Ingestion of Plasmalogen Markedly Increased Plasmalogen Levels of Blood Plasma in Rats

Megumi Nishimukai, Takuya Wakisaka, and Hiroshi Hara*

Division of Applied Bioscience, Graduate School of Agriculture, Hokkaido University, Sapporo 060-8589, Japan

ABSTRACT: Plasmalogens, a subclass of phospholipids, are widely distributed in human and animals, and are taken into the body as food. However, no data exist on the intestinal absorption or fate of ingested plasmalogen. Here, we determined whether dietary plasmalogen is absorbed and whether blood and tissue concentrations increased in normal male Wistar rats by using four separate experiments. Phospholipids containing more than 20 wt% of plasmalogen extracted from the bovine brain were incorporated into test diets (10–15 wt%). In experiment 1, we estimated the absorption rate by measuring the plasmalogen vinyl ether bonds remaining in the alimentary tract of rats after the ingestion of 2 g of test diet containing 91 μmol plasmalogen. The absorption rate of plasmalogen was nearly 80 mol% after 4 h, comparable to the total phospholipid content in the test diet. In experiment 2, we observed no degradation of the plasmalogen vinyl ether bonds under *in vitro* conditions simulating those of the stomach and small intestinal lumen. In experiment 3 we confirmed a comparable absorption (36 mol%) by using a closed loop of the upper small intestine in anesthetized rats 90 min after injecting a 10 wt% brain phospholipid emulsion. Feeding a test diet containing 10 wt% brain phospholipids for 7 d increased plasmalogen concentration threefold in blood plasma and by 25% in the liver; however, no increases were seen in blood cells, skeletal muscle, brain, lungs, kidneys, or adipose tissue (experiment 4). We concluded that dietary plasmalogen is absorbed from the intestine and contributes to a large increase in plasmalogen levels in blood plasma.

Paper no. L9063 in *Lipids 38,* 1227–1235 (December 2003).

Plasmalogens are a subclass of phospholipids with vinyl ether double bonds in the *sn*-1 position (1-*O*-alk-1′-enyl). These lipids are widely distributed through human and animal tissues and in anaerobic bacteria; the average proportion of plasmalogens in the total phospholipids in the human body is about 18% (1). The brain, heart, lungs, muscle, and red blood cells contain relatively high levels of ethanolamine plasmalogens (2).

Although the role of plasmalogen is not fully understood, many reports suggest that plasmalogen is a structural component of the cell membrane that maintains cell membrane dynamics (3), and that it also has various functions within the cells. A protective role against oxidative stress has been proposed, especially in cerebral and cardiac tissues in which plasmalogen levels are higher than in other tissues (4). Plasmalogen also may be an endogenous lipidemic antioxidant, as the vinyl ether double bond is sensitive to oxidative agents (5–8). Further, the antioxidative effects of plasmalogen on lipoprotein (9,10) and its role in lipoprotein metabolism (11) have been reported. These earlier studies suggest that increases in plasmalogen levels in plasma lipoprotein protect against cholesterol oxidation and lower the incidence of coronary heart disease. Recently, Farooqui *et al.* (12) reported that plasmalogen deficiency is involved in nerve degeneration in Alzheimer's disease.

Several studies have shown that dietary alkylacylglycerol, another ether-linked lipid, is absorbed and incorporated as plasmalogen in tissues and red blood cell membranes (13), and alkylacylglycerol supplementation was found to restore tissue plasmalogen deficiency (14). However, no data have been reported on the absorption of dietary alkenyl phospholipids or plasmalogen, nor are data available on the fate of plasmalogen vinyl ether double bonds after the ingestion of foods that are common sources of plasmalogens (15).

The aims of the present study were (i) to examine the absorption of plasmalogen in bovine brain phospholipids (BPL) incorporated into test diets, and (ii) to determine whether the ingestion of plasmalogen increased plasmalogen levels in blood plasma, blood cells, and body tissues.

EXPERIMENTAL PROCEDURES

Animals and diets. Male Wistar rats (Japan SLC, Hamamatsu, Japan), weighing about 150 g, were given free access to deionized water and a semipurified stock diet (Table 1; 16–19) for a 7-d acclimation period. Four separate experiments were then performed with the acclimated rats. All rats used in the experiments were housed individually in stainless-steel cages with mesh bottoms. The cages were kept in a room in which the temperature (22–24°C), RH (40–60%), and lighting (lights on: 0800–2000 h) were controlled. This study was approved by the Hokkaido University Animal Committee, and animals were maintained in accordance with the Hokkaido University guidelines for the care and use of laboratory animals.

The test diets used in experiments 1 and 4 were casein–sucrose-based semipurified diets containing test lipids. Three test lipids were used in experiments 1, 2 (150 g lipid/kg diet), and 4 (100 g lipid/kg diet): soybean oil (SO; Wako Pure Chemical Industries, Tokyo, Japan), purified soybean phosphatidylcholine [SPL, more than 95 wt% phosphatidylcholine

*To whom correspondence should be addressed at Division of Applied Bioscience, Graduate School of Agriculture, Hokkaido University, Kita-9, Nishi-9, Kitaku, Sapporo 060-8589, Japan.
E-mail: hara@chem.agr.hokudai.ac.jp

Abbreviations: BPL, brain phospholipids; SO, soybean oil; SPL, soybean phosphatidylcholine.

TABLE 1
Composition of Stock and Test Diets

		Test diets		
	Stock diet[a]	SO diet	SPL diet	BPL diet
	g/kg diet			
Casein[b]	250	250	250	250
Lipids[c]				
Corn oil	50			
SO		150 or 100		
SPL			150 or 100	
BPL				150 or 100
Mineral mixture[d]	40	40	40	40
Vitamin mixture[e]	10	10	10	10
Granulated vitamin E[f]	1.0	1.0	1.0	1.0
Choline bitartrate	4.0	4.0	4.0	4.0
Sucrose	to make 1 kg		to make 1 kg	

[a]The stock diet was given during the acclimation and recovery periods.
[b]Casein (ALACID; New Zealand Dairy Board, Wellington, New Zealand).
[c]In the stock diet, corn oil was used as a lipid. Three test lipids (SO, soybean oil; SPL, soybean PC; BPL, brain phospholipids) were added to the test diets as described in the Experimental Procedures section and in Table 2. Lipid levels were 150 g/kg diet in experiment 1 and 100 g/kg diet in experiment 4. Retinyl palmitate (7.66 μmol/kg diet) and ergocalciferol (0.0504 μmol/kg diet) were added to the lipids.
[d]The mineral mixture was prepared according to the method established at the AIN-76 Workshop held in 1989 (13). The mixture supplied the following minerals (mg/kg diet): Ca, 4491; P, 2997; K, 3746; Mg, 375; Fe, 100; I, 0.32; Mn, 10.0; Zn, 34.7; Cu, 6.00; Na, 4279; Cl, 6542; Se, 1.05; Mo, 1.00; Cr, 0.50; B, 0.50; V, 0.25; Sn, 2.00; As, 1.00; Si, 20.0; Ni, 1.00; F, 2.72; and Co, 0.20.
[e]The vitamin mixture was prepared in accordance with the AIN-76 mixture (14) except that menadione and L-ascorbic acid were added at 5.81 μmol/kg diet (15) and 284 mmol/kg diet (16), respectively.
[f]Vitamin E granules (Juvela; Eisai Co., Tokyo, Japan) supplied 423 μmol of all-*rac*-α-tocopheryl acetate per kilogram of diet.

(PC); EPIKURON200; Lucas Meyer GmbH, Hamburg, Germany], and BPL containing more than 20 wt% plasmalogen. Diet compositions are shown in Tables 1 and 2.

TABLE 2
Composition of Test Lipids

	Test lipids			
	BPL	SPL	SO	Corn oil
Phospholipid class (wt%)				
PC	50.8	98.0		
PE	44.1	Traces		
Others[a]	5.1	Traces		
Phospholipid subclass (mol%)				
Alkenylacyl	22.0	1.7		
Alkylacyl	1.0	1.4		
Diacyl	76.5	95.0		
Lysophospholipids	—	0.9		
Others	0.5	1.0		
FA (mol%)				
14:0	9.0	—	—	—
16:0	22.8	13.5	12.3	13.4
18:0	17.6	3.1	3.7	1.7
18:1	34.9	7.8	25.5	29.3
18:2n-6	1.3	68.2	52.3	54.9
18:3n-3	—	7.3	6.1	0.6
20:1	2.9	—	—	—
20:4n-6	3.9	—	—	—
22:5n-3	3.0	—	—	—
22:6n-3	4.5	—	—	—

[a]PI + PS + sphingomyelin. For abbreviations see Table 1. Dash, not detected.

BPL were extracted by the method of Folch (20) with some modifications. Briefly, homogenized bovine brain (approximately 400 g/brain) was completely washed with acetone and then with ethanol, and lipids were extracted from the washed brain homogenate using petroleum ether. After complete removal of the solvent, phospholipids were extracted by diethyl ether and again washed with acetone. The plasmalogen and total phospholipid contents in the extracted BPL fractions were 22.2 and 89.1 wt%, respectively, in experiment 1 and 27.9 and 93.2 wt%, respectively, in the other experiments.

Experiment 1 (dietary plasmalogen absorption in fasted rats). Acclimated rats were fasted for 1 d and then divided into three groups of six rats each. Each group of rats was fed one of the test diets described above (2 g diet/rat). After 2 or 4 h, aortic blood was collected from anesthetized rats (pentobarbital sodium, 50 mg/kg body weight; Abbott Laboratories, North Chicago, IL) into a heparinized syringe, and the rats were sacrificed. The stomach, small intestine, cecum, and colon and their contents were removed after ligation of both ends of each organ and stored at –40°C until analysis.

Experiment 2 (degradation of plasmalogen in the alimentary tract). To evaluate the survival of plasmalogen in the stomach after ingestion of a diet containing plasmalogen, 3 vol of 0.1 mol HCl/L was added to the test diet used in experiment 1 (BPL) and then incubated at 37°C for 1 h. We also examined the degradation rate of plasmalogen in a 10 wt% BPL emulsion under the same conditions as the test diet.

To evaluate the degradation of plasmalogen in the small and the large intestinal lumens, contents of the entire small and large intestines of four acclimated rats were collected between 1000 and 1100 h. The freshly prepared and pooled contents of the small intestine or cecum of four rats were added to a phosphate buffer (8 mmol phosphate/L, pH 7.4) containing BPL (final volume, 34 mL; 94 μmol plasmalogen and 360 μmol phospholipids). The cecal contents were gently suspended in the buffer and incubated under nitrogen gas at 37°C. Three small samples of the reaction mixture containing the contents of the small intestine or cecum were taken after 0, 0.5, 1, 2, or 4 h incubation.

Experiment 3 (absorption of plasmalogen from a closed loop of the small intestine). Six acclimated rats were anesthetized (pentobarbital sodium, 40 mg/kg body weight) and the upper small intestine was drawn out from a midline incision. A closed intestinal loop was made by ligating the pylorus and the mid small intestine (45 cm distal from the ligament of Treitz); 5 mL of BPL emulsion (100 g/L, 57.3 μmol plasmalogen/5 mL) emulsified with 10 g/L sodium taurocholate was then injected into the loop and the midline incision was closed. After 90 min, the rats were killed by withdrawing the aortic blood, and the closed loop with contents was removed. The contents were collected and the mucosa of the loop was also completely scraped away and collected. Both were stored at –40°C until subsequent analyses.

Experiment 4 (effects of feeding a diet containing plasmalogen for 7 d). Acclimated rats were divided into three groups of six rats and fed the test diets shown in Table 1 for

7 d. These test diets contained SO, SPL, or BPL (100 g/kg diet) as described above. Body weight and food consumption were measured every day. On the last day, aortic blood was collected from rats under pentobarbital anesthesia and was heparinized. The liver was removed after saline perfusion from the portal vein, and the brain, lungs, kidneys, heart, gastrocnemius muscle, and epididymal fat pad were removed and stored at −40°C. The stomach with its contents was also removed. The contents were collected and the pH was measured after weighing. All rats were killed between 1000 and 1130 h.

Analytical methods. The heparinized blood collected in experiments 1 and 4 was separated into plasma and blood cells by centrifugation, and the blood cells were then washed twice with saline. The frozen stomach, cecum, and colon were opened by cutting the wall, and the total contents were collected. Contents of the small intestine were collected together with the mucosa by applying pressure from outside the intestinal wall after thawing (21).

Total lipids in the blood plasma and cells, gastrointestinal contents, organs, and tissues were extracted with chloroform/methanol/saline (10:5:3, by vol) (22).

Plasmalogen in the BPL and in the total lipids extracted in the chloroform–methanol solution were measured by the iodine addition method (23–25). Briefly, an iodine solution (0.37 mmol/L) was added to the extracted lipid solution. After 10 min at room temperature, the reaction mixture was diluted with 95% ethanol. In this method, the plasmalogen vinyl ether double bonds reacted specifically with the iodine in the presence of methanol. As the iodine reacted with the vinyl ether moiety, the reduction of absorbance at 355 nm was measured photometrically. We observed that the BPL plasmalogen concentrations estimated by the iodine addition method were very closely correlated ($r = 0.984$) with those measured by the *p*-nitrophenylhydrazone method (26), in which plasmalogen was degraded to fatty aldehyde with 0.3 mol/L sulfuric acid. We also measured free fatty aldehyde in the liver by using the *p*-nitrophenylhydrazone method with 5 mmol/L sulfuric acid. Specificity of the iodine addition method for double bonds of the vinyl ether moiety in plasmalogen was very high; that is, iodine consumption by soybean lecithin and cholesterol was only 0.14 and 0.07 mol%, respectively, of that by plasmalogen. This method is convenient for tracing the vinyl ether double bonds in plasmalogen.

The phospholipid classes of the BPL and SPL were separated by TLC (chloroform/methanol/ammonia water, 65:35:8, by vol) and quantified after charring with sulfuric acid.

The phospholipid subclasses of the BPL and SPL were analyzed by HPLC. To do so, the BPL or SPL (approximately 5 mg) was suspended in 1.5 mL of Tris/HCl buffer (0.1 M, pH 7.0) containing 0.01 mmol $CaCl_2$. After the addition of 2 mL of diethyl ether and phospholipase C (*Bacillus cereus,* EC 3.1.4.3; Sigma, St. Louis, MO), the sample was vigorously mixed at room temperature for 3 h. After the diethyl ether layer was collected and evaporated, the sample was reacted with anhydride benzoic acid and 4-dimethylaminopyridine at room temperature for 3 h. Diradylglycerobenzoates were separated by HPLC (Waters 2695 separation module; Waters, Milford, MA) with TSK Silica gel 60 (4.6 × 250 mm; Tosoh, Tokyo, Japan) into three subclasses. The mobile phase was hexane/dichloromethane/acetonitrile (90:7.5:0.75, by vol) with a flow rate of 0.8 mL/min. Detection was performed at 230 nm using a Waters 2996 photodiode array detector (27).

Test lipids were hydrolyzed by KOH in ethanol, and their FA composition was measured by GLC (Shimadzu GC-14A; Shimadzu, Tokyo, Japan) with a CBP20-M25-025 capillary column (25 m i.d., 0.25 mm; Shimadzu), FID, and helium as carrier gas after methylation with 5% HCl–methanol. Initial temperature was 170°C, followed by an increase of 2°C/min to 250°C (28).

Phospholipid concentration was measured using a phosphate assay or choline oxidase procedure (Phospholipid-test Wako and Phospholipid B-test Wako; Wako Pure Chemical Industries, Osaka, Japan). Triglyceride (TG) and cholesterol concentrations in the plasma and liver were also assayed by enzymatic procedures (Triglyceride G-test Wako and Cholesterol C-test Wako; Wako Pure Chemical Industries).

Calculations and statistical analysis. We calculated the molar amounts of plasmalogen and phospholipids by taking the average M.W. of SPL, BPL, and plasmalogen in BPL as 777.3, 770.1, and 729.5, respectively.

In experiment 1, plasmalogen and phospholipid absorption rates were estimated from the disappearance rates of both lipids from the alimentary tract by using the following equation:

$$\text{absorption (\%)} = \frac{\text{[ingested plasmalogen or phospholipids (μmol)} - \text{remaining plasmalogen or phospholipids (μmol)]}}{\text{ingested plasmalogen or phospholipids (μmol)}} \times 100 \quad [1]$$

The remaining dietary lipids were estimated by subtracting the corresponding average values of the SO group from the values of the SPL or BPL group. The reliability of this method of estimation is discussed in the Discussion section below.

The results were analyzed by two-way (Table 3, Figs. 1 and 2) or one-way (Figs. 3–5) ANOVA. Duncan's multiple range test was used to determine whether mean values were significantly different ($P < 0.05$) (29). Correlation coefficients for the relationships between the results of the iodine addition method and the *p*-nitrophenylhydrazone method were assessed by the least squares method (30). These statistical analyses were performed by using the general linear model procedure of SAS (SAS version 6.07; SAS Institute Inc., Cary, NC).

RESULTS

Phospholipid classes and subclasses in the BPL. Test lipid compositions are shown in Table 2. The major phospholipid classes in the BPL were determined by TLC to be PC and phosphatidylethanolamine (PE), whereas the subclasses detected by HPLC were alkenylacyl (22.0 mol%), alkylacyl (1.0 mol%), and diacyl (76.5 mol%). No lysophospholipids were detected.

Experiment 1 (dietary plasmalogen absorption in fasted rats). In experiment 1, rats consumed the 2-g portions of the test diet within the first 10 min. However, considerable

TABLE 3
Amounts of Plasmalogen and Phopholipids in the Gastrointestinal Tracts and Their Contents[a] 2 and 4 h After Feeding 2 g of 15% Lipid Diets Containing SO, SPL, and BPL (45.5 mmol ethanolamine plasmalogen/kg diet)

	Plasmalogen (μmol)			Phospholipids (μmol)		
Position	2 h	4 h	*P* values	2 h	4 h	*P* values
Stomach						
SO	1.00 ± 0.16^{c}	0.82 ± 0.12^{c}	Lipid (L) <0.001	10.8 ± 2.7^{d}	11.5 ± 1.5^{d}	Lipid (L) <0.001
SPL	0.74 ± 0.17^{c}	0.56 ± 0.17^{c}	Time (T) <0.001	97.3 ± 12.4^{b}	18.1 ± 9.7^{d}	Time (T) <0.001
BPL	37.1 ± 1.62^{a}	7.01 ± 1.85^{b}	L × T <0.001	169 ± 9.4^{a}	64.8 ± 7.0^{c}	L × T <0.001
Small intestine						
SO	2.00 ± 0.49^{b}	1.45 ± 0.09^{b}	Lipid (L) <0.001	16.0 ± 1.0^{c}	17.1 ± 2.0^{c}	Lipid (L) <0.001
SPL	1.63 ± 0.10^{b}	1.44 ± 0.18^{b}	Time (T) 0.413	35.2 ± 2.9^{b}	17.9 ± 1.6^{c}	Time (T) 0.044
BPL	10.9 ± 1.49^{a}	9.43 ± 2.15^{a}	L × T 0.828	52.9 ± 7.0^{a}	46.7 ± 7.0^{a,b}	L × T 0.124
Cecum						
SO	4.01 ± 1.50^{b}	4.52 ± 1.07^{b}	Lipid (L) 0.007	6.55 ± 1.14^{b}	6.51 ± 0.94^{b}	Lipid (L) <0.001
SPL	5.40 ± 0.59^{b}	5.97 ± 1.56^{b}	Time (T) 0.072	5.04 ± 0.36^{b}	7.17 ± 1.40^{b}	Time (T) 0.003
BPL	6.11 ± 0.95^{b}	10.5 ± 1.24^{a}	L × T 0.196	6.11 ± 0.95^{b}	26.8 ± 5.08^{a}	L × T 0.020
Colon						
SO	0.415 ± 0.060	0.470 ± 0.092	Lipid (L) 0.381	0.667 ± 0.248	0.856 ± 0.242	Lipid (L) 0.807
SPL	0.307 ± 0.065	0.271 ± 0.098	Time (T) 0.646	0.697 ± 0.299	0.592 ± 0.227	Time (T) 0.432
BPL	0.337 ± 0.054	0.445 ± 0.211	L × T 0.808	0.592 ± 0.102	1.08 ± 0.486	L × T 0.606
Total						
SO	7.43 ± 1.60^{c}	7.26 ± 1.07^{c}	Lipid (L) <0.001	34.1 ± 3.1^{c}	35.9 ± 2.5^{c}	Lipid (L) <0.001
SPL	8.08 ± 0.79^{c}	8.23 ± 1.80^{c}	Time (T) <0.001	138 ± 11.3^{b}	43.7 ± 10.6^{c}	Time (T) <0.001
BPL	54.4 ± 2.19^{a}	27.4 ± 3.25^{b}	L × T <0.001	232 ± 10.6^{a}	139 ± 16.5^{b}	L × T <0.001

[a]Values are means ± SE (n = 6). These values contain mucosal plasmalogen and phospholipids. Values in the same position not sharing a common roman superscript letter differ significantly, $P < 0.05$, where "position" includes (e.g.) "Stomach" × "Plasmalogen" values. For other abbreviations see Table 1.

amounts of the test diet containing SPL were spilled. Intake of the test diets was 2.0 ± 0.0 g in the SO and BPL groups and 1.5 ± 0.1 g in the SPL group. Total phospholipids ingested were 289 (± 19) and 353 μmol in the SPL and BPL groups, respectively, and plasmalogen intake was 92 μmol in the BPL group.

Molar amounts of plasmalogen and phospholipids in the stomach, small intestine, cecum, and colon are shown in Table 3. The values in the SO group show the amounts of endogenous plasmalogen and phospholipids detected, including those from the mucosa, bile–pancreatic juice, and intestinal bacteria. The plasmalogen level in the cecal contents was the highest among all gastrointestinal segments in the SO group. No increases in plasmalogen and phospholipid levels in the colonic contents were observed in the BPL group. Plasmalogen and phospholipid levels in the cecal contents were higher in the BPL group than in the other groups 4 h after ingestion.

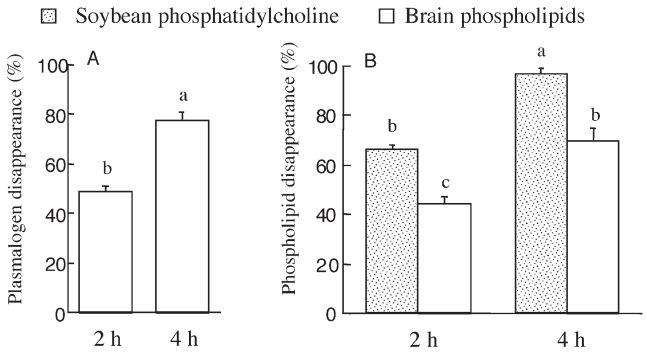

FIG. 1. Plasmalogen (A) and phospholipid (B) disappearance (absorption) from the entire alimentary tract in two groups of animals after ingestion of either 2 g of test diet containing brain phospholipids (BPL) or soybean phosphatidylcholine (SPL) in experiment 1. Details are provided in the Materials and Methods section. Each value shown is the mean ± SEM for six rats. *P* values estimated by one-way ANOVA for the plasmalogen disappearance rate were <0.001. *P* values estimated by two-way ANOVA for the phospholipid disappearance rate were <0.001 for lipid (P) and time (T), and 0.595 for P × T. Mean values not sharing a common letter are significantly different between groups ($P < 0.05$). (A) From animals fed the BPL diet (open bar); (B) from animals fed the BPL diet (open bar) and the SPL diet (stippled bar).

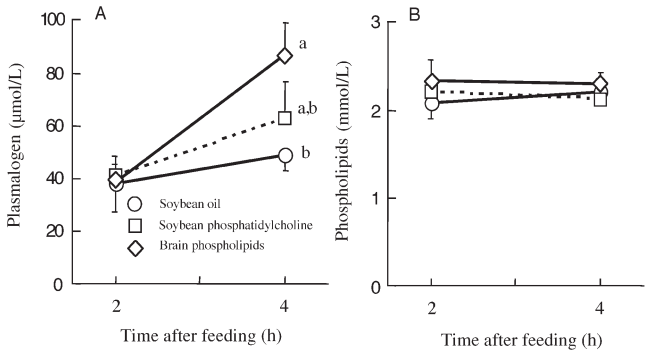

FIG. 2. Changes in plasmalogen (A) and phospholipid (B) concentrations in the blood plasma of fasted rats after feeding 2 g of test diet containing 150 g test lipid/kg diet in experiment 1. Test lipids were soybean oil (SO) (○), SPL (□), and BPL (◇). Each value shown is the mean ± SEM for six rats. *P* values estimated by two-way ANOVA for plasmalogen were 0.168 for lipid (P), 0.002 for time (T), and 0.171 for P × T. *P* values for phospholipids were 0.397 for lipid (P), 0.967 for time (T), and 0.757 for P × T. Mean values for times not sharing a common roman letter are significantly different between groups ($P < 0.05$). For abbreviations see Figure 1.

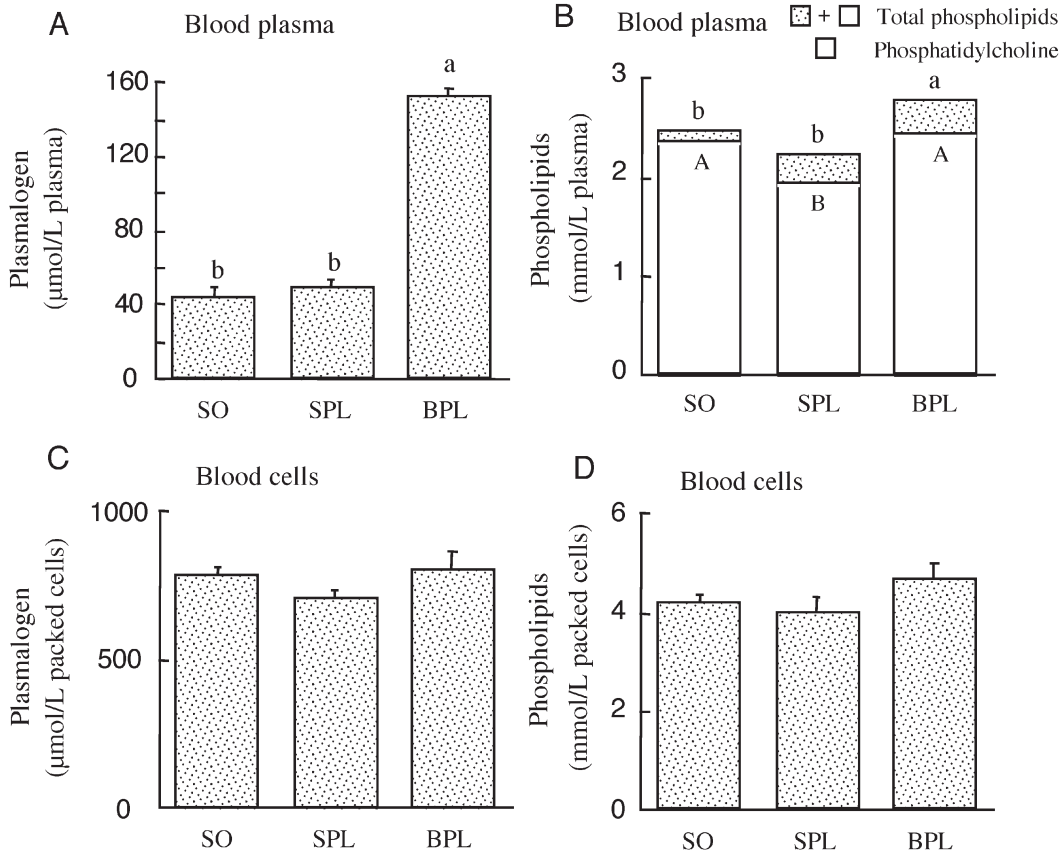

FIG. 3. Plasmalogen (panels A and C) and phospholipid (panels B and D) concentrations in the blood plasma (panels A and B) and blood cells (panels C and D) after feeding test diets containing 100 g test lipid/kg diet for 7 d in experiment 4. Test lipids were SO, SPL, and BPL. Details are provided in the Materials and Methods section. Each value shown is the mean ± SEM for six rats. *P* values were <0.001 (panel A), 0.016 and 0.022 for total and choline phospholipids (panel B), 0.435 (panel C), and 0.277 (panel D). Mean values not sharing a common roman letter are significantly different between groups ($P < 0.05$). For abbreviations see Figures 1 and 2.

The gastric content of phospholipids was higher in the BPL group than in the SPL group at 2 and 4 h. The proportions of phospholipids remaining in the gastrointestinal tract were 44.4 ± 2.62 and 15.2 ± 2.02 mol% of the total ingested lipids at 2 and 4 h, respectively, in the BPL group, and the solids contents in the stomach were 50.0 ± 1.90 and 15.9 ± 0.29 wt% at 2 and 4 h, respectively, in the BPL group, values that were comparable to those in the SO group.

Plasmalogen disappearance (absorption), as estimated from the lipids remaining in the entire alimentary tract, was approximately 50 mol% 2 h after feeding, and that amount was increased to nearly 80 mol% after 4 h (Fig. 1A). Phospholipid absorption in the BPL group was lower than that in the SPL group (Fig. 1B); however, the plasmalogen absorption rate was similar to that of phospholipids in the BPL group.

The plasmalogen concentration in blood plasma was unchanged 2 h after the ingestion of BPL. However, the concentration increased from 2 to 4 h and was two times higher than that after ingestion of SO (Fig. 2A). The total phospholipid concentration in plasma was not affected by time or diet (Fig. 2B).

Experiments 2 and 3 (examination of the fate of plasmalogen under in vitro *and* in situ *conditions).* No degradation of the plasmalogen vinyl ether bonds in the BPL diet was seen after the addition of 3 vol of 0.1 mol HCl/L solution to the diet and incubation at 37°C for 1 h (recovery rate was 100%). The pH of the medium was 3.6 after incubation. However, in the case of the emulsified BPL solution, 80 mol% plasmalogen was degraded under the same acid conditions. The pH of the medium containing 10 wt% BPL emulsion was 1.3 after incubation.

Plasmalogen in the emulsified BPL maintained its vinyl ether structure after 2 h incubation with the postprandial contents of the small intestine of rats, whereas the concentration of plasmalogen mixed and cultured with the cecal bacteria was reduced by 0.99, 7.73, and 16.0 mol% of the initial concentration at 1, 2, and 3 h after the start of the anaerobic culture at 37°C (each value was the mean of three observations). Recoveries of plasmalogen in the initial mixture (0 time) in the experiments using the small intestine and the cecum were 98 and 90 mol%, respectively.

The disappearance (absorption) of plasmalogen in the BPL from the closed loop of the upper small intestine of anesthetized rats was 36.0 ± 6.08 mol%, and that of phospholipids was 24.5 ± 8.05 mol% ($n = 6$) (experiment 3).

Experiment 4 (effects of feeding a diet containing plasmalogen for 7 d). Food intake and body weight gain in the SPL group were lower than those in the SO and BPL groups.

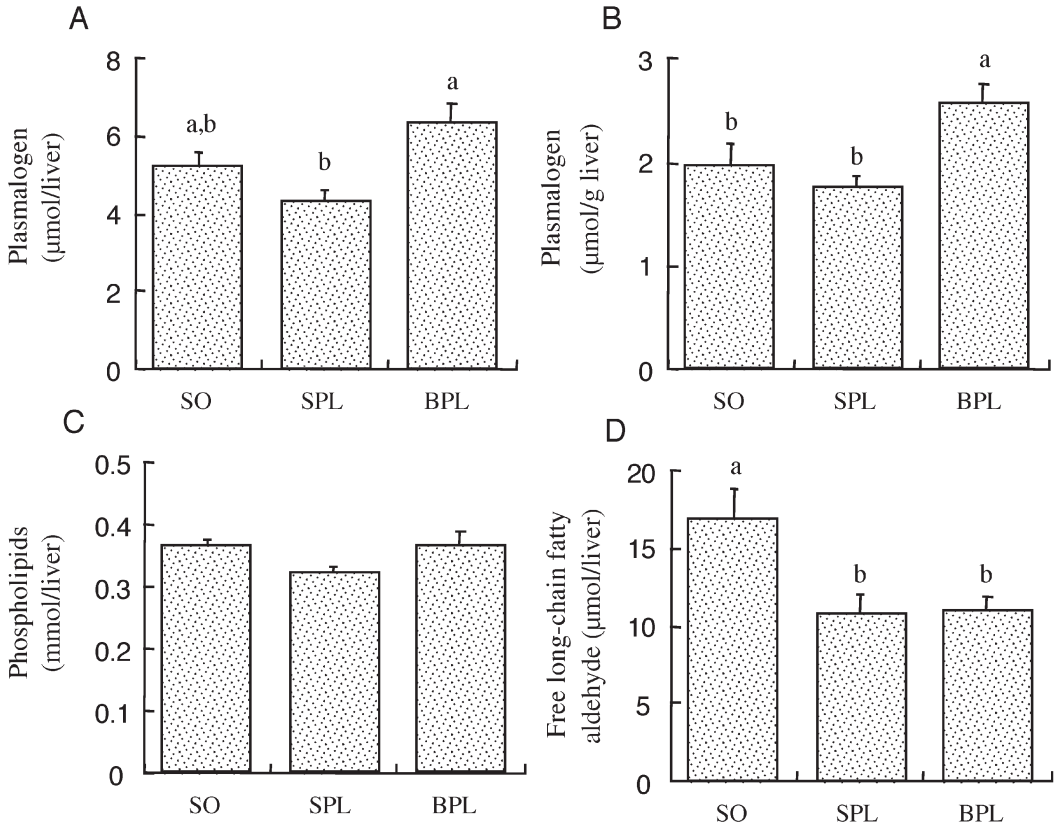

FIG. 4. Liver pools of plasmalogen (A), phospholipids (C), and free fatty aldehyde (D), and the liver concentration of plasmalogen (B) after feeding test diets containing 100 g test lipid/kg diet for 7 d in experiment 4. Test lipids were SO, SPL, and BPL. Each value shown is the mean ± SEM for six rats. *P* values were 0.007 (panel A), 0.007 (panel B), 0.094 (panel C), and 0.010 (panel D). Mean values not sharing a common roman letter are significantly different between groups ($P < 0.05$). For abbreviations see Figures 1 and 2.

Food intake in the SO, SPL, and BPL groups was 15.1, 12.0, and 14.7 g/d ($n = 6$, $P < 0.001$), respectively, and body weight gain was 7.87, 5.76, and 7.54 g/d ($n = 6$, $P < 0.001$), respectively.

Plasmalogen concentration in the blood plasma of rats fed BPL for 7 d was four times higher than that of rats fed SO or SPL (Fig. 3A). However, no differences were observed in the blood cell plasmalogen and phospholipid concentrations among the three groups (Figs. 3C and 3D). Total phospholipid concentration in the blood plasma was slightly, but significantly, higher in the BPL group than in the other groups, whereas the choline-containing phospholipid concentration was not affected by feeding the BPL diet (Fig. 3B).

In the liver, the plasmalogen concentration, but not the total phospholipid pool, was higher in the BPL group than in the SO or SPL group (Figs. 4B and 4C). The free fatty aldehyde pool was lower in rats fed SPL and BPL than in rats fed SO (Fig. 4D).

TG and cholesterol concentrations in the blood plasma were unchanged after feeding diets containing both phospholipids for 7 d (Figs. 5A and 5B). In contrast, the TG and cholesterol pools in the liver were largely decreased after feeding the SPL and BPL diets compared with those fed the SO diet (Figs. 5C and 5D).

There were no significant differences between the SO and BPL groups in the plasmalogen pools in tissues other than the liver. The average plasmalogen concentrations (sum of the SO and BPL groups, $n = 12$) were 60.5 μmol/g dry brain ($P = 0.731$, average dry weight: 308 mg), 21.8 μmol/g dry lung ($P = 0.902$, average dry weight: 182 mg), 7.32 μmol/g dry gastrocremius muscle ($P = 0.163$, average dry weight: 179 mg), 16.0 μmol/g dry kidney ($P = 0.729$, average dry weight: 347 mg), 1.51 μmol/g dry epididymal fat pad ($P = 0.409$), and 15.1 μmol/g dry heart ($P = 0.372$, average dry weight: 141 mg). The dry weight of the epididymal fat pad was lower in the BPL group than in the SO group (781 ± 63 and 981 ± 84 mg, $P = 0.039$).

The wet weight and pH of the gastric contents in the BPL group were very similar to those in the SO group. Means ± SE of both groups were 4.00 ± 0.461 g/rat ($n = 12$, $P = 0.828$) for the wet weight of the gastric contents, and 4.48 ± 0.100 ($n = 12$, $P = 0.526$) for the pH of the gastric contents.

DISCUSSION

To date, few data have been reported concerning the intestinal absorption of dietary plasmalogen or the fate of the ingested plasmalogen. In the present study, we demonstrated

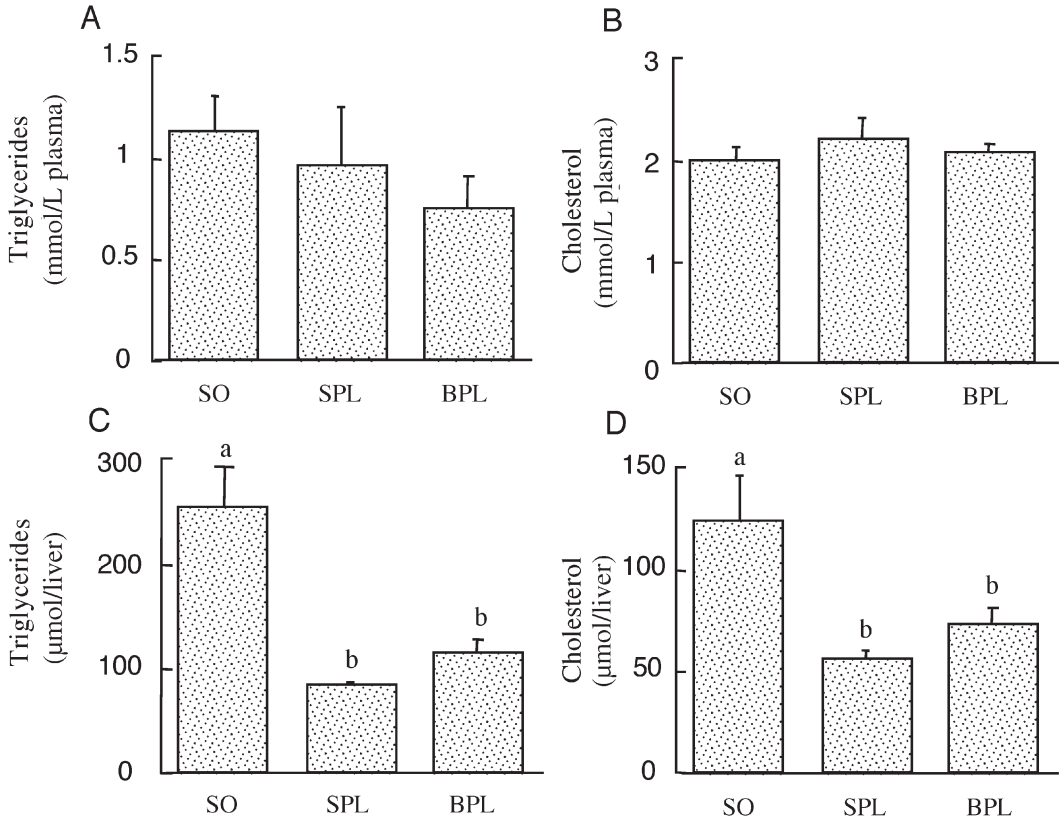

FIG. 5. Plasma concentrations of triglycerides (TG) (A) and cholesterol (B), and liver pools of TG (C) and cholesterol (D) after feeding test diets containing 100 g test lipid/kg diet for 7 d in experiment 4. Test lipids were SO, SPL, and BPL. Each value shown is the mean ± SEM for six rats. *P* values were 0.472 (panel A), 0.626 (panel), 0.001 (panel C), and 0.005 (panel D). Mean values not sharing a common roman letter are significantly different between groups ($P < 0.05$). For abbreviations see Figures 1 and 2.

that the major part of plasmalogen in BPL incorporated into a test diet disappeared from the alimentary tract 4 h after feeding; this result suggests that dietary plasmalogen is absorbed from the intestine. Vinyl ether double bonds in plasmalogen are known to be sensitive to acid. However, we showed that the acid-sensitive double bonds in the test diet were completely preserved during a 1-h exposure to excess amounts of HCl (gastric acid). This was due to the buffering action of some dietary components, such as dietary protein and some mineral salts, which prevented the pH from lowering. Actually, after incubation of a diet containing BPL with excess HCl in experiment 2, the pH was 3.6 and no degradation of the plasmalogen was observed. The pH of the stomach contents of rats was 4.5 after feeding the BPL diet in experiment 4, which was higher than the plasmalogen stability as tested by pH in experiment 2. We also found that the vinyl ether double bonds were not degraded by the contents of the small intestine. These results indicate that intestinal absorption, but not degradation, was responsible for the disappearance of ingested plasmalogen from the alimentary tract. A more important finding is the increase in plasmalogen concentration in blood plasma after feeding the test diet (Fig. 2). Also, a lower level of plasmalogen intake (approximately 2 wt% in diet) for 7 d caused a striking increase in the plasmalogen level in blood plasma, but not in blood cells (Fig. 3). We previously showed that enterally infused plasmalogen was released into the mesenteric lymph in rats cannulated into the mesenteric lymph duct (31). In the present study, we showed that the oral administration of dietary BPL in rats without lymph duct cannulation increased plasma BPL concentrations much more than in the previous study.

In the present study, plasmalogen absorption was estimated from the amount of this lipid remaining in the gastrointestinal tract together with its mucosa because the precise separation of luminal lipids from the mucosal cells was very difficult, especially in the small intestine. The amount of lipid remaining in the alimentary tract included luminal and mucosal plasmalogen derived from the diet, but endogenous lipids were excluded from our estimation of plasmalogen absorption by subtracting the values of the SO group. This means that the absorption rate in this study represents the release of vinyl ether moieties from the intestinal tract into systemic circulation.

We found that considerable amounts of plasmalogen reached the cecum, and we also observed that 16 mol% of plasmalogen was reduced by incubation with the cecal contents for 3 h. These results suggest that the cecal degradation of plasmalogen contributes in part to its disappearance from

the alimentary tract. However, the level of plasmalogen in the cecal contents was not increased 2 h after ingestion. Furthermore, the disappearance of plasmalogen from a closed loop of the upper small intestine was 36 mol% for 90 min in experiment 3. The absorption rate seems to agree with that shown in Figure 1 (50 mol% for 2 h). Thus, cecal degradation may not contribute significantly to the disappearance of plasmalogen. Also, plasmalogen was not excreted *via* the feces during the 4-h period because we observed no increase in the plasmalogen level in the colonic contents (Table 2). It has been reported that some kinds of intestinal bacteria metabolize plasmalogen (32), which agrees with our results. Also, we observed that endogenous plasmalogen, represented by the plasmalogen level in the gastrointestinal contents of the SO (control) group, was much higher in the cecum than in the other segments of the alimentary tract. This finding suggests that cecal bacteria contain plasmalogen. Anaerobic bacteria, including intestinal bacteria such as *Clostridium butyricum*, are known to be rich in plasmalogen (33).

Despite its abundance in various tissues and its importance to cell functions, few data are available on plasmalogen absorption, probably due to difficulties in the purification and quantification of intact plasmalogen. Partial hydrolysis and/or preparation of derivatives is necessary to quantify plasmalogen using chromatography (34). We adopted the iodine addition method to measure blood and tissue plasmalogen, which quantifies the vinyl ether double bonds in plasmalogen in the presence of methanol. The iodine addition method can trace the vinyl ether moiety in the lipid fraction and is suitable for the purposes of the present study. The results of this method represent the plasmalogen "activity" in the samples. Methods using isotopically labeled plasmalogen may be unable to trace intact vinyl ether double bonds, which are the most important structures of this phospholipid.

We found that the plasmalogen level in blood plasma was increased far beyond that in the control group (Fig. 3). Such increases in plasmalogen have not been reported previously. As described above, alkylacylphospholipid is converted to plasmalogen (35), which is possibly involved in the increase of plasmalogen in the blood. However, we did not find a significant amount of alkylacylphospholipid in the extracted BPL (1.0 mol%; Table 2). Thus, the increase in blood plasmalogen is derived from ingested BPL. This lipid is found in lipoprotein in the blood plasma (36). It was also reported that approximately half of the PE in lipoprotein is plasmalogen (37). As shown in Figure 3B, the total phospholipid concentration, but not the choline-containing phospholipid concentration, increased significantly after feeding a BPL diet. The increase in plasmalogen shown in Figure 3A contributed about 50% of the increase in phospholipids other than PC (Fig. 3B). The enrichment of plasmalogen in lipoprotein may reduce the susceptibility of the lipoprotein to oxidative stress and lower the incidence of coronary heart disease.

In contrast to the plasmalogen in blood plasma, feeding BPL did not increase the plasmalogen level in blood cells (Figs. 3C and 3D). The plasmalogen concentration in the blood cells was 20 times higher than that in the blood plasma (Figs. 3A and 3C), and it was reported that phospholipids in the plasma are rapidly incorporated into the red blood cell membrane (13). These results suggest that blood cell plasmalogen is maintained at a constant level.

Our results also show a small but significant increase in the liver plasmalogen concentration (Fig. 4B). Lipoproteins are synthesized in the liver, and a part of the plasmalogen in the lipoproteins may be incorporated into the liver. The increase in liver plasmalogen is possibly associated with the enrichment of plasmalogen in the blood plasma. Plasmalogen concentrations in the brain, heart, lungs, muscle, and fat tissue differed markedly, but were unchanged in spite of the large increase in blood plasma plasmalogen. Das and Hajra (35) also reported that ingested alkylglycerol is converted efficiently to plasmalogen and distributed to various tissues. However, they reported no changes in the level of plasmalogen. The level of plasmalogen in these organs or tissues may also be kept constant. The increase in plasmalogen in the blood plasma and liver described above seems to be much smaller than the amount absorbed from the intestine, as shown in Figure 1. This finding suggests the existence of high-clearance activities for the vinyl ether moiety in tissues. Recently, lysoplasmalogenase (EC 3.3.2.2, EC 3.3.2.5), by which lysoplasmalogen is degraded to fatty aldehyde, was found in the small intestine (38). The present study shows that the hepatic free fatty aldehyde pool did not increase, but rather decreased by feeding BPL. It seems that dietary plasmalogen is not degraded to free fatty aldehyde. However, we cannot exclude the possibility that the fatty aldehydes are immediately converted to fatty acids (FA) by aldehyde dehydrogenase. Thus, further work is needed to clarify the fate of absorbed plasmalogen.

Feeding a BPL diet lowered cholesterol and TG concentrations in the liver in a similar manner as feeding an SPL diet (Figs. 5C and 5D). These results show that the ethanolamine-rich phospholipid affects lipid metabolism as well as the SPL; however, the contribution of plasmalogen to the effects of BPL are not known.

We conclude that the major part of dietary plasmalogen disappeared from the intestine through intestinal absorption, not degradation into the intestinal lumen. The level of plasmalogen in blood plasma was markedly increased, which may provide increased protection against lipoprotein oxidation. In this study, we used a bovine BPL fraction as a ready source of plasmalogen; however, other sources may be available.

ACKNOWLEDGMENT

We thank Dr. Ryouta Maeba of the School of Medicine, Teikyo University, for his advice and support in the present study.

REFERENCES

1. Horrocks, L.A. (1972) Content, Composition and Metabolism of Mammalian and Avian Lipids That Contain Ether Groups, in *Ether Lipids: Chemistry and Biology* (Snyder, F., ed.), pp. 177–272, Academic Press, New York.

2. Nagan, N., and Zoeller, R.A. (2001) Plasmalogens: Biosynthesis and Functions, *Prog. Lipid Res. 40*, 199–229.
3. Lohner, K. (1996) Is the High Propensity of Ethanolamine Plasmalogens to Form Non-lamellar Lipid Structures Manifested in the Properties of Biomembranes? *Chem. Phys. Lipids 81*, 167–184.
4. Zoeller, R.A., Morand, O.H., and Raetz, C.R. (1988) A Possible Role for Plasmalogens in Protecting Animal Cells Against Photosensitized Killing, *J. Biol. Chem. 263*, 11590–11596.
5. Zoeller, R.A., Lake, A.C., Nagan, N., Gaposchkin, D.P., Legner, M.A., and Lieberthal, W. (1999) Plasmalogens as Endogenous Antioxidants: Somatic Cell Mutants Reveal the Importance of the Vinyl Ether, *Biochem. J. 338*, 769–776.
6. Zommara, M., Tachibana, N., Mitsui, K., Nakatani, N., Sakono, M., Ikeda, I., and Imaizumi, K. (1995) Inhibitory Effect of Ethanolamine Plasmalogen on Iron- and Copper-Dependent Lipid Peroxidation, *Free Radic. Biol. Med. 18*, 599–602.
7. Maeba, R., and Ueta, N. (2003) Ethanolamine Plasmalogen and Cholesterol Reduce the Total Membrane Oxidizability Measured by the Oxygen Uptake Method, *Biochem. Biophys. Res. Commun. 302*, 265–270.
8. Maeba, R., and Ueta, N. (2003) Ethanolamine Plasmalogens Prevent the Oxidation of Cholesterol by Reducing the Oxidizability of Cholesterol in Phospholipid Bilayers, *J. Lipid Res. 44*, 164–171.
9. Engelmann, B., Bräutigam, C., and Thiery, J. (1994) Plasmalogen Phospholipids as Potential Protectors Against Lipid Peroxidation of Low Density Lipoproteins, *Biochem. Biophys. Res. Commun. 204*, 1235–1242.
10. Jurgens, G., Fell, A., Ledinski, G., Chen, Q., and Paltauf, F. (1995) Delay of Copper-Catalyzed Oxidation of Low Density Lipoprotein by *in vitro* Enrichment with Choline or Ethanolamine Plasmalogens, *Chem. Phys. Lipids 77*, 25–31.
11. Mandel, H., Sharf, R., Berant, M., Wanders, R.J., Vreken, P., and Aviram, M. (1998) Plasmalogen Phospholipids Are Involved in HDL-Mediated Cholesterol Efflux: Insights from Investigations with Plasmalogen-Deficient Cells, *Biochem. Biophys. Res. Commun. 250*, 369–373.
12. Farooqui, A.A., Rapoport, S.I., and Horrocks, L.A. (1997) Membrane Phospholipid Alterations in Alzheimer's Disease: Deficiency of Ethanolamine Plasmalogens, *Neurochem. Res. 22*, 523–527.
13. Engelmann, B., Bräutigam, C., Kulschar, R., Duhm, J., Prenner, E., Hermetter, A., Richter, W.O., Thiery, J., and Seidel, D. (1994) Reversible Reduction of Phospholipid Bound Arachidonic Acid After Low Density Lipoprotein Apheresis. Evidence for Rapid Incorporation of Plasmalogen Phosphatidylethanolamine into the Red Blood Cell Membrane, *Biochim. Biophys. Acta 1196*, 154–164.
14. Das, A.K., Holmes, R.D., Wilson, G.N., and Hajra, A.K. (1992) Dietary Ether Lipid Incorporation into Tissue Plasmalogens of Humans and Rodents, *Lipids 27*, 401–405.
15. Blank, M.L., Cress, E.A., Smith, Z.L., and Snyder, F. (1992) Meats and Fish Consumed in the American Diet Contain Substantial Amounts of Ether-Linked Phospholipids, *J. Nutr. 122*, 1656–1661.
16. Reeves, P.G. (1989) AIN-76 Diet: Should We Change the Formulation? *J. Nutr. 119,* 1081–1082.
17. American Institute of Nutrition (1977) Report of the American Institute of Nutrition *ad hoc* Committee on Standards for Nutritional Studies, *J. Nutr. 107*, 1340–1348.
18. American Institute of Nutrition (1980) Second Report of the *ad hoc* Committee on Standards for Nutritional Studies, *J. Nutr. 110*, 1726.
19. Harper, A.E. (1959) Amino Acid Balance and Imbalance. 1. Dietary Level of Protein and Amino Acid Imbalance, *J. Nutr. 68*, 405–418.
20. Folch, J. (1942) Brain Cephalin, a Mixture of Phosphatides. Separation from It of Phosphatidylserine, Phosphatidylethanolamine, and a Fraction Containing an Inositol Phosphatide, *J. Biol. Chem. 146*, 35–44.
21. Kasai, T., Tanaka, T., Kiriyama, S., and Sonoyama, K. (1993) Facile Preparation of Rat Intestinal Mucosa for Assay of Mucosal Enzyme Activity, *J. Nutr. Sci. Vitaminol. 39*, 399–403.
22. Folch, J., Lees, M., and Sloane Stanley, G.H. (1957) A Simple Method for the Isolation and Purification of Total Lipides from Animal Tissues, *J. Biol. Chem. 226*, 497–509.
23. Gottfried, E.L., and Rapport, M.M. (1962) The Biochemistry of Plasmalogens. I. Isolation and Characterization of Phosphatidal Choline, a Pure Native Plasmalogen, *J. Biol. Chem. 237*, 329–333.
24. Williams, J.N., Jr., Anderson, C.E., and Jasik, A.D. (1962) A Sensitive and Specific Method for Plasmalogens and Other Enol Esters, *J. Lipid Res. 3*, 378–381.
25. Huque, T., Brand, J.G., Rabinowitz, J.L., and Gavarron, F.F. (1987) A Comparative Study of Three Methods for the Estimation of Total Plasmalogens in Lingual Taste Epithelium and Other Tissues, *Comp. Biochem. Physiol. B 86*, 135–139.
26. Pries, C., and Böttcher, C.J.F. (1965) The Determination of Free and Plasmalogen-Bound Aldehydes in Lipid Fractions, *Biochim. Biophys. Acta 98*, 329–334.
27. Nishihira, J., Ishibashi, T., Sawamura, Y., and Hosokawa, M. (1994) The Role of Ether-Linked Glycerophospholipids in Cytotoxic T Lymphocytes, *Biochem. Mol. Biol. Int. 33*, 137–146.
28. Nishimukai, M., Hara, H., and Aoyama, Y. (2003) Enteral Administration of Soybean Lecithin Enhanced Lymphatic Absorption of Triacylglycerol in Rats, *Br. J. Nutr. 90*, 565–571.
29. Duncan, D.B. (1995) Multiple Range and Multiple F Tests, *Biometrics 11*, 1–42.
30. Sokal, R.R., Rohlf, F.J., and Fujii, K. (1983) Correlation, in *Introduction to Biostatistics,* Japanese edn., pp. 320–348, Kyoritsu Shuppan, Tokyo.
31. Hara, H., Wakisaka, T., and Aoyama, T. (2003) Lymphatic Absorption of Plasmalogen in Rats, *Br. J. Nutr. 90*, 29–32.
32. Mott, G.E., and Brinkley, A.W. (1979) Plasmenylethanolamine: Growth Factor for Cholesterol-Reducing Eubacterium, *J. Bacteriol. 139*, 755–760.
33. Hagen, P.O., and Goldfine, H. (1967) Phospholipids of *Clostridium butyricum*. 3. Further Studies on the Origin of the Aldehyde Chains of Plasmalogens, *J. Biol. Chem. 242*, 5700–5708.
34. Blank, M.L., Cress, E.A., and Snyder, F. (1987) Separation and Quantitation of Phospholipid Subclasses as Their Diradylglycerobenzoate Derivatives by Normal-Phase High-Performance Liquid Chromatography, *J. Chromatogr. 392*, 421–425.
35. Das, A.K., and Hajra, A.K. (1988) High Incorporation of Dietary 1-*O*-Heptadecyl Glycerol into Tissue Plasmalogens of Young Rats, *FEBS Lett. 227*, 187–190.
36. Blank, M.L., Smith, Z.L., Cress, E.A., and Snyder, F. (1994) Molecular Species of Ethanolamine Plasmalogens and Transacylase Activity in Rat Tissues Are Altered by Fish Oil Diets, *Biochim. Biophys. Acta 1214*, 295–302.
37. Bräutigam, C., Engelmann, B., Reiss, D., Reinhardt, U., Thiery, J., Richter, W.O., and Brosche, T. (1996) Plasmalogen Phospholipids in Plasma Lipoproteins of Normolipidemic Donors and Patients with Hypercholesterolemia Treated by LDL Apheresis, *Atherosclerosis 119*, 77–88.
38. Jurkowitz, M.S., Horrocks, L.A., and Litsky, M.L. (1999) Identification and Characterization of Alkenyl Hydrolase (lysoplasmalogenase) in Microsomes and Identification of a Plasmalogen-Active Phospholipase A_2 in Cytosol of Small Intestinal Epithelium, *Biochim. Biophys. Acta 1437*, 142–156.

[Received May 6, 2002, and in final form and accepted November 4, 2003]