1-*O*-Alk-1′-enyl-2-acyl and 1-*O*-Alkyl-2-acyl Glycerophospholipids in White Muscle of Bonito *Euthynnus pelamis* (Linnaeus)

Toshiaki Ohshima*, Shun Wada and **Chiaki Koizumi**
Department of Food Science and Technology, The Tokyo University of Fisheries, Konan 4, Minato-ku, Tokyo 108, Japan

The existence of ether-linked phospholipids, including 1-*O*-alk-1′-enyl-2-acyl and 1-*O*-alkyl-2-acyl-*sn*-glycero-3-phosphocholines and ethanolamines, in bonito *Euthynnus pelamis* (Linnaeus) white muscle, was investigated by gas chromatography and gas chromatography–mass spectrometry. Chemical ionization (*iso*-butane) mass spectrometry of trimethylsilyl ethers derived from the corresponding ether-linked glycerophospholipids proved effective not only for determining molecular weights but also for structural identification based on the ions $[M - R]^+$, $[M - RO]^+$ and $[M + 1]^+$. 1-*O*-Alk-1′-enyl-2-acyl-*sn*-glycero-3-phosphocholine and ethanolamine accounted for 3.0–6.0% and 3.6–7.6% of the total glycerophospholipids, respectively. 1-*O*-Alkyl-2-acyl-*sn*-glycero-3-phosphocholine and ethanolamine were also determined for one fish and accounted for 1.4% and 0.6% of the total glycerophospholipids, respectively. The predominant long chains in the *sn*-1 position of the glycerol moieties were 16:0, 18:0 and 18:1 in the case of the alkenylacyl and alkylacyl components. Fatty acid distribution of individual glycerophospholipids was also determined.
Lipids 24, 363–370 (1989).

Ether linked phospholipids, including 1-*O*-alkyl-2-acyl-*sn*-glycero-3-phosphocholine and ethanolamine and 1-*O*-alk-1′-enyl-2-acyl-*sn*-glycero-3-phosphocholine and ethanolamine (plasmalogens), occur in various species of marine organisms (1–3). Lewis reported on the alkyl chain composition of total phospholipids of anchovy, shrimp and squid (4). Marine worms (5), abalone (6), sponges (7) and starfish (8) also contain a significant amount of plasmalogen. The gills of crustaceans such as crayfish and lobster, and the body lipids of certain species of crab, mussel, oyster and scallop are rich sources of plasmalogen (9–12). Goldfish brain (13) and garfish olfactory nervous systems (14) are well known to contain alk-1′-enylacyl glycerophospholipids as choline and ethanolamine analogues of the better-known diacyl glycerophospholipids, although their biological significance is not well understood (15). There is little information on ether-linked phospholipids in fish muscle; the presence of plasmalogen was confirmed in South African pilchard flesh by a positive Schiff reaction (16). The occurrence of plasmalogen has also been reported in carp muscle mitochondria (17), rock trout, saury, sardine, codfish (18) and dogfish (19).

In the present study, ether-linked phospholipids of fish muscle were investigated and found to be present in bonito white muscle at a relatively high level. Identification of 1-*O*-alkylacyl and 1-*O*-alk-1′-enylacyl glycerophospholipids of bonito were carried out by gas liquid chromatography (GLC) combined with chemical ionization (CI) mass spectrometry (MS) of the corresponding trimethylsilyl (TMS) ether derivatives.

*To whom correspondence should be addressed.

Abbreviations: CI, chemical ionization; CPG, choline glycerophospholipid; DMA, dimethylacetal; EI, electron impact; EPG, ethanolamine glycerophospholipid; GLC, gas-liquid chromatography; LCA, long chain aldehyde; LPC, lyso choline glycerophospholipid; LPE, lysoethanolamine glycerophospholipid; MS, mass spectrometry; O, origin; PUFA, polyunsaturated fatty acids; SF, solvent front; TLC, thin-layer chromatography; TMS, trimethylsilyl.

MATERIALS AND METHODS

Standard materials. Bovine heart ethanolamine plasmalogen and 1-palmityl glyceryl ether were purchased from Serdary Research Laboratories (London, Ontario, Canada).

Lipid extraction and fractionation. White muscle lipid of bonito, *Euthynnus pelamis* (Linnaeus), was extracted with chloroform/methanol according to the Bligh and Dyer procedure (20). The phospholipid fraction was separated from other lipids by column chromatography on Bio-Beads S-X2, using benzene as eluent (21).

Quantitation of phospholipid species. Phospholipid classes were separated from each other by two-dimensional thin-layer chromatography (TLC) on precoated Silica Gel G plates (0.25 mm thickness, Merck Japan Ltd., Tokyo) using the solvent system chloroform/methanol/water/acetic acid (65:35:2:1, v/v/v/v) in the first direction and chloroform/methanol/water (60:30:8, v/v/v) in the second direction. The spots corresponding to phospholipids were visualized by exposing the plates to iodine vapor and were each scraped off. The phospholipids were extracted from the silica gel with chloroform/methanol (2:1, v/v). Phosphorus content was determined spectrophotometrically according to the method of Bartlett (22). The ratios of 1-*O*-alkyl-2-acyl vs 1,2-diacyl compounds were estimated by measuring total fatty acids, using 13:0 methyl ester as internal standard (23).

Mild acid hydrolysis and Iatroscan thin-layer chromatography. An aliquot of a chloroform solution of the phospholipid class was evaporated to dryness in a round-bottomed flask, which was then placed in a glass tank saturated with hydrochloric acid fumes for 5 min at room temperature (24). After removal of hydrochloric acid by flushing with a nitrogen stream, the acid-hydrolyzed phospholipid in the flask was subjected to Iatroscan TLC on Chromarods S-II (Dia-Iatron Co., Tokyo, Japan) using a double development technique in the same direction with acetone first to move neutral lipids and then a mixture of chloroform/methanol/acetic acid/water to resolve polar lipids (25).

Lipid derivatization. Phospholipids were reduced with Vitride reagent (Tokyo Kasei Co., Ltd., Tokyo, Japan) to the corresponding 1-alk-1′-enyl or 1-alkyl *sn*-glycerols (26). 1-*O*-Alkyl and 1-*O*-alk-1′-enyl glycerols were separated from each other by TLC on Silica Gel G (diethyl ether/ammonia, 100:0.25, v/v) and extracted from the silica gel with chloroform/methanol (1:2, v/v). Treatment with a mixture of hexamethyldisilazane and trimethylchlorosilane in dry pyridine produced the TMS ether derivatives (27).

Dimethylacetal (DMA) derivatives of long chain aldehydes, which were liberated from the corresponding

alkenylacyl phospholipids by the mild acid hydrolysis mentioned above, were prepared by heating the aldehydes under reflux with 5% methanolic hydrogen chloride (28).

Determination of 1-O-alkyl-2-acyl, 1-O-alkenyl-2-acyl, and 1,2-diacyl chain distribution. The individual phospholipid classes were analyzed according to Mueller *et al.* (29). Our preliminary examination showed that iodine vapor used for visualizing the spots on the TLC plate could cause decomposition of the alkenylacyl phospholipids. Therefore, 2′,7′-dichlorofluorescein in ethanol was used for detection of the samples prepared for the analysis of fatty chain distribution. The purified choline and ethanolamine glycerophospholipid fractions were treated with phospholipase C (Boehringer Mannheim GmbH-Yamanouchi, Tokyo, Japan) to yield diradyl glycerols (30). The products were then acetylated (31) to the corresponding 1-*O*-alkyl-2-acyl-3-acetylglycerol, 1-*O*-alk-1′-enyl-2-acyl-3-acetylglycerol and 1,2-diacyl-3-acetylglycerol. These three acetylglycerols thus obtained were separated from each other by preparative TLC on Silica Gel G using the solvent system of petroleum ether/diethyl ether/acetic acid (90:10:1, v/v/v). The purified acetyl glycerols were saponified individually with 1 N KOH in ethanol at 85°C for one hr. Unsaponifiable matter (1-*O*-alkylglycerol or 1-*O*-alk-1′-enylglycerol) was extracted by *n*-hexane and converted to the corresponding TMS ether derivatives as mentioned above to analyze the aliphatic chain distribution in the *sn*-1 position of the glycerol moiety. The fatty acid compositions in the *sn*-1 and *sn*-2 positions of 1,2-diacyl glycerophospholipid and in the *sn*-2 position of ether-linked glycerophospholipids were analyzed as their methyl esters by GLC as mentioned below.

Gas liquid chromatography and gas chromatography-mass spectrometry (GC-MS). TMS ether derivatives of 1-*O*-alkylglycerol and 1-*O*-alk-1′-enylglycerol were analyzed by GC using a Shimadzu GC 12A instrument (Kyoto, Japan) equipped with a Supelcowax-10 fused silica open tubular capillary column (30 m × 0.25 mm i.d., Supelco Japan Ltd., Tokyo) and a Shimadzu CLH 702 split injector. The injector and the column were held at 250 and 210°C, respectively, the split ratio was 1:60, and helium was used as carrier gas at a constant inlet pressure of 1.2 Kg/cm^2.

Analysis of fatty acid methyl esters was performed on a Shimadzu GC 8APF gas chromatograph with a Chromatopack CR6A data processor. The conditions were similar to those of the TMS ether derivatives analysis, except that the column temperature was at 195°C.

Mass spectrometric analysis of the TMS ether derivatives was carried out on a Shimadzu GC-MS 9020 DF mass spectrometer fitted with a CI source to which an outlet of Supelcowax-10 column (30 m × 0.25 mm i.d.) was connected directly. The column and a Shimadzu movable on-column injector were kept isothermally at 220 and 300°C, respectively. Data acquisitional processing was carried out by an on-line Shimadzu GC-MS PAC 1100 data system. The sample, which was separated by GLC, was ionized in the CI mode by adding *iso*-butane as a reagent gas into the ion source at a pressure of about 1 Torr, using 200 eV electron beam energy, 200 μA emission current, 3 KV acceleration voltage and a source temperature of 250°C.

Electron impact (EI) mass spectra of DMA derivatives of long chain aldehydes were obtained by a Shimadzu QP 1000 quadrupole mass spectrometer with a Supelcowax-10 column (30 m × 0.25 mm i.d.), under the conditions of 70 eV electron beam energy, 3 KV acceleration energy and a source temperature of 250°C.

RESULTS AND DISCUSSION

Iatroscan TLC-flame ionization detection analyses of acid-labile phospholipid. Separations of bonito white muscle phospholipids before and after mild acid hydrolysis are compared in Figure 1. The phospholipid before acid hydrolysis showed four well-resolved peaks corresponding to choline and ethanolamine glycerophospholipids (abbreviated as CPG and EPG respectively, in Fig. 1) and lysoglycerophospholipids (LPC and LPE in the figure); the peak ascribed to LPC was larger than that of CPG and the peak ascribed to LPE was smaller than that of EPG, as had been reported previously (32,33). After mild acid hydrolysis, the peaks of LPC and LPE were large, compared with those of CPG and EPG, respectively, and a moderate peak was found in the highest moving band on the Chromarod S-II. The highest peak in Rf was consistent with that of long chain aldehydes. These changes clearly indicate that the EPG and CPG in the phospholipids were hydrolyzed with hydrochloric acid into LPE, LPC and LCA, respectively. It is well accepted that an ether bond in the alk-1′-enyl group is acid-labile, and that phospholipids having such chemical bonds are readily hydrolyzed into lysophospholipids and LCA under mild acidic condition such as saturated hydrochloric acid fumes (8). Therefore, the LPE and LPC that were produced on mild acid hydrolysis seemed to indicate the existence of alk-1-enyl ether bonds in the precursor lipid classes, EPG and CPG, of bonito white muscle phospholipids. These observations correspond well with the results of Foot and Clandinin, in which they separated those hydrolysis products of acid-labile phospholipids by Iatroscan TLC-flame ionization detection (FID) on Chromarod S-II (34).

Identification of ether-linked glycerophospholipids. Gas chromatograms of 1-*O*-alk-1′-enyl-2,3-TMS ether glycerols

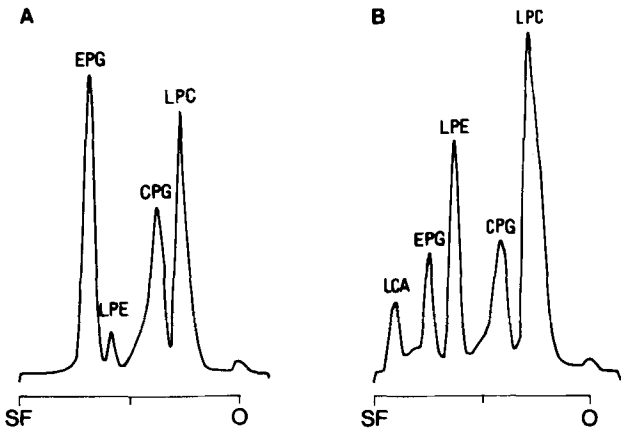
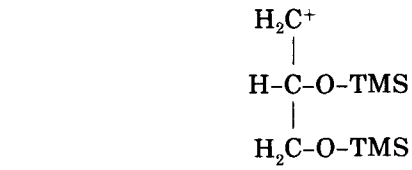
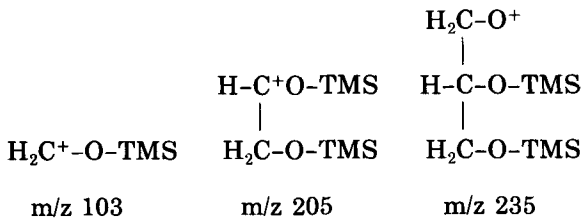


FIG. 1. Iatroscan TLC-FID chromatograms of the phospholipids of bonito white muscle before (A) and after (B) mild acidic hydrolysis. EPG, ethanolamine glycerophospholipid; LPE, lysoethanolamine glycerophospholipid; CPG, choline glycerophospholipid; LPC, lyso choline glycerophospholipid; LCA, long chain aldehyde; O, origin; SF, solvent front.

derived from a commercially available bovine heart ethanolamine plasmalogen (authentic), a laboratory-prepared bovine heart phospholipid, and a bonito white muscle phospholipid are shown in Figure 2 (A, B and C, respectively). Wood and Snyder reported that TMS ether derivatives of alkyl glycerol were successfully separated by GLC on a polar liquid phase column such as diethyleneglycol succinate polyester (35). Therefore, a Supelcowax-10 column, similar to a DEGS column in polarity of liquid phase, was used in the present study. In all samples, well resolved peaks were obtained on chromatograms: 6, 13 and 13 peaks in the authentic plasmalogen standard, bovine heart and bonito white muscle phospholipids, respectively. The CI spectrum of the most prominent peak of the authentic plasmalogen standard (No. 8 in Fig. 2A) was determined and is illustrated in Figure 3A. The spectrum showed a base peak at m/z 219 which indicates formation of the following ion by cleavage of alk-1′-enyl group from the glycerol moiety:



The relatively intense fragment peaks at m/z 103, m/z 205 and m/z 235 in Figure 3A strongly suggest that the following fragments, from the glycerol backbones in the TMS ether derivatives, are produced:



The peak at m/z 459 was considered attributable to $[M + 1]^+$, indicating the TMS ether derivative to have

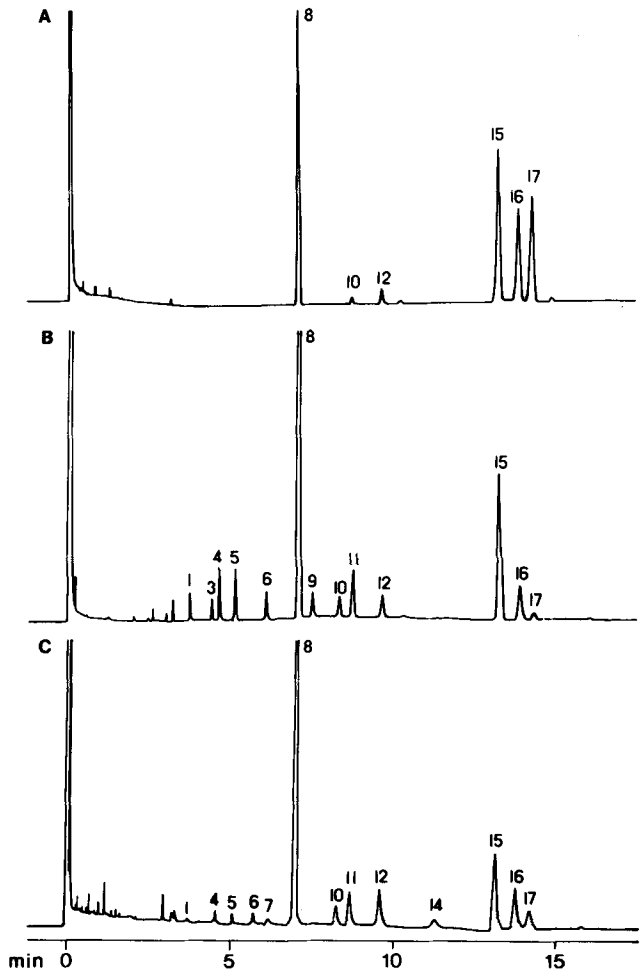


FIG. 2. GC analyses of the 2,3-TMS ether of 1-*O*-alk-1′-enyl-glycerols from (A) bovine heart muscle ethanolamine plasmalogen (authentic), (B) bovine heart muscle phospholipids (prepared in this laboratory) and (C) bonito white muscle phospholipids. See the text for peak components. Supelcowax-10 FQ column, 25 m × 0.25 mm i.d. and operated at 210°C, with helium as carrier gas.

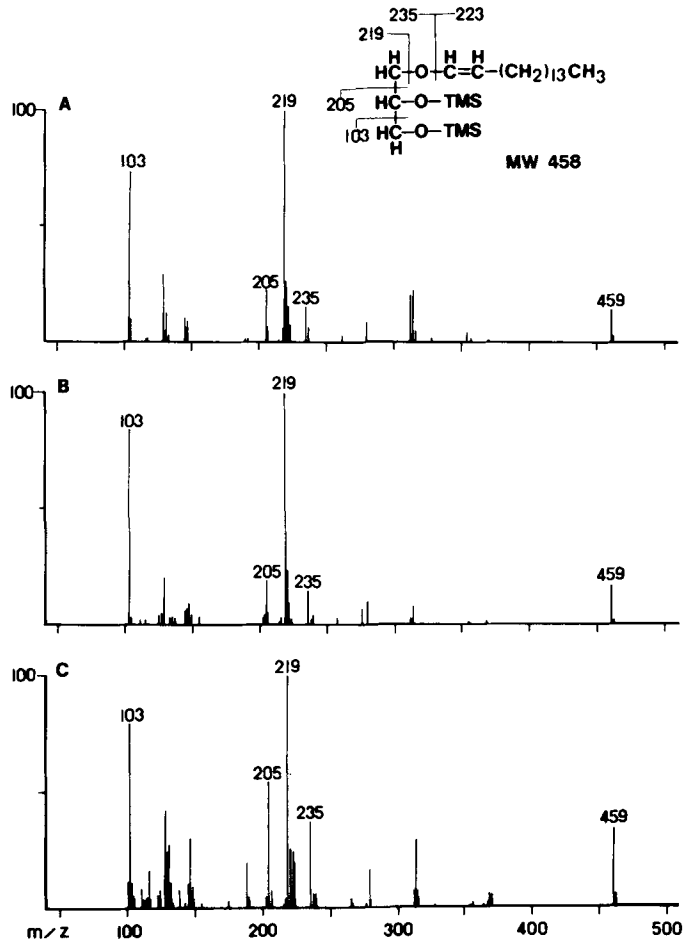


FIG. 3. CI (*iso*-butane) mass spectra of the predominant 2,3-TMS ethers of 1-*O*-alk-1′-enyl-glycerols derived from (A) bovine heart muscle ethanolamine plasmalogen (authentic), (B) bovine heart muscle phospholipids (prepared in this laboratory) and (C) bonito white muscle phospholipids. 200 eV electron beam energy, 200 μA emission current, 3 KV accelerating voltage, and source temperature of 250°C.

a molecular weight of 458. The characteristic fragmentation shown in Figure 3A definitely suggests that, in the TMS ether derivative, a hexadecenyl ether group is linked to the glycerol moiety.

Each TMS ether derivative prepared from the bovine heart phospholipid (Fig. 2B) and the bonito white muscle phospholipid (Fig. 2C) presented similar chromatographic patterns, although the former lacked peaks No. 7 and No. 14, while the latter lacked peaks No. 3 and No. 9. Of those peaks on the chromatograms, retention times (Rt) of the peaks of No. 8, 10, 12, 15, 16 and 17 were consistent with those on the chromatogram of the authentic plasmalogen standard (Fig. 2A). The CI mass spectra of three peaks numbered 8 closely corresponded to one another, as shown in Figure 3 (A, B and C).

The mass spectra in Figure 3 (A, B and C), however, did not show any specific fragment ions which offered information concerning a location of the double bond on the hexadecenyl ether group. In order to clarify this point, the alkenyl group on the glycerophospholipid was completely hydrolyzed to the corresponding LCA under mild acid conditions (8), and the chain structure of the DMA derivatives of the LCA thus obtained was explored by EI-MS.

Figures 4A and 4B show the gas chromatogram of the DMA derivatives of the LCA prepared from the bonito phospholipid by mild acid hydrolysis and the EI mass spectrum of peak No. 8 of Figure 4A, respectively. The spectrum yielded characteristic ions of $[M - 31]^+$ (due to the loss of methoxy group from the parent ion) and of m/z 75 (due to $[CH(OCH_3)_2]^+$) which usually appear in EI spectra of DMA derivatives (36). From these results, it is obvious that the predominant LCA has a structure of $CH_3(CH_2)_{14}CHO$, which is liberated with acid hydrolysis from the 1-*O*-alk-1′enyl group of plasmalogen prepared from the bonito phospholipid. In addition to this, the difference in mass number units between molecular ion and m/z 235 ion, i.e. $(M^+ - 235)$, is proposed to reflect the molecular weight of the alk-1′-enyl group; in the case of 1-*O*-alk-1′-enyl-2,3-TMS ether glycerol (Fig. 3A), mass number unit $(M^+ - 235)$, i.e. 223 in mass number unit, corresponds to the molecular weight of the ion $[CH{=}CH(CH_2)_{13}CH_3]^+$ (refer to Fig. 3A). On the basis of these results, it is concluded that 1-*O*-alk-1′-enyl glycerophospholipids certainly exist in bonito white muscle. The other components corresponding to the peaks shown in Figures 2A, 2B and 2C were identified in the same manner as follows: 1, 14:0; 3, *iso*-15:0; 4, *anteiso*-15:0; 5, 15:0, 6, *iso*-16:0; 7, *anteiso*-16:0; 9, 16:1; 10, *iso*-17:0; 11, *anteiso*-17:0; 12, 17:0; 14, *iso*-18:0; 15, 18:0; 16, 18:1; 17, 18:1 (abbreviated as carbon number:number of double bonds in the alkenyl group). The peaks numbered 2 and 13 are omitted on the chromatogram, because the alkyl components having the corresponding carbon number and number of double bonds were detected as a result of GLC analysis, as will be mentioned below (Fig. 5B).

Figure 5 compares gas chromatograms of 1-*O*-alkyl-2,3-TMS ether derivatives prepared from an authentic hexadecyl ether glycerol (A) and of bonito white muscle (B). The retention time of the most prominent peak in the chromatogram of the bonito phospholipid was consistent with that of the authentic hexadecyl ether glycerol (Figs. 5A and 5B). The CI mass spectra of these peaks are illustrated in Figure 6. The TMS ether derivatives from the bonito sample yielded m/z 461 ion (base peak) as $[M + 1]^+$, and m/z 205, m/z 219 and m/z 103 ions occurred from their glycerol backbone similarly in the case

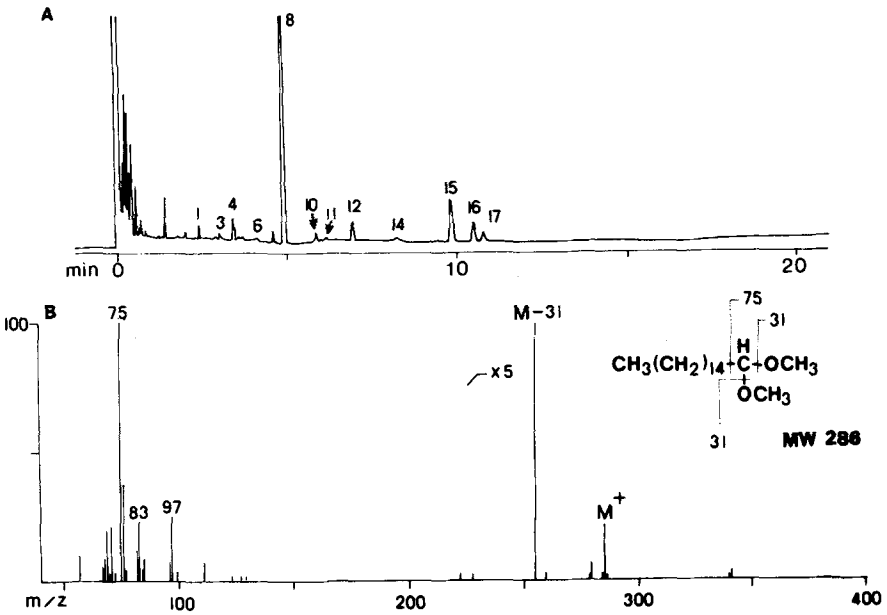


FIG. 4. GC analysis of dimethylacetal derivatives of long chain aldehydes liberated from 1-*O*-alk-1′-enyl-2-acyl glycerophospholipid of bonito white muscle by mild acid hydrolysis (A) and a typical EI mass spectrum of peak No. 8 (B). 1, 14:0; 3, *iso*-15:0; 4, *anteiso*-15:0; 6, *iso*-16:0; 10, *iso*-17:0; 11, *anteiso*-17:0; 12, 17:0; 14, *iso*-18:0; 15, 18:0; 16, 18:1; 17, 18:1. The components of No. 16 and No. 17 are positional isomers. GLC conditions are similar to those of Fig. 2, but column temperature was 195°C.

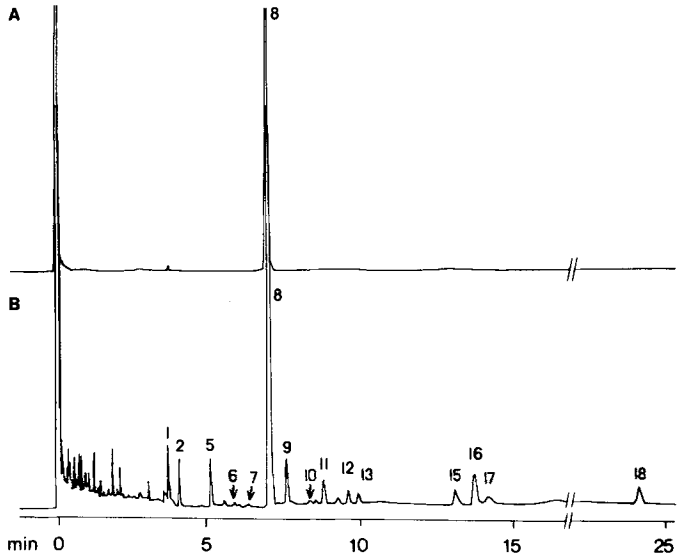


FIG. 5. GLC analyses of the 2,3-TMS ethers of 1-*O*-alkyl-glycerols derived from (A) authentic 1-*O*-hexadecyl glycerol and (B) bonito white muscle phospholipids. The analysis was performed under the conditions given in Fig. 2.

of 1-*O*-alk-1′-enyl-2,3-TMS ether derivatives, which have already been mentioned, although the 1-*O*-alkyl-2,3-TMS ether glycerol derivative did not yield a fragment ion of m/z 235. This fragmentation pattern in CI-MS of the sample prepared from bonito white muscle accorded well with that of authentic hexadecyl ether glycerol which is the component of peak No. 8 in Figure 5A. Similarly, the following results were obtained from the peak components shown in Figure 5B: 1, 14:0; 2, 14:1; 5, 15:0; 6, *iso*-16:0; 7, *anteiso*-16:0; 9, 16:1; 10, *iso*-17:0; 11, *anteiso*-17:0; 12, 17:0; 13, 17:1; 15, 18:0; 16, 18:1; 17, 18:1; 18, 20:0. The peaks numbered 16 and 17 were separated from each other as positional isomers.

Content of ether-linked glycerophospholipids. Table 1 lists the phospholipid composition of bonito white muscle. The total amount of alkylacyl glycerophospholipid and diacyl glycerophospholipid was determined in the fish samples numbered 1 to 5. Only in fish sample No. 6 were the choline and ethanolamine glycerophospholipids isolated separately from other phospholipids by column chromatography on silicic acid (37), and the proportional ratio of 1-*O*-alkyl-2-acyl glycerophospholipid and 1,2-diacyl glycerophospholipid was determined.

In the case of the choline glycerophospholipid, the alk-1′-enylacyl analogue accounted for 3.0–6.0% of the total phospholipid (5.7–11.2% of choline glycerophospholipids). The level of alkylacyl analogue was very low, only 2.4% of choline glycerophospholipids in fish sample No. 6. The most prominent component of the phospholipids seemed to be diacyl, accounting for 91.4% of choline glycerophospholipids of fish sample No. 6. In the case of ethanolamine glycerophospholipids of bonito white muscle, the amount of alk-1′-enylacyl analogue showed considerable variation, accounting for 3.6–7.6% of the

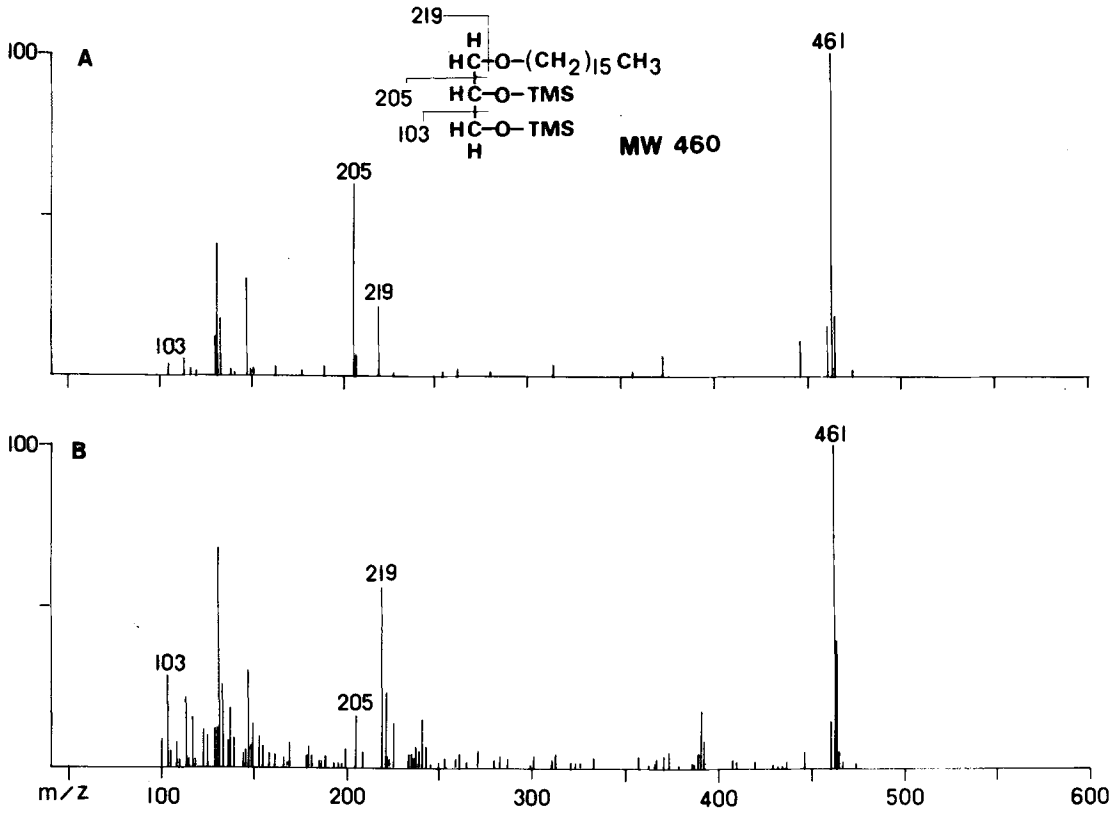


FIG. 6. CI (*iso*-butane) mass spectra of the 2,3-TMS ethers of 1-*O*-alkyl-glycerols derived from (A) authentic 1-*O*-hexadecyl glycerol and (B) bonito white muscle phospholipids. GC–MS conditions are as given in Fig. 3.

TABLE 1

Glycerophospholipid Compositions of Bonito White Muscle (as % of Recovered Phosphorus)

Lipid class	Fish sample 1	2	3	4	5	6
Choline glycerophospholipids	53.2	52.2	46.7	52.1	53.4	57.3
Alk-1'-enylacyl	5.9	3.1	3.1	3.0	6.0	3.5
Alkylacyl + diacyl	47.3	49.1	43.6	49.1	47.4	52.4[a]
Alkylacyl[b]	—	—	—	—	—	1.4[b]
Ethanolamine glycerophospholipids	17.8	24.5	22.3	21.4	25.0	23.4
Alk-1'-enylacyl	3.6	7.1	7.2	5.1	7.6	4.1
Alkylacyl + diacyl	14.2	17.4	15.1	16.3	17.4	18.7[a]
Alkylacyl[b]	—	—	—	—	—	0.6[b]
Serine glycerophospholipids	4.0	4.5	4.8	5.1	2.5	3.9
Inositol glycerophospholipids	3.8	4.4	5.0	3.6	4.4	1.3
Sphingomyelin	6.3	4.6	5.7	6.0	4.6	4.1
Lyso choline glycerophospholipids	14.2	8.7	11.8	11.4	7.5	6.8
Other	0.5	1.1	3.5	0.5	2.5	3.0

[a]The proportional ratios of alkylacyl and diacyl choline and ethanolamine glycerophospholipid were determined by GLC (see text). Value is the percentage of diacyl analogue in fish No. 6.

[b]Value is the percentage of alkylacyl analogue in fish No. 6.

TABLE 2

Fatty Chain Distribution of Choline Glycerophospholipids[a]

Fatty chain[b]	Class / Position	Diacyl *sn*-1 + *sn*-2	1-*O*-Alkyl-2-acyl *sn*-1	1-*O*-Alkyl-2-acyl *sn*-2	1-*O*-Alk-1'-enyl-2-acyl *sn*-1	1-*O*-Alk-1'-enyl-2-acyl *sn*-2
14:0		—	11.47 ± 0.75	1.46 ± 0.12	2.34 ± 0.39	1.42 ± 0.07
14:1		—	7.31 ± 0.57	—	—	—
15:0		—	5.51 ± 0.19	—	1.99 ± 0.10	1.13 ± 0.01
15:1		—	1.59 ± 0.18	—	—	—
16:0		45.81 ± 0.90	55.50 ± 1.16	15.22 ± 0.29	75.35 ± 1.20	6.93 ± 0.16
16:1n-7		1.12 ± 0.20	6.18 ± 0.14	—	—	1.35 ± 0.07
16:2n-6		—	—	—	—	3.00 ± 0.18
17:0 iso		—	1.59 ± 0.31	—	2.28 ± 0.09	—
17:0		—	—	—	2.26 ± 0.10	—
18:0		4.42 ± 0.12	—	4.96 ± 0.02	2.69 ± 0.17	2.75 ± 0.16
18:1n-9		8.57 ± 0.26	3.58 ± 0.16	16.96 ± 0.12	4.01 ± 0.03	27.49 ± 0.31
18:1n-7		1.65 ± 0.09	—	—	1.17 ± 0.17	—
20:0		—	3.37 ± 0.37	—	4.31 ± 0.02	—
20:4n-6		1.72 ± 1.35	—	3.64 ± 0.03	—	7.68 ± 0.16
20:5n-3		6.55 ± 0.24	—	3.85 ± 0.12	—	5.32 ± 0.04
21:5n-3		—	—	—	—	2.89 ± 0.20
22:5n-6		1.14 ± 0.06	—	1.75 ± 0.41	—	1.71 ± 0.37
22:6n-3		24.24 ± 0.95	—	44.59 ± 0.77	—	33.59 ± 1.63
Other[c]		4.78	3.90	7.57	3.60	4.74

[a]The data are presented as the mean ± standard deviation of three separate determinations done on the purified choline glycerophospholipid of fish No. 6.

[b]The position of double bond applies to fatty acyl chains.

[c]Other fatty acid less than 1% each of the total included 16:4n-3, 16:4n-1, 18:2n-9, 18:2n-7, 18:3n-6, 18:4n-1 and 22:5n-3.

total phospholipid (17.5–32.2% of the ethanolamine glycerophospholipids). The alkylacyl analogue was 0.6% of the total phospholipid (2.5% of the ethanolamine glycerophospholipids) as shown in fish sample No. 6 (Table 1). Of other lipid classes, lysocholineglycerophospholipid accumulated at a relatively high level (6.8 to 14.2% of the total phospholipid). These results obtained by phosphorus analysis were also supported by Iatroscan TLC-FID analysis (Fig. 1). In general, the plasmalogen content in ethanolamine glycerophospholipid is higher than that of the choline analogue. This may be a typical example, since plasmalogens in the gill lipid of two species of Pacific crab, *Cancer antennarius* and *Portanus xantusi*, were found to occur at higher levels in the ethanolamine

TABLE 3

Fatty Chain Distribution of Ethanolamine Glycerophospholipids[a]

	Class	Diacyl	1-*O*-Alkyl-2-acyl		1-*O*-Alk-1′-enyl-2-acyl	
Fatty chain[b]	Position	*sn*-1 + *sn*-2	*sn*-1	*sn*-2	*sn*-1	*sn*-2
14:0		—	6.37 ± 0.12	6.75 ± 0.71	—	—
15:0		—	—	3.47 ± 0.16	—	—
15:1		—	1.19 ± 0.04	—	—	—
16:0 iso		—	—	3.19 ± 0.27	—	—
16:0		17.64 ± 1.03	47.82 ± 0.10	40.59 ± 1.45	53.33 ± 0.35	3.65 ± 0.18
16:1n-7		—	5.37 ± 0.42	3.37 ± 0.12	—	—
16:1n-5		—	—	2.47 ± 0.18	—	—
16:2n-6		—	—	—	—	1.32 ± 0.04
17:0 iso		—	—	—	2.38 ± 0.12	—
17:0		1.61 ± 0.05	3.00 ± 0.28	2.55 ± 0.17	4.85 ± 0.24	—
16:4n-3		—	—	1.23 ± 0.14	—	—
18:0 iso		—	2.01 ± 0.32	—	1.87 ± 0.61	—
18:0		34.71 ± 0.27	15.06 ± 0.35	14.21 ± 0.15	19.44 ± 0.31	1.14 ± 0.03
18:1n-9		2.81 ± 0.04	10.84 ± 0.19	5.63 ± 0.03	10.83 ± 0.34	7.09 ± 0.17
18:1n-7		1.75 ± 0.04	6.03 ± 0.02	—	3.95 ± 0.13	—
19:0		1.03 ± 0.01	—	—	—	—
20:0		—	—	—	—	1.61 ± 0.04
20:4n-6		3.18 ± 0.05	—	1.25 ± 0.30	—	5.09 ± 0.05
20:5n-3		2.96 ± 0.03	—	1.08 ± 0.25	—	6.43 ± 0.06
22:5n-6		1.83 ± 0.05	—	—	—	1.13 ± 0.03
22:6n-3		27.94 ± 1.01	—	9.06 ± 0.70	—	63.17 ± 0.29
Other[c]		4.54	2.31	5.15	3.35	9.37

[a]The data are presented as the mean ± standard deviation of three separate determinations done on the purified ethanolamine glycerophospholipid of fish No. 6.

[b]The position of double bond applies to fatty acyl chains.

[c]Other fatty acids less than 1% each of the total included 16:4n-3, 16:4n-1, 18:2n-9, 18:2n-7, 18:3n-6, 18:4n-1 and 22:5n-3.

components (38). Also, bonito white muscle was rich in ethanolamine glycerophospholipid compared with choline analogue.

Fatty chain distributions of choline (CPG) and ethanolamine glycerophospholipids (EPG). The fatty chain distribution of CPG in bonito (fish No. 6 in Table 1) white muscle is shown in Table 2. The prominent fatty acids in the *sn*-1 and *sn*-2 positions of 1,2-diacyl CPG were 16:0, 22:6n-3, 18:1n-9 and 20:5n-3, and the percentages of polyunsaturated fatty acids (PUFA) were quite high. For 1-*O*-alkyl-2-acyl GPC, the percentages of saturated alkyl chains such as 16:0 and 14:0 were higher in the *sn*-1 position; in the *sn*-2 position PUFA were predominant. In 1-*O*-alk-1′-enyl-2-acyl CPG, the sum of monounsaturated and saturated aliphatic chains in the *sn*-1 position amounted to over 80%, while PUFA in the *sn*-2 position accounted for more than 48%.

Table 3 shows the chain distributions of EPG in the white muscle of bonito (fish No. 6 in Table 1). The prominent fatty acids of 1,2-diacyl EPG were similar to those of CPG, except that 18:0 in the former was higher, accounting for 34%. The degrees of unsaturation of the alkyl chain in the *sn*-1 position and of the fatty acyl chain in the *sn*-2 position of 1-*O*-alkyl-2-acyl EPG were lower than those of CPG; more than 66% of the alkyl chains were saturated and 22:6n-3 fatty acid amounted to only 9%. For 1-*O*-alk-1′-enyl-2-acyl EPG, the composition of the alkenyl chain in the *sn*-1 position showed a lower degree of unsaturation, although 22:6n-3 accounted for 63% of the fatty acyl chains in the *sn*-2 position. These observations made for the ether-linked glycerophospholipids of bonito white muscle agreed well with previous data on the ether-linked glycerophospholipids of rabbit alveolar macrophages (39), human platelets (23) and murine mastocytoma (40), except that PUFAs such as 22:6n-3 and 20:5n-3 were prominent in the phospholipid classes of bonito white muscle examined here.

In order to identify ether-linked glycerophospholipids, various analytical techniques, such as two-dimensional TLC (8,41), and GLC of DMA (36) and TMS ether derivatives (35), have so far been applied as conventional methods. More recently, Iatroscan TLC-FID combined with a mild acid hydrolysis procedure (34), GLC (42) and high-performance liquid chromatography (43) of *tert*-BDMS ether derivatives and fast atom bombardment mass spectrometry without any derivatization of the phospholipid (44) have been used successfully. Of these techniques, EI mass spectra of *tert*-BDMS ether derivatives, which are prepared from ether-linked glycerophospholipids, provide information concerning their molecular weights by yielding a $[M - 57]^+$ ion, differing from those of the corresponding TMS ether derivatives (42). On the other hand, the proposed CI-MS technique of TMS ether derivatives, which are prepared from ether-linked glycerophospholipids, seems to be effective not only for determining molecular weights but also for obtaining structural information on ether-linked glycerophospholipids.

In conclusion, the occurrence of novel ether-linked glycerophospholipids has been confirmed in bonito white muscle. Recent interest in the unique physiological and biological properties of 1-*O*-alkyl-2-acetyl glycerophospho-

cholines as the platelet-activating factor (23,40,45-48) also suggests a need for further studies on the molecular species of marine lipids.

ACKNOWLEDGMENTS

Masahiro Ono gave technical assistance. Financial support of a grant-in-aid for scientific research from the Ministry of Education, Science and Culture of Japan to T.O. is gratefully acknowledged. We are grateful to R. G. Ackman, Technical University of Nova Scotia, for reviewing the manuscript.

REFERENCES

1. Dembitsky, V.M. (1979) *Biology 5*, 86-90.
2. Dembitsky, V.M., and Vas'kovsky, V. Ye. (1976) *Biol. Morya 5*, 68-72.
3. Thompson, G.Y. Jr., and Lee, P. (1965) *Biochim. Biophys. Acta 98*, 151-159.
4. Lewis, R.W. (1969) *Comp. Biochem. Physiol. 31*, 725-731.
5. Pocock, D.M.E., Marsden, J.R., and Hamilton, J.G. (1969) *Comp. Biochem. Physiol. 30*, 133-136.
6. Joh, Y.G., and Hata, M. (1979) *Bull. Korean Fish. Soc. 12*, 181-189.
7. Smith, G.M., and Djerassi, C. (1987) *Lipids 22*, 236-240.
8. Vaskovsky, V.E., and Dembitsky, V.M. (1975) *J. Chromatogr. 115*, 645-647.
9. Chapell, S., and Zwingelstein, G. (1984) *Comp. Biochem. Physiol. 78B*, 363-372.
10. Nevenzel, J.C., Gibbs, A., and Benson, A.A. (1985) *Comp. Biochem. Physiol. 82B*, 293-297.
11. Chapell, S., and Benson, A.A. (1986) *Comp. Biochem. Physiol. 85B*, 507-510.
12. Roots, B.I., and Johnston, P.V. (1968) *Comp. Biochem. Physiol. 26*, 553-560.
13. Driedzic, W., Selivonchick, D.P., and Roots, B.I. (1976) *Comp. Biochem. Physiol. 53B*, 311-314.
14. Chacko, G.K., Goldman, D.E., and Pennock, B.E. (1972) *Biochim. Biophys. Acta 280*, 1-16.
15. Malins, D.C., and Varanasi, U. (1972) in *Ether Lipids, Chemistry and Biology* (Snyder, F., ed.) pp. 297-311, Academic Press, New York.
16. Silk, M.H., and De Koning, A.J. (1974) *J. Am. Oil Chem. Soc. 41*, 619-622.
17. Wodtke, E. (1981) *Biochim. Biophys. Acta 640*, 698-709.
18. Kuroda, N., and Negishi, T. (1975) *Res. Bull. Obihiro Univ. 9*, 371-374.
19. Malins, D.C. (1966) *Biochem. J. 100*, 31 p.
20. Bligh, E.G., and Dyer, W.J. (1959) *Can. J. Biochem. Physiol. 37*, 911-917.
21. Tipton, C.L., Paulis, J.W., and Pierson, M.D. (1964) *J. Chromatogr. 14*, 486-489.
22. Bartlett, G.R. (1959) *J. Biol. Chem. 234*, 466-468.
23. Mueller, H.W., Purdon, A.D., Smith, J.B., and Wykle, R.L. (1983) *Lipids 18*, 814-819.
24. Dawson, R.M.C. (1960) *Biochem. J. 75*, 45-53.
25. Ratnayake, W.M.N., and Ackman, R.G. (1985) *Can. Inst. Food Sci. Technol. J. 18*, 284-289.
26. Snyder, F., Blank, M.L., and Wykle, R.L. (1971) *J. Biol. Chem. 246*, 3639-3645.
27. Myher, J.J. (1978) in *Handbook of Lipid Research* (Hanahan, D.J., and Kuksis, A., eds.) Vol. 1, pp. 123-196, Plenum Press, New York.
28. Christie, W.W. (1982) in *Lipid Analysis* (Christie, W.W., eds.) 2nd edn., p. 58, Pergamon Press, Oxford.
29. Mueller, H.W., O'Flaherty, J.T., and Wykle, R.L. (1982) *Lipids 17*, 72-77.
30. Hanahan, D.J., and Vercamer, R. (1954) *J. Am. Chem. Soc. 76*, 1804-1806.
31. Renkonen, O. (1966) *Biochim. Biophys. Acta 125*, 288-309.
32. Ohshima, T., and Koizumi, C. (1983) *Bull. Jpn. Soc. Sci. Fish. 49*, 1205-1212.
33. Ohshima, T., Wada, S., and Koizumi, C. (1983) *Bull. Jpn. Soc. Sci. Fish. 49*, 1213-1219.
34. Foot, M., and Clandinin, M.T. (1982) *J. Chromatogr. 241*, 428-431.
35. Wood, R., and Snyder, F. (1968) *Lipids 3*, 129-135.
36. Frankel, E.N., Neff, W.E., and Selke, E. (1984) *Lipids 19*, 790-800.
37. Hanahan, D.J., Dittmer, J.C., and Warashina, E. (1957) *J. Biol. Chem. 228*, 685-700.
38. Chapell, S., Hakanson, J.L., Nevenzel, J.C., and Benson, A.A. (1987) *Lipids 22*, 76-79.
39. Sugiura, T., Masuzawa, Y., and Waku, K. (1980) *Lipids 15*, 475-478.
40. Yoshioka, S., Nakashima, S., Okano, Y., Hasegawa, H., Ichiyama, A., and Nozawa, Y. (1985) *J. Lipid Res. 26*, 1134-1141.
41. Owens, K. (1966) *Biochem. J. 100*, 354-361.
42. Myher, J.J., and Kuksis, A. (1984) *Can. J. Biochem. Cell Biol. 62*, 352-362.
43. Pind, S., Kuksis, A., Myher, J.J., and Marai, L. (1984) *Can. J. Biochem. Cell Biol. 62*, 301-309.
44. Gross, R.W. (1984) *Biochemistry 23*, 158-165.
45. Hanahan, D.J., Demopoulos, C.A., Liehr, J., and Pinckard, R.N. (1980) *J. Biol. Chem. 225*, 5514-5516.
46. Sugiura, T., Nakajima, M., Sekiguchi, N., Nakagawa, Y., and Waku, K. (1983) *Lipids 18*, 125-129.
47. Hanahan, D.J., Weintraub, S.T., Friedberg, S.J., Tokumura, A., and Ayer, D.E. (1985) *J. Lipid Res. 26*, 1345-1355.
48. Satouchi, K., Oda, M., and Saito, K. (1987) *Lipids 22*, 285-287.

[Received February 8, 1988; Revision accepted December 12, 1988]