Differential susceptibility of mono- and di-O-alkyl ether phosphoglycerides to acetolysis

Raj Kumar, Susan T. Weintraub, and Donald J. Hanahan

Departments of Biochemistry[1] and Pharmacology,[2] The University of Texas Health Science Center at San Antonio, San Antonio, TX 78284

Abstract The effectiveness of acetolysis as a tool in structural characterization of mono- and di-O-alkyl phosphoglycerides was investigated. Surprisingly, it was found that the di-O-alkyl phosphoglycerides were resistant to attack during acetolysis, whereas the mono-ether types, with a free hydroxyl function or an ester on carbon-2, were easily attacked at the glycerol-phosphate bond. On the other hand, Vitride reduction occurred readily with the mono-ether or di-ether phosphoglycerides. The implications of these findings as they relate to identification of ether phospholipids in tissues are discussed.—**Kumar, R., S. T. Weintraub, and D. J. Hanahan.** Differential susceptibility of mono- and di-O-alkyl ether phosphoglycerides to acetolysis. *J. Lipid Res.* 1983. **24:** 930–937.

Supplementary key words GLC–mass spectrometry • thin-layer chromatography • Vitride

Characterization of individual molecular species within different classes of phosphoglycerides, especially by gas–liquid chromatography–mass spectrometry (GLC–MS), is facilitated by derivatization of the compounds into a less polar form. A variety of procedures has been developed to accomplish this end. Techniques such as acetolysis (1), which is a reaction with acetic acid and acetic anhydride, enzymatic degradation with phospholipase C (2) followed by acetylation, reduction with reagents such as Vitride ($NaAlH_2(OCH_2CH_2OCH_3)_2$) (3) prior to formation of methyl (4), acetyl (5), isopropylidine (6), trimethylsilyl (7), or t-butyldimethylsilyl (8) derivatives have been used. Of these methods, the phospholipase C-acetylation technique is the mildest, but there are limitations regarding substrate specificity of this enzyme. Even though acetolysis is more severe it is often preferred over the other procedures because of its simplicity, as no further derivatization of the product is required. This technique has been successfully employed in the structure elucidation of mono ether–mono acyl phosphoglycerides (9) and diacyl phosphoglycerides (10). No information is available, however, on the use of this technique with diether phospholipids. The presence of diether phospholipids in the bovine heart (11) and the possibility that such compounds may also be present in rabbit and human platelets, polymorphonuclear neutrophils (PMN), and basophils[3] led us to study the effectiveness of this derivatization procedure. The present report describes a novel finding as regards use of this approach for characterization of authentic mono- and diether phospholipids and comparison of its use with related ester phospholipids.

EXPERIMENTAL PROCEDURE

Materials

Synthetic *rac*-1-O-octadecyl-2-O-methyl-glycero-3-phosphocholine (MGEPC) was obtained from Dr. Hansjorg Eibl and 1-O-octadecyl-2-acetyl-*sn*-glycero-3-phosphocholine (AGEPC) was purchased from Bachem Feinchemikalien (Switzerland). 1-O-octadec-9-enyl-2-O-hexadecyl-*sn*-glycero-3-phosphocholine (DEPC) was purchased from Calbiochem (Behring, CA). 1,2-Dipalmitoyl-*sn*-glycero-3-phosphocholine (DPPC) was acquired from Avanti Biochemicals (Birmingham, AL). Phosphatidylcholine (PC) was isolated from egg according to the procedure of Wells and Hanahan (12). Vitride was purchased from Alpha (Danvers, MA). Applied Science (State College, PA) was the source of the t-butyldimethylchlorosilane/imidazole reagent (TBDMCS) and 3% OV-17 on 100/120 mesh Gas-Chrom Q. The 10% SP-2330 on 100/120 mesh Chromosorb W AW was obtained from Supelco (Bellefonte, PA). Thin-layer chromatography plates (Silica Gel G, 250 μm for analytical evaluations and 500 μm for preparative proce-

Abbreviations: AGEPC, 1-O-octadecyl-2-acetyl-*sn*-glycero-3-phosphocholine; DEPC, 1-O-octadec-9-enyl-2-O-hexadecyl-*sn*-glycero-3-phosphocholine; DPPC, 1,2-dipalmitoyl-*sn*-glycero-3-phosphocholine; MGEPC, *rac*-1-O-octadecyl-2-O-methyl-glycero-3-phosphocholine; PC, phosphatidylcholine; TBDMCS, t-butyl-dimethylchlorosilane/imidazole; TBDMS, t-butyl-dimethylsilyl; GLC–MS, gas–liquid chromatography–mass spectrometry; TLC, thin-layer chromatography.

[1] R. Kumar and D. J. Hanahan.

[2] S. T. Weintraub.

[3] Kumar, R., S. T. Weintraub, and D. J. Hanahan. Unpublished observations.

TABLE 1. Cleavage of the phosphate moiety from selected phosphoglycerides by acetolysis

	MGEPC[a]	DEPC[b]	AGEPC[c]	PC[d]	DPPC[e]	PC + MGEPC	AGEPC + MGEPC
Initial phosphorus content (μg)	18	23	19	19	18	18 + 18	18 + 19
Phosphorus content after acetolysis (μg)[f]	17.1 ± 0.4	22.0 ± 0.4	0.5 ± 0.2	0.6 ± 0.2	0.5 ± 0.2	18.0 ± 1.0	19.4 ± 0.9
Percent phosphate cleaved by acetolysis	5.0	4.4	97.5	96.5	97.1	50.0	47.5

[a] *rac*-1-O-octadecyl-2-O-methyl-glycero-3-phosphocholine.
[b] 1-O-octadec-9-enyl-2-O-hexadecyl-*sn*-glycero-3-phosphocholine.
[c] 1-O-octadecyl-2-acetyl-*sn*-glycero-3-phosphocholine.
[d] Phosphatidylcholine (egg).
[e] 1,2-Dipalmitoyl-*sn*-glycero-3-phosphocholine.
[f] The numbers represent the mean ± standard deviation of five analyses for the amount of chloroform-extractable phosphorus remaining after acetolysis.

dures) were obtained from Analtech (Newark, DE) and were prewashed with the appropriate developing solvent system and then activated by heating for 0.5 hr at 140°C immediately before use. All solvents were of reagent grade.

Methods

Acetolysis. A sample containing 18 to 20 μg of P was added to a 12-ml Pyrex tube fitted with a screw-cap with a Teflon liner; 0.5 ml of acetic anhydride–acetic acid

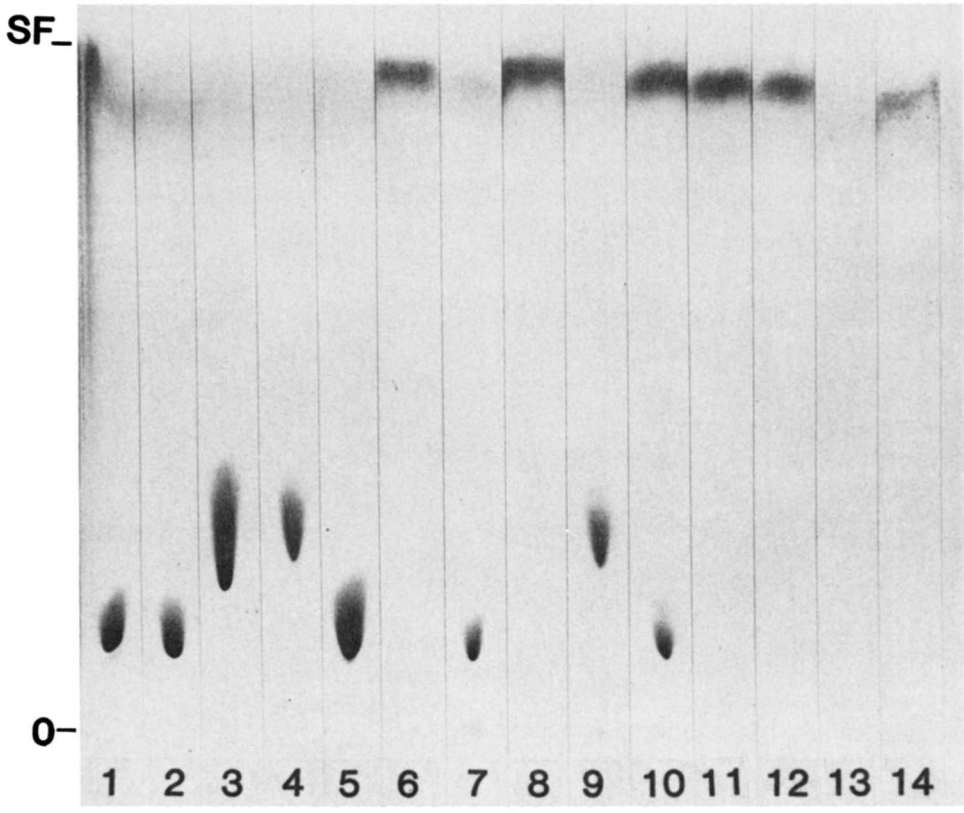

Fig. 1. Thin-layer chromatogram of selected phosphoglycerides after acetolysis using a solvent system of chloroform–methanol–water 65:35:6 (v/v/v). SF, solvent front; O, origin. Lane 1, AGEPC; lane 2, MGEPC; lane 3, egg PC; lane 4, DEPC; lane 5, AGEPC + MGEPC; lane 6, acetolysis product of AGEPC; lane 7, acetolysis product of MGEPC; lane 8, acetolysis product of egg PC; lane 9, acetolysis product of DEPC; lane 10, acetolysis product of AGEPC + MGEPC; lane 11, Vitride treatment product of AGEPC; lane 12, Vitride treatment product of MGEPC; lane 13, ether solvent blank; lane 14, Vitride treatment product of AGEPC + MGEPC.

3:2 (v/v) was added and the mixture was heated at 150°C for 5 hr in a metal heating block. The reaction mixture was allowed to cool to room temperature, 2 ml of chloroform was added and the mixture was vortexed. The lower chloroform-rich phase was washed several times with deionized water and dried over anhydrous sodium sulfate. After evaporation of the solvent by a stream of nitrogen, the residue was redissolved in a known volume of chloroform and analyzed immediately.

Vitride treatment. The method of Snyder, Blank, and Wykle (3) was employed for Vitride reduction. Samples containing between 10 and 18 μg of P in 0.1 ml of diethyl ether were treated with 0.5 ml of Vitride reagent in 15-ml glass screw-capped tubes. The sealed tubes were maintained at 37°C for 1 hr with continuous shaking in a water bath. After the addition of 6 ml of 4% acetic acid to stop the reaction, the mixture was extracted with 2 × 5-ml portions of diethyl ether. The combined ether extract was washed twice with 2 ml of deionized water. The product was purified by TLC using a solvent system of chloroform–acetone 96:4 (v/v) and derivatized as described below.

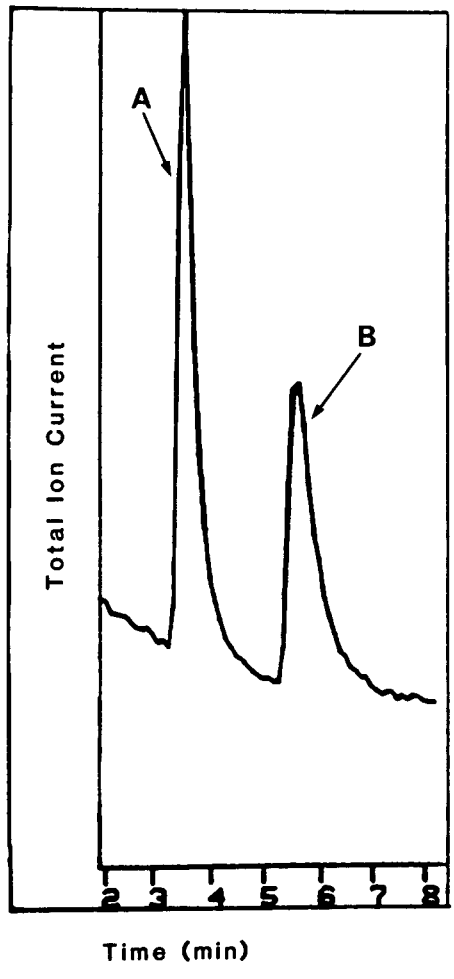

Fig. 2. Total ion current chromatogram of a mixture of 1-O-octadecyl-2-O-methyl-3-acetyl-glycerol (A) and 1-O-octadecyl-2,3-diacetyl-glycerol (B). GLC separation was accomplished with a 6 ft × 4 mm i.d. glass column packed with 10% SP-2330 at a column temperature of 275°C, as described in Experimental Procedure.

Acetylation. As a typical example 1-O-octadecyl-2-O-methyl-3-glycerol (280 μg), obtained after Vitride treatment of MGEPC, was dissolved in 0.5 ml of chloroform. Acetic anhydride (0.5 ml) and perchloric acid (0.01 ml, 12 N) were slowly added and the mixture was vortexed for 30 sec. The reaction was then stopped by placing the tube in an ice bath and adding cold water (0.45 ml) and methanol (0.5 ml). The lower organic phase was separated after centrifugation and the desired product was obtained by preparative TLC using a solvent system of chloroform–acetone 96:4 (v/v).

t-Butyl dimethylsilyl derivative formation. To a 10-ml glass screw-capped tube containing approximately 200 μg of dephosphorylated glyceride was added 100 μl of TBDMCS reagent. The reaction mixture was heated at 100°C for 15 min. After cooling, the mixture was extracted twice with 10 ml of chloroform and the lower organic layer was washed two times with 4 ml of water and dried over anhydrous sodium sulfate. The chloroform-rich phase was concentrated and used directly for GLC–MS analysis.

Phosphorus analysis. Perchloric acid digestion of samples was employed prior to phosphorus analysis by the method of Bartlett (13).

Gas–liquid chromatography–mass spectrometry. A Hewlett-Packard model 5982 mass spectrometer in combination with a Hewlett-Packard model 5710 gas chromatograph and a Hewlett-Packard model 5933 data system was utilized for these analyses. Gas chromatographic separation of acetylated compounds was accomplished with a 6 ft × 4 mm i.d. glass column packed with 10% SP-2330 on 100/120 mesh Chromosorb W AW using a helium flow rate of 30 ml/min, injector temperature of 250°C, and a column temperature of 275°C. For TBDMS derivatives, a 3 ft × 4 mm i.d. column of 3% OV-17 on 100/120 mesh Gas-Chrom Q was employed at a column temperature of 260°C. Samples were introduced into the mass spectrometer by means of a glass jet separator and glass-lined interface which was maintained at 300°C. Positive ion electron impact mass spectra were obtained from full mass scans that were recorded and stored continuously during the GLC–MS run. Selected ion retrieval traces were acquired by computer evaluation of the data.

RESULTS AND DISCUSSION

In the present study the effectiveness of the acetolysis procedure for removal of the phosphate group from

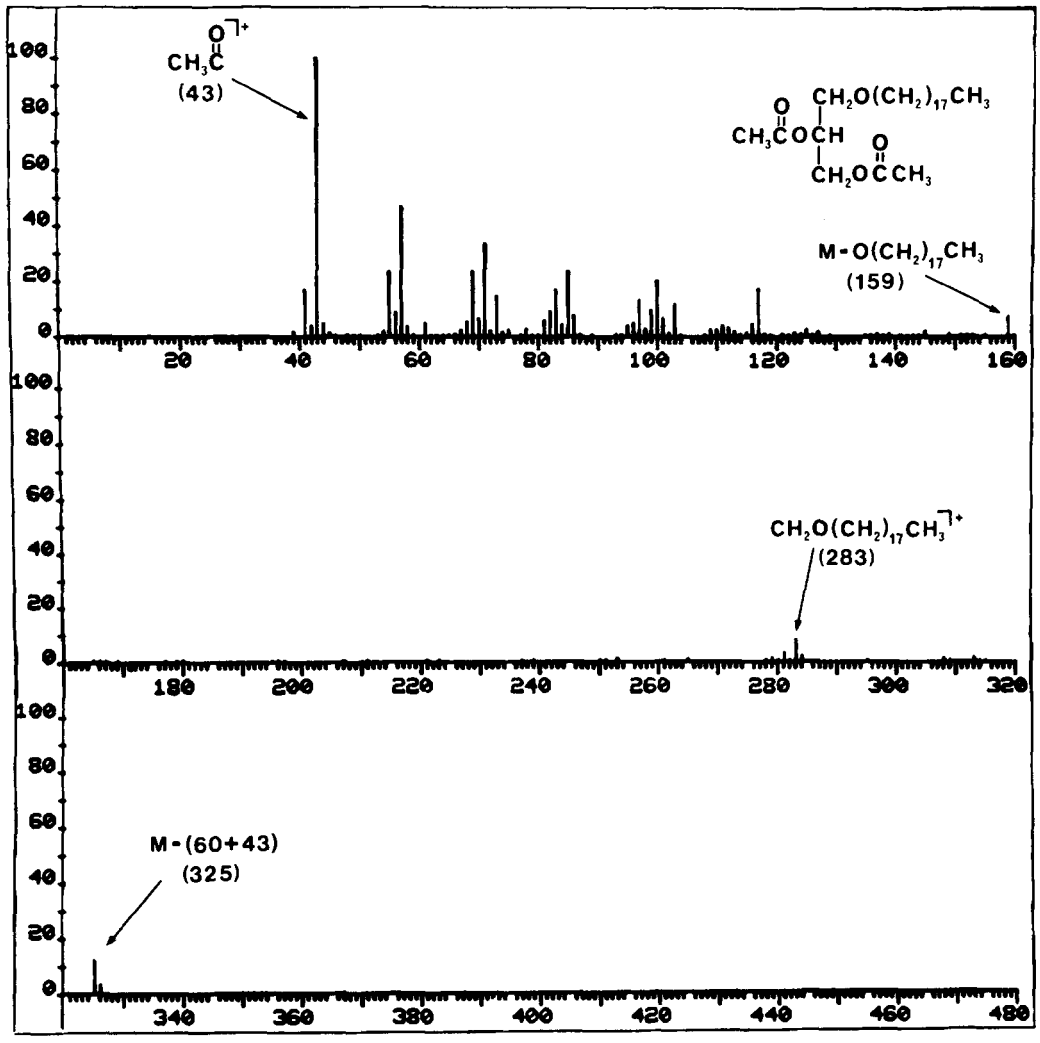

Fig. 3. Electron impact mass spectrum (70 eV) of 1-O-octadecyl-2,3-diacetyl-glycerol.

the glycerol backbone of various phosphoglycerides was investigated. In **Table 1** it can be seen that essentially quantitative cleavage of the phosphate was obtained for AGEPC, PC, and DPPC by the acetolysis technique. For MGEPC and DEPC, however, little reduction of the chloroform-soluble phosphorus was seen. When a mixture of phospholipids was subjected to acetolysis with one component of the mixture having an ether group on the 2 position of glycerol and the other component having an ester group on the 2 position, approximately 50% of the phosphorus was recovered in the chloroform-rich phase. In contrast, after treatment with Vitride, in all the compounds including the diether phosphoglycerides (data not shown), no phosphorus was detectable in the resultant chloroform-rich phase. These results suggested that, depending on the nature of the substitution on carbon 2, selective cleavage of the phosphate group in certain phosphoglycerides was possible by the acetolysis procedure, warranting further investigation of the derivatized compounds.

Thin-layer chromatographic behavior of products

Examination of the products, described above, by TLC provided an important insight into the nature of any attack on the phosphate that occurs during acetolysis. A typical chromatogram is shown in **Fig. 1** where it is clearly seen that following acetolysis MGEPC (lane 7) and DEPC (lane 9), both diether phospholipids, exhibited spots with R_f values that coincided with the corresponding untreated compounds (lanes 2 and 4, respectively). At the same time, the acetolysis products of AGEPC (lane 6) and egg PC (lane 8), i.e., glyceride acetates, moved near the solvent front. For a mixture of AGEPC and MGEPC the untreated standards comigrated as shown in lane 5. After acetolysis, however, the mixture of AGEPC and MGEPC (lane 10) yielded

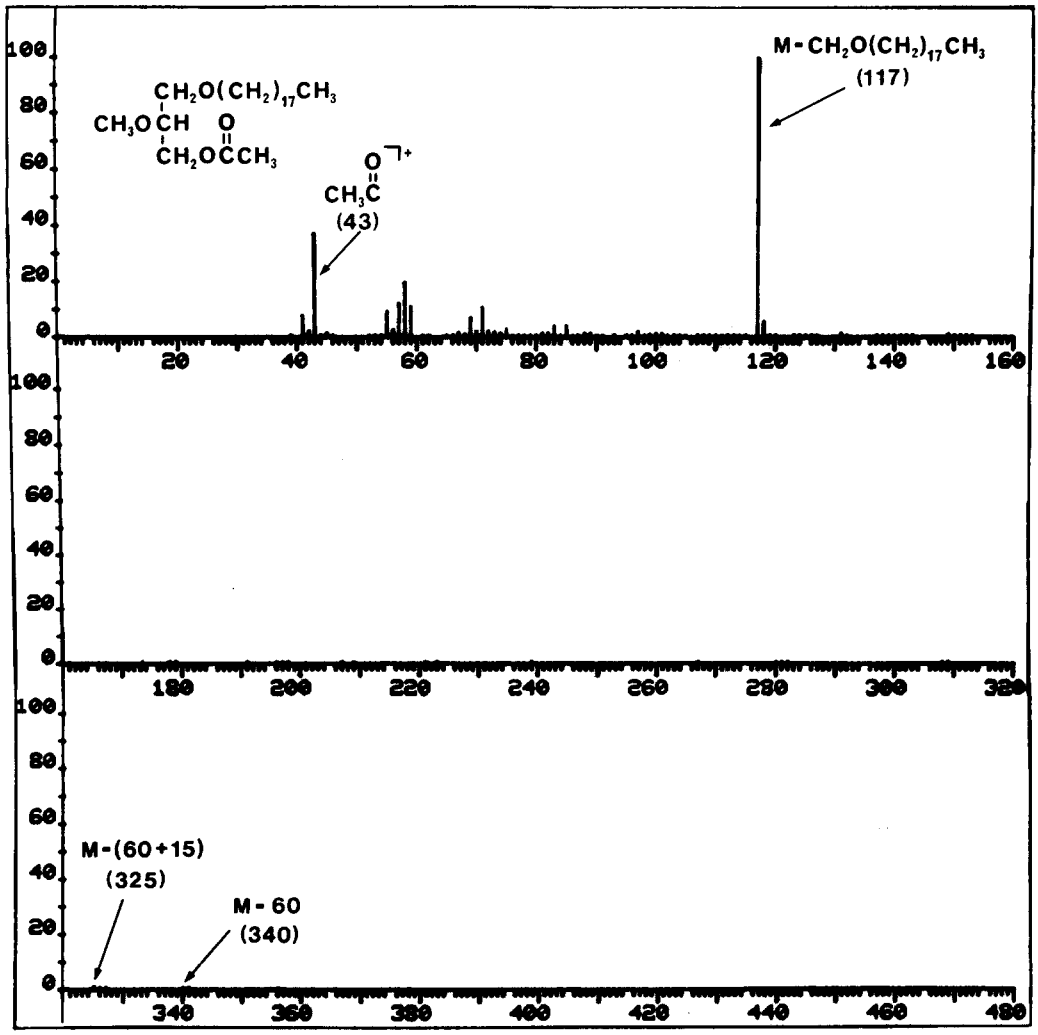

Fig. 4. Electron impact mass spectrum (70 eV) of 1-O-octadecyl-2-O-methyl-3-acetyl-glycerol.

two spots on TLC, one with an R_f value corresponding to the R_f value of untreated MGEPC, and the other migrating near the solvent front, corresponding to a glyceride. In contrast to the results of acetolysis, Vitride treatment produced derivatives in the current study that all migrated near the solvent front in the solvent system shown in Fig. 1 (lanes 11, 12, 14), appropriate for the fatty alcohols expected by this treatment.

It was also considered important to measure the amount of phosphorus that remained associated with the TLC spots attributed to the acetolysis product of AGEPC and the presumably unchanged MGEPC. To accomplish this, a mixture of AGEPC (18 μg of P) and MGEPC (18 μg of P) was subjected to the usual acetolysis procedure. Following TLC in chloroform–methanol–water 65:35:5 (v/v/v), portions of the plate of approximately 1 cm, starting at the origin, were removed by scraping, extracted with chloroform–methanol–water 1:2:0.8 (v/v/v) (14) and analyzed for phosphorus content. From the spot corresponding to MGEPC, 16 μg of P, representing 89% of the starting amount of MGEPC, was recovered, with some loss probably occurring during extraction. As regards the spot derived from AGEPC, however, no phosphorus was detected. Thus, the acetolysis procedure did not alter the TLC mobility of MGEPC and also did not significantly diminish its phosphorus content.

Gas–liquid chromatography–mass spectrometry

In order to further elucidate the nature of these chemical treatments, the mass spectra of the products were evaluated. Unfortunately, phospholipids containing long chain acyl or alkyl groups are not amenable to standard silylation procedures that render the substances sufficiently volatile for GLC–MS analysis. When acetolysis successfully removes the phosphate group,

Fig. 5. Selected ion retrieval profile derived from the GLC–MS analysis of 1-O-octadecyl-2-O-methyl-3-acetyl-glycerol. The lower trace represents the total ion current chromatogram (TI) and the upper traces are the profiles for the m/z values given; GLC conditions are the same as in Fig. 2.

however, a volatile acetyl derivative is usually obtained. If, on the other hand, the phosphate group remains attached, further treatment with a reductant such as Vitride can be used with glyceryl ethers to provide a sample that can then be derivatized for GLC–MS. To perform this evaluation for the present study two standard compounds were investigated. First, the acetolysis product of AGEPC, 1-O-octadecyl-2,3-diacetyl glycerol was analyzed. This substance had a GLC retention time of 5.5 min at 275°C on a 6 ft × 4 mm column of 10% SP-2330 (**Fig. 2,** peak B). The mass spectrum of this compound is shown in **Fig. 3.** No molecular ion peak at m/z 428 was observed. The base peak was found at m/z 43, representing the acetyl ion. Of more importance in structural elucidation, however, was the peak at m/z 325 which is formed by the loss of an acetyl group in addition to a molecule of acetic acid ([M–(43 + 60)]$^+$). Of further use in characterizing this substance was the ion at m/z 283, which is derived from cleavage of the bond between carbons 1 and 2 of the glycerol backbone, and the ion at m/z 159 which most likely represents the loss of [$O(CH_2)_{17}CH_3$] from the molecular ion.

The second compound to be analyzed by mass spectrometry for the present investigation has a methyl group in ether linkage at the number 2 carbon of a glyceryl ether phospholipid. Vitride treatment of this material produced 1-O-octadecyl-2-O-methyl-glycerol. Subsequent acetylation of this substance with acetic anhydride yielded a compound with a GLC retention time of 3.5 min under the conditions described above (Fig. 2, peak A), with a mass spectrum as shown in **Fig. 4.** Again, no molecular ion peak (m/z 400) was found. The spectrum is dominated by the peak at m/z 117 which is due to fragmentation between carbon 1 and carbon 2 of this derivative. A much smaller peak at m/z 43, attributed to acetyl, was observed. In addition, a minor peak at m/z 325 was detected which is formed by the loss of a methyl group and a molecule of acetic acid ([M–(15 + 60)]$^+$). Another ion of low intensity was found at m/z 340, representing the loss of a molecule of acetic acid ([M–60]$^+$). In order to verify that these small peaks were actually formed during the fragmentation of the substance of interest, selective ion retrieval profiles were evaluated. As can be seen in **Fig. 5,** the ion profiles for m/z 325 and m/z 340 matched that of the base peak, m/z 117 and of the total ion current chromatogram. Even though the spectrum shown in Fig. 4 could be clearly interpreted as correlating with the proposed structure, the ions that are representative of the long chain alkyl portion were very small and could be lost in samples of low concentration. It was of importance, therefore, to further characterize this compound by using a different derivative, so that complete confidence in its structure would be at hand. The derivative chosen was the t-butyldimethylsilyl ether, formed after Vitride treatment of the 2-O-methyl phospholipid. The mass spectrum of this compound is shown in **Fig. 6.** While no molecular ion was seen at m/z 472, the peak that is characteristic for TBDMS derivatives, [M–57]$^+$, was observed at m/z 415. In addition, the peak at m/z 383, representing [M–(57 + CH_3OH)]$^+$ was important in substantiating the structure of this derivative.

It was then of interest to determine if the acetylated derivative was formed after acetolysis of MGEPC. The total ion current chromatogram obtained from the GLC–MS analysis of the acetolysis products of a mixture of AGEPC and MGEPC is shown in **Fig. 7.** Evaluation of the mass spectra acquired during this scan confirmed that dephosphorylation and acetylation of AGEPC did occur as anticipated. For MGEPC, however, only a very small peak was seen at 3.5 min, signifying that a volatile

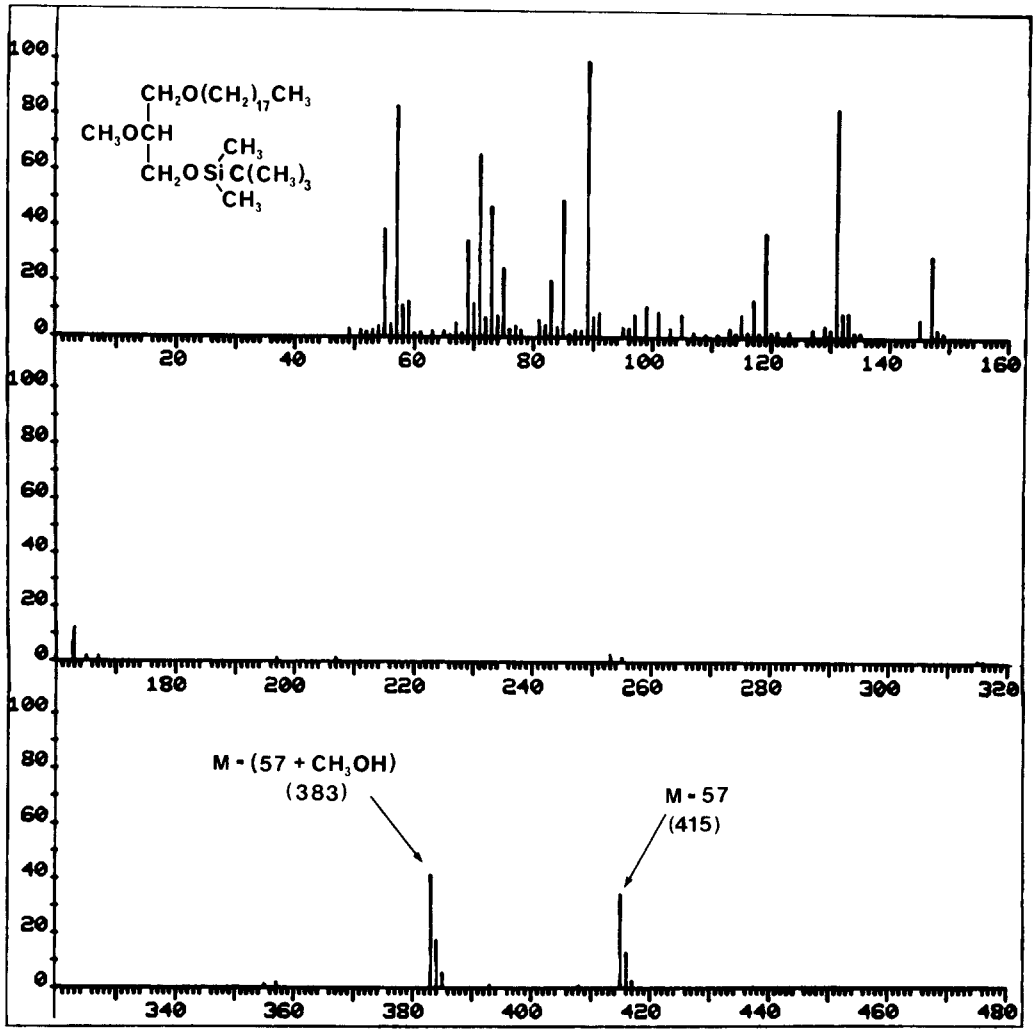

Fig. 6. Electron impact mass spectrum (70 eV) of the TBDMS derivative of 1-O-octadecyl-2-O-methyl-glycerol.

derivative was not formed. As a means of determining the relative amounts of the two substances present in the mixture, the total intensity of each respective base peak was assessed by computer integration of the appropriate selected ion retrieval profile. Following Vitride treatment of the mixture and subsequent acetylation (Fig. 2), the ratio of m/z 117 for the 2-O-methyl component to m/z 43 for the 2-O-acetyl was equal to 2.87. When this ratio was determined for the mixture after acetolysis alone (Fig. 7), the ratio of total intensity of m/z 117 to the intensity of m/z 43 was 0.08. Thus, it can be estimated that only about 3% of the MGEPC became dephosphorylated by this procedure for acetolysis.

Conclusions

The results of the phosphorus analyses in combination with the TLC and GLC–MS data indicate that diether phospholipids are resistant to derivatization by acetolysis under conditions that will satisfactorily cleave the phosphate if the substituent at C-2 is an ester. The exact mechanism of the reaction has not been determined, but unpublished results from this laboratory suggest that the presence of a free hydroxyl or an ester group at carbon number 2 is essential for the cleavage of the phosphate moiety. In agreement with this premise, the acetolysis procedure is not capable of removing the phosphate group from 1-hexadecanoyl-dihydroxyacetone phosphate (data not shown). While it is clear that an initial Vitride treatment followed by suitable derivatization would prepare these samples for analysis by GLC–MS, it would also remove any ester groups that might be present in other phospholipids in a mixture. It is, therefore, suggested, that this type of analysis be carried out by first subjecting the sample to acetolysis, followed by purification by TLC. At this point, dephosphorylated components and unreacted phosphoglycerides can be separated and recovered from the

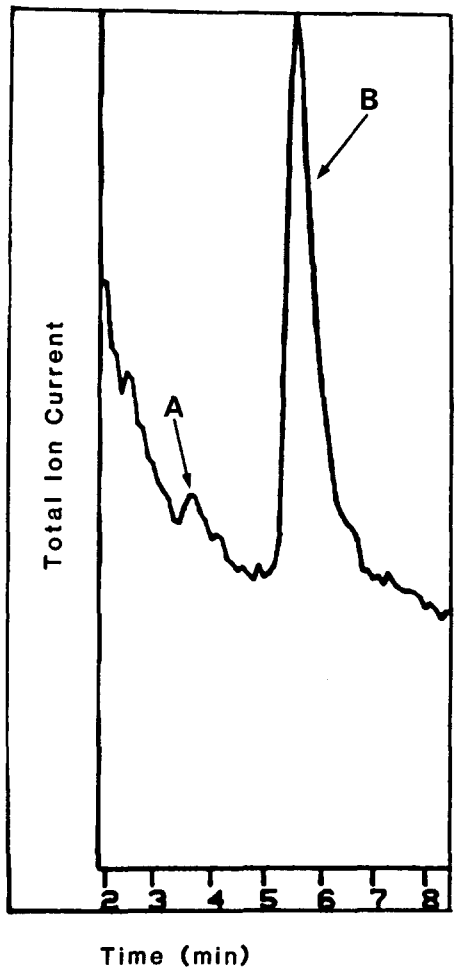

Fig. 7. Total ion current chromatogram of the products isolated after acetolysis of a mixture of AGEPC and MGEPC. GLC conditions are the same as in Fig. 2.

TLC plate, treated with Vitride if necessary, and derivatized.

It may be concluded from the present investigation that 1-O-alkyl or acyl-linked phospholipids such as AGEPC and egg PC are susceptible to phosphate cleavage by acetolysis in contrast to diether phospholipids. These results may have considerable importance in the analysis of such lipids in naturally occurring materials.

This investigation was supported by NIH Grant HL-22555 and Robert A. Welch Foundation Grant AQ-887. The authors wish to thank John T. Meredith for his technical assistance.

Manuscript received 22 February 1983.

REFERENCES

1. Renkonen, O. 1965. Individual molecular species of different phospholipid classes, part II. A method of analysis. *J. Am. Oil Chem. Soc.* **42:** 298–304.
2. Waku, K., H. Ito, T. T. Bito, and Y. Nakazawa. 1974. Fatty chain of acyl, alkenyl, and alkyl phosphoglycerides of rabbit sarcoplasmic reticulum. *J. Biochem. (Tokyo).* **75:** 1307–1312.
3. Snyder, F., M. L. Blank, and R. L. Wykle. 1971. The enzymatic synthesis of ethanolamine plasmalogens. *J. Biol. Chem.* **246:** 3639–3645.
4. Hallgren, B., and S. Larsson. 1962. Glyceryl ethers in the liver oils of elasmobranch fish. *J. Lipid Res.* **3:** 31–38.
5. Fieser, L. F. 1955. Experiments in Organic Chemistry. 3rd ed. D. C. Heath and Co., Boston, MA. 205.
6. Hanahan, D. J., J. Ekholm, and C. M. Jackson. 1963. Studies on the structure of glyceryl ethers and the glyceryl ether phospholipids of bovine erythrocytes. *Biochemistry.* **2:** 630–641.
7. Corey, E. J., and A. Venkateswarlu. 1972. Protection of hydroxyl groups as tert-butyldimethylsilyl derivatives. *J. Am. Chem. Soc.* **94:** 6190–6191.
8. Myther, J. J., and A. Kuksis. 1974. Gas chromatographic resolution of homologous monoacyl and monoalkylglycerols. *Lipids.* **9:** 382–390.
9. Satouchi, K., R. N. Pinckard, L. M. McManus, and D. J. Hanahan. 1981. Modification of the polar head group of acetyl glyceryl ether phosphorylcholine and subsequent effects upon platelet activation. *J. Biol. Chem.* **256:** 4425–4432.
10. Hasegawa, K., and T. Suzuki. 1975. Examination of acetolysis products of phosphatidylcholine by gas chromatography–mass spectrometry. *Lipids.* **10:** 667–672.
11. Marinetti, G. V., J. Erbland, and E. Stotz. 1959. Structure of beef heart plasmalogens. *J. Am. Chem. Soc.* **81:** 861–864.
12. Wells, M. A., and D. J. Hanahan. 1969. Phospholipase A from *Crotalus adamanteus* venom. *Methods Enzymol.* **14:** 178–184.
13. Bartlett, G. R. 1959. Phosphorus assay in column chromatography. *J. Biol. Chem.* **234:** 466–468.
14. Bligh, E. G., and W. J. Dyer. 1959. A rapid method of total lipid extraction and purification. *Can. J. Biochem. Physiol.* **37:** 911–917.