was performed with a pooled sample from 2–5 rats. The amount of PAF, detected by the platelet aggregation method, was corrected for the percent recovery obtained by the extraction procedure. Final recovery rate was about 30–60%. Values indicate means with standard errors.

zymosan pleurisy was similar to that of carrageenin-induced pleurisy as previously reported (15). However, several details of the exudate formation were different. In the case of the zymosan-induced pleurisy, the exudation rate, assessed by dye leakage 20 min before sacrifice at the indicated time points, showed two peaks during the time course until 96 hr. The initial peak at 0.5 hr was sharp and of short duration; whereas the second one, at around 5 hr, showed a prolonged decay. However, in the case of carrageenin-induced pleurisy, the exudation rate showed only one peak, at 5 hr, and the exudation volume peaked at around 19–24 hr (15,16).

The main mediators of the exudation at the initial peak of the zymosan pleurisy seem to be histamine and/or serotonin and PAF, as judged by the result of pretreatment with cyproheptadine and CV-6209 (Fig. 3). Suppression of the pleural exudation by cyproheptadine was more pronounced than with CV-6209, suggesting that histamine and/or serotonin may be the main contributors to the exudation at 0.5 hr, with PAF being involved to a lesser extent. Simultaneous injection of cyproheptadine and CV-6209 lowered the exudate volume and exudation rate most significantly (data not shown). Exogenous PAF has previously been reported to induce exudate formation in the pleural cavity of rats (8). The fact that indomethacin did not affect exudation would indicate that cyclooxygenase products are not involved in exudation. The mediators involved at this stage appear to be similar to those involved in PMA pleurisy (except for cyclooxygenase products), as we reported earlier that histamine, PGI_2 and PAF were the main mediators of PMA pleurisy (7–9).

The exudation represented by the second peak was suppressed by CV-6209, but not by indomethacin or cyproheptadine. Even at 5 hr, the blood level of indomethacin may be effective, since the suppression of carrageenin-induced pleural exudation at 5 hr by indomethacin in rats has been reported (16). The specificity of CV-6209 was also shown, as this agent inhibited the effect of PAF, but did not inhibit histamine-, bradykinin-, or serotonin-induced vascular permeability increases in rat skin 0.5 hr or 5 hr after injection (data not shown).

PAF activity was detected and measured in the exudate at 0.5 hr, 5 hr, and 16 hr, and the PAF amounts were found to be similar to those found in PMA pleurisy (7). However, the amount of PAF found in the supernatant of the exudate did not parallel the exudate volumes at the

respective time points. This supports the idea that PAF is just one of the mediators responsible for the exudation, and agrees with our finding that histamine and/or serotonin may foremost be responsible for the exudation seen at 0.5 hr. It remains possible that mediators other than PAF may be involved in the exudation at 5 or 16 hr. This will need to be further investigated in the future.

As for the source of the mediators at these two stages of exudation, it seems likely that in the early stage (0.5 hr) the histamine and/or serotonin may be derived from mast cells and PAF may be synthesized from resident mononuclear leukocytes in the pleural cavity. We could only define the latter cells as those containing a round nucleus and being differentiable from polymorphonuclear leukocytes, mast cells, or eosinophils by Giemsa staining. We also recognized degranulated mast cells in the 0.5 hr specimens. The source of PAF at 5 and 16 hr may be the polymorphonuclear leukocytes, as the latter cells were the dominant type in the exudate at these time points. Furthermore, we detected a significant level of PAF synthesis by rat polymorphonuclear leukocytes *in vitro* (17). However, additional work will be required to accurately define the cells that synthesize PAF.

PAF was reported to induce eosinophil accumulation in the airways of guinea pig (18); however, we did not observe eosinophil accumulation in rat pleural cavity up to 72 hr and found only a slight increase at 96 hr. The reason for this difference is not clear but may be partly attributable to a species difference.

Our results agree with the report of Martins *et al.* (19), who demonstrated that the exudation of zymosan-induced rat pleurisy after 4 hr was suppressed by the PAF antagonist WEB-2086 and who hence suggested PAF involvement. By contrast, Vannier *et al.* (20) reported that indomethacin reduced pleural exudation and leukocyte numbers in pleurisy induced by the intrapleural injection of zymosan-activated serum (0.5 mL) into rats. Our data are consistent with the former results, but not with the latter. This may be due in part because we used boiled zymosan particles without pretreatment with serum (complement). However, the particles could make contact with serum components in the pleural fluid after injection, and then a small amount of complement activation could occur initially to induce histamine release (21). The involvement of the complement components in this pleurisy model needs to be studied further. Nevertheless, we can conclude that in the zymosan pleurisy model PAF is one of the main mediators of exudation.

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REVIEW

Hexadecylphosphocholine: Preclinical and the First Clinical Results of a New Antitumor Drug[1]

Clemens Unger[a,*] and **Hansjörg Eibl**[b]

[a]Division of Hematology/Oncology, Department of Internal Medicine, University Hospital Göttingen and [b]Max-Planck-Institute for Biophysical Chemistry, D-3400 Göttingen, Federal Republic of Germany

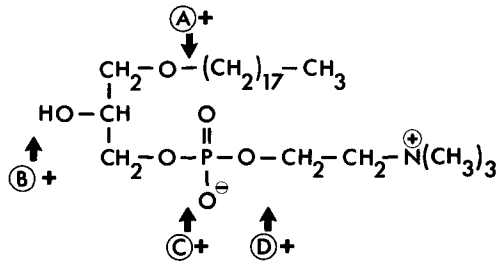
Dose-response studies on cytotoxic alkyl lysophospholipids with various chemical structures revealed that a long alkyl chain and a polar group are essential for antitumor activity. The combination of both the long alkyl chain and a phosphocholine group thus results in alkyl phosphocholines. Preclinical studies with hexadecylphosphocholine (He-PC) as a representative compound indicate distinct antineoplastic activity on leukemia cells of human origin. He-PC is highly effective in inhibiting the growth of chemically induced rat mammary carcinomas. Even more striking is the fact that a high percentage of the tumors regressed completely. In a clinical phase I trial on breast cancer patients with local recurrences, topically applied He-PC resulted in regression of skin metastases. A phase II trial for topical treatment and a phase I trial for orally applied He-PC have been initiated to further evaluate the antitumoral activity of this new compound. *Lipids 26,* **1412–1417 (1991).**

From the group of alkyl lysophospholipids, it is known that 1-*O*-alkyl-2-acetyl-*sn*-glycero-3-phosphocholine (PAF) is a thrombocyte stimulating and aggregating factor (1). The factor is mainly produced by blood cells and possesses biological activity in concentrations of as low as 10^{-10} moles/L. Furthermore, PAF has a strong blood pressure lowering effect, is produced *in vivo* during IgG induced shock and is an important mediator in allergies and inflammatory states (2).

The hydrolysis of a fatty acid chain in diacyl phospholipids by phospholipase A_2 leads to monoacyl phospholipids, the so-called lysophospholipids. A relatively constant concentration of lysophospholipids in cell membranes is maintained by the cell. This is necessitated by the fact that lysophospholipids have potent surface active properties and can destroy cell membranes at critical concentrations (approximately 20 mole% of cell membrane phospholipids) (3,4).

A number of observations made in recent years have underlined the importance of lysophospholipids in cell biology. Lysophospholipids stimulate macrophage phagocytosis (5,6), enhance the humoral immune response, and influence the development of delayed hypersensitivity reactions (7). Furthermore, lysophospholipids alter the surface properties of erythrocytes and thereby their agglutinating ability (8). They also promote cell fusion (9) and have been shown to affect the activity of several enzymes *in vitro* (10–12).

As already mentioned, the low and non-toxic level of lysophospholipids naturally present in biological membranes is maintained by the interplay of several phospholipid metabolizing enzymes. Under physiological conditions, membrane destruction and cell lysis do not occur. Correspondingly, the metabolic half-life of these structures is very short. For this reason, in the early 1970s, Westphal, Eibl and Weltzien synthesized ether analogs of lysophospholipids which were largely protected from attack by phospholipid metabolizing enzymes (13–16).



SCHEME 1. Chemical structure of alkyl lysophosphocholines and various attack sites of phospholipid metabolizing enzymes. A, alkyl cleavage enzyme; B, acyltransferase; C, phospholipase C; D, phospholipase D.

Scheme 1 shows the chemical structure of an ether lysolecithin and potential attack sites of phospholipid metabolizing enzymes. The replacement of the ester bond in the *sn*-1 position by an ether linkage endows this lysophospholipid with novel biological properties—it becomes antiproliferative and cytotoxic towards tumor cells, while its physicochemical properties remain largely unchanged. The cytotoxic properties can be considerably enhanced by substituting the *sn*-2 hydroxyl group, which results in compounds which cannot be reacylated. These structural characteristics are represented in the alkyl lysophospholipid prototype 1-*O*-octadecyl-2-*O*-methyl-*rac*-glycero-3-phosphocholine (ET18-OCH_3). This ether lipid exhibits an unusually broad spectrum of biological

[1]Based on a paper presented at the Third International Conference on Platelet-Activating Factor and Structurally Related Alkyl Ether Lipids, Tokyo, Japan, May 1989.

*To whom correspondence should be addressed at the Division of Hematology/Oncology, Department of Internal Medicine, University Hospital Göttingen, Robert-Koch-Str. 40, D-3400 Göttingen, Federal Republic of Germany.

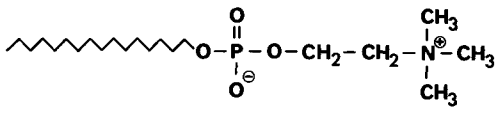
Abbreviations: ALP, alkyl lysophospholipids; CMC, criticl micelle concentration; DMBA, 7,12-dimethylbenzanthracene; ET-18-OCH_3, 1-*O*-octadecyl-2-*O*-methyl-*rac*-glycero-3-phosphocholine; G-3-PC, glycero-3-phosphocholine; GM-CSF, granulocyle macrophage-colony stimulating factor; He-PC, hexadecyl phosphocholine; ID_{50}, 50% inhibition dosage; LD_{50}, half lethal dosage; Log p, oil/water partition coefficient; MNU, methylnitrosourea; PAF, 1-*O*-alkyl-2-acetyl-*sn*-glycero-3-phosphocholine.

properties such as macrophage activation to cytotoxic effector cells, malignant cell differentiation, inhibition of tumor cell invasiveness in normal tissues and direct cytotoxic effects on tumor cells *in vitro* and *in vivo*. Comprehensive overviews have been presented by Berdel and Munder (17,18). Based on *in vivo* investigations, the direct cytotoxic effect of ET18-OCH_3 appears to be due to its accumulation in tumor cells. According to investigations by Snyder's group, many neoplastic cells are lacking an *O*-alkyl cleavage enzyme (19). It was suggested that lack of the enzyme in tumor cells may lead to an accumulation of alkyl lysophospholipids (ALP) in tumoral tissue, whereas normal cells would be able to metabolize ALP (18). However, more detailed studies on various tumor cells with different levels of *O*-alkyl cleavage enzyme showed that ET18-OCH_3 levels were not controlled by *O*-alkyl cleavage enzyme (20) and that ET18-OCH_3 is not a substrate for the *O*-alkyl cleavage system (21).

In recent years our Göttingen group has intensively studied the structure-activity relationship of alkyl lysophospholipids and has analyzed the structural requirements which determine the substrate properties of the most important phospholipid metabolizing enzymes, e.g., alkyl cleavage enzyme, acyl transferases and the phospholipases C and D (20–30). In summary, the results indicate that cytotoxic alkyl lysophospholipids have to fulfill two requirements: (i) reacylation in the *sn*-2-position must be prevented; and (ii) the substrate characteristics for phospholipase C and/or D must be preserved. These principles were translated and simple molecules which possess the suggested minimal structural requirements for cytotoxic activity were synthesized (31).

ALKYL PHOSPHOCHOLINES: A NEW CLASS OF ANTITUMORAL AGENTS

According to the rationale mentioned above, we prepared molecules which have a long alkyl chain directly connected to the phosphocholine moiety. Compared to the alkyl lysophospholipids, the alkyl phosphocholines lack the glycerol backbone, and they are only metabolized by action of the phospholipases C or D. All of the following investigations were carried out with hexadecylphosphocholine (He-PC) (Scheme 2).



SCHEME 2. Chemical structure of hexadecylphosphocholine (He-PC) as a representative of alkyl phosphocholines.

Preclinical investigations: physical properties. The physical properties of hexadecylphosphocholine were compared to 1-palmitoyl-*sn*-G-3-PC, which represents a lysophospholipid that occurs naturally in biomembranes (Table 1). The Table summarizes the critical micellar concentrations (CMC) and values for the hemolysis of human erythrocytes and for the oil/water partition. The similarity of the values for micelle formation and for hemolysis is not surprising, as both mechanisms depend on a common property—the surface activity which involves formation of micelles, but also results in the reorganization of lipid bilayer.

TABLE 1

Physical Properties of Hexadecylphosphocholine and 1-Palmitoyl-*sn*-Glycero-3-Phosphocholine

Parameter	Hexadecylphosphocholine	1-palmitoyl-*sn*-G-3-PC
log p[a] (olive oil/water)	−1.48	−1.52
log CMC[b] [mol/L]	−5.10	−5.22
log 50% hemolysis[c] [mol/L]	−5.22	−5.28

[a] Logarithm of the olive oil/water partition coefficient.

[b] Logarithm of the critical micelle concentration.

[c] Logarithm of the 50% hemolysis value after incubation of human erythrocytes with the compounds for 5 min.

As known from previous reports, micelle formation and the hemolytic properties of lysophospholipids critically depend on the length of the carbon chain (3,29). This particularly applies to the oil/water partition coefficients. Thus, the physical properties of hexadecylphosphocholine closely resemble those of the naturally occurring membrane lysophospholipid 1-palmitoyl-*sn*-G-3-PC.

However, despite the similarity of their physical properties, the two compounds behave quite differently with respect to antitumor activity. Whereas hexadecylphosphocholine is antitumor active, 1-palmitoyl-*sn*-G-3-PC is not. This suggests that the antineoplastic activity of hexadecylphosphocholine cannot be explained by its surface active property.

Cell experiments. The *in vitro* cytostatic and cytotoxic activity of He-PC was determined on four human leukemic cell lines: HL60 and U937 (promyelocytic leukemia) Raji (Burkitt's lymphoma) and K562 (erythroleukemia). The cells were incubated in RPMI medium at different concentrations of He-PC for up to 72 hr. The 50% growth inhibition dosage (ID-50) after 48-hr incubation was calculated by the loss of [^{3}H]thymidine incorporation into cellular DNA. The trypan blue dye exclusion assay was used to determine the half lethal dosage (LD-50). *In vitro* investigations of He-PC revealed concentration dependent cytotoxicity and growth inhibition for HL-60 cells (Fig. 1). ID-50 and LD-50 values (Table 2) were calculated from dose response curves after 48 hr of incubation when cell growth was in logarithmic phase. The data indicate that HL60 and U937 cells were highly sensitive, Raji were moderately sensitive and K562 were of low sensitivity in respect to He-PC. Susceptibility ranking of the four cell lines was similar in both test systems and corresponded to results obtained for alkyl lysophosphocholines such as ET18-OCH_3 in earlier experiments (20,21). It has to be stressed again that the cytotoxic effects observed in these experiments cannot be explained by the surface active properties of hexadecylphosphocholine. In fact, co-cultured erythrocytes remained viable up to the highest doses used (40 μg/mL, 72 hr) and did not show hemolysis.

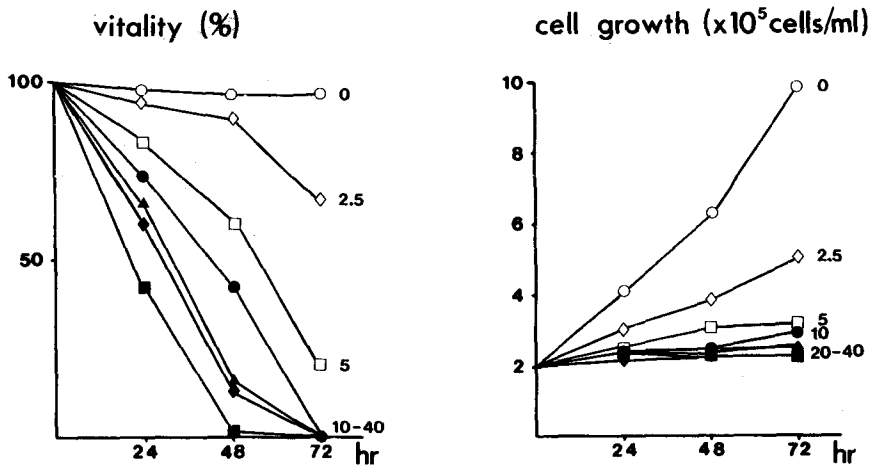


FIG. 1. He-PC dose dependent toxicity and inhibition of proliferation of the HL60 leukemia cell line. Cells (2×10^5) were cultured for 24, 48 and 72 hr in the presence of 2.5 to 40 μg/mL of He-PC. At each point in time, the number of total cells was counted and the number of dead and alive cells was determined. Data represents the mean for 3–6 independent experiments; S.D.s were less than ± 10%.

TABLE 2

Differential Sensitivity of Leukemia Cell Lines to Hexadecylphosphocholine[a]

Cell line	He-PC (μg/mL)	
	ID_{50}	LD_{50}
HL 60	5.0	7.7
U 937	3.0	16.0
Raji	10.0	26.0
K 562	15.0	>40.0

[a]Cells were incubated for 48 hr. ID_{50} values were obtained from [^{3}H]thymidine incorporation. LD_{50} values were obtained by trypan blue dye exclusion test.

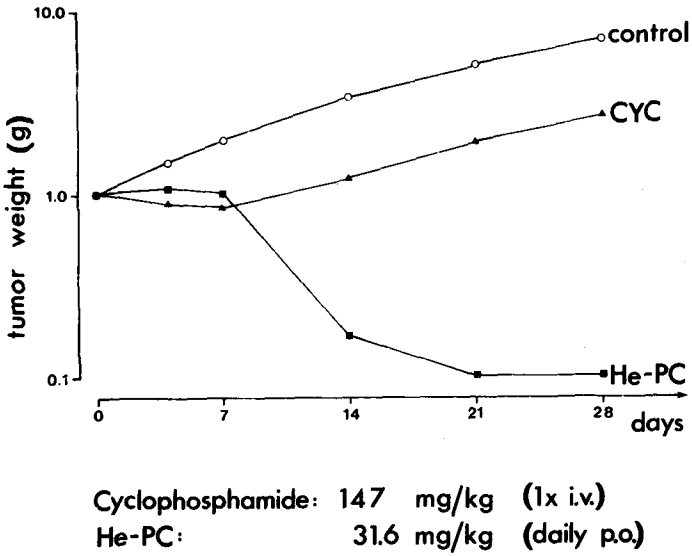


FIG. 2. Antineoplastic activity of He-PC in comparison to cyclophosphamide on DMBA induced rat mammary carcinoma. Dosages: He-PC 31.6 mg/kg body wt daily p.o. for 28 days; cyclophosphamide 147 mg/kg body wt as a single i.v. injection (maximally tolerated dosage).

Animal experiments. It has long been known that alkyl lysophospholipids (e.g., ET18-OCH_3) have reproducible cytostatic effects on chemically induced, autochthonous breast carcinomas in rats (32). In a series of experiments, two alkyl lysophospholipids (ET18-OCH_3 and the thioether BM41.440) were compared with octadecyl phosphocholine and hexadecyl phosphocholine on methylnitrosourea (MNU)-induced breast carcinoma in the rat (33,34). The optimally nontoxic dose was administered orally q.d. over a period of five weeks. All substances tested in this series showed distinct antitumor activity. Of particular interest is the fact that there was no mortality in the groups treated with alkyl phosphocholines, whereas in the groups treated with the alkyl lysophospholipids (ALP) 13% and 17% of the animals died. Another interesting observation was the complete tumor remission in the group treated with hexadecyl phosphocholine and octadecyl phosphocholine. This finding motivated us to further investigate the antineoplastic activity of He-PC in another carcinogen induced breast carcinoma (DMBA) in the rat (35). Also in this tumor model, the oral administration of He-PC in concentrations of 4–46 mg/kg BW led to a dose-dependent tumor growth inhibition and led to a high number of complete remissions at doses above 20 mg/kg BW. Figure 2 shows the effect of He-PC (31.6 mg/kg BW q.d., orally) compared to the maximal tolerable dose of cyclophosphamide (147 mg/kg BW once-time dose i.v.). One can see that in the first few days cyclophosphamide retards tumor growth, but after a week the tumor continues to grow. In the case of He-PC therapy, after about three weeks, complete tumor remission is achieved. Interestingly, all animals in which there was a complete disappearance of macroscopic tumors, suffered a relapse within a short time after cessation of therapy. Whether or not this represented regrowth of previously suppressed tumors, or the development of new tumors, is currently under investigation. In all experiments, tolerance of the

He-PC was extremely high and toxic effects were not observed. It must also be pointed out that hexadecyl phosphocholine shows no hematotoxicity (35). Leukocyte count in blood remains unchanged, but after one week the leukocyte count increases at doses of 46 mg/kg BW, remains constant for the remainder of the therapy, and then returns to normal after discontinuation of treatment. Microscopic studies revealed that the leukocyte increase can be attributed to mature granulocytes. The cause for the leukocyte increase remains unknown. It may be due to a cell redistribution from tissues to vasculature as could be observed after glucocorticoid therapy. On the other hand, granulocyte macrophage-colony stimulating factor (GM-CSF) release might be the cause.

Finally, it must be stressed that the activity of He-PC against experimental tumors appeared to be extremely selective. No response was seen against transplantable rat tumors such as DS-carcinosarcoma, the leukemias L5222, L1210, P388 or the Lewis lung carcinoma (35). Recently, however, we found that a human head and neck tumor transplanted into nude mice was highly sensitive to He-PC (data not shown).

Biodistribution and He-PC metabolism. Biodistributional studies after oral administration of He-PC in mice showed that He-PC is absorbed at high rates from the intestine. Based on this, clinical oral treatment regimens appear feasible (36). This finding is in line with other reports showing that ET18-OCH_3 and the thioether BM41.440 are well absorbed from the intestinal tract (37,38). The highest accumulation of He-PC was found in liver, kidney and lung, which is similar to what was observed for ET18-OCH_3.

The metabolism of He-PC was investigated in the mouse liver (36). After oral administration of He-PC labelled in the polar head group, the time-dependent formation of radiolabelled choline, phosphocholine and 1,2-diacyl-glycerophosphocholine was observed, which suggests the importance of phospholipases C and D in He-PC catabolism. Studies on the hydrolysis of He-PC *in vitro* carried out with phospholipase C *(bacillus cereus)* and phospholipase D (partially purified from cabbage) support the finding that He-PC is a substrate for these enzymes. Formation of 1,2-diacyl-glycerophosphocholine, labelled in the polar head group, is consistent with earlier results obtained with ET18-OCH_3 (24,25). In the latter study, we were able to demonstrate that the labelled phosphocholine group was transferred from ET18-OCH_3 to 1,2-diacylglycerol resulting in the formation of phosphatidylcholine.

Clinical investigations. The favorable results of the animal experiments on chemically induced breast carcinomas in the rat motivated us to use hexadecylphosphocholine as the first of the alkyl phosphocholines in clinical trials. After completion of the toxicological studies, two forms of administration were selected for clinical use. Firstly, topical application for treatment of skin metastases of the cutaneously metastasizing breast carcinoma (39) and, more recently, oral administration for testing the tolerance and effectiveness on solid metastasizing tumors.

Topical application. For the topical application, hexadecylphosphocholine was dissolved in a mixture of 1-*O*-alkylglycerols with C_3 (1-*O*-propylglycerol), C_6 (1-*O*-hexylglycerol) and C_9 (1-*O*-nonylglycerol) chain lengths in water. The resulting mixture called KASKADE for short (Table 3) is slightly viscous and can be applied to the skin easily. 1-*O*-Alkylglycerols were chosen as carrier for He-PC, because the amphiphilic molecules can permeate the membrane and thereby change membrane permeability. We were able to demonstrate this using the blood brain barrier model (40). For example, cyclophosphamide has an uptake index of 11% for rat brain. However, if cyclophosphamide is injected together with propylglycerol, butylglycerol or pentylglycerol into the arteria carotis of the rat, the brain uptake index is increased with increasing chain length (up to C_9) of the alkylglycerol carrier (40). Similar results were obtained with other cytostatic agents. As a possible mechanism of action, we suspect that short chain alkylglycerols can cause a short-term membrane perturbation and, as a consequence, cause increased permeability for the drugs. A second favorable characteristic of alkylglycerols is their bactericidal and fungicidal activity (39). Here, too, the longer chain alkylglycerols exhibit a greater antimicrobial effect. It should also be mentioned that the 1-*O*-alkylglycerols used in the formulation have some antineoplastic properties of their own (41) which might add to the therapeutic effects of the solution for topical treatment.

TABLE 3

Composition of KASKADE for the Topical Treatment of Skin Metastases[a]

Compound	% (w/v)
Nonylglycerol	17
Hexylglycerol	17
Propylglycerol	33
Water	33

[a]20–80 mg He-PC/mL Kaskade.

Within the framework of the clinical phase I trial, local recurrences on the skin (partialiy exulcerated) in patients with metastatic breast carcinoma were treated with local cascade administration. After written consent, the substance was applied once a day for five days. In the case of proven local tolerance, the substance was applied twice a day for further treatment. The patient characteristics can be seen in Table 4. Altogether, 25 patients were treated in the phase-I trial, 24 were evaluated. All patients were extensively pre-treated. We observed three complete remissions, four partial remissions, eight no changes and nine progressive diseases. The good local tolerance of the mixture was striking. Cutaneous adverse effects observed were average dryness of the skin, epithelial scaling and, in individual cases, itching.

Reports on three of the patients treated. Patient 1. A 36-year-old woman with multiple, flat skin metastases in the peripheral area which had been treated with radiation therapy. Diameter of metastases was up to 2 cm; in the middle a large, solid node was visible. Chemotherapy was not possible as a consequence of hemolytic uremic syndrome. This patient showed a complete regression of the skin metastases, including the solid node, after eight weeks of topical treatment. The histological results from a skin biopsy after three months of treatment revealed normal architecture with a slight increase in collagen content.

TABLE 4

Patient Characteristics and Response to Therapy

	No.
Patient characteristics	
Number of patients entered	25
Evaluable	24
Pretreatment	
Surgery	22
Radio-, chemo- and hormone therapy	18
Radio- and chemotherapy	3
Radio- and hormone therapy	2
Radiotherapy only	1
Response to therapy	
Complete remission	3
Partial remission	4
No change	8
Progressive disease	9

TABLE 5

Pharmacokinetic Parameters of Hexadecylphosphocholine in Rats After a Single Oral Dose of 200 mg/kg Body Weight[a]

Parameter	
AUC, 0-240 hr (mg × hr × L^{-1})	6200
λ_z	0.0072
C_{max} (mg × L^{-1})	39.2
t_{max} (hr)	48
$t_{/2}$ (hr)	96
Cl (mL × min^{-1})	0.53
V_d (L × kg^{-1})	4.7

[a]AUC, area under the curve; λ_z, rate constant for terminal elimination; C_{max}, maximum serum concentration; t_{max}, time of maximum serum concentration; Cl, clearance; $t_{/2}$, half-life for the terminal elimination, V_d, distribution volume.

Patient 2. A 45-year-old woman had marked exulcerated local recurrence. She died five weeks after treatment was initiated. After five weeks of local therapy increasing epithelial proliferation occurred in the periphery of the exulceration. This patient's histological findings of an untreated area of skin showed large tumor cell clusters extending into the subcutaneous layer. In the treated area, one could see heavily damaged tumor cell clusters.

Patient 3. A 68-year-old patient had undergone a bilateral mastectomy with a marked local recurrence. After an 8-week course of therapy, a partial remission of metastases had occurred.

The general local tolerance of the cascade is striking. Systemic adverse effects are completely absent. It should be particularly stressed that neither hematotoxic nor other blood chemistry changes could be observed. Recently, a multicenter study on this type of topical skin carcinoma therapy has been initiated in the Federal Republic of Germany.

Oral administration. The goal of this phase I study was to test the tolerance and effectiveness of orally administered hexadecylphosphocholine in the treatment of advanced solid tumors. This study was just initiated, so we can only report on initial impressions. Before starting the phase I trial, pharmacokinetic investigations of He-PC in the rat after oral administration were carried out. A high performance liquid chromatography (HPLC) method was worked out to determine serum He-PC levels (42). The sensitivity of the method is approximately 5 nmol He-PC/mL serum. Table 5 shows the pharmacokinetic data of He-PC in the rat after oral administration.

The very long half-life of 96 hr is particularly noteworthy. Maximum levels after oral administration of 200 mg/kg He-PC were reached after 48 hr. This characteristic of hexadecylphosphocholine clearly differs from that of the alkyl lysophospholipids which are under current clinical study. It is also noteworthy that the one-time administration per week of the chosen dose led to a high percentage of complete remissions in DMBA induced breast carcinoma of the rat (35).

In the clinical pilot study, seven patients were treated with He-PC orally over a course of eight weeks. Two patients received a daily dose of 50 mg, five a dose of 100 mg. Until now, there has been no evidence of systemic toxicity; leukocytes in the peripheral blood also remained unaffected. We are planning to increase the dose in increments of 50 mg to reach maximal tolerable levels.

Evaluation and perspectives. Ether lipids and ether lysophospholipids are similar in structure to natural membrane components. Their antitumoral effect is one of the most interesting characteristics. The substances are completely different from the classical antitumor drugs, as their primary target appears to be the plasma membrane. As far as we know, they are not mutagenic or carcinogenic, nor do they possess immune suppressive properties. In addition, our work on the alkyl phosphocholines has shown that selective antitumoral effect can be achieved in animals which opens up new perspectives for a possible, specific treatment of individual tumors in man. A further interesting aspect is their possible molecular mode of action in that He-PC probably interferes with signal transmission from the cell membrane to the cell nucleus (manuscript in preparation). Particularly in the last years, the connection between phospholipid metabolism and signal transduction on cellular membranes has become quite evident. Research in the area of ether lipids is certainly only in the early stages of development, and further studies are necessary to clarify the potential significance of these compounds in cancer therapy.

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Comparison of Selective Cytotoxicity of Alkyl Lysophospholipids[1]

W.R. Vogler[a,*], A.C. Olson[a], S. Okamoto[a], M. Shoji[a], R.L. Raynor[a], J.F. Kuo[a], W.E. Berdel[a], H. Eibl[b], J. Hajdu[c] and H. Nomura[d]

[a]Emory University School of Medicine, Atlanta, Georgia 30322, [b]Max-Planck-Institut für Biophysikalische Chemie, 3400 Göttingen, Germany, [c]California State University, Northridge, California 91330 and [d]Takeda Chemical Industries, Ltd., Osaka 532, Japan

Alkyl lysophospholipids have been shown to be cytooxic to a number of neoplastic tissues. One, ET-18-OCH_3, has been used to selectively purge leukemic cells from mixtures with normal marrow progenitor cells, *in vitro* and *in vivo*. We have measured the 50% inhibitory (IC_{50}) effect of a series of ether lipids (EL) on leukemic cells (HL60, K562, Daudi, KG-1, KG-1a) and normal marrow progenitor cells. Cells were incubated with varying concentrations of EL for 4 hr and assayed for viability, [^{3}H]thymidine incorporation and clonogenicity in semi-solid media. The effect on protein kinase C (PKC) activity was assayed for each compound. Compounds tested included three glycerophosphocholine analogs—ET-18-OCH_3, ET-16-$NHCOCH_3$, and BM 41.440. In addition, a lipoidal amine, CP 46665, an ethyleneglycolphospholipid, AEPL, and four single chain alkylphosphocholine analogs, $HePC_2$, $HePC_3$, $HePC_4$ and $HePC_6$ were also tested. During the period of incubation, the cells remained viable (>70%) as judged by trypan blue dye exclusion. The glycerophosphocholines were the most active and showed the highest therapeutic index. The lipoidal amine was active, but toxic to normal marrow progenitor cells. The ethyleneglycolphospholipid was active against HL60, but not against the other cell lines. The single chain alkylphosphocholine analogs were less active. All of the compounds inhibited PKC activity; however, the glycerophosphocholines were the most inhibitory.
***Lipids* 26, 1418–1423 (1991).**

Alkyl lysophospholipids (ALP) are analogs of lysophosphatidylcholine which have been shown to be cytotoxic to a variety of neoplastic tissues *in vitro* (1–6). In addition, some antitumor activity has been demonstrated in humans (7). There has been some degree of selective cytotoxicity to neoplastic tissues and sparing of normal tissue (8). We have shown this selectivity in an animal model and an *in vitro* model by treating mixtures of normal bone marrow and leukemic cells *in vitro* with ET-18-OCH_3 and demonstrating elimination of leukemic cells and preservation of normal hematopoietic stem cells and progenitor cells (9–11).

The mechanism of action of this group of compounds is not clearly understood, but the site of action appears to be the cell membrane. Synthesis of membrane phospholipids, phosphatidylcholine, phosphatidylethanolamine and phosphatidylinositol are inhibited by ET-18-OCH_3 (12–15). Protein kinase C (PKC) activity and phorbol ester stimulation of PKC in tumor cells are inhibited by ET-18-OCH_3 (16), BM 41.440 (17) and the alkylphosphocholine analogs (18). $HePC_2$ inhibits inositol phosphate formation and PKC activity (19). Inhibition of (sodium plus potassium)-activated adenosine triphosphatase and the sodium pump has been described (20). Thus, multiple membrane associated enzymes are inhibited.

Modification of one of the more active ALP (ET-18-OCH_3) has resulted in a number of derivatives with varying degrees of antitumor activity *in vitro* (21–23). In studies on cell lines and fresh leukemic cells from patients, we observed a range of activity with ET-18-OCH_3. To determine the relationship of structure to activity, a number of ether lipids were tested and compared to ET-18-OCH_3. These consisted of three glycerophosphocholine analogs, a lipoidal amine, an alkyl ethylene glycophospholipid and four single chain alkylphosphocholine analogs. These compounds were tested against a spectrum of leukemic cell lines for antitumor activity, normal bone marrow progenitor cells to assess selectivity, and PKC to assess inhibitory activity.

MATERIALS AND METHODS

Compounds. Figure 1 shows the structures of the compounds and gives the letters assigned to each. ET-18-OCH_3 (A1), synthesized as described elsewhere (24–26), was obtained from Medmark Chemicals (Grünwald, Germany). ET-16-$NHCOCH_3$ (A2) was synthesized as previously described (27). 1-Hexadecylthio-2-methoxymethyl-2-deoxy-*rac*-glycero-3-phosphocholine, BM 41.440 (A3) was obtained from Boehringer Mannheim (Mannheim, Germany). 1-[2′-3′-(Di-n-decyloxy)-n-propyl]-4-aminomethyl-4-phenyl piperidine (CP-46665) (B) was a gift from Dr. K. Jensen, Pfizer Central Research (Groton, CT). 2-[2′-(Dodecyloxy)ethoxyl]ethyl 2-pyridinioethyl phosphate (AEPL) (C) was obtained from Takeda Chemical Industries, Ltd. (Osaka, Japan). Hexadecylphosphocholine (D1), hexadecylphospho-3-(*N,N,N*-trimethylamino)propanol (D2), hexadecylphospho-4-(*N,N,N*-trimethylamino)-butanol (D3), and hexadecylphospho-6-(*N,N,N*-trimethylamino)hexanol (D4) were prepared as previously described (28). The compounds were dissolved in RPMI 1640 medium (Gibco Laboratories, Grand Island, NY) containing 10% fetal bovine serum (FBS) (Gibco Laboratories), sterilized by acrodisc filtration (0.22 μm; Gelman Sciences, Inc., Ann Arbor, MI) and stored at −20°C until used.

Leukemic cell lines. The cell lines used in these studies were the myelocytic cell line HL60, obtained from Dr. Robert Gallo, NIH (Bethesda, MD) (29); the K562 cell line, obtained from Dr. Bismark Lozzio, University of Tennessee (Knoxville, TN) (30); the Daudi human B-cell leukemic

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*To whom correspondence should be addressed at P.O. Box AE, Emory University, Atlanta, Georgia 30322.

Abbreviations: AEPL, 2-[2′-dodecyloxy)ethoxy]ethyl 2-pyridinioethyl phosphate; ALP, alkyl lysophospholipid; BM 41.440, 1-*O*-hexadecylthio-2-methoxymethyl-2-deoxy-*rac*-glycero-3-phosphocholine; CP-46665, 1-[2′,3′-(di-n-decyloxy)-n-propyl]4-aminomethyl-4-phenyl-piperidine; EL, ether lipid; ET-16-$NHCOCH_3$, 1-*O*-hexadecyl-2-acetamido-2-deoxy-*sn*-glycero-3-phosphocholine; ET-18-OCH_3, 1-*O*-octadecyl-2-*O*-methyl-*rac*-glycero-3-phosphocholine; FBS, fetal calf serum; $He\text{-}PC_2$, hexadecylphosphocholine; $He\text{-}PC_3$, hexadecylphospho-(*N,N,N*-trimethylamino)propanol; $He\text{-}PC_4$, hexadecylphospho-(*N,N,N*-trimethylamino)butanol; $He\text{-}PC_6$, hexadecylphospho-(*N,N,N*-trimethylamino)hexanol; IC_{50}, 50% inhibitory activity; PKC, protein kinase C.

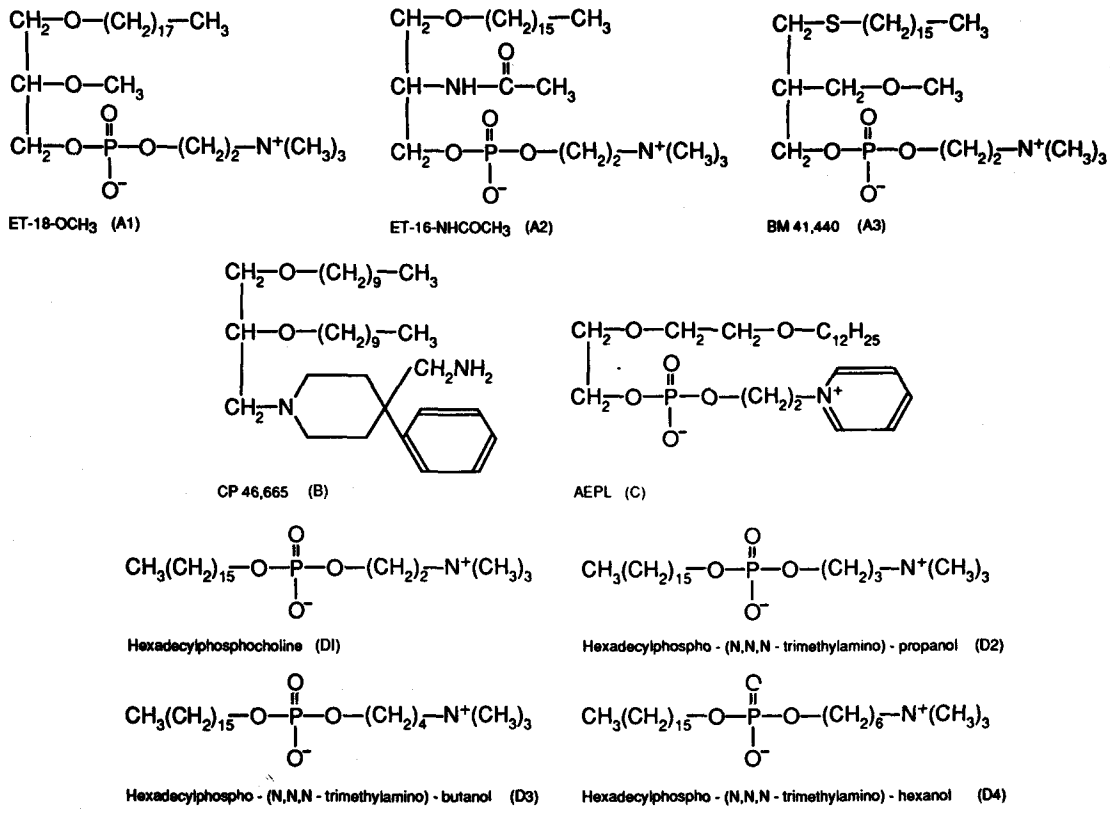


FIG. 1. Structures of ether lipids.

cell line, obtained from American Type Culture Collection (Rockville, MD) (31); and the myeloblastic leukemic cell lines KG-1 and KG-1a, kindly provided by Dr. H. Phillip Koeffler, University of California (Los Angeles, CA) (32, 33). All cells were continuously cultured at 37°C in 5% CO_2 in RPMI 1640 medium and 10% FBS (except KG-1, which required 20% FBS), and passaged twice weekly. Cells were in log phase of growth at the time the experiments were conducted.

Normal bone marrow cells. Cells were obtained by aspiration from posterior iliac crests of normal donors for allogeneic bone marrow transplantation giving informed consent or from bone marrow fragments following orthopedic procedures. Mononuclear cells were separated by Ficoll-Hypaque (Histopaque-1077, Sigma Chemical Co., St. Louis, MO).

Cytotoxicity assays. Two $\times$ 10^6 cells/mL were incubated with varying concentrations of EL for 4 hr at 37°C in RPMI 1640 medium, 10% FBS, 1% PSN or PS Antibiotic Mixture (Gibco) and 1% glutamine (RPMI-C). Following incubation, the cells were washed three times in RPMI 1640 medium, counted and resuspended in RPMI-C at a concentration of 1 $\times$ 10^6/mL. Viability was determined by trypan blue dye exclusion. Cells wre diluted in RPMI 1640-C for tritiated thymidine incorporation and clonogenicity in methylcellulose. For tritiated thymidine incorporation, 0.4 μCi of [*methyl*-^{3}H]thymidine (sp. act. 6.7 Ci/mmol) was incubated with 4 $\times$ 10^5 cells in 200 μL medium in triplicate or quadruplicate for 18 hr n 5% CO_2 at 37°C or incubated for 1 hr with 0.4 μCi of higher specific activity thymidine (89–90 Ci/mmol). Following incubation, the cells were harvested with a cell harvester, and upon addition of scintillation fluid, counted in a scintillation counter at an efficiency of 90%. The counts were expressed as CPM/4 $\times$ 10^5 cells.

Clonogenicity of cultured leukemic cells in methylcellulose was carried out as previously described (11). Briefly, 500 cells (1 $\times$ 10^4 for KG-1 and KG-1a) in RPMI-C were plated in triplicate in medium containing 0.8% methylcellulose, 30% FBS, 0.3% bovine serum albumin, 5 $\times$ 10^{-5}M mercaptoethanol, 5.6 $\times$ 10^{-8} M selenium and, in some instances, 5% phytohemagglutinin leucocyte conditioned medium (PHA-LCM or HPCM). The total volume was 1 mL. The plates were incubated in moist 5% CO_2 at 37°C for 7–14 days and colonies (>40 cells) scored.

Normal marrow progenitor cells were assayed in triplicate by a modification of the method of Fauser and Messner (34), as previously described (11), and counted on day 14. The total number of progenitors was defined as the sum of mixed colonies (GEMM), erythroid colonies (CFU-E), erythroid bursts (BFU-E), granulocyte-macrophage colonies (GM-CFU) (>40 cells) and GM clusters (<40 cells).

PKC assay. The activity of PKC was assayed as previously described (35). Briefly, PKC was partially purified from pig brain extract or purified to homogeneity from rat brain extracts (36,37). The reaction mixtures (0.2 mL) contained 5 μmol of piperazine-*N,N'-bis*(2-ethanesulfonic acid) (pH 6.5), 2 μmol $MgCl_2$, 5 μg of

phosphatidylserine, 40 μg of histone H1, 0.06 μmol of ethylene glycol-*bis*(β-aminoethyl ether)-*N,N,N'N'*-tetraacetic acid (EGTA), with or without 0.1 μmol of $CaCl_2$, 1.0 nmol of [γ-^{32}P] ATP (containing about 1 × 10^6 cpm) and various kinds and concentrations of EL. The reaction was carried out at 30°C for 5 min.

Statistical methods. The IC_{50} was defined as the concentration of EL causing 50% inhibition of the control value for the assay employed. Because of some variability in growth patterns of the cell cultures, the means of repeated experiments were averaged and plotted as percent of control for each dose. The IC_{50} was either read off the graph or calculated by the least-squares method. Triplicate samples were run in each assay and all assays were repeated a minimum of three times.

RESULTS

Figure 2 gives the IC_{50} for thymidine incorporation of eight of the compounds. Compound B (CP-46665) was not tested. As can be seen, the more active compounds contained the glycerol backbone. KG-1 was only tested with A1 (ET-18-OCH_3) and A2 (ET-16-$NHCOCH_3$), and the latter was more inhibiting. The reverse was observed in KG-1a, and C (AEPL) was essentially inactive.

Figure 3 shows the IC_{50} for the clonogenic assay. Colony formation by KG-1 cells was quite variable, usually yielding poor colony growth, and the data are not interpretable. The plating efficiency for each of the cell lines was as follows: HL60, 25.9 ± 12%; K562, 49.1 ± 14%; Daudi, 26.7 ± 9%; and KG-1a 11.7 ± 5%. In general, the glycerol EL were more active in HL60, K562 and Daudi. In KG-1a, A1 was more active than A2.

Figure 4 gives the IC_{50} for PKC activity. As shown, the glycerophospholipids were more active than the single chain hexadecylphosphocholine analogs. The lipoidal

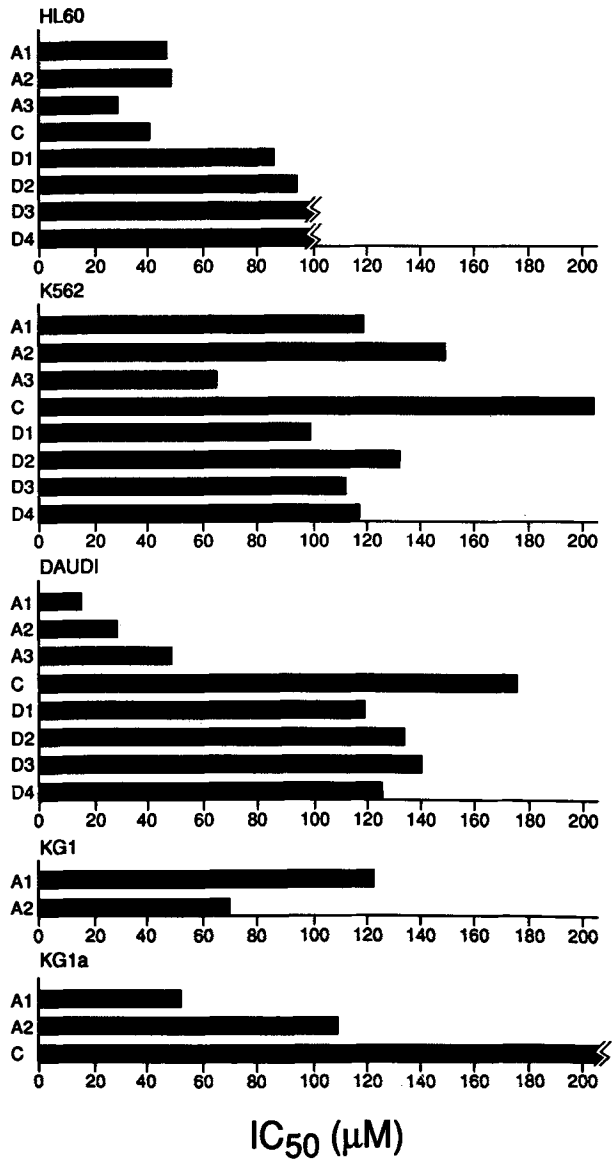


FIG. 2. Effects of EL on tritiated thymidine incorporation by leukemic cell lines. Plotted are the mean IC_{50} (μM) for each compound tested. See text for identification of compounds.

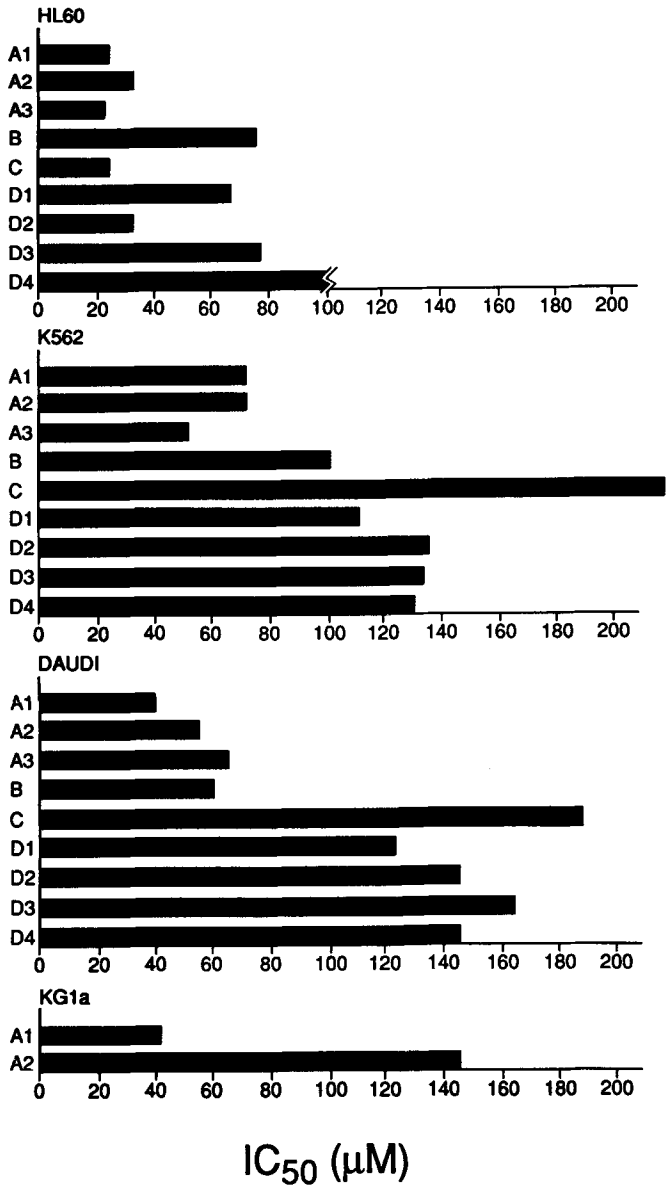


FIG. 3. Effect of EL on clonogenic assays in leukemic cell lines. Plotted are the mean IC_{50} (μM) for each compound. See text for identification of compounds.

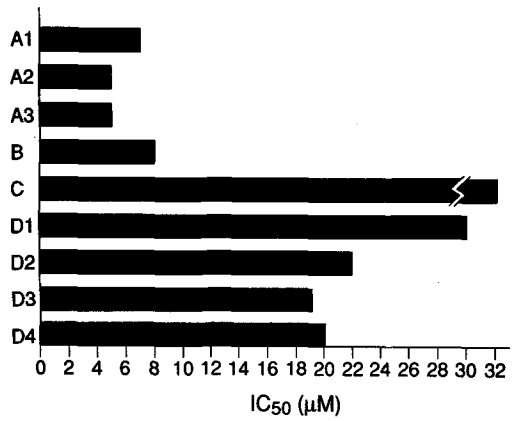


FIG. 4. Effect of EL on protein kinase C activity as described in text. Plotted are the IC_{50} for each compound tested.

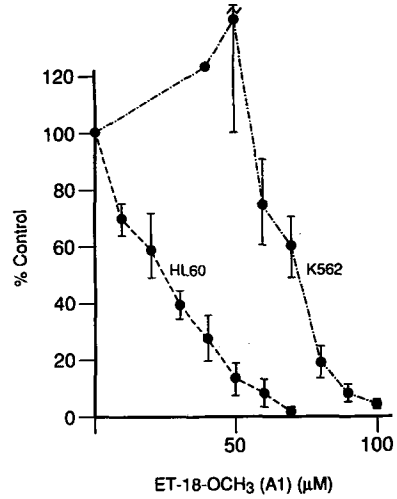


FIG. 6. Patterns of inhibition on colony formation by compound A1 on HL60 and K562 cells. Plotted are the mean and standard errors of 3–5 experiments per point.

amine (B) was also more active, but the ethylene compound (C) was relatively inactive with an IC_{50} of 177 μM.

The effect of these compounds on normal marrow progenitor cells is shown in Figure 5. As can be seen, except for the lipoidal amine, compound B, the IC_{50} was greater than 150 μM. There were no significant changes with increasing doses of drugs in the distribution of the types of colonies observed (data not shown).

Table 1 gives the calculated clonogenic therapeutic index for all of the compounds in HL60, K562 and Daudi cell lines. These were computed by dividing the IC_{50} for normal marrow progenitor cells by the IC_{50} for each of the cell lines for each of the drugs. As can be seen, the glycerol and ethylene compounds in general gave higher therapeutic ratios than the alkylphosphocholine analogs. Compound A3 gave the highest therapeutic index in all three cell lines. The lipoidal amine (compound B) was clearly toxic to normal cells.

There were two types of inhibitory patterns observed in the clonogenic assays. The first was a linear dose related inhibition. The second pattern was a non-linear inhibition characterized by an initial rise or plateau followed by rapid lysis. Figure 6 demonstrates these patterns with compound A1 in HL60 and K562 cells. In those instances, the IC_{50} was determined from the graph.

TABLE 1

Calculated Therapeutic Index[a]

Compounds	Cell Lines		
	HL60	K562	Daudi
A1 (ET-18-OCH_3)	6.08	2.03	3.65
A2 (ET-16-$NHCOCH_3$)	8.15	3.58	4.74
A3 (BM 41.440)	16.78	7.92	5.94
B (CP-46665)	0.74	0.55	0.95
C (AEPL)	24.56	2.57	3.28
D1 ($HePC_2$)	2.88	1.74	1.57
D2 ($HePC_2$)	12.00	2.91	2.75

[a]Ratio of IC_{50} of normal progenitor cells (CFU) *vs.* clonogenic leukemic cell lines.

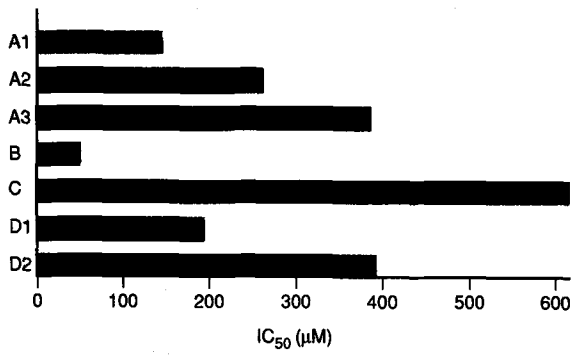


FIG. 5. Effect of EL on normal marrow progenitor cells. The IC_{50} of 3–5 separate marrow specimens were averaged and plotted for each compound. D3 and D4 were not tested because of limited antitumor activity.

At the IC_{50}, viability, as determined by trypan blue dye exclusion, was greater than 75% in all cell lines with all EL concentrations except Daudi which was 66% after exposure to compound A1. Despite the fact that the majority of cells present were viable, there was a significant effect on clonogenic cells.

DISCUSSION

This investigation attempted to explore relationships between structures and function in a series of ether lipids. Assays used included thymidine uptake as a measure of DNA synthesis, colony formation in semi-solid media as a measure of the leukemic stem cell and normal progenitor cells and PKC activity to assess potential mechanisms in regulation of cell growth.

Two myelogenous leukemic cell lines were employed. One, the HL60 cell line, was sensitive to A1, the reference compound which appeared from our own data and the literature to be the most active compound (8,38,39). The other, K562, was known to be resistant to the reference compound (6). Two other myeloid cell lines, KG-1 and KG-1a, differ in that the former can be induced to differentiate by phorbol esters (33) and the other cannot. Lastly,

Daudi represents a lymphoid leukemic cell line. Because of some difficulty in colony growth of KG-1 and KG-1a cells, only limited data are available.

From the results presented, A3 (BM 41.440) proved to be the most active compound and gave the highest therapeutic ratio in all of the cell lines tested. Although the ethylene compound (C) was active in HL60, it was inactive against K562 and Daudi, suggesting a limited spectrum of activity. The hexadecylphosphocholine analogs differ in the distance between the phosphorus and the tertiary ammonium group. The shorter the distance, the more active the compounds. The lesser activity, when compared to the A-groups, may be due to the relatively shorter alkyl chain, and synthesis of compounds with chainlengths of 18 to 20 carbons is in progress.

These agents are of interest because of their antitumor activity, in which they appear to be active through mechanisms differing from those of other chemotherapeutic agents. More importantly, they appear to be selectively toxic to neoplastic tissues sparing normal marrow progenitor cells. The reasons for the relative selectivity of the toxicity are poorly understood. The initial observation that ET-18-OCH_3 was more rapidly metabolized due to cleavage of the *O*-alkyl ether linkage in normal marrow cells and myelocytes of chronic myelocytic leukemia compared to leukemic blast cells (40,41) has not been confirmed in subsequent studies (6,15,42). Uptake of radiolabelled ET-18-OCH_3 and its concentration in the membrane of sensitive HL60 cells was greater than in the resistant K562 cells or normal granulocytes (6). The biochemical mechanisms involved remain to be elucidated.

Previous studies have indicated that the ether linkage or thio linkage in the *sn*-1 position of glycerophosphocholines is important for antitumor activity. The length of the alkyl chain in *sn*-1 is important, C_{16} or C_{18} compounds are more active (43). Substitution in *sn*-2 with a methoxy or acetamido group increases antitumor activity. Our data would suggest that the glycerophosphocholine-type compounds are more active than ethylene or alkylphosphocholine analogs. It is of interest that $HePC_2$, which lacks the glycerol backbone, shows antineoplastic activity (44,45). Further modification of their structure may enhance their activity.

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Stereospecific Synthesis of Antitumor Active Thioether PAF Analogs[1]

Suresh K. Bhatia[2] and Joseph Hajdu*
Department of Chemistry, California State University, Northridge, Northridge, California 91330

A novel stereospecific synthesis of antitumor active thioether analogs of platelet-activating factor (PAF) is reported. The synthesis is based upon: i) the use of *D*-serine to provide the chiral center for the construction of the optically active phospholipid molecule; ii) development of the *sn*-1-thioalkyl function via thioacetate displacement of methanesulfonate-activated primary hydroxyl group followed by alkylation of the *sn*-1-thiolate function; and iii) introduction of the phosphocholine moiety through the 2-chloro-2-oxo-1,3,2-dioxaphospholane/trimethylamine sequence. The entire scheme relies on the use of a single protecting group. The synthetic thioether phospholipid 1-*S*-hexadecyl-2-*N*-acetamidodeoxy-*sn*-glycero-3-phosphocholine has been shown to be a potent antitumor active phospholipid, exhibiting tumor cytotoxicity against a lymphoblastoid lymphoma (Li-A) cell line and a malignant histiocytic (DHL-4) cell line of human origin at the same level of potency as ET-18-OMe and 1-*O*-octadecyl-2-*N*-acetamidodeoxy-*sn*-glycero-3-phosphocholine. The synthetic method described has a great deal of flexibility, providing a convenient general route to a wide range of thioether PAF analogs.
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Stereospecific synthesis of antitumor active platelet-activating factor (PAF) analogs is one of the key steps toward elucidating the structural basis of their biological mechanism of action (1). Specifically, a number of structurally modified alkyl ether derivatives of PAF have been shown to exhibit selective cytotoxicity against a series of different human cancer cells (1–3). These synthetic phospholipid compounds appear to act at the cell membrane inhibiting phosphatidylcholine biosynthesis (4), protein kinase C activity (5), as well as phorbol ester stimulation of protein kinase C (6). While most early attempts to prepare antitumor active phospholipids have led to *racemic* mixtures of the target compounds (1–3), studies which employed enantiomerically pure ether lipids seem to indicate significant differences both in potency and selectivity of the respective stereoisomers (7,8). Therefore, availability of facile and efficient synthetic methods for the preparation of structurally variable chiral PAF analogs is a prerequisite for establishing structure-activity relationships, as well as for the design of new phospholipid compounds with targeted antineoplastic activities.

Thioether analogs of platelet-activating factor represent a particularly useful series of anticancer phospholipids, since in contrast to the corresponding *sn*-1-*O*-alkyl derivatives, the sulfur compounds have been reported to possess lower platelet-aggregating potency and show less immunosuppression, two noted side effects in antileukemic phospholipid chemotherapy (9). Indeed, one such compound, Ilmofosine (BM 41,440, 1-hexadecylthio-2-methoxymethyl-*rac*-glycero-3-phosphocholine), is currently in clinical trials as a potential anticancer drug (10). As part of our research toward developing new synthetic methods for the preparation of phospholipid derivatives (11–15) we have recently introduced a stereospecific sequence leading to 1-thioalkyl-2-acylaminodeoxy-*sn*-glycero-3-phosphocholines (1) (13). In this paper we describe the synthesis in detail and demonstrate the applicability of the method to the preparation of other structurally related phospholipid analogs.

MATERIALS AND METHODS

General methods. Melting points were determined on a Mel-Temp apparatus (Laboratory Devices, Hollistow, MA) and are uncorrected. IR spectra were recorded on a Beckman Acculab 2 spectrophotometer Beckman Instruments (Fullerton, CA). ^{1}H NMR (internal Me_4Si) were taken on an IBM NR-80 instrument. Optical rotations were determined either on a Perkin Elmer 241 MC (Perkin Elmer, Norwalk, CT) or Rudolph AutoPol III polarimeter. 4-(Dimethylamino)pyridine, triphenylchloromethane, acetyl chloride, palmitoyl chloride, *p*-nitrophenyl chloroformate, sodium methoxide and potassium thioacetate were obtained from Fluka Chemical Corporation (Ronkonkoma, NY). *D*-serine from Sigma Chemical Co. (St. Louis, MO) and trimethylamine from Kodak Laboratory Chemicals (Rochester, NY) were used as received. Tetradecanol, methanesulfonyl chloride and hexadecyl iodide were obtained from Aldrich Chemical Co. (Milwaukee, WI). Anhydrous trifluoroacetic acid (Pierce Chemical Co., Rockford, IL), methanol (Burdick and Jackson, Muskegon, MI) and anhydrous diethyl ether (Mallinckrodt, St. Louis, MO) were used as received. Acetonitrile (Burdick and Jackson) and triethylamine (Fluka) were dried over activated Linde 4A molecular sieves (Fluka). Benzene (Burdick and Jackson) was kept over sodium wire; chloroform and methylene chloride (Mallinckrodt) were distilled from phosphorus pentoxide (Baker Chemical Co., Phillipsburg, NJ) just prior to use. 2-Chloro-2-oxo-1,3,2-dioxaphospholane was prepared by procedures in the literature (16). Column chromatography was carried out with silica gel 60 (70–230 mesh ASTM, E.M. Laboratories, Cherry Hill, NJ). The silica gel support was constantly kept at 120°C and was cooled to room temperature only prior to use in a desiccator over P_2O_5. Thin-layer chromatography was carried out on Whatman MK6F plates. The phospholipids were visualized by molybdic acid spray (17) and by charring (50% aq.

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[2]Present address: Biomolecular Engineering Branch Naval Research Laboratory, Washington, D.C. 20375-5000.
*To whom correspondence should be addressed.

sulfuric acid). Trityl compounds were visualized by UV light, and all other compounds were detected by iodine vapor. The thiols were additionally visualized by dipping into methanolic solution of 5,5′-dithio*bis*(2-nitrobenzoic acid) (18). Elemental analyses were performed by Galbraith Laboratories, Inc. (Knoxville, TN) and by Desert Analytics (Tucson, AZ).

N-*triphenylmethyl*-D-*serine methyl ester* (4). Triethylamine (6.39 mmol) was added to a cooled (ice-water bath) suspension of *D*-serine methyl ester hydrochloride (4.65 g, 30.0 mmol) in 70 mL dry chloroform. After 5 min, triphenylmethyl chloride (8.65 g, 31.0 mmol) was added and the resulting solution was stirred at room temperature for 24 hr. The solution was washed with water (3 × 50 mL), the organic layer was dried over anhydrous $MgSO_4$ and the solvent was flash-evaporated. The oily residue was triturated with hexane. The solidified product was filtered and dried in vacuum, yielding 9.9 g (91.4%) of chromatographically pure triphenylmethyl ether 4 R_f($CHCl_3$/CH_3OH, 98:2, v/v) 0.48. Crystallization from chloroform/petroleum ether gave a white solid; m.p. 141-2°C.^{1}H NMR ($CDCl_3$) δ 3.29 (*s*, 3H,OCH_3), 3.59 (*m*, 3H, OCH_2CHN), 7.20-7.45 (*m*, 15H, phenyl); $[\alpha]_D^{23}$ −17.63 (c 1.6, CH_3OH/$CHCl_3$, 1:4 v/v) *Anal.* Calcd. for $C_{23}H_{23}NO_3$:C, 76.43; H, 6.41, N, 3.87. Found: C, 76.64, H, 6.24; N, 3.76.

N-*triphenylmethyl*-O-*methanesulfonyl*-D-*serine methyl ester* (5). Methanesulfonyl chloride (1.26 g, 11.0 mmol) was added to a cooled (ice-water bath) solution of alcohol 4 (4.36 g, 10.0 mmol) in 50 mL anhydrous methylene chloride containing triethylamine (1.21 g, 12.0 mmol) and the resulting mixture was stirred at 0°C for 2 hr. Thin-layer chromatography showed disappearance of the starting material. The solution was washed three times with 50 mL water followed by brine (50 mL), dried over $MgSO_4$, and the solvent was flash-evaporated at 30°C to give 4.0 g (91%) of methanesulfonate (5) as a viscous oil: R_f($CHCl_3$) 0.4; ^{1}H NMR ($CDCl_3$) δ 2.97 (*s*, 3H, O_3SCH_3), 3.27 (*s*, 3H, OCH_3), 3.60-4.38 (*m*, 4H, OCH_2CHN, NH), 7.22-7.44 (*m*, 15H, phenyl). The compound is unstable on storage; therefore, it was used as soon as possible for the next reaction step.

N-*triphenylmethyl*-S-*acetyl*-D-*cysteine methyl ester* (6). A solution of mesylate 5 (3.9 g, 8.88 mmol) in dry acetonitrile (80 mL) was stirred with $KSCOCH_3$ (1.71 g, 15.0 mmol) at room temperature for two days. The dark brown precipitate that formed was filtered and the acetonitrile was evaporated *in vacuo*. The residue was treated with 100 mL chloroform and filtered. The chloroform solution was washed with 1% aq. $NaHCO_3$ (60 mL) and saline (60 mL) and dried over anhydrous $MgSO_4$. Evaporation of the solvent gave a brown semisolid as a chromatographically pure product 6 (5.15 g, 84.6%): R_f ($CHCl_3$) 0.59; ^{1}H NMR ($CDCl_3$) δ 2.39 (*s*, 3H, $SCOCH_3$), 3.18 (*s*, 3H, OCH_3), 3.28-3.51 (*m*, 4H, $NCHCH_2SCO$, NH), 7.24-7.46 (*m*, 15H, phenyl) which was used directly for the next reaction.

N-*triphenylmethyl*-D-*cysteinol* (7). Lithium aluminum hydride (0.5 g, 13-15 mmol) in one portion was added to a cooled solution (0°C, ice-water bath) of ester 6 (3.15 g, 7.52 mmol) in dry ether (150 mL). The mixture was stirred at 0°C for 30 min, then at room temperature for 3 hr. Excess hydride was decomposed by slow addition of water at 0°C. The insoluble inorganic material was filtered over a pad of celite and washed with ether. The combined etheral layer was washed with 60 mL saline and dried over anhydrous $MgSO_4$. The solvent was evaporated *in vacuo* and the residue was chromatographed on freshly activated silica gel using chloroform. The product 7 was obtained as a semisolid (1.78 g, 68%) R_f($CHCl_3$) 0.60; ^{1}H NMR ($CDCl_3$) δ 1.73 (*br s*, 1H, OH), 2.13 (*br s*, 1H, SH), 2.76 (*m*, 2H, CH_2S), 3.27-3.50 (*m*, 3H, OCH_2CHN), 7.24-7.50 (*m*, 15H, phenyl), which was used for the next step as soon as possible.

S-*hexadecyl*-N-*triphenylmethyl*-D-*cysteinol* (8). A solution of compound 7 (1.78 g, 5.0 mmol) in absolute methanol (50 mL) containing sodium methoxide (0.28 g, 5.2 mmol) was stirred with hexadecyl iodide (1.76 g, 5.0 mmol) at room temperature for 16 hr. The methanol was then evaporated *in vacuo*, the residue was suspended in 50 mL water and extracted with 50 mL chloroform. The organic layer was washed with saline and dried over $MgSO_4$. Evaporation of the solvent gave the crude hexadecyl thioether 8, which was chromatographed on freshly activated silica gel using chloroform to give analytically pure product 8 (1.8 g, 61%) as a strongly hygroscopic viscous oil: ^{1}H NMR ($CDCl_3$) δ 0.88 (*t*, 3H, CH_3), 1.25 [*s*, 28H, $(CH_2)_{14}$], 2.14-2.22 (*m*, 5H, CH_2SCH_2, OH), 2.75-3.58 (*m*, 4H, OCH_2CHN, NH), 7.24-7.51 (*m*, 15H, phenyl); $[\delta]_D^{23}$ −20.72 (c 1.53, CH_3OH/$CHCl_3$ 1:4 v/v). *Anal.* Calcd. for $C_{38}H_{55}NOS$: C, 79.52; H, 9.66; N, 2.44; S, 5.59. Found: C, 79.64; H, 9.62; N, 2.29; S, 5.52.

1-Thiohexadecyl-2-N-*triphenylmethylaminodeoxy*-sn-*glycerol-3-phosphocholine* (10). 2-Cloro-2-oxo-1,3,2,-dioxaphospholane (0.64 g, 4.5 mmol) in dry benzene (5 mL) was added to a cooled solution (0°C) of alcohol 8 (2.4 g, 4.2 mmol) in dry benzene (95 mL) containing triethylamine (0.465 g, 4.6 mmol). The reaction mixture was stirred at room temperature for 24 hr. The precipitated triethylamine hydrochloride was filtered off and the solvent was removed under reduced pressure. The resulting semisolid, 2-(1-*S*-hexadecyl-2-*N*-triphenylmethylaminodeoxy-*sn*-glyceryl)-2-oxo-1,3,2-dioxaphospholane 9, formed as a single phosphate positive product ^{1}H NMR ($CDCl_3$) δ 0.87 (*t*, 3H, CH_3), 1.26 [*s*, 28H, $(CH_2)_{14}$], 2.14-2.22 (*m*, 4H, CH_2SCH_2) 3.10 (*m*, 2H, HCNH), 3.60-4.34 (*m*, 6H $(CH_2O)_3$), 7.24-7.52 (*m*, 15H, phenyl). This cyclic phosphotriester intermediate is moisture sensitive and readily undergoes hydrolytic decomposition; therefore, it was used directly for the next step.

The semisolid obtained (9) was suspended in dry acetonitrile (50 mL) and transferred to a pressure bottle. The pressure bottle was cooled in a dry-ice bath and to this, anhydrous trimethylamine (3 mL) was added. The content of the pressure bottle was heated at 65°C in an oil bath for 20 hr. Acetonitrile was then removed and crude product was chromatographed on activated silica gel with $CHCl_3$/CH_3OH/H_2O (65:25:4, v/v/v) to give analytically pure phospholipid 10 (1.9 g, 61.3%) as a strongly hygroscopic white solid. ^{1}H NMR ($CDCl_3$) δ 0.88 (*t*, 3H, CH_3) 1.25 [*s*, 28H, $(CH_2)_{14}$], 2.10 (*m*, 4H, CH_2SCH_2), 2.92-4.15 [*m*, 16H with *s* at 3.24 $N(CH_3)_3$, OCH_2CHN, OCH_2CH_2N]; $[\alpha]_D^{23}$-10.84° (c 1.31, CH_3OH/$CHCl_3$ 1:4, v/v); R_f($CHCl_3$/CH_3OH/water, 65:25:4, v/v/

v) 0.52. *Anal.* Calcd. for $C_{43}H_{67}N_2O_4PS \cdot \frac{1}{2}H_2O$: C, 69.04; H, 9.16; N, 3.74; P, 4.15; S, 4.28. Found: C, 68.86; H, 9.33; N, 3.67; P, 3.86; S, 4.39.

*1-Thiohexadecyl-2-*N*-acetamidodeoxy-*sn*-glycero-3-phosphocholine (1)*[3].

i) By detritylation-acylation method (Scheme I). Anhydrous CF_3COOH (0.4 g, 3.5 mmol) from a freshly opened vial was added to a solution of compound **10** (1.0 g, 1.35 mmol) in dry chloroform (30 mL). The resulting solution was stirred at room temperature for 1 hr. Thin-layer chromatography showed complete disappearance of the starting material (**10**). 4-(Dimethylamino)pyridine (0.465 g, 3.81 mmol) and acetyl chloride (0.120 g, 1.5 mmol) were added to this solution and the reaction mixture was stirred at room temperature for 20 hr. The chloroform was then evaporated and the residue was passed through Rexyn I-300 (25 mL bed volume) with $CHCl_3/CH_3OH$/water (4:5:1, v/v/v) using 60 mL total volume of eluant. Ten-mL fractions were collected and analyzed for phospholipid. The phosphate-positive fractions were combined, the solvent was evaporated *in vacuo*, and the residue was redissolved in benzene and freeze-dried. Finally, the freeze-dried product was dissolved in $CHCl_3/CH_3OH$/water (65:25:4, v/v/v) and chromatographed on activated silica gel to afford 0.45 g (62%) analytically pure phospholipid **1** as a hygroscopic white solid. ^{1}H NMR ($CDCl_3$) δ 0.88 (*t*, 3H, CH_3), 1.25 [*s*, 28H, $(CH_2)_{14}$], 1.98 (*s*, 3H, $NCOCH_3$), 2.52–2.64 (*m*, 4H, CH_2SCH_2), 3.34 [s, 9H, $N(CH_3)_3$], 3.74–4.27 (*m*, 7H, $NCHCH_2O$, OCH_2CH_2N); $[\alpha]_D^{23}$ −4.07° (c 1.08, $CH_3OH/CHCl_3$, 1:4, v/v), + 7.57° (c 1.4, CH_3OH). *Anal.* Calcd. for $C_{26}H_{55}N_2O_5PS \cdot H_2O$: C, 56.09; H, 10.32; N, 5.03; P, 5.56; S, 5.76. Found: C, 56.03; H, 10.22; N, 4.65; P, 5.50; S, 5.69.

ii) By the acylation-phosphorylation method (Scheme II). HCl was bubbled through a solution of chloroform-methanol (1:1, 100 mL) for a period of 45 min. Trityl compound **8** (2.2 g, 3.84 mmol) was added to this and the resulting solution was stirred at room temperature for 1 hr. The solvent was removed under reduced pressure, and the residue was treated with 150 mL saturated K_2CO_3 and 150 mL ether. After stirring for 30 min, the ether layer was separated, washed with aq. K_2CO_3 (100 mL), brine (100 mL), and then dried over anhydrous $MgSO_4$. The crude product was chromatographed on silica gel (20 g), eluting first with $CHCl_3/CH_3OH$ (95:5, v/v), then with $CHCl_3/CH_3OH$ (9:1, v/v). The amino alcohol (**12**) was obtained as a white solid (1.06 g, 84%) which was freeze-dried from benzene prior to the next step. ^{1}H NMR ($CDCl_3$ + D_2O) δ 0.87 (*t*, 3H, CH_3), 1.26 [*s*, 28H, $(CH_2)_{14}$], 2.56 (*m*, 4H, CH_2SCH_2), 3.0–3.6 (*m*, 3H, $NCH\text{-}CH_2O$); $[\alpha]_D^{23}$ + 21.13 (c 1.41, $CH_3OH/CHCl_3$, 1:4, v/v).

A solution of alcohol **12** (0.600 g, 1.81 mmol) in dry chloroform (25 mL) containing 4-(dimethylamino)pyridine (0.230 g 1.89 mmol) and acetyl chloride (0.141 g, 1.79 mmol) was stirred at room temperature for two days. The solvent was removed under reduced pressure and the residue was chromatographed on silica gel (20 g) with $CHCl_3/CH_3OH$ (94:6, v/v). The *N*-acetyl compound **13** (0.480 g, 71%) was obtained as a white solid: ^{1}H NMR ($CDCl_3$) δ 0.87 (*t*, 3H, CH_3), 1.25 [*s*, 28H, $(CH_2)_{14}$], 2.02 (*s*, 3H, $COCH_3$), 2.45–2.76 (*m*, 4H, CH_2SCH_2), 3.75–4.01 (*m* 4H $NCHCH_2O$, OH), 5.97 (*m*, 1H, NH); $[\alpha]_D^{23}$ + 6.92 (c 1.2, $CH_3OH/CHCl_3$, 1:4, v/v).

A suspension of alcohol **13** (0.430 g, 1.16 mmol) in dry benzene (30 mL) containing triethylamine (0.136 g, 1.34 mmol) and 2-chloro-2-oxo-1,3,2-dioxaphospholane (0.180 g, 1.27 mmol) was stirred at room temperature for two days. The precipitated triethylamine hydrochloride was filtered off and the filtrate was evaporated to dryness. The semisolid residue was transferred to a pressure bottle with anhydrous acetonitrile (25 mL), and trimethylamine (3 mL) was added to the mixture. The pressure bottle was sealed and the solution was kept at 65°C in an oil bath for two days. The solvent was then evaporated and the residue was chromatographed on silica gel using $CHCl_3/CH_3OH$/water (65:25:4, v/v/v) as eluant. The fractions containing the pure phospholipid (**1**) were combined, the solvent was evaporated and the product freeze-dried from benzene. Compound **1** was obtained as a hygroscopic white solid (0.322 g, 52%). Spectroscopic data for this compound are given above.

Compounds **1′** and **1″** with the corresponding palmitoyl and tetradecyloxycarbonyl substitutents at the *sn*-2 position were prepared by employing the same experimental conditions as described for the acetyl compounds.

*1-Thiohexadecyl-2-*N*-palmitoylaminodeoxy-*sn*-glycero-3-phosphocholine (1′).*

i) *By detritylation-acylation method (Scheme I).* Compound **1′** was obtained as a white hygroscopic solid in 51% yield from **10**. ^{1}H NMR ($CDCl_3$) δ 0.87 [t 6H, $(CH_3)_2$] 1.25 [*s*, 54H, $(CH_2)_{27}$], 2.13 (*br t*, 3H, $COCH_2$), 2.48–2.78 (*m*, 4H, CH_2SCH_2), 3.33 [*s*, 9H, $N(CH_3)_3$], 3.78–4.37 (*m*, 7H, $NCHCH_2O$, OCH_2CH_2N); $[\alpha]_D^{23}$ + 9.62° (c 1.32, CH_3OH); *Anal.* Calcd. for $C_{40}H_{83}N_2O_5PS \cdot 3H_2O$: C, 60.88; H, 11.37; N, 3.55; P, 3.92; S, 4.06. Found: C, 60.59; H, 11.53; N, 3.54; P, 3.78; S, 4.44.

ii) *By the acylation-phosphorylation method (Scheme II).* The *N*-palmitoyl analog of **13** was obtained in 64% yield from the corresponding amino alcohol precursor (**12**). Compound **13′** was isolated as a hygroscopic solid ^{1}H NMR ($CDCl_3$) δ 0.87 [*t*, 6H, $(CH_3)_2$] 1.25 [*s*, 54H, $(CH_2)_{27}$], 2.12 (*br t*, 2H $COCH_2$), 2.44–2.75 (*m*, 4H, CH_2SCH_2), 3.73 (*m*, 3H, $NCHCH_2O$); $[\alpha]_D^{23}$ + 9.58 (c 1.2 CH_3OH-$CHCl_3$, 1:4, v/v).

Phosphorylation of the *sn*-2-palmitoyl alcohol (**13′**) with 2-chloro-2-oxo-1,3,2-dioxaphospholane, followed by ring-opening with trimethylamine as outlined above afforded the corresponding phospholipid **1′** in 41% yield. Spectroscopic data for this compound are given above.

*1-Thiohexadecyl-2-*N*-tetradecyloxycarbonylaminodeoxy-*sn*-glycero-3-phosphocholine (1″).*

i) By detritylation-acylation method (Scheme I). Compound **1″** was obtained in 57% yield from **10**. ^{1}H NMR ($CDCl_3$ + D_2O) δ 0.88 [*t*, 6H, $(CH_3)_2$], 1.25 [*s*, 52H, $(CH_2)_{26}$], 2.52–2.65 (*m*, 4H, CH_2SCH_2), 3.37 [*s*, 9H, $N(CH_3)_3$], 3.74–4.32 (*m*, 9H, OCH_2CHN, OCH_2CH_2N, $NCOOCH_2$); $[\alpha]_D^{23}$ + 6.36° (c 1.21, CH_3OH). *Anal.* Calcd.

[3]For the synthesis of *rac-(1)* and D-*(2)* from cysteine see Reference 26.

for $C_{39}H_{81}N_2O_6PS•3H_2$): C, 59.21; H, 11.08; N, 3.54; P, 3.91; S, 4.05. Found: C, 59.11; H, 11.05; N, 3.51; P, 3.87; S, 3.87.

ii) *By the acylation-phosphorylation method (Scheme II): 1-Thiohexadecyl-*N*-tetradecyloxycarbonylaminodeoxy-*sn*-glycerol (13″).* A solution of alcohol 12 (0.500 g, 1.51 mmol) in 20 mL anhydrous chloroform containing 4-(dimethylamino)pyridine (0.195 g, 1.6 mmol) and *p*-nitrophenyl tetradecyl carbonate (14, 0.568 g, 1.5 mmol) was kept at room temperature for 30 hr. The solution was diluted with chloroform (50 mL) and washed with 5% K_2CO_3 until the aqueous solution became colorless. The organic layer was washed with saline, dried over anhydrous $MgSO_4$, and the solvent was evaporated. The crude product (13″) was chromatographed on silica gel (20 g) with $CHCl_3/CH_3OH$ (94:6 v/v) to afford the target compound as a crystalline solid (13″, 0.610 g, 71%). ^1H NMR ($CDCl_3$) δ 0.87 [*t*, 6H, $(CH_3)_2$], 1.25 [*s*, 52H, $(CH_2)_{26}$], 2.46–2.75 (*m*, 4H, CH_2SCH_2), 3.77–4.12 (*m*, 5H, OCH_2CHN, $NCOOCH_2$); $[\alpha]_D^{23}$ + 7.54.

p-*Nitrophenyl tetradecyl carbonate (14).* Tetradecanol (2.14 g, 10 mmol) was added to a solution containing *p*-nitrophenyl chloroformate (2.2 g, 10.9 mmol) in 70 mL dry chloroform. 4-(Dimethylamino)pyridine (1.342 g, 11.0 mmol) was added and the reaction mixture was stirred at room temperature for 16 hr. The solvent was then evaporated under reduced pressure and the residue was chromatographed on silica gel with chloroform-hexane (3:1). The product (14, 3.7 g, 97.5%) was obtained as a liquid which solidified on standing. ^1H NMR ($CDCl_3$) δ 0.87 (*t*, 3H, CH_3), 1.26 [*s*, 24H, $(CH_2)_{12}$], 4.28 (*t*, 2H, OCH_2), 7.27–8.33 (*dd*, H_{arom}). *Anal.* Calcd. for $C_{21}H_{33}NO_5$: C, 66.47; H, 8.76; N, 3.69. Found: C, 66.62; H, 8.87; N, 3.64.

*1-Thiohexadecyl-2-*N*-tetradecyloxycarbonylamino-*sn*-glycero-3-phosphocholine (1″).* A suspension of alcohol 13″ (0.50 g, 0.88 mmol) in dry benzene (30 mL) containing triethylamine (0.110 g, 1.1 mmol) and 2-chloro-2-oxo-1,3,2-dioxaphospholane (0.142 g, 1.0 mmol) was stirred at room temperature for two days. The precipitated triethylamine was filtered, and the solvent was evaporated *in vacuo*. The residue was transferred into a pressure bottle with anhydrous acetonitrile (30 mL), and trimethylamine (3 mL) was added while the mixture was cooled in a dry-ice bath. The pressure bottle was sealed and the solution was kept at 65°C for two days. The solvent was then evaporated and the product (1″) was chromatographed on activated silica gel with $CHCl_3/CH_3OH$/water (54:25:4 v/v/v). The product was then freeze-dried from benzene to give 0.320 g (49.3%) analytically pure phospholipid 1″. Spectroscopic data for the product (1″) are given above.

RESULTS AND DISCUSSION

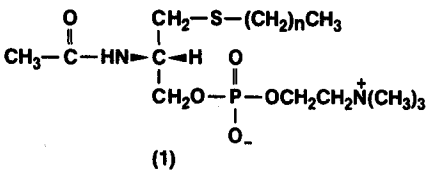
The structural design of (1) and (2) as our target compound has relied on studies showing that: i) the antitumor activity of 1-*O*-octadecyl-2-acetamidodeoxy-*sn*-glycero-3-phosphocholine (2, n = 17) is comparable to that of ET-18-OMe (1-*O*-octadecyl-2-*O*-methyl-*rac*-glycero-3-phosphocholine) (1,7); and ii) the 1-*S*-alkyl analogs of ET-18-OMe and related 1,2-dialkylphospholipids appear to exceed the tumor cytotoxicity of the 1-*O*-alkyl series yet, at the same time, exhibit diminished platelet-aggregating activity (9,19). Thus, by replacing the *sn*-1-*O*-alkyl substituent of (1) with the corresponding thioether function, we sought to achieve high antineoplastic potency with decreased immunosuppressive and platelet activating capacity.

Our approach to the synthesis of (1) is based on construction of the chiral phosphodiester molecule around the α-carbon of *D*-serine using a sequence of reactions that keeps the stereochemistry of the optically active center unchanged (Scheme I). Based on the structural relationship between the starting amino acid (3) and the target phospholipid (1), the synthesis must involve: i) reduction and thioalkylation at the incipient *sn*-1-position; ii) selective *N*-acylation of the amino group at the secondary carbon; and iii) phosphorylation of the hydroxymethyl function with subsequent elaboration of the phosphodiester head group.

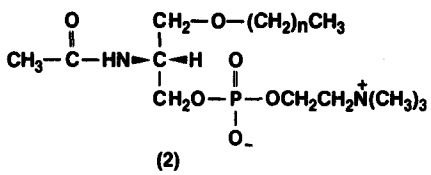
In order to achieve these objectives, we first prepared *N*-trityl-*D*-serine methyl ester (4). This intermediate allows facile manipulation of the primary carbon functions for developing the thioalkyl and phosphocholine residues, respectively. Thus, activation of the alcohol function by treatment of (4) with methanesulfonyl chloride/triethylamine in chloroform gave the corresponding methanesulfonate (5) in excellent yield (91%). Compound (5) was then allowed to react with potassium thioacetate in anhydrous acetonitrile at room temperature for introduction of the sulfur atom at the incipient *sn*-1-position of the target phospholipid (84%). Reduction of both the thioester and the carbomethoxy functions of (6) with lithium aluminum hydride gave the γ-hydroxy thiol (7) in one step (68%). This compound (7) was then selectively *S*-alkylated using hexadecyl iodide/1 equiv. sodium methoxide in methanol (75%).

The sequence here employed for elaboration of the thioether function via thioacetate-displacement, followed by reduction and subsequent alkylation, turned out to be a convenient route to sulfur-substituted lipid derivatives, since the intermediates are readily obtained, isolated and purified. The alternative method involving direct coupling between the methanesulfonate (5) and sodium hexadecyl thiolate (20) appeared to be much less efficient.

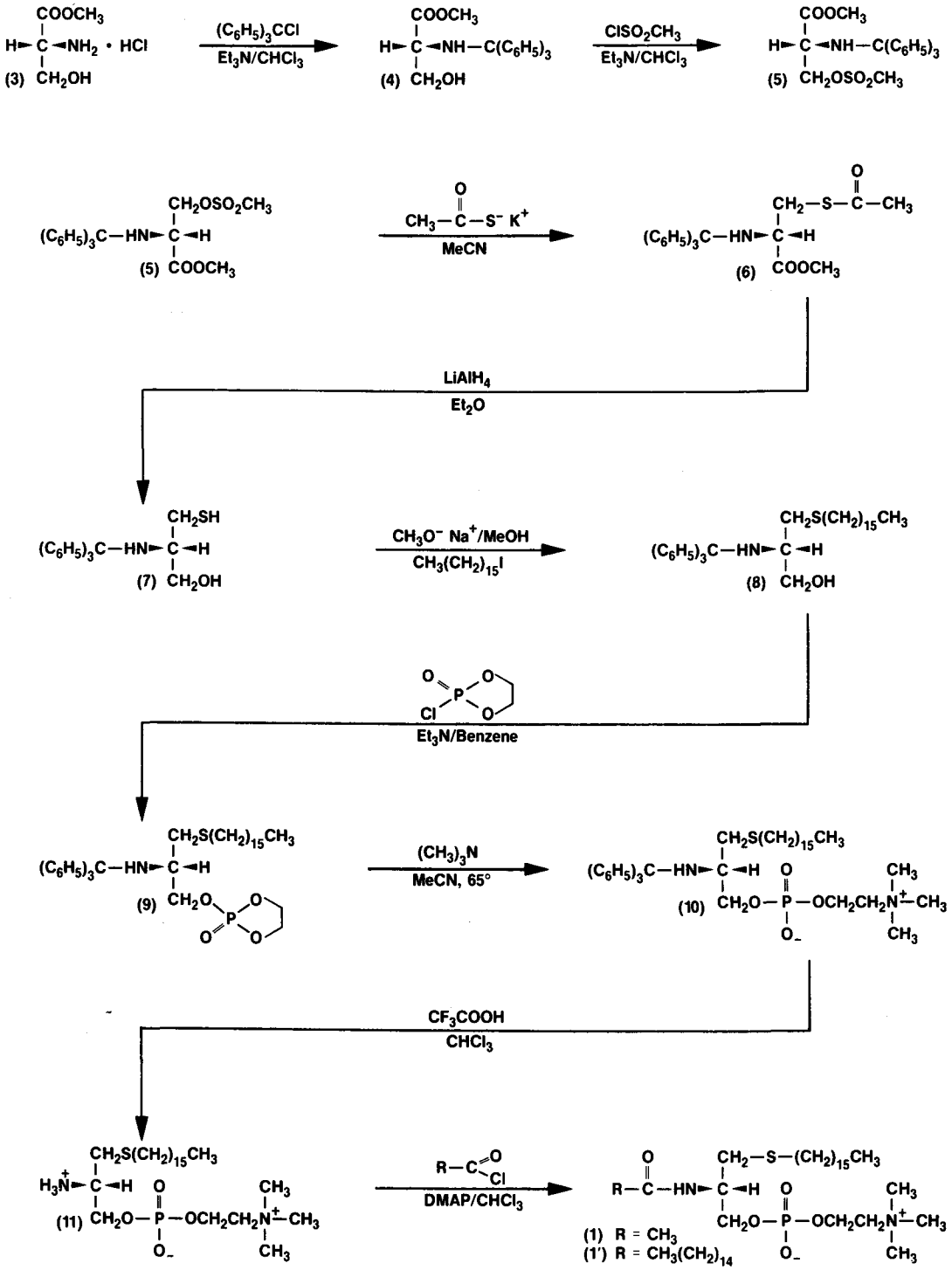
Phosphorylation of alcohol 8 with 2-chloro-2-oxo-1,3,2-dioxaphospholane in benzene in the presence of 1 equiv. triethylamine, followed by treatment of the cyclic triester (9) with anhydrous trimethylamine in



(1)



(2)



SCHEME I. Synthesis of 1-thiohexadecyl-2-acetamidodeoxy-*sn*-glycero-3-phosphocholine.

acetonitrile at 65°C for 16 hr gave the corresponding phosphocholine (10). The compound (10) obtained as a single phosphate-positive product from the reaction mixture, was readily purified by silica gel chromatography to give a strongly hygroscopic white solid (61%). Detritylation of (10) was carried out with anhydrous trifluoracetic acid in chloroform, and the resulting aminophospholipid (11) was acetylated *in situ* using acetyl chloride/4-(dimethylamino)pyridine at room temperature, to afford the target PAF analog 1 in 62% yield.

The main synthetic strategy that has emerged from Scheme I is the use of the *N*-trityl protecting group for the preparation of substituted *sn*-2 aminodeoxyglycerophospholipids. This intermediate is likely to become

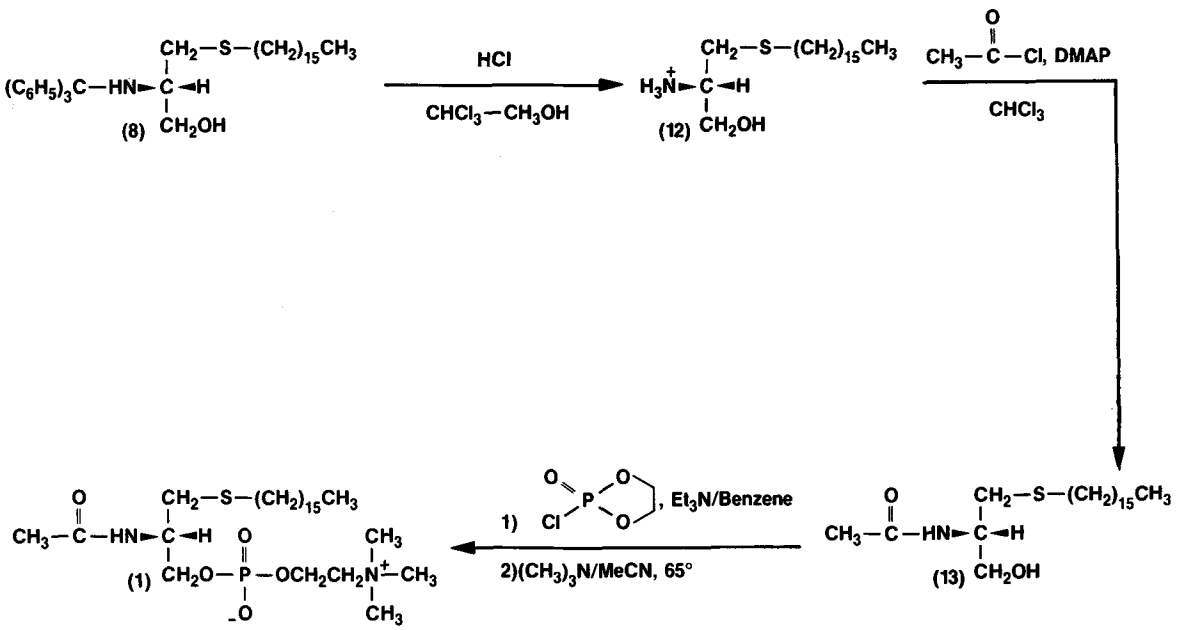
a useful precursor for the synthesis of other structurally related derivatives, where electrophilic neighboring group activation might be required. Specifically, in contrast to carbonyl-carrying protecting groups, the *N*-trityl function eliminates the possibility of intramolecular side-reactions (*e.g.*, oxazoline formation; refs. 21,22) potentiated by the nucleophilic carbonyl oxygen of *t*-butoxycarbonyl- or carbobenzoxy-substituted amino derivatives. Furthermore, *N*-trityl protected amino alcohols provide hydrophobic substrates that turned out to be highly suitable for efficient phosphorylation. In addition, the resulting *N*-trityl phospholipids are readily purified by silica gel chromatography, yielding much better recoveries than the corresponding aliphatic derivatives (23,24). Finally, due to the ease with which the amino group is deprotected, the desired *sn*-2 substituent of the target phospholipid can be introduced after the polar headgroup had been elaborated.

We have also explored the use of an alternative strategy for the synthesis of 1 through introduction of the *sn*-2 acyl substituent prior to elaboration of the phosphocholine moiety of the target phospholipid. As shown in Scheme II, compound 8 was first detritylated with HCl in anhydrous methanol/chloroform solution to give the corresponding amino alcohol (12, 84%), which was then selectively *N*-acetylated (25) in 71% yield. The product, suspended in dry benzene, was phosphorylated with 2-chloro-2-oxo-1,3,2-dioxaphospholane/triethylamine at room temperature for two days. Ring opening of the ethylene-bridged phosphodiester portion of the molecule by anhydrous trimethylamine in acetonitrile at 65°C for two days yielded compound (1). The phospholipid product was isolated by filtration and purified by silica gel chromatography (52%).

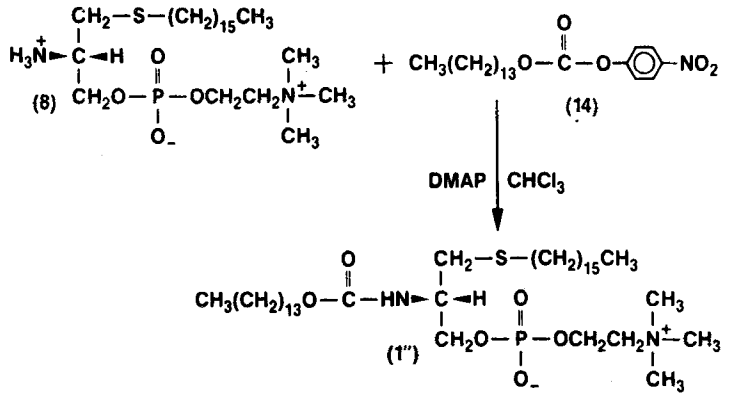
The sequence outlined in Scheme II provides an efficient alternative route to a number of thioether phospholipids, with two important constraints: i) the nature of the *sn*-2 substituent must be committed prior to phosphorylation; and ii) the functional group introduced in the 2-position must be stable under the phosphorylation conditions. It should be pointed out that the key to successful application of the sequence is to provide rigorously anhydrous conditions both during *N*-acylation of the amino alcohol (12) and phosphorylation of the corresponding amide (13). Specifically, anhydrous conditions are required for preferential *N*-acylation in converting compound 12 to 13, preventing the formation of the corresponding diacyl compound (23). The latter has been shown to occur in the reaction mixture as the minor product, isolated in 10–15% yield. Similarly, efficient phosphorylation of alcohol 13 greatly depends on anhydrous conditions; even traces of water appear to cause substantial decrease in yield of 1, most likely due to oxazoline formation (21,22) at the incipient *sn*-3 position.

Synthesis of related analogs. Availability of the *sn*-2 amino intermediate (11) from the sequence described in Scheme I prompted us to extend the scope of the synthesis to the preparation of related derivatives by varying the *sn*-3 substituents. Along these lines we have prepared two closely related *sn*-2 analogs: i) the corresponding long-chain amide (1′); and ii) an *sn*-2 carbamoyl-(tetradecyloxycarbonylamino)phosphocholine 1″. Thus, electrophilic trapping of the sn-2 amino group of 11 by *p*-nitrophenyl tetradecyl carbonate (14)/4-dimethylaminopyridine in chloroform afforded 1″ in 57% yield, (eq. [1]).

The *sn*-2 palmitoyl compound (1′) was obtained under similar reaction conditions (51%). Therefore, it appears that in analogous reactions using other functionalized electrophilic segments, new structurally re-



SCHEME II. Alternative synthesis of 1-thiohexadecyl-2-acetamidodeoxy-*sn*-glycero-3-phosphocholine via acylamino alcohol intermediate.



lated phospholipid derivatives might be preparable. As introduction of the *sn*-2 substituents occurs in the final step of the synthesis, functional groups, that are sensitive to the experimental conditions involved in phosphorylation or interfere with elaboration of the polar headgroup may now be incorporated.

Antitumor activity of compound (1). Comparison of the antineoplastic activity of 1-thiohexadecyl-2-acetamideodeoxy-*sn*-glycero-3-phosphocholine (1) to those exhibited by the 1-*O*-alkyl ether PAF analog (2) and ET-18-OMe indicates that the three compounds are about equipotent. Preliminary studies using a lymphoblastoid lymphoma cell line (Li-A) and a malignant histiocytic cell line (DHL-4) of human origin indicate that both cell lines have about the same sensitivity to the thioether PAF-analog (1) as to the two reference compounds, which are among the most active anticancer phospholipids to date. These results seem to suggest that structurally related analogs of compound (1) might become suitable candidates for future studies of antineoplastic phospholipids.

In conclusion, it might be noted that the strength of the synthetic methods presented is in their simplicity and efficiency; flexibility in terms of the substituents that can be introduced; and applicability to the development of other structurally variable thioether PAF analogs. Synthetic work along these lines is currently under way in our laboratory.

ACKNOWLEDGMENTS

We thank Professor W.E. Berdel of the Technical University of Munich, Germany, for determination of the antitumor activity of compound (1). We are grateful to the National Institutes of Health (DK36215 and CA 46750) for financial support.

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Antitumor Activity of Ilmofosine (BM 41.440) in the [3]Lewis-Lung Carcinoma Model[1]

Dieter B.J. Herrmann[a,*], Hans-George Opitz[a] and Paul G. Munder[b]

[a]Boehringer Mannheim GmbH, Department of Immunopharmacology, 6800 Mannheim and [b]Max-Planck-Institute for Immunobiology, 7800 Freiburg, Germany

Ilmofosine (1-hexadecylthio-2-methoxymethyl-1,3-propanediol-phosphocholine, BM 41.440) is a thioether phospholipid with cytostatic/cytotoxic properties. The antineoplastic activity of this compound was investigated *in vivo* in the [3]Lewis-lung carcinoma system. [3]Lewis lung tumor-bearing C57Bl/6 mice were treated with 0.625 to 40 mg Ilmofosine/kg per day *p.o.* either from days 1 to 9 or from days 11 to 28 after intrafoot-pad tumor cell inoculation. Ilmofosine caused a significant dose-related response on tumor growth and metastases, expressed in terms of tumor diameter, tumor weight, survival time and number of metastases-free animals as compared to sham-treated and positive (cyclophosphamide) controls. The results suggest that direct cytostatic/cytotoxic effects, rather than immune-modulatory mechanisms, preferentially contribute to the antitumor activity of Ilmofosine *in vivo*.
***Lipids* 26, 1431–1436 (1991).**

Alkyl lysophospholipids (ALP) are synthetic analogues of naturally-occurring lysophosphatidylcholine, which is a minor but important intermediate in the continuous exchange and renewal of phospholipids in cellular membranes (1, 2). ALP represent a new class of antitumor agents (3,4).

Ilmofosine is a thioether lysophosphatidylcholine analogue which has recently been tested in clinical phase I tolerability studies in adult patients with refractory cancers (5,6). The physiological lysophosphatidylcholine molecule was modified at two sites. Firstly, the ester bond at the *sn*-1 position of the glycerol moiety was replaced by a thioether linkage that is not cleaved by phospholipases; secondly, the hydroxyl group at the *sn*-2 position was replaced by a methoxymethyl group, thereby preventing acylation at position 2 of the lysophospholipid *via* acyltransferases (7,8).

The most frequently considered principal mechanisms to account for the antitumor activity of ALP *in vivo* are the direct and selective cytostatic and cytotoxic effects on malignant cells (9–13) and/or an induction of tumoricidal, immuno-competent cells of the monocyte/macrophage lineage (4,9,14). The molecular mechanism by which ALP deploy their direct tumor cell destructive effect appears to be attributable to a very selective interference of these compounds which cellular phosphatidylcholine metabolism (12,15–17), but it remains uncertain whether this effect is related to a specific inhibition of protein kinase C (PKC) activity (18,19). It was the aim of the present study to shed light on the mode of action of the thioether phospholipid derivative Ilmofosine *in vivo*.

MATERIALS AND METHODS

Compounds. Ilmofosine was synthesized by Boehringer Mannheim GmbH (Mannheim, Germany). Details on the synthesis of this and related alkyl lysophospholipids (ALP) have been published (7). Figure 1 shows the chemical structure of Ilmofosine. The compound was supplied as a crystalline, white powder. The chemical purity of each batch used for the experiments was greater than 98%. Cyclophosphamide (Endoxan^R), used as a reference compound, was obtained from ASTA Pharma AG (Bielefeld, Germany). Ilmofosine and cyclophosphamide were dissolved in phosphate-buffered saline (PBS) immediately before use and diluted as required.

Animals. Syngeneic C57Bl/6 mice, 2–3-months-old, with a body weight of 20–25 g, were used in all experiments. The animals were obtained from our own specific pathogen-free (SPF) unit and were kept under standard laboratory conditions (room temperature, 23 ±1°C; humidity, 50–70%; day/night rhythm, 12 hr, 6 a.m. to 6 p.m.). Food (Altromin^R 1324) and water were provided *ad libitum*.

[3]Lewis-lung carcinoma model. The [3]Lewis-lung carcinoma was maintained by serial passage in C57Bl/6 mice, transplanting 1×10^6 viable tumor cells *s.c.* into the axilla. Seven days later, the animals were killed, the tu-

BM 41.440

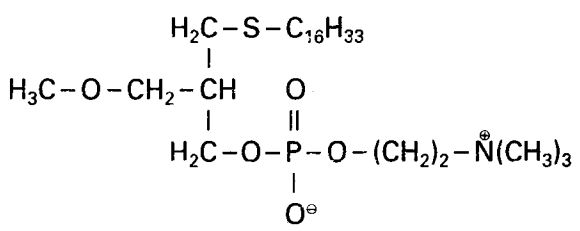


FIG. 1. Structure of 1-*S*-hexadecylthio-2-methoxymethyl-1,3-propanediol-3-phosphocholine (Ilmofosine, BM 41.440).

[1]Based on a paper presented at the Third international Conference on Platelet-Activating Factor and Structurally Related Alkyl Ether Lipids, Tokyo, Japan, May 1989.

*To whom correspondence should be addressed at Boehringer Mannheim GmbH, Department of Immunopharmacology, Sandhofer Strasse 116, 6800 Mannheim 31, Germany.

Abbreviations: ALP, alkyl lysophospholipid(s); BM 41.440, 1-*S*-hexadecylthio-2-methoxymethyl-1,3-propanediol-phosphocholine (Ilmofosine); ET-18-OCH_3, 1-*O*-octadecyl-2-*O*-methyl-*rac*-glycero-3-phosphocholine; FCS, fetal calf serum; MNU, methylnitrosourea; MethA, 3-methylcholanthrene; MST, mean survival time; PAF, platelet-activating factor, 1-*O*-alkyl-2-acetyl-*sn*-glycero-3-phosphocholine; PBS, phosphate-buffered saline (43.85 g NaCl, 1.10 g $NaH_2PO_4 \times H_2O$ and 11.95 g $Na_2HPO_4 \times 12H_2O$ dissolved in 5 L of water, double-distilled, at pH 7.2, 5 N NaOH); PKC, protein kinase C; SPF, specific pathogen free.

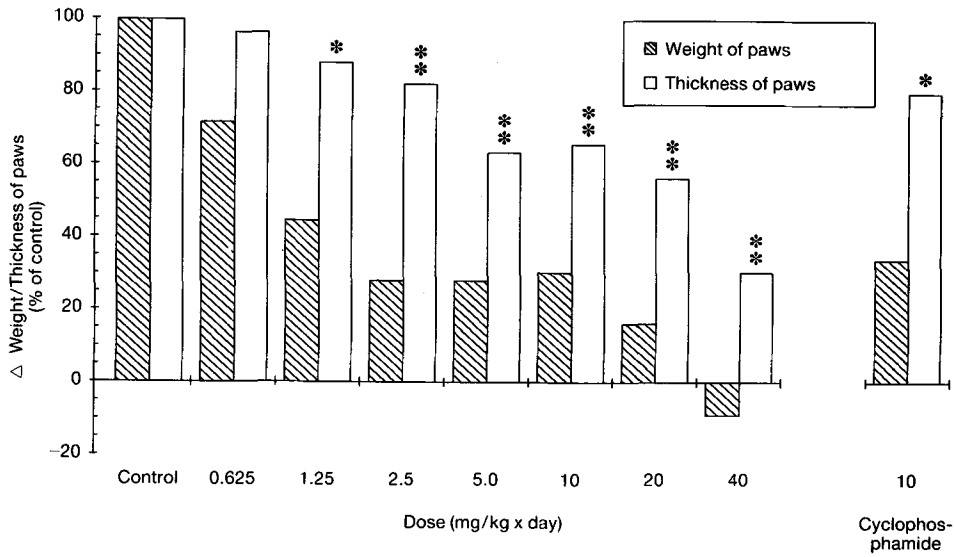


FIG. 2. Effect of Ilmofosine on primary ^{3}Lewis-lung carcinoma growth. Female C57Bl/6 mice, n = 20, were inoculated with 1 × 10^6 tumor cells/mouse on day 0. Surgical removal of the primary tumor was done on day 10. Animals were daily treated *p.o.* from day 1 through day 9 (Schedule I). For mean weight and mean diameter of normal, uninoculated hind paws of C57Bl/6 mice refer to Materials and Methods. *, p≤0.01; **, p≤0.001.

mors excised, and a single cell suspension was prepared by a combined mechanical and enzymatic technique according to Treves *et al.* (20). Briefly, after removal of necrotized parts, tumor fragments were minced with scissors into small pieces which were then suspended in 50 mL cold PBS under continuous slight stirring for 15 min at room temperature. Subsequently the supernatant was sucked off and the sediment trypsinized (15 min, 37°C) with 50 mL of a 0.25% solution of trypsin (Boehringer Mannheim GmbH). After spontaneous sedimentation, the supernatant was discarded and the remaining tissue fragments trypsinized again (30 min, 37°C). Trypsinization was stopped by adding 10 mL fetal calf serum (FCS). The cell suspension was filtered through a stainless steel sieve, underlayered with 5 mL FCS and then centrifuged for 10 min at 1,500 rpm. The cell pellet was washed twice with ice-cold PBS. Cell viability was determined by the trypan blue dye exclusion test and was usually greater than 90%.

Trypan blue dye exclusion test. The trypan blue dye exclusion test for checking cell viability was performed according to the method of Hudson and Hay (21).

Tumor transplantation and surgical removal. In order to study the effect of test and standard compounds, washed, viable ^{3}Lewis-lung carcinoma cells (1 × 10^6 cells/mouse) were injected into the right hind foot pad of female C57Bl/6 mice in a volume of 0.05 mL PBS. Primary tumor growth in controls and in drug-treated mice was determined by caliper measurements of the diameters of the tumor-bearing hind legs. Within 8 to 10 days the inoculated foot usually reached a diameter of 0.5–0.6 cm. In some experiments, tumors were then surgically removed by amputating the primary tumor-bearing hind leg just above the knee joint under phenobarbital anaesthesia (22). The weights of amputated, primary tumor-bearing hind-feet were determined. The differences in weight and diameter, respectively, in comparison to feet of non-tumor-bearing C57Bl/6 mice represent a measurement for primary tumor growth (mean weight and mean diameter ± SD of normal, uninoculated hind paws of C57Bl/6 mice: 134 ± 0.5 mg, 2.1 ± 0.1 mm, n = 10).

Drug treatment schedules. In a first series of experiments, animals were daily treated orally with Ilmofosine, cyclophosphamide or with the drug-free vehicle (PBS), respectively, from day 1 through day 9 after tumor cell inoculation, *i.e.,* treatment was stopped 1 day prior to amputation of the tumor-bearing hind leg on day 10 (Schedule I). In a second set of experiments, treatment started on day 11, *i.e.,* 1 day after amputation, through day 28 after tumor cell inoculation (Schedule II). Ilmofosine and cyclophosphamide were administered by gavage in 0.2 mL PBS. The *in vivo* experiments were usually terminated on day 55 or day 60. Animals treated according to Schedule I were randomly assigned to treatment and control groups immediately after tumor cell inoculation. Mice treated according to Schedule II were randomized 1 day after amputation prior to start of the treatment.

Examination of pulmonary metastases. All animals were examined for pulmonary metastases by injecting diluted India ink into the trachea before fixation of the whole lung, as described in detail by Wexler (23). Metastases appeared as white nodules against black-stained normal lungs.

Statistics. Statistical comparisons of *in vivo* results *vs.* the appropriate negative (PBS) or positive (cyclophosphamide) control groups were performed according to the Mann-Whitney test by using the RS/1 program (BBN Software Products Corp., Cambridge, MA) and a Hewlett-Packard/Vectra ES computer (Microsoft GmbH, Aschheim/Dornach, Germany).

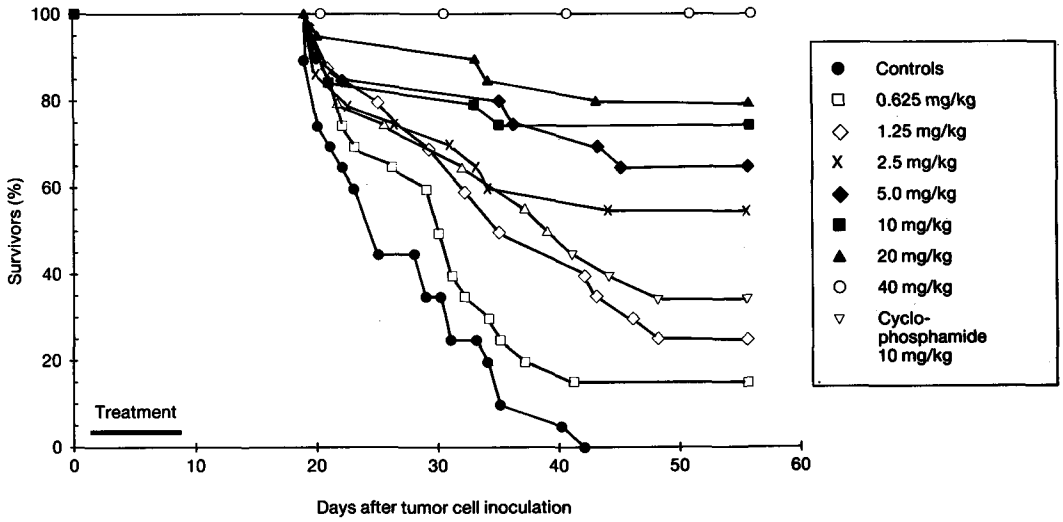


FIG. 3. Influence of Ilmofosine on survival time of ^{3}Lewis-lung carcinoma-bearing mice. Same experiment as shown in Figure 2.

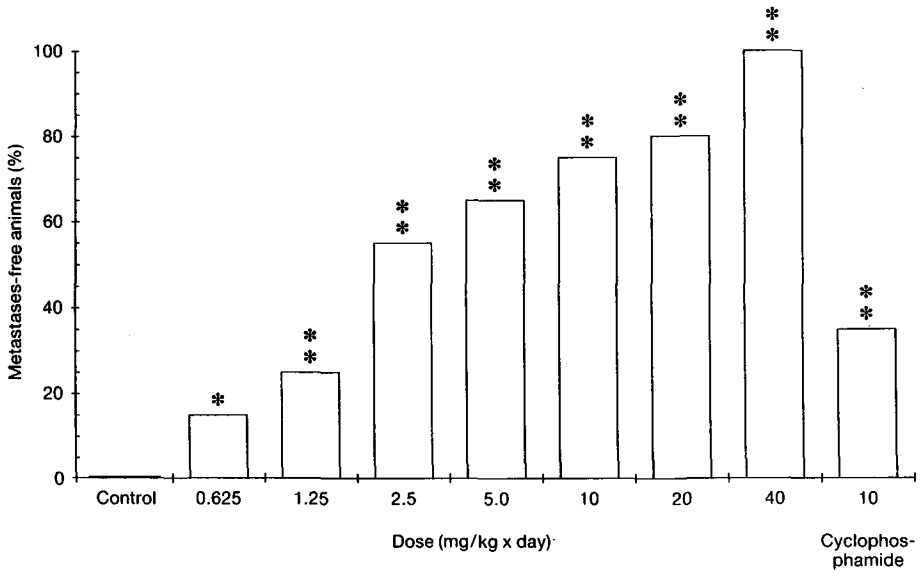


FIG. 4. Influence of Ilmofosine on the number of metastases-free animals. Same experiment as shown in Figure 2. *, $p \leq 0.01$; **$p \leq 0.001$.

RESULTS

The first series of experiments were aimed at testing the influence of Ilmofosine on the growth and metastatic spread of the primary tumor in the ^{3}Lewis-lung model. Groups of 20 mice were inoculated *s.c.* with 1×10^6 ^{3}Lewis-lung tumor cells into the foot pad of the right hind leg. Animals were then treated with doses ranging from 0.625 to 40 mg/kg daily *p.o.* from day 1 through day 9 after tumor cell inoculation. Corresponding control mice were either treated with PBS or cyclophosphamide (10 mg/kg × day). Figures 2–4 show the dose dependent effect on several tumor parameters from a characteristic experiment. These parameters included: i) primary tumor growth, expressed as reduction in weight and thickness of inoculated hind paws (Fig. 2); ii) survival time (Fig. 3); and iii) cumulative numbers of metastases-free animals (Fig. 4).

From this type of experiment (Figs. 2–4) it can be seen that the therapeutic effect of Ilmofosine is clearly dose-related. Even doses as low as 1.25 and 2.5 mg/kg resulted in a significant inhibition of primary tumor growth ($p \leq 0.01$ and $p \leq 0.001$, respectively; Fig. 2). Cyclophosphamide (10 mg/kg × day) also inhibited primary tumor growth, but was always less effective as compared to 5 mg/kg Ilmofosine. This dose of cyclophosphamide was selected based on results of preceding dose response experiments, and proved always to induce survival of 30–40% of the animals under the experimental conditions used (Fig. 3).

In accordance with its inhibitory effect on primary tumor growth, Ilmofosine dose-dependently increased

TABLE 1

Dose-Dependent Effect of Ilmofosine on Mean Survival Time in the ^{3}Lewis-Lung Carcinoma Model

Compound	Dose (mg/kg × day)	MST (Days) [a] Schedule I [b] (Days 1–9)	MST (Days) [a] Schedule II [c] (Days 11–28)
Control (PBS)	—	24	24
Cyclophosphamide	10	39	34
Ilmofosine	0.625	30	27
	1.25	35	36
	2.5	> 55	38
	5.0	> 55	> 60
	10	> 55	> 60
	20	> 55	> 60
	40	> 55	> 60

[a]MST, mean survival time, as calculated from Figures 3 and 5.

[b]Schedule I, daily treatment *p.o.*, days 1–9 after tumor cell inoculation.

[c]Schedule II, daily treatment *p.o.*, days 11–28 after tumor cell inoculation.

survival time in the same experiment (Fig. 3). Control mice began to die from metastases 18–19 days after tumor cell inoculation; 50% of the animals died within 24–26 days, and none of the control animals was alive after 40–42 days. Even the lowest doses of Ilmofosine tested were able to increase mean survival time (Table 1). Cyclophosphamide (10 mg/kg × day) also increased mean survival time up to 39 days as compared to sham-created controls (24 days), but was always less effective compared to 2.5 mg/kg Ilmofosine (Table 1). All 20 animals which were treated daily with 40 mg/kg *p.o.* from day 1 through day 9 after tumor cell inoculation survived the whole observation period up to 55 days. Furthermore, the cumulative numbers of metastases-free animals increased dose-dependently in the same experiment (Fig. 4).

The next set of experiments was aimed at evaluating the therapeutic efficacy of Ilmofosine in this model when treatment started 1 day after removal of the primary tumor by amputation of the tumor-bearing hind leg. The ^{3}Lewis-lung tumors were surgically removed after having reached a diameter of about 0.5–0.6 cm, *i.e.*, on day 10 after tumor cell inoculation. After randomization the animals were then treated with doses of Ilmofosine ranging from 0.625 to 40 mg/kg daily *p.o.* from day 11 through day 28. Once again, cyclophosphamide served as positive control. Figures 5 and 6 show the dose-dependent influence of Ilmofosine on survival time and on the number of metastases-free animals from the same characteristic experiment, respectively.

As can be seen from Figures 5 and 6, as well as Table 1, Ilmofosine again caused a dose-dependent increase of survival time and in the number of metastases-free animals over the total observation period of 60 days as compared to sham-treated and positive (cyclophosphamide) controls.

In all *in vivo* experiments described here Ilmofosine was well tolerated. No noticeable toxic effects and no deaths from toxicity were observed.

DISCUSSION

1-*O*-Alkyl and 1-*S*-alkyl lysophospholipids are structurally related to the membrane component 2-lysophosphatidylcholine as well as to platelet-activating factor (PAF), a naturally occurring lipid mediator, which appears to be involved in the mediation of acute inflammatory and allergic reactions (24,25). Some of these synthetic ether phospholipids previously have been shown to possess antitumor activity *in vitro* (9–12) and *in vivo* (9,14,26). The explanations for these antineoplastic effects include: i) direct cytotoxicity (9,12); ii) generation of tumoricidal immunecompetent cells such as macrophages (9,14); iii) anti-invasiveness (27) and anti-metastatic properties (4,9,14); and iv) induction of differentiation (28,29).

One of these compounds, the thioether lipid Ilmofosine (BM 41.440), has recently been tested in short- and long-term clinical phase I tolerability studies in refractory cancer patients (5,6). Ilmofosine exerts antitumor activity in several *in vivo* tumor systems, including the methylcholanthrene-induced fibrosarcoma (MethA), the L 1210 leukemia, the ^{3}Lewis-lung carcinoma (4) and the methylnitroso-urea (MNU)-induced mammary carcinoma (30). Compared to 1-*O*-alkyl lysophospholipids, thioether-type compounds such as Ilmofosine have certain advantages—for instance, lack of platelet aggregating properties (31,32), a higher therapeutic index *in vitro* (13), and pharmacokinetic detectability at clinically relevant concentrations (5,33).

The question, whether or not the known antitumor effect of Ilmofosine *in vivo* may be attributable to direct cytotoxicity and/or be mediated by the activation of immune-competent cells, such as macrophages or natural killer cells, prompted us to extend our former investigations (4,34) on the activity of this compound in the ^{3}Lewis-lung carcinoma model *in vivo*.

The *in vivo* data presented here indicate that the thioether phospholipid Ilmofosine shows an almost linear and reproducible, dose-dependent dose response relationship in the^{3}Lewis-lung carcinoma model. This is true for all tumor parameters monitored, *i.e.*, primary tumor volume, expressed in terms of weight and thickness of tumor-bearing paws, mean survival time, and numbers of metastases-free animals. Oral administration of Ilmofosine immediately after tumor cell inoculation as well as immediately after amputation of the primary tumor proved to be effective at clinically relevant doses (5,6). At the time of surgical removal of the primary tumor-bearing hind leg, *i.e.*, 10 days after tumor cell inoculation, the metastatic spread in the ^{3}Lewis-lung system has already occurred and tumor cells can be found in the lungs (35; also D.B.J. Herrmann, unpublished results). Thus, Ilmofosine exerts a direct inhibitory effect on the local metastatic growth in the lungs, resulting in a dose-dependent increase in the number of metastases-free animals and, subsequently, in a dose-dependent increase in mean survival time.

Stimulation of cellular or humoral host immune mechanisms frequently show a "bell-shaped" dose-response curve with a decrease of activity at higher dose levels. Therefore, the *in vivo* data shown here make it very unlikely that the postulated immune-modulating mechanisms of ALP (3,9,14) significantly contribute to the

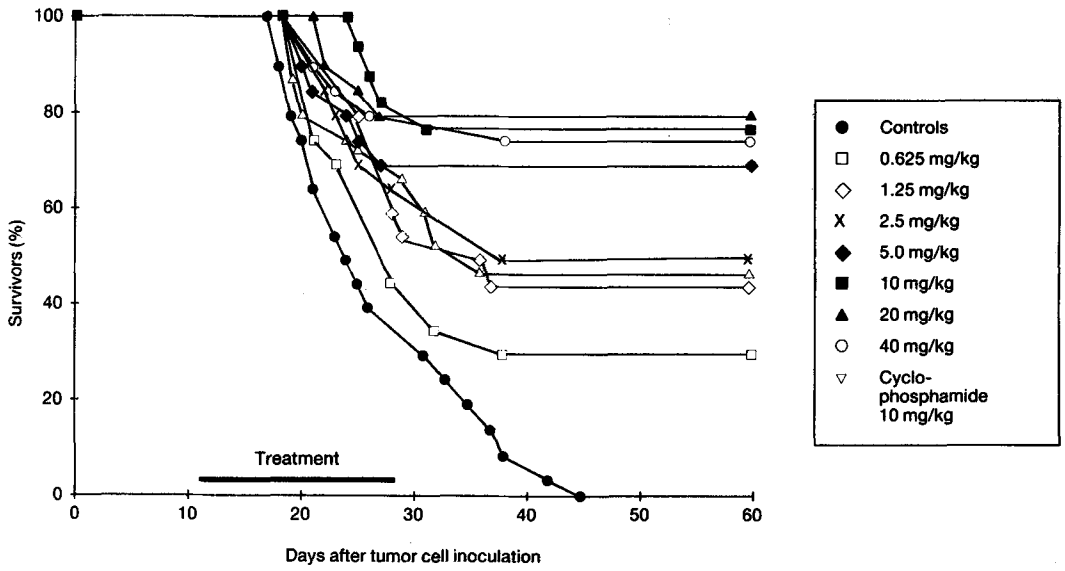


FIG. 5. Influence of Ilmofosine on survival time of ^{3}Lewis-lung carcinoma-bearing mice under an adjuvant treatment schedule. Female C57Bl/6 mice, n = 20, were inoculated with 1×10^6 tumor cells/mouse on day 0. Surgical removal of the primary tumor was done on day 10. Animals were daily treated *p.o.* from day 11 through day 28 (Schedule II).

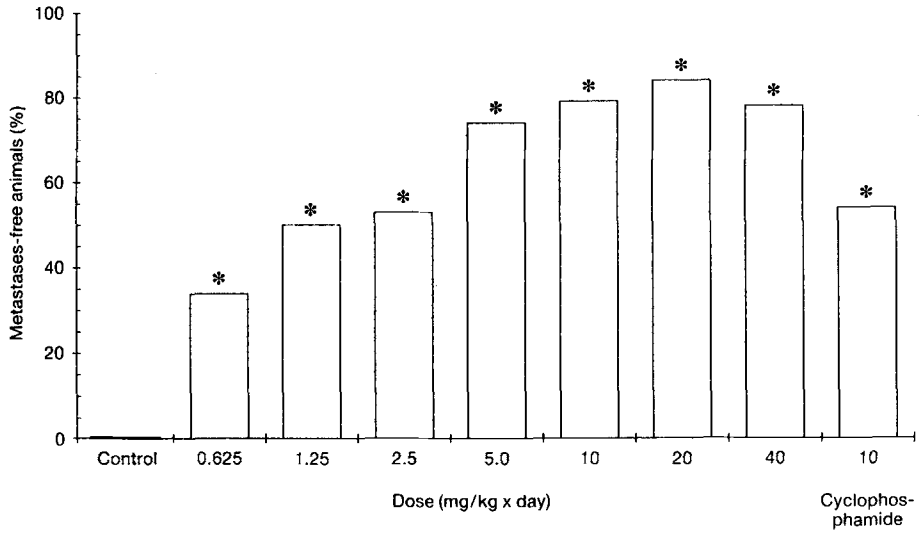


FIG. 6. Influence of Ilmofosine on the number of metastases-free animals. Same experiment as shown in Figure 5. *p≤0.001.

antitumor activity of Ilmofosine, at least in this *in vivo* tumor system.

Our results are well in accordance with the dose-dependent anti-tumor activity of Ilmofosine in the methylnitroso-urea (MNU)-induced mammary carcinoma model (30), and with our recent findings in a methylcholanthrene (MethA)-induced fibrosarcoma model (36). Studies on the antitumor activity of ET – 18 – OCH_3, a structurally related ALP, also revealed dose-related effects in different *in vivo* models, including MethA sarcoma (37), MNU-induced mammary carcinoma (30,38), myeloma MPC-11 (3,9), and L 1210 leukemia (39). Moreover, after a comprehensive evaluation, Talmadge *et al.* (40) did not find convincing evidence for immunomodulatory properties of ALP in mice.

Among all the animal models for testing new anticancer drugs *in vivo*, the ^{3}Lewis-lung carcinoma system is of particular relevance because some of its biological features are very similar to human solid tumors. The ^{3}Lewis-lung carcinoma that arose spontaneously in 1951 in a C57Bl/6 mouse (41) is highly malignant and metastasizes regularly to the lungs (41–43), probably due to its lack of immunogenicity in syngeneic recipients (44,45). Furthermore, this tumor is, as are human solid cancers, characterized by a primary resistance to a majority of chemotherapeutics (46-50).

Ilmofosine was very well tolerated in all *in vivo* experiments reported here. This confirms our earlier observations made during preclinical toxicology studies where a high LD_{50} value of 615 mg/kg was determined for oral

administration of the drug to mice (4). Good tolerance also was seen in the first clinical short- and long-term tolerability studies in cancer patients (5,6). No systemic toxicities were observed (5, 6). Therefore, Ilmofosine appears to have a broad therapeutic range compared to conventional cytostatics.

In conclusion, the data presented here strongly suggest that the therapeutic activity of Ilmofosine *in vivo* relates to its direct cytostatic/cytotoxic effect against tumor cells rather than to immunomodulatory properties as reported for ALP, mainly based on *in vitro* studies. If our observations can be extrapolated to humans, the present results will have a major impact on the designs of further clinical phase II studies. Low toxicity, lack of mutagenicity (4), together with its dose-dependent antitumor and antimetastatic activity, observed in this study under a post-surgical, oral treatment schedule make Ilmofosine an interesting candidate for adjuvant cancer chemotherapy.

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1-β-D-Arabinofuranosylcytosine Conjugates of Ether and Thioether Phospholipids. A New Class of Ara-C Prodrug with Improved Antitumor Activity[1]

Chung Il Hong[a,*], Charles R. West[a], Ralph J. Bernacki[b], Cameron K. Tebbi[c] and Wolfgang E. Berdel[d]

Departments of [a]Neurosurgery, [b]Experimental Therapeutics and [c]Pediatrics, Roswell Park Cancer Institute, Buffalo, New York 14263, and [d]Department of Hematology and Oncology, Free University of Berlin, Berlin 45, Germany

The 1-β-D-arabinofuranosylcytosine (ara-C) conjugates 1-*O*-alkyl (ether) and 1-*S*-alkyl (thioether) phospholipids, being analogues of ara-CDP-*sn*-1,2-*O*-dipalmitoylglycerol (1), showed significant antitumor activity against L1210 and P388 leukemia *in vivo*. The more active conjugates include the 1-*O*-alkyl analogues, ara-CDP-*rac*-1-*O*-hexadecyl-2-*O*-palmitoylglycerol (2) and ara-CDP-*rac*-1-*O*-octadecyl-2-*O*-palmitoylglycerol (3), and the corresponding 1-*S*-alkyl analogues, ara-CDP-*rac*-1-*S*-hexadecyl-2-*O*-palmitoyl-1-thioglycerol (4) and ara-CDP-*rac*-1-*S*-octadecyl-2-*O*-palmitoyl-1-thioglycerol (5, Cytoros). The conjugates were formulated by sonication, in which the conjugates existed as discs (size 0.01–0.04 μm). Among the conjugates of the three different phospholipids, the 1-*S*-alkyl analogues 4 and 5 displayed the strongest antitumor activity against L1210 leukemia in mice, followed by the 1-*O*-alkyl (2 and 3) and the 1-*O*-acyl (1) analogues. The 1-*S*-alkyl analogue 5 was considerably more effective than the 1-*O*-acyl analogue 1 against myelomonocytic WEHI-3B leukemia in mice. Conjugate 5 (Cytoros) showed a significant therapeutic activity in mice with colon 26 carcinoma, M5076 sarcoma, and C-1300 neuroblastoma. Furthermore, this agent inhibited liver metastases of M5076 sarcoma. Conjugates 3 and 5 also inhibited the metastasis of 3-Lewis lung carcinoma to the lungs of mice. Cytoros (5) and its analogues, with other ether and thioether phospholipids, appear to offer increased therapeutic benefit to mice with tumors.

***Lipids* 26, 1437–1444 (1991).**

Favorable activity of 1-β-D-arabinofuranosylcytosine (ara-C) conjugates of nontoxic 1-*O*-acylphospholipids such as ara-CDP-L-DP (1) (1–3) and antineoplastic properties of certain ether and thioether phospholipids such as ET-18-OCH_3 (4) and BM 41.400 (5) led us to synthesize a series of ara-C conjugates of 1-*O*-alkyl (ether) and 1-*S*-alkyl (thioether) phospholipids (6–8). The rationale is that the conjugates are not only new prodrugs of ara-C, but also may generate two cytotoxic groups with different target sites in neoplastic cells. Most of them displayed significant antitumor activity against L1210 and P388 leukemia *in vivo* (6,8). The conjugates of ether and thioether phospholipids significantly inhibited 3-Lewis lung carcinoma (3-LL) growth and also increased survival of mice bearing these tumor cells (9,10).

This paper reports on the therapeutic effects of the selected ara-C conjugates from the three different phospholipids (Fig. 1), and also describes further effects of the more active conjugates on various animal solid tumor models.

MATERIALS AND METHODS

Drugs. The chemical structures of the selected conjugates are depicted in Figure 1. The conjugates include: i) the 1-*O*-acyl analogue, ara-CDP-L-DP (1); ii) the 1-*O*-alkyl analogues, ara-CDP-DL-PCA (2) and ara-CDP-DL-PBA (3); and iii) the 1-*S*-alkyl analogues, ara-CDP-DL-PTCA (4) and ara-CDP-DL-PTBA (Cytoros, 5). Details on synthesis (2,6, 11,12) and chemical properties (2,6,11–13), and preliminary antitumor activity data (1–3,6–14) have been published. The conjugates were synthesized in 30% yield by condensation of ara-CMP morpholidate with the phosphatidic acids in anhydrous pyridine (6,12). The conjugate was formulated by sonication in sterile water followed by filtration through a 0.22-μm membrane filter into a sterile receiving container using a laminar flow cabinet (13).

Animals and tumors. DBA/2J mice (male), weighing 23–30 g, in groups of five or six were inoculated *i.p.* with 1×10^6 L1210 lymphoid leukemia cells (or *i.c.* with 1×10^5 L1210 cells), and each drug was given *i.p.* once daily as a single or multiple injections, starting at day 1 as outlined previously (8,15). Survival time and animal weights were recorded and the %ILS (% increase in life span) was compared with the median survival of the untreated mice. Statistical significance was determined by using the Cox-Mantel test. The data calculations were performed using software prepared previously (16).

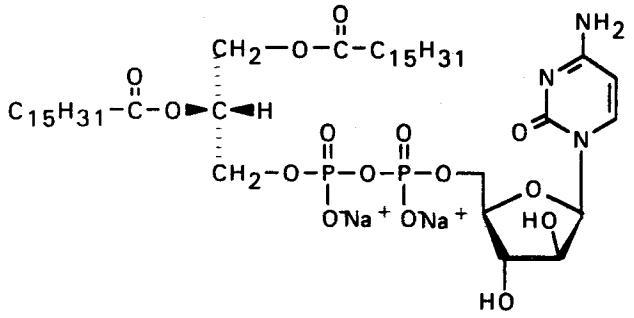
For antitumor studies with animal solid tumor models, such as colon 26 carcinoma and M5076 carcinoma, the methodology was described previously (17). A 50-mg piece of the tumors was transplanted *s.c.* by trochar implant into the abdominal flank of female BALB/C or C57BL/6 mice (wt 20–25 g). Drugs were administered *i.p.* or *i.v.* on day 7, or on days 7 and 14. Tumor growth, animal weight, and survival were checked using procedures described previously (16,17). Data were analyzed, where required, by analysis of variance (ANOVA) and by the Mann-Whitney

[1]Based on a paper presented at the Third International Conference on Platelet-Activating Factor and Structurally Related Alkyl Ether Lipids, Toyko, Japan, May 1989.

*To whom correspondence should be addressed at Department of Neurosurgery, Roswell Park Cancer Institute, Elm and Carlton Streets, Buffalo, NY 14263.

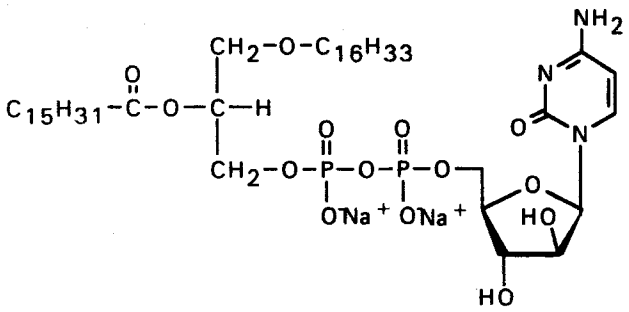
Abbreviations: ANOVA, analysis of variance; ara-C, 1-β-D-arabinofuranosylcytosine; ara-CDP-DL-PBA, ara-CDP-*rac*-1-*O*-octadecyl-2-*O*-palmitoylglycerol; ara-CDP-DL-PCA, ara-CDP-*rac*-1-*O*-hexadecyl-2-*O*-palmitoylglycerol; ara-CDP-DL-PTBA (Cytoros), ara-CDP-*rac*-1-*S*-octadecyl-2-*O*-palmitoyl-1-thioglycerol; ara-CDP-DL-PTCA, ara-CDP-*rac*-1-*S*-hexadecyl-2-*O*-palmitoyl-1-thioglycerol; ara-CDP-L-DP, ara-CDP-1-β-D-arabinofuranosylcytosine 5′-diphosphate-*sn*-1,2-*O*-dipalmitoylglycerol; ara-CMP morpholidate, 1-β-D-arabinofuranosylcytosine 5′-monophosphoromorpholidate; BM 41.440, 1-hexadecylmercapto-2-methoxymethyl-propyl-3-phosphocholine; CDP-diacylglycerol, cytidine 5′-diphosphate-*sn*-1,2-*O*-diacylglycerol; ET-18-OCH_3, 1-*O*-octadecyl-2-*O*-methyl-*rac*-glycero-3-phosphocholine; i.c., intracerebral; i.p., intraperitoneal; %ILS;; % increase in life span; 3-LL, 3-Lewis lung carcinoma; s.c., subcutaneous.

Diacyl

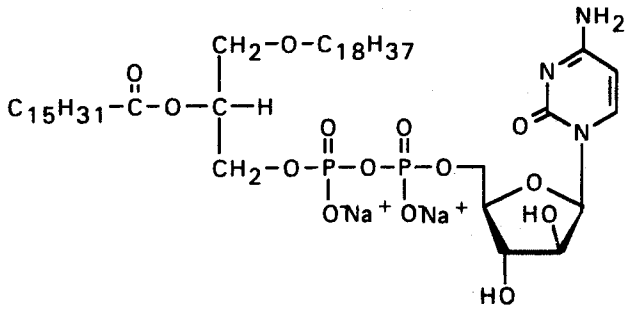


1, ara-CDP-L-DP

Ether

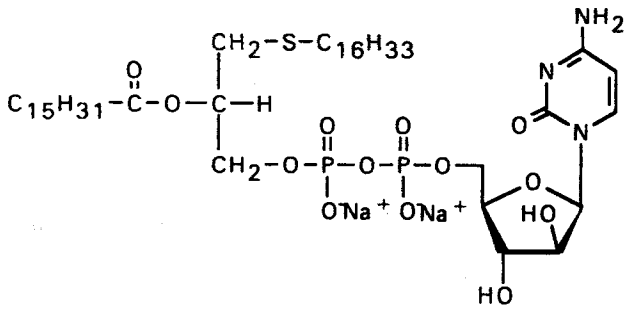


2, ara-CDP-DL-PCA

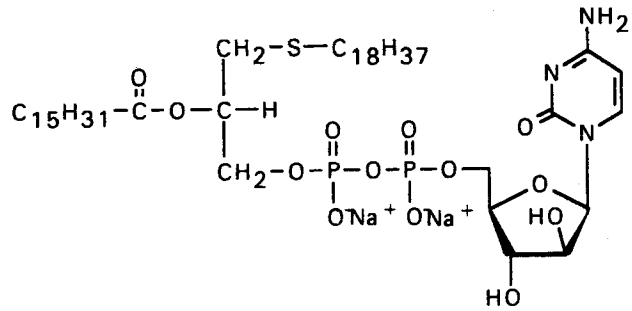


3, ara-CDP-DL-PBA

Thioether



4, ara-CDP-DL-PTCA



5, ara-CDP-DL-PTBA
(Cytoros)

FIG. 1. Chemical structures of ara-C conjugates of various phospholipids.

U test. The 0.05 level of probability was taken as significant.

The antitumor study with C-1300 neuroblastoma was carried out following the published method (18). A/Strong Ros mice (wt 20–25 g) were inoculated *s.c.* in the scruff of the neck with 2×10^6 C-1300 neuroblastoma cells on day 0, and drug was injected *i.p.* once every 10 days, a total of 6 times, starting on day 3. Tumor growth, animal weight and survival were checked as outlined previously (18).

For metastases experiments of 3-LL, the viable cells

(1×10^6) in 0.05 mL PBS were injected *s.c.* into the left hind foot pad of C57BL/6 mice (wt 20–25 g). Within 7–10 days, the injected foot bore a tumor with a diameter of 0.6 cm. The tumors were then surgically removed under pentobarbital anesthesia. Drugs were given *i.p.* after randomization on days 1–5 after the operation. The end-point of the experiments was either death from metastasis or survival (10).

RESULTS

Drug formulation. The conjugates in sonicated solution existed as discs with diameters varying from 0.01 μm to 0.04 μm as judged by electron microscopy after negative-staining or freeze-fracture (13,19). The formulation, when stored at 3–4°C, was chemically stable for more than six months.

L1210 lymphoid leukemia. Table 1 summarizes the effects of the ara-C conjugates of 1-*O*-acyl, 1-*O*-alkyl (ether) and 1-*S*-alkyl (thioether) phospholipids against *i.p.* implanted L1210 lymphoid leukemia in DBA/2J mice according to the procedure outlined in the NCI protocols (15), with some modifications (8). Both the optimal single doses (300–400 mg/kg) and the five consecutive doses (40–80 mg/kg/day × 5) produced significant antitumor activity with increases in life span by 215–371% as compared with survival of the control group ($p < 0.01$). Ara-CDP-L-DP (1), ara-CDP-DL-PTCA (4) and ara-CDP-DL-PTBA (Cytoros, 5) gave one out of six long-term suvivors >45 days. In Figure 2, the antileukemic effectiveness of ara-CDP-L-DP, ara-CDP-DL-PBA, and ara-CDP-DL-PTBA are compared. The results indicate that the conjugate of thioether phospholipid, ara-CDP-DL-PTBA (Cytoros), showed stronger therapeutic efficacy than did the others. In fact, 26 out of 90 animals treated with a single dose of ara-CDP-DL-PTBA (400 mg/kg) survived more than 45 days (Fig. 3). Furthermore, it appeared that toxicity, as reflected in weight loss, was low (Fig. 3).

Table 2 summarizes the effects of ara-CDP-L-DP, ara-CDP-DL-PCA, and ara-CDP-DL-PTBA against *i.c.* implanted L1210 lymphoid leukemia in DBA/2J mice. The optimal single doses (300–500 mg/kg) produced significant antitumor activity with ILS of 200–229%. Multiple courses of drug therapy resulted in no gain in animal life span. However, five consecutive doses (60 mg/kg/day × 5) of ara-CDP-DL-PTBA produced two long-term survivors out of six animals.

Colon 26 carcinoma. Administration (*i.p.*) of a single dose of ara-CDP-DL-PTBA on day 7 to BALB/C female mice, implanted *s.c.* in the abdominal flank with colon 26 carcinoma on day 0 resulted in significant (>100% ILS) increases in life span and decreases in tumor size (Table 3). Median life span more than doubled (day 15 to 33) following the *i.p.* administration of 200–600 mg/kg of ara-CDP-DL-PTBA. Additionally, tumor growth was significantly retarded in the drug-treated animals with half maximal growth tumor size occurring more than three days later (16.5 to 20 days or more). Table 4 shows the effects of *i.v.* administration of ara-CDP-DL-PTBA on day 7, or on days 7 and 14, to the tumor bearing mice. The *i.v.* drug therapy also resulted in tumor size reduction, but little increase in animal lifespan. Treatment with a single dose (300 mg/kg) resulted in some reduction in tumor sizes (84% control tumor size at day 21), but with minimal effect on animal lifespan (ILS 21%). Multiple courses of drug therapy (300 mg/kg/day, days 7 and 21) resulted in improved tumor size reduction (58% control tumor size at day 21) and gain in animal life-span (ILS 84%). Two toxic deaths at day 7 and the loss in weight may be attributable to greater drug toxicity by *i.v.* administration *vs. i.p.*

M5076 sarcoma. Table 5 shows the effects of *i.p.* administration of ara-CDP-DL-PTBA on day 7 to C57BL/6

TABLE 1

Antitumor Activity Against *i.p.* Implanted L1210 Lymphoid Leukemia in DBA/2J Mice[a]

Compound	Treatment schedule (qd)	Optimal dose[b] mg(μmole)/kg/day	Survival days[c] Median (T/C)	% ILS[d]	45-Day survivors
ara-C	1	400(1544)	8.0/7.0	14	0/6
	1-5	200(822)	16.0/7.0	129	0/6
ara-CDP-L-DP	1	300(293)	20.5/6.5	215	1/6
	1-5	40(39)	30.0/8.0	275	1/6
ara-CDP-DL-PCA	1	400(407)	27.5/7.0	293	0/6
	1-5	80(81)	25.5/7.0	264	0/6
ara-CDP-DL-PBA	1	400(392)	25.0/7.0	257	0/6
	1-5	100(98)	23.0/7.0	264	0/6
ara-CDP-DL-PTCA	1	400(400)	27.5/7.0	293	1/6
	1-5	80(80)	33.5/7.0	379	1/6
ara-CDP-DL-PTBA (Cytoros)	1	400(389)	28.5/7.0	307	1/6
	1-5	80(79)	33.0/7.0	371	1/6

[a] DBA/2J mice (23–38 g) were implanted *i.p.* with 1×10^6 L1210 leukemia cells on day 0. Treatments (*i.p.*) were initiated 24 hr after tumor inoculation. Animal survival was checked daily until death or 45 days.

[b] Dose producing greatest increase in life span.

[c] Statistical significance was determined by using the Cox-Mantel test, and they are all statistically significant with $p < 0.01$.

[d] Calculated based on survivors according to the NCI protocols (15).

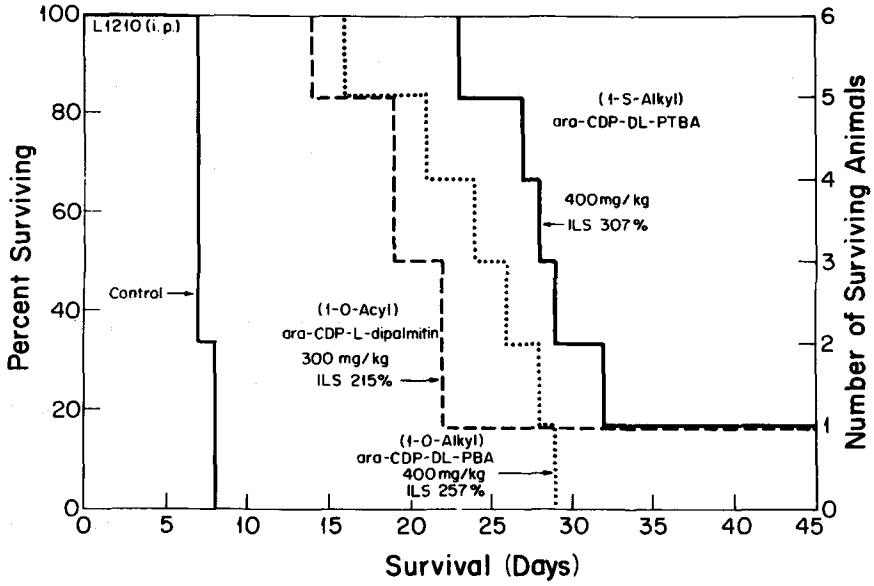


FIG. 2. Effects of changes in *sn*-1 substituent of the conjugate on antitumor activity against *i.p.* implanted L1210 lymphoid leukemia in DBA/2J mice. Experimental details are the same as described in Table 1.

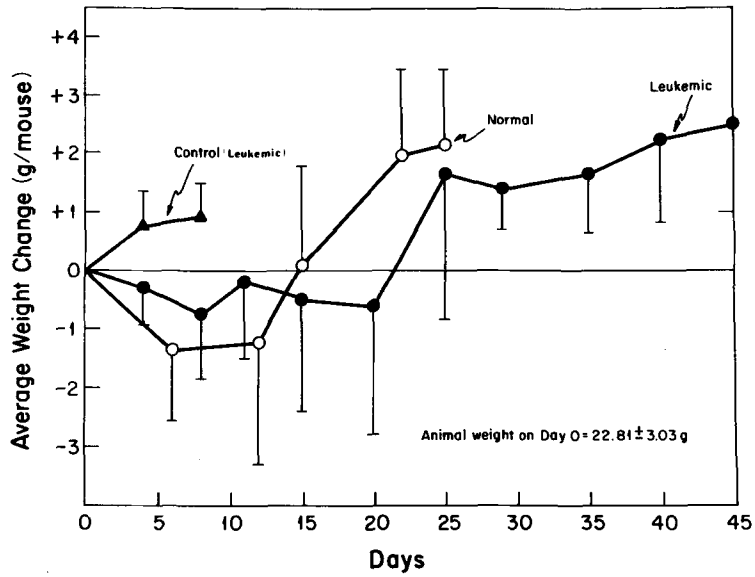


FIG. 3. Average weight changes of normal and *i.p.* inoculated L1210 leukemic DBA/2J mice during *i.p.* treatment with ara-CDP-DL-PTBA (qd 1, 400 mg/kg). Leukemic DBA/2J male mice (av. wt 22.81 ± 3.03 g, total 90 animals) and non-leukemic normal mice (total 5) received drug on day 1. Control leukemic mice (total 54) received saline. All of the normal mice survived more than 45 days. Out of 90 treated leukemic mice, 26 survived more than 45 days.

mice implanted *s.c.* with M5076 sarcoma. A single dose of area-CDP-DL-PTBA (200 or 400 mg/kg) resulted in negligible increases in life span of the tumor bearing mice. The higher dose (800 mg/kg) was severely toxic and caused early death. However, a significant therapeutic effect of drug administration was noted on tumor size. There was also a corresponding decrease in liver metastases observed on day 33, as noted by a decrease in the number of macroscopic tumor foci in liver from animals administered 400 mg/kg drug.

C-1300 neuroblastoma. Administration (*i.p.*) of multiple courses of drug therapy (250 mg/kg/10 days × 6), starting on day 3 to A/Strong Ros male mice, implanted *s.c.* in the scruff of the neck with C-1300 neuroblastoma on day 0, resulted in tumor-free treated animals at least for four weeks as compared to the control ($p < 0.001$) (Fig. 4).

TABLE 2

Antitumor Activity Against *i.c.* Implanted L1210 Lymphoid Leukemia in DBA/2J Mice[a]

Compound	Treatment schedule (qd)	Optimal dose[b] mg(μmole)/kg/day	Survival days[c] Median (T/C)	% ILS[d]	45-Day survivors
ara-C	1-5	300(1233)	16.0/7.0	129	0/6
	1-9	100(411)	14.0/7.0	100	0/6
ara-CDP-L-DP	1	300(293)	21.5/7.0	200	0/6
	1,5,9	133(134)	20.0/8.0	150	0/6
	1-5	80(80)	22.0/8.0	175	0/5
	1-9	50(50)	16.0/7.0	147	0/5
ara-CDP-DL-PCA	1	500(508)	23.0/7.0	229	0/6
	1,5,9	167(162)	21.0/7.0	200	0/5
	1-5	100(102)	22.0/7.0	214	0/6
ara-CDP-DL-PTBA	1	400(389)	24.0/7.5	220	0/5
	1,5,9	167(162)	22.0/7.5	193	0/5
(Cytoros)	1-5	60(58)	21.5/7.5	187	0/6
	1-9	50(49)	19.5/7.0	179	0/6

[a]DBA/2J mice (23-30 g) were implanted *i.c.* with 1×10^5 L1210 leukemia cells on day 0. Treatments (*i.p.*) were initiated 24 hr after tumor inoculation. Animal survival was checked daily until death or 45 days.

[b]Dose producing greatest increase in life span.

[c]Statistical significance was determined by using the Cox-Mantel test, and they are all statistically significant with $p<0.01$.

[d]Calculated based on survivors according to the NCI protocols (15).

TABLE 3

Effect of *i.p.* ara-CDP-DL-PTBA (Cytoros) in BALB/C Mice with *s.c.* Colon 26 Carcinoma[a]

Dosage (mg/kg)	Survival parameters Range (days)	Median (days)	Mean ± SE (days)	% ILS[b]	Half-maximal tumor size[c] (days)
Control	12-32	15	19.8 ± 2.4	0	16.5
200	15-33	33	28.6 ± 3.5	120	20[d]
400	28-41	33	33.4 ± 2.1[d]	120	20[d]
600	13-45	34	30.2 ± 6.9	126	23[d]

[a]BALB/C female mice (20-25 g, 10 controls or 5 drug treated mice per dosage group) were implanted *s.c.* by trochar with a 50-mg piece of tumor on day 0. On day 7 drug was administered *i.p.* Tumor growth was monitored twice per week by caliper measurements of two perpendicular diameters and tumor mass was estimated algebraically (17). Mortality was checked daily for 60-65 days.

[b]%ILS are based on median survival times.

[c]Time to reach half maximal size achieved in the control group.

[d]$p<0.05$.

TABLE 4

Effect of *i.v.* ara-CDP-DL-PTBA (Cytoros) in BALB/C Mice with *s.c.* Colon 26 Carcinoma[a]

Dosage (mg/kg)	Treatment schedule	Survival data (days) Range	Median	Mean	%ILS[b]	Mean tumor size (g) Day 14	Day 21
Control	—	14-30	19	21	0	1.2	1.9
300	day 7	20-49	23[c]	30	21	0.6	1.6
300	days 7, 14	7-35	35[d]	30[d]	84	0.6	1.1

[a]On day 7 or 14 drug was administered *i.v.* Other experimental details are the same as described in Table 3.

[b]%ILS are based on median survival times.

[c]$p<0.05$.

[d]Excluding 2 toxic deaths at day 7.

TABLE 5

Effect of *i.p.* ara-CDP-DL-PTBA (Cytoros) in C57BL/6 Mice with *s.c.* M5076 Sarcoma[a]

Dosage (mg/kg)	Survival parameters				Half maximal tumor size[c] (days)	No. of metastatic nodules in liver[d] median (range)
	Range (days)	Median (days)	Mean ± SE (days)	%ILS[b]		
Control	37–53	43	43.8 ± 2.2	—	26.5	175 (14–450)
200	33–56	46	45.4 ± 4.0	6	31.5[e]	170 (10–380)
400	39–68	46	48.0 ± 5.2	6	32[e]	0 (0–85)[f]
800	13–60	17	24.2 ± 9.0	ND[g]	>34	ND[g]

[a]Female C57BL/6 mice (20-25 g) were implanted *s.c.* in the abdominal flank with 50 mg piece of tumor with a trochar on day 0. Other experimental procedures were similar to those described in Table 3 except 10 animals were used per group.

[b]%ILS are based on median survival times.

[c]Time to reach maximal tumor size achieved in the control group.

[d]On day 33 five animals from each group were sacrificed and the number of macroscopic tumor nodules in the liver were counted using a dissecting microscope.

[e]$p<0.01$.

[f]$p<0.05$.

[g]ND, not determined.

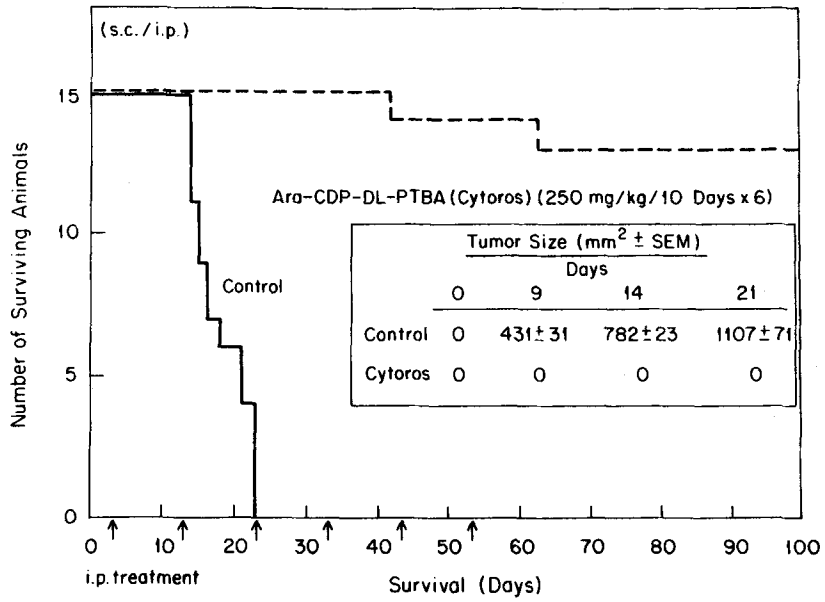


FIG. 4. Therapeutic effects of ara-CDP-DL-PTBA on *s.c.* implanted C-1300 neuroblastoma in A/Strong Ros mice. Mice (20–25 g) were inoculated *s.c.* in the scruff of the neck with 2×10^6 tumor cells on day 0. Drug was injected *i.p.* once every 10 days, a total of 6 times, starting on day 3. Experimental details were as described previously (18).

The survival of this group was significantly greater than the mean of 18 ± 3.7 days for the control group ($p < 0.001$). Weight loss was observed in treated animals, ranging from 10–20% of body weight ($p < 0.001$).

3-LL metastasis. Figure 5 shows the effects of ara-CDP-DL-PBA, ara-CDP-DL-PTBA and equimolar mixtures of ara-C and ET-18-OCH_3 on 3-LL metastasis by giving the drugs as adjuvant chemotherapy shortly after removal of the primary tumor. Administration (*i.p.*) of five consecutive daily doses (80–100 mg/kg/day; days 1–5 postoperatively) of ara-CDP-DL-PBA and ara-CDP-DL-PTBA produced moderate therapeutic effects as measured by an increase in survival time and absolute numbers of surviving animals in comparison to controls. Equimolar

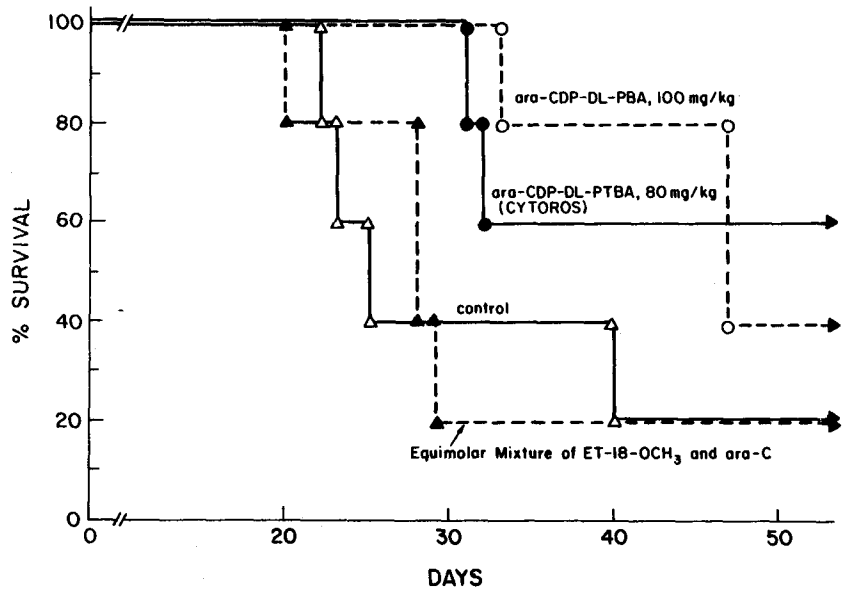


FIG. 5. Therapeutic effects on metastatic 3-LL. C57BL/6 female mice (20–25 g, 5 mice per group) were implanted *s.c.* into the left hind foot pad with 1×10^6 tumor cells on day 0. On days 7–10, the tumors (diameter 0.6 cm) were surgically removed. Drugs were given *i.p.* for five consecutive days shortly after the primary tumor was removed. Equimolar mixture of ara-C and ET-18-OCH_3 was 1:1 mixture of 98.8 mole/kg of each compound, which was the same dose level of ara-CDP-DL-PBA, 100 mg (98.8 μmole/kg. Further details have been outlined previously (10).

mixtures of ara-C and ET-18-OCH_3 were less effective than the conjugates.

DISCUSSION

The conjugates used in this study are amphiphiles (20) that form micelles in water. Upon sonication, the conjugates appear as discs (size 0.01–0.04 μm) when observed by electron microscopy after negative-staining or freeze-fracture (13,19); similar discs are formed by CDP-diacylglycerol (21). The conjugates in micellar form were chemically stable when stored at 3–4°C for a period of more than six months (13). Thus, the conjugates can be formulated safely in micellar solution and stored in a refrigerator.

All of the conjugates shown in Figure 1 displayed a significant therapeutic activity in L1210 leukemic mice. Among them, the thioether phospholipid conjugate, ara-CDP-DL-PTBA (Cytoros), revealed the strongest antitumor activity. Ara-CDP-DL-PTBA also showed considerably higher efficacy than the 1-*O*-acyl phospholipid conjugate, ara-CDP-L-DP, against myelomonocytic WEHI-3B leukemia in mice (22).

Ara-CDP-DL-PTBA also displayed a significant therapeutic activity in mice with various solid tumors such as colon 26 carcinoma, M5076 sarcoma, C-1300 neuroblastoma and 3-LL (5). Multiple doses of ara-CDP-DL-PTBA produced greater therapeutic benefits in mice with colon 26 carcinoma (Table 4) and C-1300 neuroblastoma (Fig. 4). Furthermore, this agent inhibited liver metastases of M5076 sarcoma as noted by the decreased number of macroscopic tumor foci in the treated animals (Table 5). The drug also inhibited the metastases of 3-LL to the lungs of mice as demonstrated by an increase in survival time and in the number of surviving animals (Fig. 5).

The mode of action of the ara-C conjugates remains largely unknown. They may act as lipophilic prodrugs with sustained release of ara-C because of the effectiveness by a single dose treatment (Tables 1 and 2), or they may facilitate transfer of the active moiety across the tumor cell membranes because of their amphiphilic nature. However, there is experimental evidence that the administration of ara-CDP-DL-PTBA leads to a greater intracellular retention of ara-CTP than that resulting from ara-C (23). Some other possible pharmacologically favorable properties of the conjugates include resistance to hydrolysis by cytidine deaminase (1), rapid interaction with serum lipoproteins (24), rapid uptake by cells (25), and effects on lipid biosynthesis (26,27). In addition, the conjugates of 1-*O*-alkyl (ether) and 1-*S*-alkyl (thioether) phospholipids may enhance their therapeutic activity by releasing an additional cytotoxic moiety, namely a 1-*O*-(or-*S*-)alkyl lysophospholipid (28,29). Thus, superior antitumor activity of ara-CDP-DL-PTBA compared to that of ara-CDP-L-DP may be attributed, in part, to the release of two cytotoxic compounds, *viz.* ara-C and 1-*S*-alkyl lysophospholipid. From the present and previous studies (6,8), we can draw the conclusion that the conjugate should resemble the naturally occurring CDP-diacylglycerols which are immediate precursors of phosphatidylinositol (30,31), and that long aliphatic side chains, together with the 1-*O*-alkyl- or 1-*S*-alkyl linkage and long fatty acyl group at *sn*-2 position of glycerol, seem to enhance activity. Further studies are necessary to elucidate the mode of action of these agents.

In summary, the enhanced therapeutic activity of ara-CDP-DL-PTBA (Cytoros) and its 1-*O*-alkyl analogues and their broad therapeutic spectra in both mouse leukemia and solid tumor models suggest that clinical investigations would be warranted.

ACKNOWLEDGMENTS

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Induction of Differentiation of Human Myeloid Leukemia HL-60 Cells by Novel Nonphosphorus Alkyl Ether Lipids[1]

Yoshio Honma[a,*], Takashi Kasukabe[a], Motoo Hozumi[a], Hiroshi Akimoto[b] and Hiroaki Nomura[b]

[a]Department of Chemotherapy, Saitama Cancer Center Research Institute, Saitama-362 and [b]Central Research Division, Takeda Chemical Ind. Ltd., Osaka-532, Japan

We synthesized a new series of nonphosphorus alkyl ether glycerolipids, in which the 2-acetyl group of platelet-activating factor was replaced by a pyrimidin-2-yl group and the 3-phosphocholine portion by an ω-(substituted ammonio)ethoxyethyl side-chain including ω-thiazolio-, imidazolio- and pyridinio groups with or without a carboxyl substituent, respectively (compound I–XI). Their effects on cell proliferation and differentiation of human myeloid leukemia HL-60 cells were examined. Incubation of HL-60 cells with these cationic and zwitterionic alkyl ether lipids inhibited proliferation of HL-60 cells with IC_{50} values ranging from 10 to 500 ng/mL. The cells were induced by the lipids to differentiate into morphologically and functionally mature granulocytes. Among the compounds we tested, 1-octadecyl-2-pyrimidinyl-3-[3-(5-carboxylatepentyl)imidazolioethoxyethyl]glycerol (compound I) was the most effective in inducing differentiation of HL-60 cells. Compound I showed on a molar basis, an inhibitory effect on the leukemic cells over 50 times greater than did 2-(2-dodecyloxyethoxy)ethyl 2-pyridinioethyl phosphate, the antileukemic alkyl ether phospholipid.

Lipids 26, 1445–1449 (1991).

Several human and murine myeloid leukemia cells can be induced to differentiate *in vitro* into mature granulocytes and macrophages by various inducers (1,2). Syngeneic mice inoculated with myeloid leukemia M1 cells all died of leukemia, but their survival was significantly enhanced by injection of several differentiation inducers including an alkyl ether phospholipid (3). These findings suggest that treatment of differentiation inducers could be a new approach to the control of leukemia.

Some long-chain alkyl ether phospholipids induced differentiation of human myeloid leukemia HL-60 cells and mouse myeloid leukemia M1 cells (4,5). Although the experimental therapeutic effects of these ether phospholipids were definite, the therapeutic index was not sufficient to be acceptable for clinical trials in the treatment of leukemia (6).

To search a new antitumor agent with a better drug therapeutic index, we synthesized a new series of alkyl ether lipids, 1-*O*-octadecyl-2-*O*-substituted glycerolipids with a 3-(ω-(trimethylammonio)ethoxyethyl group and found that the highest *in vitro* antiproliferative activity was obtained when the 2-substituent was the pyrimidin-2-yl group (7). Based on this study, we continued functional modifications at the polar head base of the 1-*O*-octadecyl-2-*O*-pyrimidinyl glycerolipids by replacing the ω-trimethylammonio group with a thiazo, imidazolio or pyridinio group with or without a carboxyl substituent. These procedures yielded two groups of compounds, zwitterionic (seven) and cationic (five) ones, depending upon whether the polar head base was a heteroaromatic quaternary ammonium group with or without a carboxylate function. This paper deals with the potent growth-inhibitory effects of these new alkyl ether glycerolipids and their ability to induce differentiation of human myeloid leukemia cells.

MATERIALS AND METHODS

Chemicals. The compounds used in this study were 1-*O*-octadecyl-2-*O*-pyrimidin-2-yl 3-*O*-ω-substituted ethoxyethyl glycerols in which a quaternary heteroaromatic ammonio group (thiazolio, imidazolio and pyridinio) with or without a carboxyl moiety was introduced at the ω-position of the 3-polar head side-chain (compounds I–XI). The other compound used was 1-*O*-octadecyl-2-*O*-acetonyl-3-[2-[2-(3-(5-carboxypentyl)imidazolio)ethoxyl]ethyl]glycerol (compound XII). These cationic (compound III, IV, V, VIII and IX) and zwitterionic alkyl ether lipids (compounds I, II, VI, VII, X, XI and IX) were synthesized in our laboratories (7). The purity and chemical structure of each compound were confirmed by thin-layer chromatography (TLC), nuclear magnetic resonance (NMR) and elemental analysis.

Cells and cell culture. Human myeloid leukemia HL-60, K562, ML-1, HEL, U937 and KU812 cells were maintained in RPMI-1640 medium supplemented with 10% fetal bovine serum at 37°C in a humidified atmosphere of 5% CO_2 in air (8–12). Bone marrow cells were obtained from the femurs of 8-week-old mice. Immature myeloid cells were enriched using Lympholyte-M, a separation medium for isolation of mouse lymphocytes, at a density of 1.087–1.088 g/mL (Cedarlane Laboratories, Hornby, Canada). Bone marrow nucleated cells were cultured in RPMI-1640 medium supplemented with 10% fetal bovine serum, 50 units/mL recombinant GM colony-stimulating factor and 50 units/mL recombinant interleukin 3 (Genzyme Co., Boston, MA). The cells were seeded in suspension at a concentration of 3×10^5/mL in a multidish (Nunc, Roskilde, Denmark). All the test compounds were dissolved in absolute ethanol and added to the culture (final ethanol concentration, 0.1 to 0.5%). This concentration range of ethanol had no apparent effect on cell growth or differentiation.

Assay of cell growth and the properties of differentiated cells. Cells (10^5/mL) were suspended in 2 mL of culture medium and were cultured with or without alkyl ether lipids in a multidish (Nunc). The cell number was counted with a Model ZBI Coulter Counter (Coulter Electronics,

[1]Based on a paper presented at the Third International Conference on Platelet-Activating Factor and Structurally Related Alkyl Ether Lipids, Tokyo, Japan, May 1989.

*To whom correspondence should be addressed at Saitama Cancer Center, Research Institute, Ina-machi, Kitaadachi-gun, Saitama 362, Japan.

Abbreviations: ET18-OMe, 1-*O*-octadecyl-2-*O*-methyl-*rac*-glycero-3-phosphocholine; NBT, nitroblue tetrazolium; NMR, nuclear magnetic resonance; ST-023, 1-*O*-tetradecyl-2-*O*-methyl-*rac*-glycero-3-phosphocholine; ST-029, 2-(2-octadecyloxyethoxy)ethyl 2-pyridinioethyl phosphate; TLC, thin-layer chromatography.

Hialeah, FL) 6 days after treatment. Nitroblue tetrazolium (NBT) reduction was assayed as reported previously (4). The percentage of cells containing intracellular blue-black formazan deposits was then determined by examination of at least 200 cells. Lysozyme activity was determined by a lysoplate method with lysoplates containing 1% agar, 67 mM sodium phosphate buffer (pH 6.6), 50 mM NaCl, and heat-killed *Micrococcus lysodeikticus* (0.5 mg/mL) (4). The percentages of cells that were morphologically similar to mature granulocytes were determined in smears treated with May-Gruenwald-Giemsa stain.

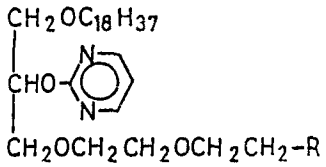
RESULTS

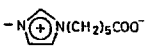
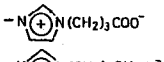
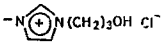
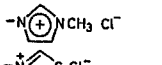
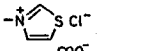
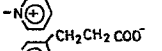
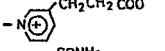
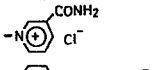
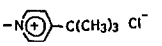
We synthesized novel alkyl ether lipids which differ from known alkyl lysophospholipids in that they contain no phosphoric acid ester group. In the course of screening, we found that these lipids induced differentiation of HL-60 cells. Then we examined the effects of various analogs on growth inhibition and differentiation of HL-60 cells (Table 1). The growth-inhibiting effects of the compounds were estimated by determining the concentrations required to reduce the cell number to half that of untreated cultures (IC_{50}). Nitroblue tetrazolium (NBT) reduction is a typical functional marker of differentiation of HL-60 cells (13), and the cells were cultured with IC_{50} concentration of each compound for assay of NBT reduction. These 1-*O*-octadecyl-2-*O*-pyrimidin-2-yl glycerolipids which are characterized by the absence of a phosphoryl group in the 3-polar head side-chain (compounds I–XI) showed dramatically potent activities on growth and differentiation of HL-60 cells, as compared to alkyl ether phospholipids including the reference compound, ET18-OMe (4,5). Among the compounds tested, most of the cationic lipids showed greater activity than those with the zwitterionic structure, with a few exceptions. The maximal activity was observed with the *N*-methylimidazolio compound (IV) with the IC_{50} value of 10.5 ng/mL. However, the *n*-carboxypentylimidazolio compound (I) with the IC_{50} value of 96.7 ng/mL was more effective than compound IV in inducing NBT reduction of the cells (Table 1). This led us to synthesize and test further analogs of compound I having an appropriate substituent at position 2 and/or 3 of the glycerol backbone. A change in the structure of the polar head base (ω-heteroaromatic ammonio group) did not yield a more favorable inducer of cell differentiation than compound I, although only a limited number of compounds was tested. Figure 1 shows the chemical structures of the compounds obtained by modification of the substituent at position 2 (compound XII) and the repeated ethyleneoxy unit at position 3 (compound X and XI). These compounds did not show any significant increase in either the growth inhibition or the ability to induce NBT reduction as compared to compound I (Table 2). Consequently, among the compounds tested, compound I was the most effective in inducing NBT reduction of the cells.

Lysozyme activity also is a typical marker of functional differentiation of the cells (4). Lysozyme activity was induced in a dose-dependent manner by compound I, as was

TABLE 1

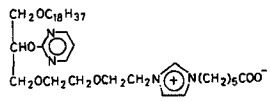
Effect of Various Nonphosphorus Ether Lipids on Growth and Differentiation of HL-60 Cells



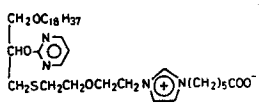
Compound no.	R	Growth inhibition IC_{50} (ng/mL)[a]	Induction of differentiation NBT reduction (%)[b]
I		96.7	48.3
II		499.6	34.9
III		69.6	35.0
IV		10.5	30.1
V		283.8	31.7
VI		409.7	34.3
VII		225.0	20.3
VIII		171.5	17.0
IX		82.4	22.6

[a]IC_{50}, concentration resulting in half the number of control generations.

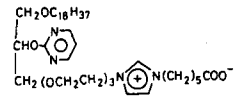
[b]For assay of NBT reduction, HL-60 cells were cultured with the IC_{50} concentration of each compound for 6 days. Means for three separate experiments.



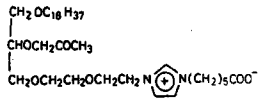
Compound I



Compound X



Compound XI



Compound XII

FIG. 1. Chemical structures of compound I and analogs.

TABLE 2

Effect of Analogs of Compound I on Growth and Differentiation of HL-60 Cells[a]

Compound	Growth inhibition IC_{50} (ng/mL)	Induction of differentiation NBT reduction (%)
I	96.7	48.3
X	141.7	31.2
XI	237.5	39.6
XII	412.5	35.5

[a]HL-60 cells were treated with analogs (Fig. 1), as shown in Table 1.

NBT reduction (Fig. 2). When HL-60 cells were cultured without inducers, most of them were promyelocytic, whereas when the cells were cultured with increasing concentrations of the compound, mature granulocytes appeared and increased in number (Fig. 3). After culture for 6 days, more than 90% of the cells were induced to differentiate into myelocytic and more mature cells.

The growth-inhibitory effect of the new ether lipid was compared with known ether lipids. Figure 4 shows compound I was more effective in inhibiting growth of HL-60 cells than the previously known alkyl phospholipids (ET18-OMe, ST-023 and 029). The inhibitory effect of compound I was about 50 times greater on a molar basis than that of the dodecyl ethyleneglycophospholipid (ST-029), which had been proved to be therapeutically effective on leukemic mice in our laboratories (6).

The chemical structure of compound I is quite different from those of alkyl ether phospholipids, and this difference was expected to be reflected in differences in antileukemic properties. Therefore we examined the growth-inhibitory effect of this compound on several human myeloid leukemia cell lines (ML-1, HEL, KU812, U937 and K562) in direct comparison with alkyl ether lysophospholipid (ST-023). K562 cells are known to be resistant to ET18-MOe alkyl ether phospholipid (14). Figure 5 and Table 3 show that normal mouse bone marrow cells are fairly resistant to compound I, as well as ST-023, and compound I represents a similar selective effect on several

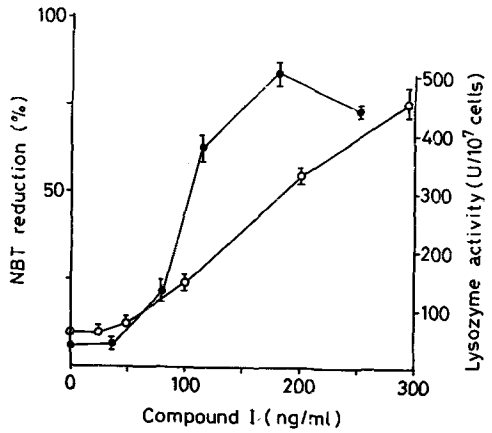


FIG. 2. Induction of lysozyme activity and NBT reduction in HL-60 cells by compound I. HL-60 cells were treated with various concentrations of compound I for 6 days. ●, % of cells showing NBT reduction. O, lysozyme activity. One unit (U) of activity was defined as the amount equivalent to 1 μg of egg white lysozyme.

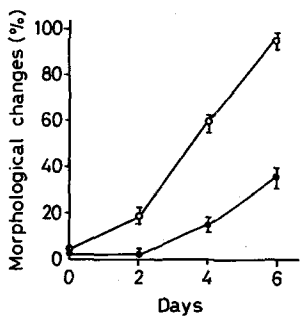


FIG. 3. Effect of compound I on morphological changes of HL-60 cells. Cells were cultured with 150 ng/mL of compound I for the times indicated. O, myelocytes and more mature cells; ●, segmented and banded neutrophils.

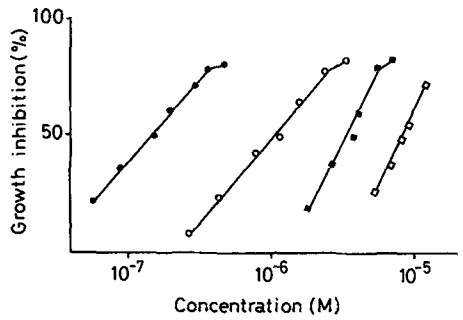


FIG. 4. Growth-inhibitory activity of several alkyl ether lipids on HL-60 cells. HL-60 cells were cultured with various concentrations of ether lipids for 6 days, and then the numbers of viable cells were determined. Means for three separate experiments. ●, compound I; O, 1-*O*-octadecyl-2-*O*-methyl-*rac*-glycerophosphocholine (ET18-OMe); ■, 1-*O*-tetradecyl-2-*O*-methyl-*rac*-glycerophosphocholine (ST-0.23); □, 2-[2-(dodecyloxy)ethoxy]ethyl 2-pyridinioethyl phosphate (ST-029).

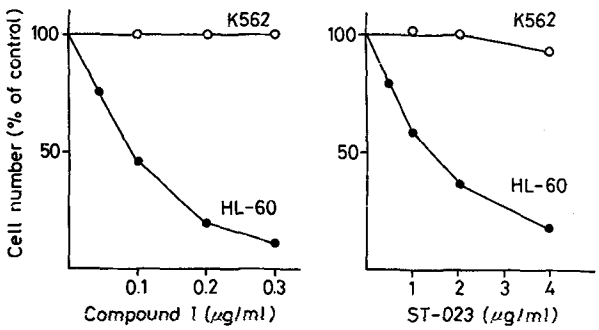


FIG. 5. Effect of compound I on growth of human myeloid leukemia HL-60 and K562 cells. Cells were treated with various concentrations of compound I or ST-023 for 6 days and then cell numbers were determined. ●, HL-60 cells; O, K562 cells.

TABLE 3

Effect of Compound I and ST-023 on Growth of Several Human Myeloid Leukemia Cell Lines[a]

Cell line	Inhibition of cell growth (IC_{50})	
	Compound I (ng/mL)	ST-023 (μg/mL)
HL-60	97 ± 2	1.6 ± 0.3
ML-1	110 ± 3	1.8 ± 0.2
HEL	121 ± 3	1.9 ± 0.3
KU812	104 ± 3	1.7 ± 0.2
U937	275 ± 5	1.7 ± 0.2
K562	>1,000	>10
Mouse bone marrow cells	875 ± 9	>10

[a]Cells were treated with various concentrations of compound I or ST-023 for 6 days, and then cell numbers were determined. Means ± SD for three separate experiments.

human leukemia cell lines although it was much less active against K562 cells. This inhibition pattern was similar to that of ST-023 (4).

DISCUSSION

Aiming to obtain a better therapeutic inducer of human leukemia, we synthesized a new class of nonphosphorus alkyl ether lipids, cationic (compound III, IV, V, VIII and IX) and zwitterionic types (compound I, II, VI, VII, X, XI and XII), and examined their growth-inhibitory activity and ability to induce differentiation of HL-60 cells. The structural features of the present alkyl ether lipids are: (i) a long-chain alkyl ether linked to position 1 of the glycerol backbone, (ii) an ether-linked pyrimidin-2-yl group at position 2 (in the case of compound XII, the acetoacetyl group was introduced), and (iii) the absence of a phosphate group and the presence of an ω-(heteroaromatic quaternary ammonio)ethoxyethyl unit in the polar head side-chain at position 3 of glycerol.

The ether lipids examined were much more potent than the alkyl ether lysophospholipids (ET18-OMe and ST-023) in inhibiting cell growth and inducing cell differentiation on a molar concentration basis. This emphasizes the importance of the nonphosphorus alkyl ether lipids in this series. An increase in growth-inhibiting activity appeared to be associated with the metabolic stability of the lipids which is attributed to the absence of a phosphate group and the presence of an ω-(substituted ammonio)ethoxyethyl function in place of the phosphocholine moiety. In view of the substrate specificities of phospholipases (A_1, A_2, C and D) (15), the present class of ether lipids should be resistant to the action of phospholipases and thus can be expected to accumulate more readily in the cell membrane than alkyl lysophospholipids, as the latter are thought to be cleaved by phospholipases C and D (16). Recently, we reported that a labeled nonphosphorus alkyl ether lipid,[2] similar in structure to ET18-OMe, was not degraded at all (no formation of radiolabeled choline or choline phosphate) during incubation with HL-60 cells, when compared with an alkyl ether phospholipid (17). These results suggest that the nonphosphorus alkyl ether lipids are metabolically stable and cannot be substrates for phospholipases C and D.

Marked individual variation occurs among different human leukemias with respect to their sensitivity to the antiproliferative effect of alkyl lysophospholipids (18,19). This series of alkyl glycerolipids has similar target specificity on proliferation of human myeloid leukemia cells (Table 3). Compound I showed high inhibitory activities, except against K562 cells, and the potencies against HL-60, ML-1, HEL, and KU812 cells were more than 15 times higher than that of ST-023. The results suggest that the mode of action of the new types of lipids may be similar to that of alkyl lysophospholipids.

Our findings showed that the cationic ether lipids were generally more inhibitory against leukemia cells than those with zwitterionic structure, presumably due to a higher affinity of the cationic type for malignant cells. Compound I and IV inhibited the proliferation of HL-60 cells at 10^{-7} and 10^{-8} M, respectively. The high efficiency of the lipids in inhibiting cell growth may suggest the presence of specific binding sites for the lipids, which would presumably be located on the surface of the plasma membrane of HL-60 cells (20). The initial target site still remains to be identified.

Alkyl lysophospholipids and the analogs have aroused increasing interest as a new class of potential anticancer agents. Clinical pilot studies of several alkyl ether lipids have been undertaken on patients suffering from a variety of malignant diseases (21,22). In general, alkyl ether lipids tend to inhibit proliferation of certain types of leukemia cells at a low concentration and to inhibit the growth of normal bone marrow cells only at a high concentration (4,23). The difference in response of leukemic and normal cells indicates the potential advantages of using alkyl ether lipids as antileukemic agents. Our preliminary experiments have shown that compound I is active against *in vivo* experimental tumors (S180 and MM46) with less acute toxicity in mice when compared with ST-023 (in preparation). Further *in vivo* studies on the antileukemic properties of compound I are underway.

[2]1-*O*-octadecyl-2-*O*-methyl-3-*O*-(ω-trimethylammonioethoxyethyl)-glycerol, tritium-labeled in the *N*-methyl group of the 3-polar-head side-chain.

ACKNOWLEDGMENTS

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PREFACE

This special *Lipids* issue represents a thorough, state-of-the-art account on platelet activating factor (PAF) and structurally related alkyl lysophospholipids (ALP). The present collection contains some hundred peer-reviewed research articles and reviews by the world's leading authorities in this field. The contributions are based on papers and posters which were originally presented at the Third International Conference on Platelet-Activating Factor and Structurally Related Alkyl Ether Lipids held in Tokyo, Japan, in May 1989. Most of the original research papers and focused in-depth review articles have since been updated and provide a detailed overview of the state of current research on ether lipid mediators covering biochemical, cell biological, physiological and pathological aspects of PAF function as well as the role of ether lipids (ALP) in cancer therapy.

The present volume covers a wide range of specialized topics including PAF metabolism and its regulation; PAF and PAF analogs and their metabolic effects; PAF analysis by spectrometric and immunological assay; PAF receptor and PAF antagonists; cell biology of PAF; PAF and the cerebral system; PAF, the cardiovascular system, and hypotension; PAF, the respiratory system, allergy, and asthma; PAF and the renal system; PAF and the gastrointestinal system; the pathophysiology of PAF; and alkyl lysophospholipids in cancer therapy. This timely collection is the most comprehensive compendium of peer-reviewed papers published in the PAF field to date.

I wish to express my sincere thanks to Professor Shoshichi Nojima, Chairman of the Conference, and to the Members of the Organizing Committee for their commitment and for making the Conference a most memorable experience. Thanks are also due to the colleagues who have co-edited the papers. I am deeply indebted to the many referees for their enormous efforts and their expert advice; without them peer-review would not have been possible. My cordial thanks also to Dr. Barbara Malewicz, Kerry McParland and Kerry Ruck at the Hormel Institute for their tireless efforts and encouragement, and to Annette Wall and the entire *Lipids* Production Staff for their extraordinary dedication and care. Last but not least, I would like to thank each of the authors for their contributions and for their cooperation and patience in revising and updating their papers. This special issue is the result of a special team effort. All involved can be proud of it.

This special issue of *Lipids*, which is also available as hardcover copy, should become a key reference source for researchers in a broad range of biochemical, biological and biomedical disciplines concerned with the role of PAF, ALP, and related ether lipid mediators and their respective modes of action.

Wolfgang J. Baumann
Editor