Characterization of the alkyl ether species of phosphatidylcholine in bovine heart

E. L. Pugh, M. Kates, and D. J. Hanahan[1]

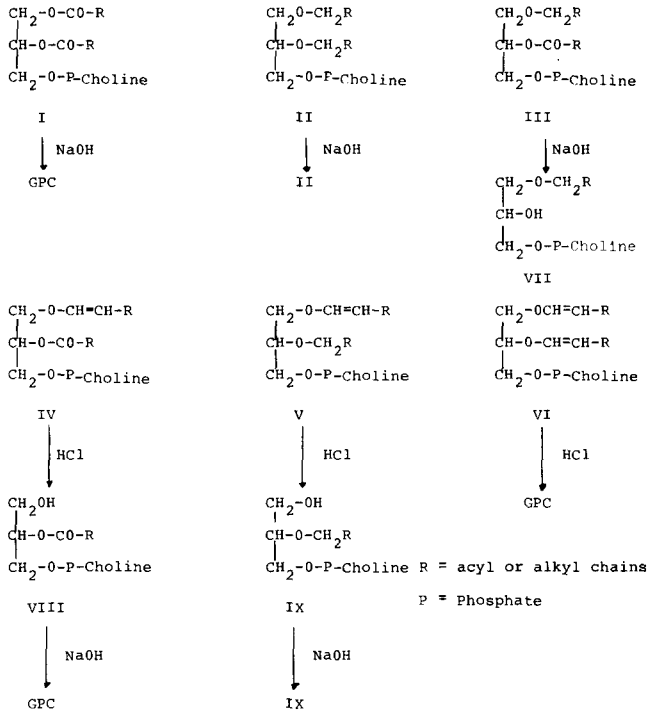
Department of Biochemistry, University of Ottawa, Ottawa, Ontario, Canada, K1N 6N5; and Department of Biochemistry, University of Texas, Health Science Center at San Antonio, TX 78284

Abstract The molecular types of lecithin in beef heart lipids were investigated by a combination of mild hydrolytic procedures and silicic acid chromatography. The major species of *sn*-glycero-3-phosphorylcholine (GPC) were found to be: diacyl-GPC (57%), alk-1-enyl, acyl-GPC (39%), alkyl acyl-GPC (3%), and dialkyl-GPC (<1%). No di-alk-1-enyl-GPC or alk-1-enyl alkyl-GPC were detected. The derived monoalkyl- and dialkyl-glycerols were characterized by their infrared spectra and alkyl chain compositions.

Supplementary key words plasmalogens · monoether-monoesters · dialkyl ether · di-alk-1-enyl ether · alk-1-enyl alkyl ether

Glycerol ether-containing lipids were discovered about fifty years ago (1) and since that time have been found to be widespread in nature (2). The major ether-containing lipids have been identified as 1-*O*-alkyl-glyceryl monoether derivatives, such as batyl, chimyl, selachyl alcohols and the plasmalogenic 1-*O*-alk-1-enyl glyceryl ethers (2). However, dialkyl glyceryl ethers are also known to occur in extremely halophilic bacteria, the lipids of which are derived from 2,3-di-*O*-phytanyl-*sn*-glycerol (3–7). In a preliminary report, Popovic (8) presented chromatographic evidence for the presence of dialkyl glyceryl ethers in hydrolysates of human heart lipids, but did not characterize the glycerol diethers further. Marinetti and Stotz (9), however, isolated and identified dialkyl glyceryl ether in hydrolysates of hydrogenated beef heart lipids.

Since 1-*O*-alkyl-glyceryl monoethers in animal tissues occur largely in the phosphatidylcholine fraction (2), we have carried out a detailed chemical examination of the lecithin fraction of beef heart lipids for the diacyl (I), the saturated dialkyl ether (II), the acyl alkyl monoether (III), and the plasmalogen (alk-1-enyl acyl) (IV) species (**Scheme 1**). The possible occurrence of the alk-1-enyl alkyl (V) and di-alk-1-enyl (VI) ether species was also investigated.



Scheme 1. Hydrolytic procedures for analysis of lecithin species.

Species I, II, and III would be stable to mild acid hydrolysis; subsequent mild alkaline deacylation would convert I to glycerophosphorylcholine (GPC) and III to 1-monoalkyl-GPC (VII) but would not affect II (see Scheme 1). Species IV and V would be hydrolyzed by the mild acid treatment to the

Abbreviations: GPC, *sn*-glycero-3-phosphorylcholine; PC, phosphatidylcholine; "lecithin" as used here is phosphatidylcholine containing either two acyl groups, one acyl and one alk-1-enyl group, one acyl and one alkyl group, or two alkyl groups; TLC, thin-layer chromatography; GLC, gas–liquid chromatography.

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2-monoacyl-GPC (VIII) and the 2-monoalkyl-GPC (IX), respectively, and species VI would be completely hydrolyzed to GPC. Subsequent mild alkaline deacylation would convert VIII to GPC but would not affect IX.

Thus, analysis of the lipid products of mild acid and subsequent mild alkaline hydrolysis should reveal the species composition of the beef heart lecithin in terms of components I to VI. The data presented here indicate that the saturated ether species II (dialkyl) and III (alkyl acyl) most likely exist but rule out the presence of detectable amounts of types V (alk-1-enyl alkyl) and VI (dialk-1-enyl).

MATERIALS AND METHODS

Aluminum oxide AG-7 was a product of Bio-Rad Laboratories, Richmond, CA and silicic acid, 100–200 mesh, of Mallinckrodt, St. Louis, MO. Total weight was determined by drying the samples in high vacuum for 6 hr, and lipid phosphorus was assayed by the technique of Bartlett (10). 1-*O*-Alkyl glycerols were prepared from the ether-containing phospholipids by the acetolysis procedure described earlier (11) and were then converted to the isopropylidene derivatives by the method of Hanahan, Ekholm, and Jackson (12). Infrared spectra were measured on carbon tetrachloride or chloroform solutions in a Unicam SP 1000 infrared spectrophotometer.

Extraction of total lipids

Three fresh bovine hearts, weighing 3–5 lb each (obtained from Ottawa Beef Co. Ltd.) were used as starting material in three separate experiments. In a typical experiment, a beef heart (3 lb) obtained fresh from the slaughter house was trimmed of fat, cut into small pieces and ground in a meat grinder. All operations were carried out on ice at 4°C unless otherwise stated. The coarsely ground meat (780 g) was divided into seven lots and each lot was blended in a Waring blender with 350 ml of methanol–chloroform 2:1 (v/v) for 1 min. The homogenates were combined, shaken, and left at room temperature for 1–2 hr. The mixture was then filtered through coarse filter paper and Pyrex glass wool on a Buchner funnel and the cell residue was washed with 500 ml of methanol–chloroform 2:1. The combined filtrates were diluted with 825 ml of chloroform, followed by 825 ml of water, transferred to a separatory funnel, and the phases were allowed to separate overnight. The chloroform phase was separated, diluted with benzene, and concentrated in vacuo to dryness, and the residual lipid material was dissolved in chloroform (50 ml) and stored at 4°C. The yield of total lipids was 15.5 g (19.9 mg/g fresh tissue).

Isolation and purification of lecithin from beef heart lipids

Total beef heart lipids were separated into neutral lipids and phospholipids by acetone precipitation as described elsewhere (13). Lecithin was then isolated from the phospholipid fraction through the successive use of aluminum oxide and silicic acid chromatography. The aluminum oxide chromatographic separation was conducted by a modification of the procedure of Luthra and Sheltawy (14) in which lecithin and sphingomyelin were eluted with chloroform–methanol 66:34 (v/v) and then phosphatidylethanolamine and cardiolipin were removed together using the solvents chloroform–methanol–water 25:25:4 (v/v) and 10:10:3 (v/v), successively. The lecithin–sphingomyelin fraction was rechromatographed on a column of silicic acid and elution was carried out with chloroform–methanol 9:1 (v/v), 3:2 (v/v), and 1:4 (v/v), successively. Lecithin was eluted with chloroform–methanol 3:2 and migrated as a single spot on TLC (**Fig. 1**), giving positive tests (13) for plasmalogen (Schiff reagent), phosphorus (molybdate–H_2S0_4), and choline (Dragendorff). The purified lecithin had a phosphorus content of 4.0% and showed the expected infrared spectrum (13); yield, 3.0 g (3.8 mg/g fresh tissue).

Acid treatment of the purified lecithin from beef heart

Beef heart lecithin (35 mg phospholipid phosphorus) in 20 ml of chloroform was treated with 2 ml of 4 N HCl. The mixture was shaken vigorously by hand for 1–2 min and then transferred rapidly to a separatory funnel and washed three times with 20 ml of methanol–water 10:9 (v/v). The chloroform phase was usually acid-free after the second wash. The chloroform-soluble fraction containing the acid-stable lipids and free aldehydes was diluted with benzene and taken to dryness in a rotary evaporator. The effectiveness of this treatment in releasing free aldehydes from the plasmalogens was checked by thin-layer chromatography using the solvent system chloroform–methanol–water 65:35:5 (v/v) (see Fig. 1). Free and bound aldehydes were detected, respectively, by spraying with the Schiff reagent before and after spraying with $HgCl_2$ and sodium bisulfite (13). The acid-stable lecithin and

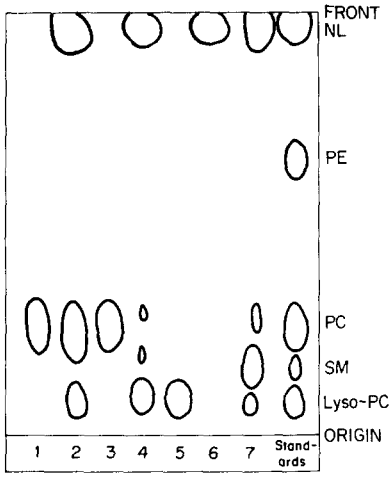

Fig. 1. Thin-layer chromatogram on silica gel H of lipid fractions from beef heart. *1*) Purified lecithin isolated by alumina and silicic acid column chromatography (starting material); *2*) chloroform-soluble products of mild acid treatment of lecithin; *3*) acid-stable lecithin isolated from mixture *2* by silicic acid column chromatography; *4*) chloroform-soluble products of mild alkaline deacylation of acid-stable lecithin; *5*) acid-stable lysolecithin isolated from mixture *2* by silicic acid chromatography; *6*) chloroform-soluble products of mild alkaline deacylation of acid-stable lysolecithin; *7*) chloroform-soluble products of mild acid plus alkaline hydrolysis of total beef heart lipids. Solvent: chloroform–methanol–water 65:35:5 (v/v). Standards: lyso-PC, lysolecithin; SM, sphingomyelin; PC, lecithin; PE, phosphatidylethanolamine; NL, neutral lipids.

lysolecithin were isolated by silicic acid chromatography (as described above).

Base treatment of the acid-stable lecithin and lysolecithin

Acid-stable lecithin and lysolecithin were subjected to mild alkaline deacylation with 0.2 N NaOH in methanol for 60–90 min, according to the procedure previously described (13). Acid- and alkali-stable lecithin and lysolecithin were separated by preparative TLC on silicic acid (Fig. 1).

Acid–base treatment of total lipids of beef heart

Total beef heart lipids (31 mg) in 1 ml of chloroform were treated with 0.1 ml of 4 N HCl followed by subjection of the chloroform-soluble products to mild alkaline hydrolysis, as described above. The final chloroform-soluble products were examined by TLC (Fig. 1).

Isolation of unsaponifiable material from beef heart

Total beef heart lipids (3.1 g) were heated under reflux in 30 ml of 2.5% anhydrous HCl (0.7 N) in methanol for 5 hr (3, 13); 5 ml of 7 N aqueous sodium hydroxide was then added and refluxing was continued for 2 hr. The mixture was diluted with 60 ml of 90% methanol and the unsaponifiable material (containing sterols, aldehyde dimethyl acetals, etc., and any monoether or diether present) was extracted with several portions of petroleum ether (bp 30–60°C); any emulsions were broken by centrifugation. The yield of total unsaponifiable material was 182 mg/g total lipids (18%). After extraction of the unsaponifiable material, the hydrolysate was acidified with 2.6 ml of 6 N HCl and the free fatty acids were extracted with petroleum ether. The yield of fatty acids was 600 mg/g total lipids (60%). After extraction of the fatty acids, the hydrolysate (35.5 ml) was diluted with chloroform (31.5 ml) and then with water (22.5 ml) to form a two-phase mixture. The lower chloroform phase was withdrawn, diluted with benzene, and evaporated to dryness in vacuo; the residue was redissolved in chloroform (5 ml). This fraction should contain any monoalkyl ether analogue of lysophosphatidic acid formed from monoalkyl ether analogues of glycerophospholipids; dialkyl ether analogues of glycerophospholipids are hydrolyzed under the above conditions to di-*O*-alkyl glycerols (3, 13), which appear in the unsaponifiable fraction. The yield of monoether analogue of lysophosphatidic acid (identified by TLC in $CHCl_3$–methanol–90% acetic acid, 30:4:20; R_f 0.54) was 8 mg/g total lipids (0.8%).

Chromatography

For separation of glycerophospholipids, lipids were chromatographed on silica gel H plates (0.25 mm, analytical; 1 mm, preparative) with chloroform–methanol–water 65:35:5 (v/v) as solvent.

The unsaponifiable lipids were analyzed by thin-layer chromatography on silica gel H plates developed in either chloroform or chloroform–ether 20:1 (v/v). For preparative separation of the unsaponifiable fraction, up to 40 mg of lipid material was streaked on silica gel H plates (1 mm thick layers) which were developed in the chloroform–ether solvent. As markers, authentic samples of long-chain aldehyde dimethyl acetals, fatty acid methyl esters, cholesterol, and 1,2-di-*O*-octadecyl glycerol (15) were used.

Gas–liquid chromatography was carried out on a 6 ft column of 10% butanediolsuccinate polyester on Gas-Chrom A at 185°C and a gas flow rate of 60 ml/min in a Carlo-Erba gas chromatograph. Authentic standards of alkyl chlorides (see below) and isopropylidene derivatives of 1-*O*-alkyl glycerols (12) were used to calibrate the column.

Cleavage of the di-*O*-alkyl glycerol with boron trichloride (3)

A solution of the beef heart di-*O*-alkyl glycerol (1 mg) in 5 ml of chloroform was cooled to 0°C and flushed with boron trichloride gas. The reaction flask was tightly stoppered and allowed to stand at room temperature for 24 hr. The mixture was then concentrated under a stream of nitrogen and the residual oil was dissolved in petroleum ether and washed with water. The petroleum ether phase was analyzed both by TLC and by GLC. As controls, 1,2-di-*O*-hexadecyl, 1,2-di-*O*-octadecyl, 1,2-di-*O*-octadecenyl, and 1,2-di-*O*-phytanyl glycerol (6,15) were cleaved with boron trichloride in the same manner and the petroleum ether phases obtained were used as standards for hexadecyl, octadecyl, octadecenyl, and phytanyl chlorides, respectively.

RESULTS

Characterization of products from acid treatment of beef heart lecithin

Brief acid treatment of the purified beef heart lecithin (Fig. 1, lane 1), as described above, proceeded smoothly with no emulsion problems and with quantitative recovery of lipid phosphorus in the chloroform-soluble lipids (**Table 1**). The lipid reaction products were shown by thin-layer chromatography to contain two phosphorus-positive compounds with R_f values corresponding to those of lecithin and lysolecithin and a third non-phosphorus containing Schiff-positive component that chromatographed at or near the solvent front in the polar solvent system (Fig. 1, lane 2) and accounted for all of the aldehyde in the original purified lecithin fraction. The acid-stable lecithin and lysolecithin contained no detectable plasmalogen and accounted for 61% and 39%, respectively, of the original lipid phosphorus (Table 1). The quantitative recovery of lipid phosphorus and the absence of plasmalogen in the lecithin and lysolecithin obtained after acid treatment rule out the occurrence of significant amounts of di-alk-1-enyl-GPC (VI) in beef heart lipids.

The acid-stable lecithin and lysolecithin, isolated as described in the Methods section, were chromatographically pure (Fig. 1, lanes 3 and 5, respectively); the lecithin had a phosphorus content of 4.02% and the lysolecithin had 6.07% P (Table 1). Infrared spectra of the two lipids gave the expected patterns and no plasmalogen was detected in either fraction.

TABLE 1. Lipid products of mild acid and alkaline hydrolysis of beef heart lecithin[a]

Products		Distribution of Lipid-P, % of Total Lecithin-P
Mild acid hydrolysis		
Total PC	↗ Acid Stable-PC (I + II + III)[b]	61.3
	↘ Acid-stable lyso-PC (VIII + IX, from IV and V, resp.)	39.9
Mild acid + alkaline hydrolysis		
I,II,III	↗ Acid-Alkali-stable PC (II)	3.8[b]
	↘ Acid-Alkali-stable lyso PC (VII from III)	
VIII + IX →	Acid-alkali-stable lyso-PC (IX from V)	0.0

[a] Beef heart lecithin (300 mg, containing 12.0 mg lipid-P) was treated with acid as described in Methods section and the chloroform-soluble products (12.8 mg lipid-P; 107% recovery) were then fractionated by silicic acid chromatography. The isolated acid-stable lecithin [160 mg, containing 6.44 mg lipid-P (4.02% P)] and lysolecithin [69 mg, containing 4.19 mg lipid-P (6.07% P)] were then separately treated with mild alkali and the chloroform-soluble products were assayed for lipid-P and examined by TLC (see Fig. 1).

[b] Included traces of sphingomyelin.

Characterization of products from base treatment of acid-derived lysolecithin

The acid-stable lysolecithin (Fig. 1, lane 5) was treated with mild alkali and the lipid products examined by thin-layer chromatography showed only one spot that was phosphorus-negative and ran at the solvent front in a polar solvent (Fig. 1, lane 6) and that chromatographed as fatty acid methyl ester (R_f 0.78) in the neutral lipid solvent described above. No phosphorus was detectable in this material (Table 1), and the infrared spectrum showed a typical pattern for methyl esters, which was confirmed by comparison with the infrared spectrum of pure methyl palmitate. The observations indicate that the lysolecithin formed by acid treatment of the original lecithin was a monoacyl-GPC and was derived from the plasmalogen (IV). These observations further rule out the possibility of the occurrence of significant amounts of an alk-1-enyl alkyl species of lecithin (V).

Products from base treatment of acid-derived lecithin

The acid-stable lecithin (Fig. 1, lane 3), after treatment with alkali, was shown to contain three phosphorus-positive components (Fig. 1, lane 4) which represented 3.8% of the starting lecithin (6.3% of

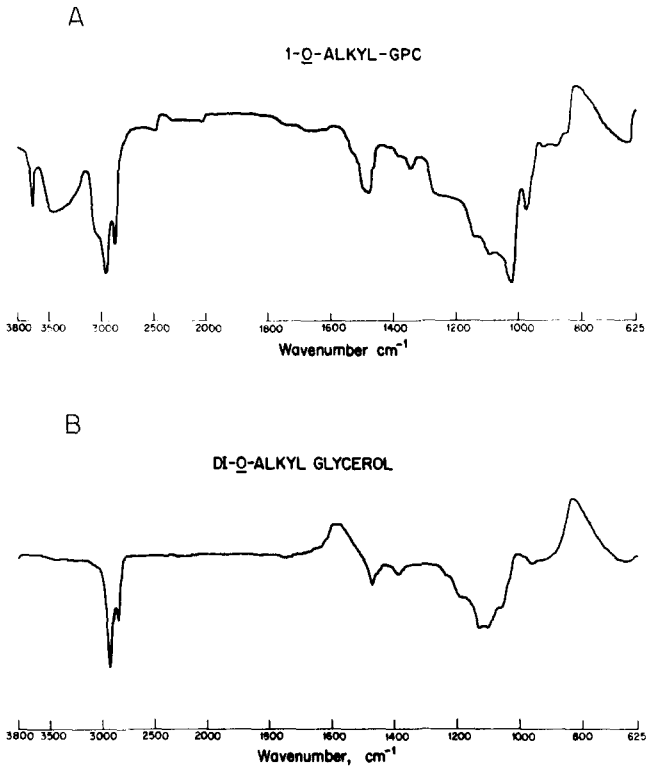

Fig. 2. Infrared spectrum of: *A*, 1-*O*-alkyl-GPC (VII) obtained after base treatment of acid-stable lecithin (see Methods); sample concentration 1% (wt/vol) in chloroform. *B*, Di-*O*-alkyl glycerol isolated from the unsaponifiable lipids of beef heart; sample concentration 0.58% (wt/vol) in carbon tetrachloride.

acid-stable lecithin) (Table 1). Of these, the major component was the most polar one having R_f value (0.1), phosphorus content (6.32% P), and infrared spectrum (**Fig. 2A**) corresponding to those of 1-*O*-alkyl-GPC. Acetolysis of the latter yielded 1-*O*-alkyl glycerols in which the distribution of chain lengths (determined by gas–liquid chromatography of the isopropylidene 1-*O*-alkyl glycerol derivatives) was shown to be: 14:0, 1.7%; 15:0, 5%; br-16:0, 5%; 16:0, 61%; 18:0, 8%; 18:1, 24%. The isolation of a 1-*O*-alkyl-GPC after base treatment of the acid-stable lecithin shows that the original sample must have contained a monoacyl monoalkyl species (III).

The two other acid- and alkali-stable phosphorus-containing compounds were relatively minor components (Fig. 1, lane 4). One of these compounds was identified as sphingomyelin by its mobility on TLC (Fig. 1, lane 4) and by its infrared spectrum; the other was tentatively identified as a di-*O*-alkyl-GPC (II) on the basis of the following observations: R_f value on TLC identical to that of the original lecithin (Fig. 1, lane 4); acid and base stability; presence of choline as shown by the positive reaction with the Dragendorff reagent; and the presence of absorption bands at 1095 cm^{-1} (C–O–C ether), 1250 cm^{-1} (P = 0), and 1065 cm^{-1} (P–O–C), consistent with the spectrum reported for synthetic di-*O*-octadecyl-GPC (16). The phosphorus content was also consistent with the proposed di-*O*-alkyl-GPC structure (found, 3.65% P; calculated for the dioctadecyl ether analogue of lecithin (16), 3.97% P).

The same three products, namely 1-*O*-alkyl-GPC, sphingomyelin, and (presumably) di-*O*-alkyl-GPC were also the only products observed after acid–base treatment of total beef heart lipids (Fig. 1, lane 7). Only the 1-*O*-alkyl glycerol, but no detectable amount of di-*O*-alkyl glycerol, was found in the neutral lipid fraction (see above) either before or after drastic saponification, indicating the presence of monoalkyl-diacyl glycerols but not dialkyl-acyl glycerols.

Identification of the di-O-alkyl glycerol moiety of di-O-alkyl-GPC

The di-*O*-alkyl glycerol moiety of the presumed di-*O*-alkyl-GPC was isolated from the unsaponifiable lipids (see Methods section) by preparative thin-layer chromatography (yield, 0.5 mg/g total lipids) and was found to have a R_f value identical to that of synthetic 1,2-di-*O*-octadecyl glycerol (15) in two solvent systems: R_f 0.45 in chloroform–ether 20:1; R_f 0.31 in chloroform. The beef heart dialkyl glycerol also had an infrared spectrum (Fig. 2*B*) identical to that reported earlier (15) for di-*O*-octadecyl glycerol, with strong absorption bands characteristic of a glycerol diether (ether C–O–C, 1120 cm^{-1} and primary alcoholic C–O, 1050 cm^{-1}), as well as strong CH_2 and CH_3 absorption bands (2930, 2850, 1465 and 1370 cm^{-1}) and a weak band corresponding to OH (3450 cm^{-1}). No significant band corresponding to ester (C = 0, 1730 cm^{-1}) was apparent.

The beef heart di-*O*-alkyl glycerol was further characterized by cleavage with boron trichloride which yielded a petroleum ether-soluble product with a R_f on silica gel H TLC identical to that of standard octadecyl chloride (R_f 0.70 in chloroform–ether 20:1). When analyzed by GLC, the boron trichloride products showed one major peak (peak 1, 84%) with relative retention identical to that of authentic octadecyl chloride and only minor or trace peaks corresponding to C_{16} (peak 2), C_{14} (peak 3) and C_{12} (peak 4) chains (**Table 2**). Thus the major

diether component in beef heart lipids is identified as the di-*O*-octadecyl derivative of glycerol, suggesting that the major di-*O*-alkyl-GPC may be largely the di-*O*-octadecyl analogue, previously synthesized (16). However, an insufficient amount of the dialkyl-GPC was available for further comparison with the synthetic material.

DISCUSSION

Data presented in this paper show that lecithin from bovine heart is composed of at least four species. Two of these are the well-known diacyl ester (I) and plasmalogen (IV) forms. Another species containing one acyl chain and one saturated ether chain (III) was isolated as the 1-*O*-alkyl-GPC derivative (VII) and was shown to contain alkyl chains ranging from C_{14} to C_{18} with 16:0 (61%) and 18:1 (24%) being the most predominant. The occurrence of small amounts (3–5%) of monoalkyl monoacyl lecithins in beef heart with similar alkyl chains had been reported previously in several studies (17–19), and this is now confirmed by the present findings.

A di-*O*-alkyl-GPC species (II) was also isolated from beef heart and the di-*O*-alkylglyceryl moiety was shown to contain only saturated alkyl chains ranging from C_{12} to C_{18} with 18:0 (84%) being the major chain (Table 2). The major di-*O*-alkyl-GPC component is thus most likely a di-*O*-octadecyl-GPC. The absence of any significant amount of unsaturation in the dialkyl glycerols is noteworthy, in contrast to the presence of a considerable amount of 18:1 chain in the 1-*O*-alkyl glycerols.

Since we now know that neither the alk-1-enyl alkyl ether type (V) nor the di-alk-1-enyl ether type (VI) lecithin occurs in beef heart, the di-*O*-alkyl glycerol (largely di-C_{18}) isolated by Marinetti and Stotz (9) from hydrolysates of hydrogenated beef heart lipids must have arisen from a dialkyl ether type phospholipid, thus lending further evidence for the presence of a di-*O*-alkyl-GPC (II) in beef heart.

From the present data (Table 1) the four main species of lecithin may be calculated to be present in the following proportions: diacyl species (I), 57%; plasmalogens (IV), 39%; and mono-*O*-alkyl monoacyl (III) plus di-*O*-alkyl (II), 3.8%. The di-*O*-alkyl type (II) alone is estimated to be about 0.2% of the total lecithin fraction, on the basis of the amount of glycerol diether isolated from the total lipids. The alk-1-enyl alkyl ether type (V) is considered to be absent in beef heart lipids, on the basis of the present studies showing that no phospholipid-P was obtained after mild alkaline hydrolysis of the acid-stable lysolecithin fraction.

TABLE 2. Gas–liquid chromatographic identification of alkyl chlorides derived from the di-*O*-alkyl glycerol moiety of beef heart lipids

Alkyl Chloride Peak	Identity	Relative Retention[a]	Area %
1	12:0	0.076	7
2	14:0	0.16	8
3	16:0	0.33	1
4	18:0	0.63	84

[a] Retention of compounds relative to methyl octadecanoate on butanediol succinate polyester at 185°C. Retention of methyl octadecanoate, 11 min. Relative retention of standard alkyl chlorides: 16:0, 0.32; 18:0, 0.63; 18:1, 0.71; phytanyl chloride, 0.49.

The question concerning the possible role of the small amounts of monoether and diether lecithins in cellular membranes of heart is an intriguing one that must await the results of further experimentation.

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