Mild alkali-stable phospholipids in chicken egg yolks: characterization of 1-alkenyl and 1-alkyl-*sn*-glycero-3-phosphoethanolamine, sphingomyelin, and 1-alkyl-*sn*-glycero-3-phosphocholine

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Abstract Chicken egg yolk phospholipids were subjected to mild alkaline hydrolysis. The resulting alkali-stable phospholipids were characterized by chemical, chromatographic, and enzymatic methods. Two major phospholipids, 1 - *O* - alkyl - *sn* - glycero - 3 - phosphoethanolamine and sphingomyelin; and two minor phospholipids, 1-*O*-alkenyl-*sn*-glycero-3-phosphoethanolamine and 1-*O*-alkyl-*sn*-glycero-3-phosphocholine; were identified. The sphingomyelins were converted into ceramides by enzymatic hydrolysis with phospholipase C. Ceramides derived from sphingomyelins with non-hydroxy fatty acids (99% of total ceramides) consisted predominantly of the N-palmitoyl, N-stearoyl, and N-nervonyl species. Ceramides derived from sphingomyelins with hydroxy fatty acids (1% of total ceramides) consisted almost exclusively of the N-α-hydroxyeicosanoyl species. The long chain bases of ceramides derived from both species of sphingomyelins (hydroxy and non-hydroxy fatty acids) consisted of 97% D-*erythro*-sphing-4-enine and 3% D-*erythro*-sphinganine. 1-*O*-alkyl-*sn*-glycero-3-phosphoethanolamine and 1-*O*-alkyl-*sn*-glycero-3-phosphocholine were converted to corresponding 1-*O*-alkyldiacetylglycerols. The 1-*O*-alkyldiacetylglycerols derived from both ether phospholipids consisted largely of the hexadecyl and octadecyl species. Smaller quantities of the heptadecyl, *cis*-9-octadecenyl and eicosanyl derivatives were also present.—**Do, U. H., and S. Ramachandran.** Mild alkali-stable phospholipids in chicken egg yolks: characterization of 1-alkenyl and 1-alkyl-*sn*-glycero-3-phosphoethanolamine, sphingomyelin, and 1-alkyl-*sn*-glycero-3-phosphocholine. *J. Lipid Res.* 1980. **21:** 888–894.

Supplementary key words ceramides · D-sphing-4-enine · D-sphinganine

Since chicken egg yolks have been an important source of phospholipids, phospholipids of egg yolks have been studied extensively. However, only few data are available on the classes and composition of egg yolk phospholipids formed by mild alkaline hydrolysis of total egg phospholipids. One so-called "alkali-stable" phospholipid, 1-*O*-alkyl GPE, was isolated from egg yolk and was characterized by Carter, Smith, and Jones (1) in 1958. From elementary analysis they predicted that 1-*O*-alkyl GPE consisted primarily of 1-*O*-hexadecyl and 1-*O*-octadecyl GPE. Another alkali-stable phospholipid known to be present in egg yolks is sphingomyelin. Karlsson (2) and Fujino and his associates (3) have studied its composition and determined its long chain base and fatty acid composition. However, these studies were preliminary and by no means complete. Thus, the purpose of the present investigation was to isolate and further characterize alkali-stable egg phospholipids by use of chemical, chromatographic, and enzymic techniques. The data presented here indicate the existence of at least two other alkali-stable phospholipids in egg yolks, 1-*O*-alkenyl GPE and 1-*O*-alkyl GPC. Analysis shows the presence of heptadecyl, 9-octadecenyl, and eicosanyl species, in addition to the hexadecyl and octadecyl species reported by Carter et al. (1), among the constituents of these ether phospholipids. The isolation and characterization of sphingomyelin containing hydroxy fatty acids is also reported.

MATERIALS AND METHODS

Materials

Authentic fatty acids, α- and β- hydroxy fatty acids, their methyl esters, bovine brain ceramides, long-chain bases, sphingomyelin, cholesterol, PE, PC,

Abbreviations: GPE, *sn*-glycero-3-phosphoethanolamine; GPC, *sn*-glycero-3-phosphocholine; GLC, gas–liquid chromatography; TLC, thin-layer chromatography; TMS, trimethylsilyl; sphing-4-enine (sphingosine), 1,3-dihydroxy-2-amino-4-*trans*-octadecene; sphinganine (dihydrosphingosine), 1,3-dihydroxy-2-amino octadecane; PC, phosphatidylcholine; PE, phosphatidylethanolamine; IR, infrared; DNPH, dinitrophenylhydrazine; AHTT, 4-amino-5-hydrazino-1,3,4-triazole-3-thiol.

lysoPC, *O*-alkyl glyceryl ethers, mono-, di- and triacylglycerols, hexamethyldisilazane–chlorotrimethylsilane–pyridine 3:1:9 (v/v/v), 14% (w/v), BF_3 in methanol, 0.5 N methanolic sodium methoxide, acetic anhydride, specific spray reagents (4), HI-FLOSIL (60–200 mesh) silica gel, silica gel 60 extra pure (70–230 mesh), and silica gel TLC plates (G, H, and 2% $Na_2B_4H_7$-impregnated G) were obtained from Applied Science Division, Milton Roy Company Laboratory Group, State College, PA. Chicken eggs were purchased from a local grocery store in State College, PA. Lithium aluminum hydride ($LiAlH_4$) and phospholipase C (EC 3.1.4.3 from *Clostridium perfringens*) were purchased from Aldrich Chemical Company, Metuchen, NJ. DL-sphing-4-enine and sphinganine were obtained from Sigma Chemical Company, St. Louis, MO. IR spectra (KBr pellets) were obtained with a Perkin-Elmer 5800 high-resolution IR spectrophotometer (Norwalk, CT).

Isolation and purification of alkali-stable phospholipids from egg yolks

Egg yolks (10 kg) were extracted with 10 l of chloroform–methanol 2:1 for 2 hr at room temperature. The extracts were partitioned by adding 0.2 volume of water (5). After the acetone precipitation at 4°C overnight (6), the yield of crude polar lipids was 452 g. Mild alkaline hydrolyses of the lipids were carried out by the method of Sweeley (7); 452 g of crude polar lipids yielded gave 44.9 g of alkali-stable lipids. Crude alkali-stable lipids (33 g) were applied to 600 g of a HI-FLOSIL column and eluted successively with 3 l of chloroform–methanol, 1 l of chloroform–methanol 4:1, 1 l of chloroform–methanol 1:1, 2.5 l of chloroform–methanol 1:3; 3 l of chloroform–methanol–water 25:75:4 and 2 l of chloroform–methanol–2N NH_4OH 25:75:3. Chloroform–methanol–water 25:75:4 eluted 1-*O*-alkenyl and 1-*O*-alkyl GPE. Chloroform–methanol–2N NH_4OH 25:75:3 eluted sphingomyelins and lysoPC. Those fractions containing 1-*O*-alkenyl and 1-*O*-alkyl GPE (3.76 g) were repurified by column chromatography on silica gel 60 (180 g). Early fractions eluted with chloroform–methanol 1:1 were rich in 1-*O*-alkenyl GPE. Later fractions eluted with chloroform–methanol 1:3 contained only 1-*O*-alkyl GPE. The fractions rich in 1-*O*-alkenyl GPE were further purified by preparative TLC (development with chloroform–methanol–water 65:25:4). Fractions containing sphingomyelins and lysoPC were also repurified by column chromatography on silica gel 60 in essentially the same manner as 1-*O*-alkenyl and 1-*O*-alkyl GPE. Early fractions contained only sphingomyelins while later fractions contained both sphingomyelins and lysoPC. Those fractions rich in lysoPC were again subjected to mild alkaline hydrolysis, in the manner already described. After hydrolysis, the sample was treated with a twofold excess of 12N HCl for 30 min to cleave any remaining alkenyl groups present. After partitioning by the method of Folch, Lees, and Sloane Stanley (5), pure 1-*O*-alkyl GPC was isolated from the lower organic layer by preparative TLC, in the manner already described.

Preparation and fractionation of ceramides

Phospholipase C from *Clostridium perfringens* (15 mg) converted 2.1 g of purified egg sphingomyelin to ceramides and phosphorylcholine (8). The IR spectrum of the ceramides was virtually identical to a published spectrum of egg ceramides (9) and showed strong absorption at 965 cm^{-1} (the *trans*-double bond), 1640, and 1550 cm^{-1} (amide bond). There was virtually no absorption at 1730 cm^{-1} (aliphatic carboxylic ester).

The total ceramides (2.2 g) were applied to a column containing 100 g of HI-FLOSIL and eluted successively with 0.8 l of chloroform, 0.6 l of 2% methanol in chloroform, and 1 l of 5% methanol in chloroform. Ceramides containing non-hydroxy fatty acids were eluted with 2% methanol in chloroform while ceramides with hydroxy fatty acids were eluted with 5% methanol in chloroform. The yield of pure non-hydroxy and hydroxy ceramides was 1.54 g and 15 mg, respectively.

Hydrolysis of ceramides

A sample of ceramides (2.1 g) dissolved in 150 ml of 0.5 N methanolic potassium hydroxide was boiled under reflux for 18 hr (9). The ether extracts containing crude long chain bases were subsequently purified by silicic acid column chromatography according to the procedure of Barenholz and Gatt (10). The yield of pure D-*erythro*-sphing-4-enine was 0.58 g and its melting point was 71–76°C. Its N-acetyl derivative had a melting point of 85–86°C (Literature 11; 85–87°C).

Ceramides (50 mg) were also treated with 5 ml of 0.5 N methanolic sodium methoxide and 1 ml of benzene in a sealed glass tube for 30 min at 75–85°C (12). Evaporation of the ether extracts gave fatty acid methyl esters.

Preparation and acetylation of 1-*O*-alkyl glycerols from ether phospholipids

The method of Carter and coworkers (1) was employed. Alternatively, 1-*O*-alkyl glycerols were also obtained by direct reduction of 1-*O*-alkyl GPE and 1-*O*-alkyl GPC with lithium aluminum hydride as described by Snyder (15).

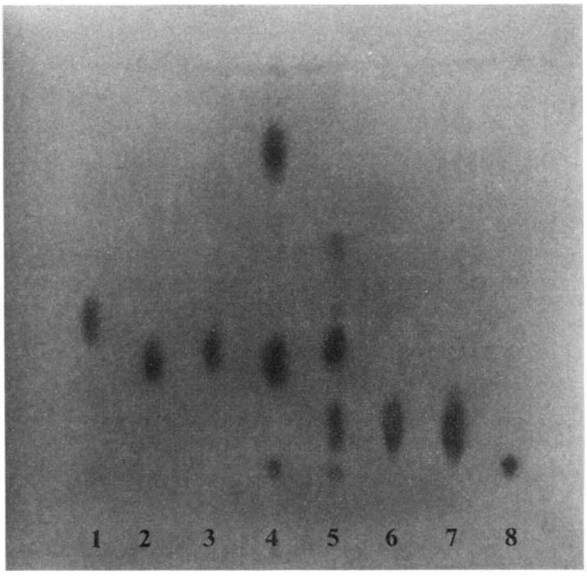

Fig. 1. Separation of alkali-stable lipids by thin-layer chromatography. Lane 1, egg yolk 1-*O*-alkenyl GPE; lane 2, egg yolk 1-*O*-alkyl GPE; lane 3, bovine brain lyso PE; lane 4, lipid standards cholesterol, PE, PC, and lyso PC from the solvent front to the origin; lane 5, crude egg yolk alkali-stable lipids before column purification; lane 6, standard bovine sphingomyelin; lane 7, egg yolk sphingomyelin; lane 8, egg yolk 1-*O*-alkyl GPC. The thin-layer was silica gel H PREKOTE (Applied Science Division), the solvent was chloroform–methanol–acetic acid–water 50:25:7:3 (v/v/v/v) and 50% aqueous sulfuric acid solution was used for detection.

The crude 1-*O*-alkyl glycerols (100 mg each) were applied to a silica gel 60 column and eluted successively with 0.2 l of pure chloroform, 0.2 l of 2% methanol in chloroform, and 5% methanol in chloroform. Up to 50 mg of standard or purified *O*-alkyl glycerols was converted to the corresponding diacetates by treatment with 1 ml of acetic anhydride and 0.2 ml of pyridine at 80°C for 1 hr and then at room temperature overnight (1).

Analytical chromatography

Various alkali-stable lipids were chromatographed on silica gel H plates with the following solvent systems: chloroform–methanol–water (65:25:4, chloroform–methanol–15 M NH_4OH 65:35:5, and chloroform–methanol–acetic acid–water 50:25:7:3 (13). Specific spray reagents, ninhydrin for amino groups, molybdenum blue for phospholipids, Dragendorff reagent for choline group, and DNPH and AHTT (14) for aldehydes, were used to identify functional groups (4). *O*-Alkyl glycerols and corresponding diacetates were separated by TLC on 2% sodium tetraborate-impregnated silica gel G plates developed in either petroleum ether–ether–acetic acid 40:60:1 or chloroform–methanol 95:5 (15). Authentic 1-*O*-hexadecyl glycerol, 2-*O*-hexadecyl glycerol, and their diacetates were employed as reference compounds.

The TMS derivatives of N-acetyl long chain bases and hydroxy fatty acid methyl esters were prepared by the method of Gaver and Sweeley (11). Fatty acid methyl esters, TMS derivatives of long chain bases, and diacetyl derivatives of *O*-alkyl glycerols were analyzed by GLC on a Hewlett-Packard 7610 or Perkin-Elmer 3920B gas chromatograph, both equipped with flame ionization detectors. The fatty acid methyl esters were identified by comparison of retention times with authentic fatty acid methyl esters before and after micro-hydrogenation. Similarly, N-acetyl TMS derivatives of long chain bases were identified by comparison with N-acetyl TMS derivatives of DL-sphinganine and sphing-4-enine. *O*-alkyl glycerols were identified by comparison of their diacetyl derivatives with those of standard 1-*O*-alkyl glycerols (14:0, 16:0, 18:0, 18:1, and 20:0) and 2-*O*-alkyl glycerols (16:0 and 18:0).

Identification of other compounds, for which standards were not available, was based on calculations of predicted retention times derived from semilogarithmic plots of retention times versus chain length (12). Calculation of the composition was based on the triangulation.

RESULTS

Characterization and properties of alkali-stable phospholipids

Alkali-stable phospholipids could be separated to some extent by TLC on silica gel G plates by development with either chloroform–methanol–water 65:25:4, or chloroform–methanol–15 M NH_4OH 65:35:5. However, as shown in **Fig. 1,** complete separation could be achieved by TLC on silica gel H plates by development with chloroform–methanol–acetic acid–water 50:25:7:3.

Examination of the mixture of crude alkali stable lipids by TLC *before* column chromatographic fractionation (Fig. 1, lane 5) showed the presence of three organic phosphorus-positive compounds, with R_f values corresponding to the standard lipids, sphingomyelin (Fig. 1, lane 6), PC, and lysoPC (Fig. 1, lane 4). A fourth and minor phosphorus-positive compound (Fig. 1, lane 5), which migrated slightly ahead of standard PC, was isolated after column chromatography and preparative TLC. This phospholipid (Fig. 1, lane 1) was positive to ninhydrin and also to DNPH or 6N HCl followed by AHTT, suggesting that it possessed a free amino group and a bound aldehyde group. Its IR spectrum (**Fig. 2A**) was consistent with 1-*O*-alkenyl GPE structure. For example, peaks at 1650 cm^{-1} (C=C vinyl ether

stretching), 1250 cm^{-1} (C—O—C vinyl ether stretching) and 720 cm^{-1} (—CH= deformation), were all characteristic of a 1-*O*-alkenyl (plasmalogen) ether (4). In addition, the spectrum contained strong CH_2 and CH_3 absorption bands (2920, 2850, 1470, and 1370 cm^{-1}), strong phosphate bands at 1220 cm^{-1} (P=O), 1080 cm^{-1} (P—O^-) and 1000 cm^{-1} (P—O—C), a strong NH_2 band (1570 cm^{-1}) and a broad band around 3250 cm^{-1} due to a hydroxyl group. However, its spectrum was devoid of aliphatic carboxylic ester absorption (1700–1750 cm^{-1}).

A major alkali-stable phospholipid (Fig. 1, lane 2) had the same R_f value as the standard PC. However, the lipid was ninhydrin-positive and did not give positive reactions with aldehyde- or choline-specific reagents. Its IR spectrum (Fig. 2B) showed a definite peak at 1130 cm^{-1} which was characteristic of long chain glyceryl ether (1, 4), a CH_2 deformation peak at 1470 cm^{-1}, and strong phosphate bands at 1220, 1075, and 1025 cm^{-1}, but no peaks due to carboxylic ester or amide. The spectrum was in excellent agreement with the IR spectrum of egg yolk 1-*O*-alkyl GPE described by Carter et al. (1). Both 1-*O*-alkyl GPE and 1-*O*-alkenyl GPE were not hydrolyzed by phospholipase C from *Clostridium perfringens* after 24 hr at room temperature.

The other major egg alkali-stable phospholipid (Fig. 1, lane 7) exhibited chromatographic behavior identical to that of standard bovine sphingomyelin (Fig. 1, lane 6) on TLC plates in the three developing solvent systems. It also showed positive reactions with the choline-specific reagent and the molybdenum blue reagent. The purified egg sphingomyelin was completely converted to ceramides and phosphocholine by phospholipase C overnight at room temperature. Its IR spectrum was virtually identical to that of bovine brain sphingomyelin published by Rouser et al. (16).

The most polar egg alkali-stable phospholipid (Fig. 1, lane 8) migrated together with the authentic lysoPC in the three solvent systems tested. This lipid gave positive tests with molybdenum blue and Dragendorff reagents. Its resistance to 2 N KOH or concentrated HCl suggested that it had an alkyl glyceryl ether bond in the molecule. The presence of such a functional group was supported by the presence of a peak at 1130 cm^{-1} due to C—O—C alkyl ether stretching in its IR spectrum. The IR spectrum of egg 1-*O*-alkyl GPC was virtually identical to that reported by Pugh, Kates, and Hanahan (6) for bovine heart 1-*O*-alkyl GPC. In addition to the alkyl ether peak at 1130 cm^{-1}, peaks due to CH_3, CH_2, phosphate, and hydroxyl groups were also present in its spectrum. Egg 1-*O*-alkyl GPC showed

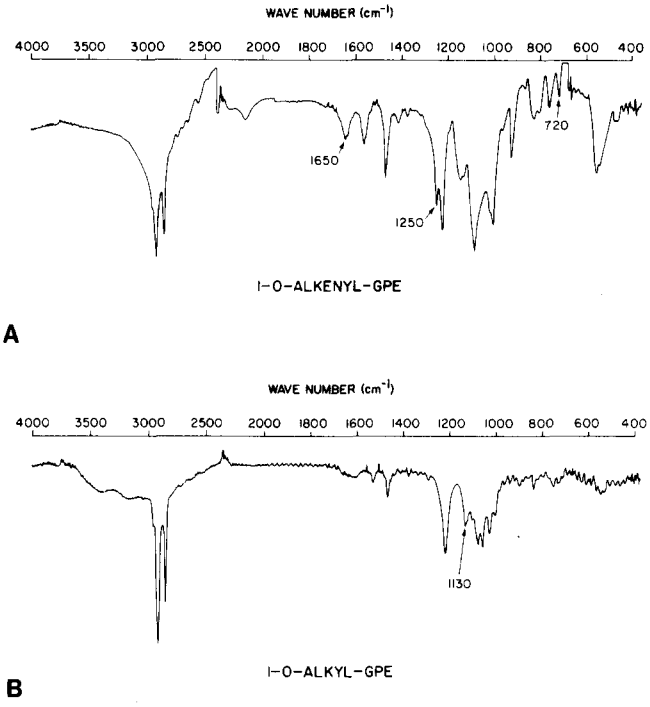

Fig. 2. Infrared spectra of egg yolk ether phospholipids. A) 1-*O*-Alkenyl GPE, sample concentration, 2% in KBr; B) 1-*O*-Alkyl GPE, sample concentration, 3% in KBr.

no detectable signs of hydrolysis by phospholipase C from *Clostridium perfringens* even after 48 hr at room temperature.

Characterization of ceramides derived from enzymic hydrolysis of sphingomyelins

The TLC analysis of the ceramides obtained by phospholipase C hydrolysis of egg yolk sphingomyelins is illustrated in **Fig. 3.** Standard bovine brain ceramides (Fig. 3, lane 3) consisting of ceramides with a) saturated dihydroxy base-normal fatty acids, b) unsaturated dihydroxy base-normal fatty acids, c) saturated dihydroxy base-hydroxy fatty acids, and d) unsaturated dihydroxy base-hydroxy fatty acids, are shown for comparison (7, 17). The total egg ceramides were fractionated by column chromatography into two groups, nonhydroxy ceramides (Fig. 3, lane 4) and hydroxy ceramides (Fig. 3, lane 5). The major spot in Fig. 3, lane 4 (lower spot) had the same mobility as the bovine brain ceramide comprised of unsaturated dihydroxy base-normal fatty acids. Attempts to isolate the minor ceramide in Fig. 3, lane 4 (upper spot) by repeated preparative TLC on 2% sodium tetraborate-impregnated silica gel G plates were not successful. However, it is well-known that the solvent system chloroform–methanol 95:5 permits TLC separation of ceramides on the basis of the presence or absence of the *trans*-double bond in the long chain

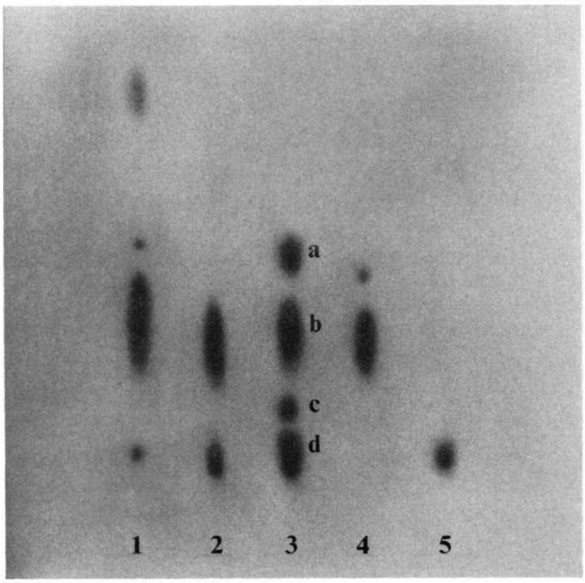

Fig. 3. Thin-layer chromatogram of ceramides dervied from egg yolk sphingomyelins by hydrolysis with phospholipase C. Lane 1, crude egg yolk ceramides from enzymic reaction; Lane 2, column fractions 9–10 eluted with 5% methanol in chloroform; Lane 3, standard bovine brain ceramides: a) saturated dihydroxy base-normal fatty acids, b) unsaturated dihydroxy base-normal fatty acids, c) saturated dihydroxy base-hydroxy fatty acids, and d) unsaturated dihydroxy base-hydroxy fatty acids, from the top to the origin; Lane 4, purified egg yolk non-hydroxy ceramide; Lane 5, purified egg yolk hydroxy ceramide. The thin-layer was silica gel G impregnated with 2% sodium tetraborate; the solvent was chloroform–methanol 95:5 (v/v); and 50% aqueous sulfuric acid solution was used for detection.

bases. Furthermore, the chain length or degree of unsaturation of the fatty acid moieties has little effect on the mobility of ceramides when borate-impregnated plates are used (7, 17). Therefore, the minor egg ceramide in Fig. 3, lane 4 was assumed to be derived from saturated dihydroxy base-normal fatty acids, since its R_f value was identical to that of the corresponding bovine brain ceramide in Fig. 3, lane 3. Finally, the slow-moving compound in Fig. 3, lane 5 had an R_f value identical to that of the bovine brain ceramide containing unsaturated dihydroxy base-hydroxy fatty acids. The existence of at least three different species of ceramides as the essential components of egg sphingomyelin was further supported by GLC analysis of the fatty acids and long chain bases derived from them.

Composition of fatty acids and long chain bases of sphingomyelins

Egg ceramides were hydrolyzed under mildly basic conditions to the corresponding long chain bases and fatty acids. The TLC analysis of crude long chain bases showed only two ninhydrin positive spots whose R_f values were identical to those of authentic sphing-4-enine and sphinganine. The compound corresponding to sphing-4-enine was the predominant base. Only small amounts of the compound corresponding to sphinganine were found. The long chain bases from both hydroxy and nonhydroxy ceramides were quantitatively analyzed by GLC as their N-acetyl TMS derivatives. The long chain bases of both types of ceramides consisted of 97% sphing-4-enine and 3% sphinganine. **Table 1** shows the fatty acid composition of nonhydroxy and hydroxy ceramides.

Characterization of glyceryl ether phospholipids

The two egg yolk glyceryl ether phospholipids, 1-*O*-alkyl GPC and GPE, were converted to 1-*O*-alkyl glycerols by two different methods: acetolysis followed by saponification or lithium aluminum hydride reduction. The crude alkyl glycerols were purified by column chromatography and were converted to diacetyl derivatives. These were subsequently analyzed by GLC on both polar SILAR 10C and non-polar SE-30 columns. A typical chromatogram obtained with a SILAR 10C column is shown in **Fig. 4.** The small peak X eluted just before the major peak (18:0) was an unknown impurity and was not considered in the calculation of alkyl chain composition since repeated column chromatography removed more than 95% of the impurity. The distribution of alkyl moieties in 1-*O*-alkyl GPE and 1-*O*-alkyl GPC is shown in **Table 2.** Both ether phospholipids contained hexadecyl and octadecyl groups as the major

TABLE 1. Fatty acid composition of non-hydroxy and α-hydroxy ceramides from egg yolk sphingomyelins

Non-Hydroxy Fatty Acids[a]	Mole %	Hydroxy Fatty Acids[c]	Mole %
14:0	1.0	α-OH 14:0	0.1
16:0	84.3	α-OH 16:0	0.5
18:0	5.2	α-OH 18:0	0.2
19:0	0.2		
20:0	1.0	α-OH 20:0	98.5
21:0	0.2		
22:0	1.5	α-OH 22:0	0.6
22:1	0.5		
23:0	0.6		
24:0	0.7	α-OH 24:0	0.1
24:1	4.5		
Others[b]	0.3		
Total	100.0		100.0

[a] Methyl esters of non-hydroxy fatty acids were separated by gas–liquid chromatography on a glass column (4 mm i.d. × 1.8 m) packed with 3% SILAR 10C on Gas-Chrom Q 100/120 mesh; column temperature, 180°C; nitrogen flow rate, 40 ml/min.

[b] Others indicate fatty acids 15:0, 16:1, and 18:1.

[c] Trimethylsilyl ether derivatives of methyl esters of hydroxy fatty acids were separated by gas–liquid chromatography on a glass column (4 mm i.d. × 1.8 m) packed with 3% SE-30 on Gas-Chrom Q 80/100 mesh; column temperature, 210°C; nitrogen flow rate, 40 ml/min.

alkyl moieties. Small amounts of heptadecyl, 9-octadecenyl and eicosanyl groups were also present.

DISCUSSION

Four alkali-stable phospholipids have been completely separated on ordinary TLC plates by proper choice of an adsorbent (Silica H rather than G) and an acidic solvent system (Fig. 1). Complete separation of two molecular species of intact ether phospholipids, 1-*O*-alkyl and 1-*O*-alkenyl GPE, on ordinary TLC plates has not been previously described. The use of high resolution IR spectrometry greatly facilitated the characterization of these ether phospholipids.

The presence of a minor alkali-stable phospholipid moving ahead of 1-*O*-alkyl GPE on the TLC plate was quite unexpected. The purified phospholipid gave a positive ninhydrin test and its acid hydrolysate contained ethanolamine and phosphoethanolamine. Since Renkonen (18) isolated the *O*-methylated N-dinitrophenylated derivatives of 1-*O*-alkenyl 2-acyl GPE on silver nitrate-impregnated silica gel G in addition to the well-known 1-*O*-alkyl 2-acyl and 1,2-diacyl GPE from egg yolk lipids, the minor phospholipid was suspected as the 1-*O*-alkenyl GPE surviving mild alkaline hydrolysis followed by the adjustment of pH to 5. A portion of the suspected phospholipid was spotted on a TLC plate and was exposed to HCl fumes (15). When the plate was developed with chloroform–methanol–15 M NH_4OH 65:35:5, free fatty aldehyde was detected at the solvent front. Another portion of the phospholipid was completely hydrogenated with platinum oxide (15) and the product was mixed with authentic 1-*O*-alkyl GPE. When this mixture was analyzed by TLC with the acidic solvent system described in Fig. 1, a single spot was observed upon detection. These results, resistance to phospholipase C from *Clostridium perfringens* and the spectrum in Fig. 2A, confirm the presence of 1-*O*-alkenyl GPE in egg yolk.

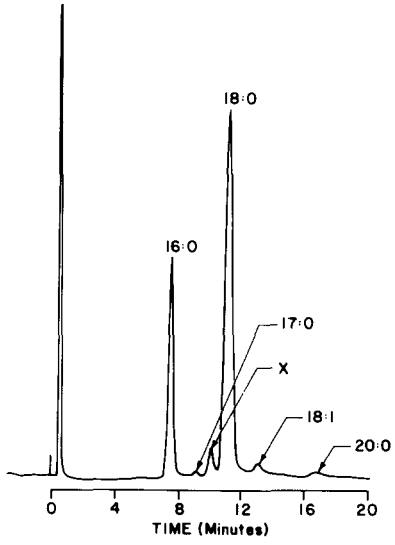

Fig. 4. Gas–liquid chromatogram of 2,3-diacetyl 1-*O*-alkyl glycerols derived from egg yolk ether phospholipids. Conditions for chromatography were as follows: 3% SILAR 10C glass column (4 mm i.d. × 1.8 m) on Gas-Chrom Q 100/120 mesh; column temperature, 200°C; nitrogen flow rate, 40 ml/min.

TABLE 2. Distribution of glyceryl ethers from egg yolk 1-*O*-alkyl GPE and 1-*O*-alkyl GPC[a]

Side Chain	Weight %[b]	
	1-*O*-Alkyl-GPE[c]	1-*O*-Alkyl-GPC[c]
16:0	28.6	29.5
17:0	0.8	1.3
18:0	65.7	63.2
18:1	4.1	4.4
20:0	0.8	1.6
Total	100.0	100.0

[a] Diacetyl derivatives of 1-*O*-alkyl glycerols derived from 1-*O*-alkyl GPE and 1-*O*-alkyl GPC were analyzed by gas–liquid chromatography on a glass column (4 mm i.d. × 1.8 m) packed with 3% SILAR 10C on Gas-Chrom Q 100/120 mesh; column temperature, 200°C; nitrogen flow rate, 40 ml/min.

[b] Percentages represent the means of duplicate determinations on two different samples. The agreement between duplicate determinations and between different samples was 5% for the major components and 10% for the minor components.

[c] See text footnote for the abbreviations.

The position of attachment of the alkyl moieties to glycerol in egg ether phospholipids was determined by chromatographic methods. Thus, when the *O*-alkyl glycerols derived from $LiAlH_4$ reduction of egg ether phospholipids were analyzed by TLC on a sodium tetraborate-impregnated silica gel G plate with two solvent systems described in Methods, the *O*-alkyl glycerols derived from egg ether phospholipids migrated together with the authentic 1-*O*-alkyl glycerols. Authentic 2-*O*-alkyl glycerols migrated ahead of authentic 1-*O*-alkyl glycerols in these solvent systems. In another experiment, when 1-*O*-alkyl and 2-*O*-alkyl glycerol diacetates were quantitatively analyzed by GLC, base line separation of these positional isomers was achieved on a SILAR-10C column. When *O*-alkyl glycerol diacetates derived from egg ether phospholipids were analyzed under the identical conditions, only 1-*O*-alkyl glycerol diacetates were detected. These chromatographic results indicate that the alkyl moieties are exclusively located at the *sn*-1 position of ether phospholipids. The results are in excellent agreement with the chemical and nuclear magnetic resonance studies by Carter et al. (1).

The striking similarity between the alkyl chain composition of 1-*O*-alkyl GPE and that of 1-*O*-alkyl GPC (Table 2) suggests that a common precursor, 1-*O*-alkyl dihydroxyacetone phosphate (19), may be involved in the biosynthesis of major portions of these phospholipids. Since PE and PC constitute more than 88% of the total phospholipids in egg yolk (20), these ether phospholipids would include hydrolytic products of 1-*O*-alkyl or 1-*O*-alkenyl 2-acyl GPE and GPC, as well as any "native" 1-*O*-alkyl or 1-*O*-alkenyl GPE and GPC.

The observed pattern of the nonhydroxy fatty acid distribution of egg yolk sphingomyelin in Table 1 is characteristic of sphingomyelin (2, 3, 7) in general, i.e., a high concentration of palmitic acid, moderate concentrations of stearic and nervonic acids, and small amounts of other saturated and monoenoic fatty acids. Furthermore, the data on fatty acid and long chain base composition presented here are in good agreement with the preliminary report of Fujino and coworkers (3), who presented chromatographic evidence that N-palmitoyl-D-sphing-4-enyl-phosphocholine was the major molecular species of egg yolk sphingomyelin. The present study (Table 1) also reveals that egg yolks contain N-α-hydroxyeicosanoyl-D-sphing-4-enyl phosphocholine as a minor molecular species of sphingomyelin. Such species of sphingomyelin derived from hydroxy fatty acids have been isolated from bovine rennet stomach (21).

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