A novel approach to structure proof of glyceryl ether-containing glycerophospholipids. Base-catalyzed methanolysis of platelet-activating factor (AGEPC) at 60°C

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Abstract A novel reaction was explored in which synthetic platelet-activating factor, 1-O-alkyl-2-acetyl-sn-glycero-3-phosphocholine (AGEPC), upon treatment with 1 N NaOH in methanol at 60°C for 20 min, sequentially released the acetyl group, then the choline moiety with concomitant formation of the monomethyl ester of 1-O-alkyl-glycero-phosphoric acid. A mechanism is proposed in which a transient cyclic phosphate intermediate is formed and then attacked by a CH_3O moiety to yield a mixture of the sn-2 and sn-3 methyl esters. Proof of structure of the monomethyl ester derivative was achieved through the use of thin-layer chromatography, aluminum oxide chromatography, and examination of the trimethylsilyl derivative of the monomethyl ester by gas–liquid chromatography–mass spectrometry. Replacement of the acyl group on the 2 position with an ethyl or methyl residue completely prevented any attack by 1 N NaOH in methanol at 60°C. Sphingomyelin was not attacked and only acetate removal was noted with 1-O-alkyl-2-acetyl-sn-glycero-3-phosphoethanolamine under similar conditions. The significance of these findings as they relate to the influence of substituents on the chemical and biological reactivity of AGEPC is discussed. —**Hanahan, D. J., S. T. Weintraub, S. J. Friedberg, A. Tokumura, and D. E. Ayer.** A novel approach to structure proof of glyceryl ether-containing glycerophospholipids. Base-catalyzed methanolysis of platelet-activating factor (AGEPC) at 60°C. *J. Lipid Res.* 1985. **26:** 1345–1355.

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The biochemical importance of glyceryl ether-containing lipids in metabolic processes in mammalian cells has assumed a new dimension with the discovery of a potent glycerophospholipid chemical mediator, platelet-activating factor (PAF). This substance has been identified as 1-O-alkyl-2-acetyl-sn-glycero-3-phosphocholine (AGEPC), in which the alkyl group is primarily composed of long chain hydrocarbon (16:0, 18:0) residues (1–3). This lipid exhibits high potency at levels in the range of 1×10^{-10} M to 1×10^{-11} M toward washed rabbit platelets in initiating aggregation and in causing serotonin release (1–3). It also has been shown to have hypotensive activity (4). The high biological activity together with the observation that very low amounts of this factor are generated in stimulated phagocytic cells (5) has emphasized the need for more refined and definitive assay and structure proof procedures than are available at present. Recently, Kumar, Weintraub, and Hanahan (6) reported that a diether glycerophospholipid, i.e., 1-O-alkyl-2-O-methyl-sn-glycero-3-phosphocholine was resistant to acetolysis whereas an analogous compound, the 2 acetyl derivative (AGEPC), was easily attacked. This observation intimated that current structure proof techniques might not be adequate for identification of these types of naturally occurring compounds.

In the course of this research study designed to provide a more efficient route to structural analysis of naturally produced platelet-activating factor, a novel reaction was observed in which base-catalyzed methanolysis of 1-O-alkyl-2-acetyl-sn-glycero-3-phosphocholine led to the rapid release of choline and also produced a unique acidic phosphorus-containing product. The various aspects of this reaction are described in detail.

Abbreviations: TMS, trimethylsilyl; DMS, dimethylsilyl; TLC, thin-layer chromatography; GLC–MS, gas–liquid chromatography–mass spectrometry; AGEPC, acetyl glyceryl ether phosphocholine; GEPC, glyceryl ether phosphocholine.

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EXPERIMENTAL

Materials

1-O-Octadecyl-2-acetyl-*sn*-glycero-3-phosphocholine (18:0 *sn*-3 AGEPC), 1-O-octadecyl-(lyso)-*sn*-glycero-3-phospho-choline (18:0 *sn*-3 GEPC), 1-O-octadec-9-*cis*-enyl-2-acetyl-*sn*-glycero-3-phosphocholine (18:1 (9) *sn*-3 AGEPC) 1-O-octadec-9-*cis*-enyl-(lyso)-*sn*-glycero-3-phosphocholine (18:1 (9) *sn*-3-GEPC) and 1-O-octadecyl-2-O-methyl-*rac*-glycero-3-phosphocholine (18:0 2-O-methyl-*rac*-GEPC) were products of Bachem Feinchemikalien, Bubendorf, Switzerland.

The following compounds were synthesized in the Lipids Research Laboratories at The Upjohn Company, Kalamazoo, MI (D. E. Ayer, unpublished procedures): 1-O-octadec-10-*cis*-enyl-2-acetyl-*sn*-glycero-3-phosphocho-line (18:1 (10) *sn*-3 AGEPC); 1-O-octadecyl-2-O-ethyl-*sn*-glycero-3-phosphocholine (18:0-2-O-ethyl *sn*-3 GEPC); 1-O-octadecyl-3-acetyl-*sn*-glycero-2-phosphocholine (18:0 *sn*-2 AGEPC); 3-O-octadecyl-2-acetyl-*sn*-glycero-1-phos-phocholine (18:0 *sn*-1-AGEPC) was prepared from a starting material of 3-O-octadecyl-2-hexadecanoyl-*sn*-glycero-1-phosphocholine. The latter compound was subjected to base-catalyzed methanolysis at room temperature for 25 min; the resulting deacylated derivative was isolated by thin-layer chromatography and reacted with acetic anhydride in the presence of catalytic amounts of perchloric acid to yield the corresponding acetylated derivative. This product was purified by thin-layer chromatography. At the same time a series of analogs of 18:0 *sn*-3 AGEPC were synthesized in which the number of methylenes between the phosphate and quaternary nitrogen on the polar head group were varied: *a*) 1-O-octadecyl-2-acetyl-*sn*-glycero-3-phosphoric acid-3'-trimethylammonium propyl ester (U66983); *b*) 1-O-octadecyl-2-acetyl-*sn*-glycero-3-phos-phoric acid-6'-trimethylammonium hexyl ester (U66985); *c*) 1-O-octadecyl-2-acetyl-*sn*-glycero-3-phosphoric acid-10'-trimethylammonium decyl ester (U66982).

Dimyristoyl phosphatidylcholine was purchased from Avanti Biochemicals, Birmingham, AL. 1-O-Octadecyl-2-N-acetamido-*sn*-glycero-3-phosphocholine (18:0 2-NAc-*sn*-3-GEPC) was a generous gift from Dr. J. Hadju (Boston University). A diether glycerophospholipid, 1-O-octadec-*cis*-9-enyl-2-O-hexadecyl-*sn*-glycero-3-phosphocho-line (DEPC) was purchased from Calbiochem (La Jolla, CA). 1-O-Alkyl-2-acetyl-*sn*-glycero-3-phosphoethanolamine (AGEPE) was kindly provided by Dr. Raj Kumar.

Phosphorus assay

Lipid phosphorus was determined by the method of Bartlett (7), subsequent to perchloric acid digestion of the lipid sample.

Base-catalyzed methanolysis procedure

In the usual instance, 1 to 5 mg of lipid sample was dissolved in 100 μl of methanol and 1.0 ml of freshly prepared 1 N NaOH in methanol was added. This mixture was then incubated at the desired temperature, i.e., 25°C, 40°C, or 60°C, for varying periods of time. At the end of the incubation, the mixture was cooled at 15°C in an ice bath, 1.1 ml of 1 N HCl was added, and the contents were mixed by vortexing. At this point, 1.0 ml of chloroform was introduced and the mixture was subjected to vigorous vortexing for 15 to 20 sec. The chloroform-rich and water-methanol-rich phases were separated by careful pipetting. The lower chloroform-rich phase was washed with methanol-water 10:9 (v/v) until the washes were neutral. The latter were combined with the original water-soluble fraction and saved. The chloroform-soluble fraction was evaporated to dryness under nitrogen and the residue was dissolved in the desired volume of methanol.

Thin-layer chromatography

Precoated silica gel G plates, 250 μm and 500 μm in thickness, were obtained from Analtech (Newark, NJ). The plates were pre-developed in a solvent system of chloroform-methanol-water 65:35:6 (v/v/v), air-dried, and stored in a desiccator at room temperature. Immediately prior to use these plates were placed in an oven at 100°C for 3 min, cooled to room temperature, and lipid samples were then applied. The most frequently used solvent system for separation of the intact lipids and their base-treated products was chloroform-methanol-water 65:35:6 (v/v/v).

Aluminum oxide chromatography

A modification of the procedure of Rhodes and Lea (8) was employed in which Bio-Rad aluminum oxide (AG-7) was adjusted to Grade II Brockman as per manufacturer's directions and then packed in a column measuring 8 mm × 145 mm. Three solvent systems were utilized, the first of which was chloroform-methanol 1:1 (v/v) for removal of neutral glycerophospholipids such as the choline-containing types. The other solvents used in succession were ethanol-chloroform-water 5:2:1 (v/v/v) and ethanol-chloro-form-water 5:2:2 (v/v/v) to remove the acidic glycerophos-pholipids.

TMS derivative preparation

The lipid sample, weighing in the range of 50 to 75 μg, was transferred in methanol into a glass tube fitted with a Teflon-lined screw-cap. The solvent was removed by a stream of nitrogen and the residue was treated with 50 μl of dry benzene and 50 μl of a freshly prepared solution containing 2% trimethylchlorosilane (Pierce Chemical Co., Rockford, IL) and 98% N-O-*bis*(trimethylsilyl)trifl-uoroacetamide (Pierce Chemical Co.). The reaction tube was capped and the contents were heated at 60°C for 30 min, cooled to room temperature, and an appropriate aliquot was used directly for analysis by GLC-MS.

Choline assay

Water-soluble choline was assayed essentially as described by Kates (9). If total lipid-bound choline was to be determined, the lipid sample was subjected to reflux in 1 N HCl for 4 to 6 hr and the water-soluble hydrolyzate was assayed directly for choline.

Preparation of 1-O-alkyl-2-acetyl-sn-glycero-3-phosphoric acid (AGEPA) and 1-O-alkyl-2-(lyso)-sn-glycero-3-phosphoric acid (lyso GEPA)

AGEPA was obtained through the action of phospholipase D (*Streptomyces chromofuscus*, Calbiochem-Behring) on AGEPC, using slight modifications of the procedures described by Kates (9) and by Satouchi et al. (10). The calcium salt of AGEPA was separated by preparative thin-layer chromatography (silica gel G) using a solvent system of chloroform–methanol–28% ammonium hydroxide 65:35:6 (v/v/v) and the area at the origin of the chromatogram was extracted with a solvent mixture of chloroform–methanol–0.2 N HCl 1:2:0.8 (v/v/v). Addition of chloroform and water to this extract and vortexing allowed recovery of AGEPA in the lower chloroform-rich layer. The latter extract was washed with methanol–water 1:1 (v/v) until the washes were neutral.

The same procedure was used to prepare lyso-GEPA from lyso-GEPC (1-O-alkyl-2-(lyso)-sn-glycero-3-phosphocholine. Each reaction proceeded smoothly and to completion within 2 hr at 23°C.

Mass spectrometry

Mass spectrometric analyses were performed on a Finnigan-MAT 212 mass spectrometer in combination with an INCOS 2200 data system. The electron impact mass spectra were obtained with an accelerating voltage of 3 kV, an electron energy of 70 eV, and an ion source temperature of 250°C.

RESULTS

Characteristics of the base-catalyzed methanolysis reaction

When 1-O-octadecyl-2-acetyl-sn-glycero-3-phosphocholine (18:0 sn-3 AGEPC) was subjected to treatment with 1 N NaOH in methanol, the chemical nature of the products of the reaction was dramatically dependent on temperature and, to some extent, on time. At room temperature, and over a time period from 5 to 60 min, base-catalyzed methanolysis of AGEPC led only to removal of the acetyl group with the resultant formation of the corresponding lyso derivative, i.e., 1-O-alkyl-2-(lyso)-sn-glycero-3-phosphocholine. When the temperature of the reaction was raised to 60°C, however, the following sequence of events occurred. First there was removal of the acetyl

moiety, then choline release and concomitant formation of a lipid phosphorus-containing derivative.

When the reaction was conducted in 0.1 N or 0.25 N NaOH in methanol at 40°C, only deacetylation of AGEPC resulted even after a period of 35 to 40 min. On the other hand, treatment of AGEPC with 1 N NaOH in methanol at 40°C led to deacetylation and a significant release of choline (80% of starting level) and formation of a chloroform-soluble lipid mixture, which contained lyso-GEPC and another choline-free, phosphorus-containing lipid.

The types of changes seen after methanolysis at 60°C are illustrated in the thin-layer chromatogram presented in **Fig. 1A**.

Characterization of the lipid product formed at 60°C in 1 N NaOH in methanol

General observations. As shown in Fig. 1A, lane 4, the major lipid phosphorus product (I), identified below as the monomethyl ester of 1-O-alkyl-glycerophosphoric acid, formed after reaction with 1 N NaOH in methanol at 60°C, migrated farther (R_f 0.49) on silica gel G than
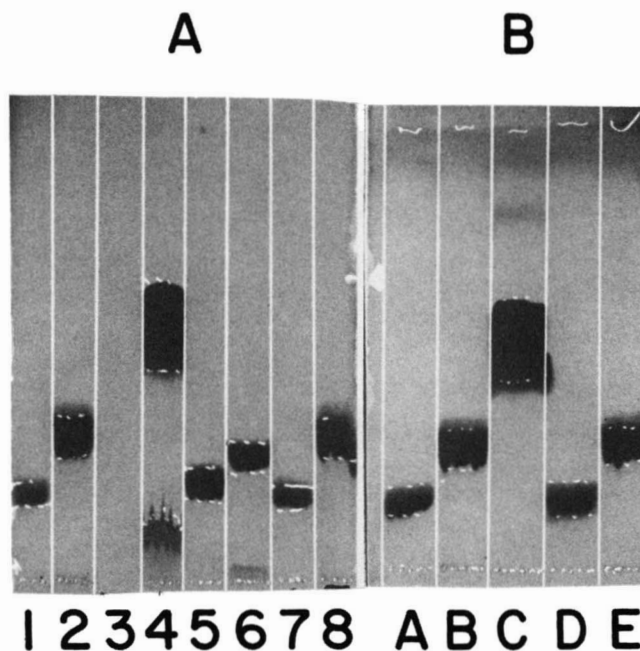
Fig. 1. Thin-layer chromatographic profile of phospholipids subjected to base-catalyzed methanolysis at 60°C. Chromatography was accomplished on silica gel G plates using a solvent system of chloroform–methanol–water 65:35:6 (v/v/v). The individual lanes in each of the two plates, A and B, contained the following: Plate A: 1, lysolecithin standard; 2, spingomyelin standard; 3, blank; 4, chloroform soluble product from 18:0 sn-3 AGEPC treated with 1 N NaOH in methanol at 60°C for 20 min; 5, 18:0 sn-3 AGEPC (untreated); 6, 18:0 2-O-ethyl-sn-3-AGEPC subjected to base treatment at 60°C for 30 min (same R_f value for treated as well as untreated sample); 7, lysolecithin standard; 8, sphingomyelin subjected to base treatment at 60°C for 2 hr (see lane 2 for non-treated sample). Plate B: A and D, lysolecithin standard: B and E, sphingomyelin standard; C, major product from lane 4, plate A, after purification by aluminum oxide chromatography (see text for details).

did the starting compound AGEPC, R_f 0.23 (Fig. 1A, lane 5). A minor phosphorus-containing component (II) was always found at the origin, but represented less than 5% of the total phosphorus. Component I was subjected to TLC in neutral and basic solvent systems and exhibited R_f values that supported the presence of an acidic function (data not shown). Further evidence that I was indeed acidic was provided by the observations that it streaked in an acid solvent system (TLC) and that all the choline was released from the starting material (see Table 1).

Proof that component I contained a free hydroxyl function was obtained by treatment of I with acetic anhydride in the presence of trace amounts of perchloric acid (11). A major derivative was formed which migrated on silica gel G (Merck) at an R_f 0.57, compared to an R_f 0.48 for I, using a solvent system of chloroform–methanol–water 65:35:6 (v/v/v).

In order to further study the chemical nature of I, purification by chromatographic procedures was necessary. Use of thin-layer chromatography was not the procedure of choice. This decision was based on the observation (A. Tokumura, personal communication) that both the calcium salt of 1-O-acyl-(lyso)-glycerophosphate and its corresponding monomethyl ester exhibited identical R_f values. Hence, a more selective route to the isolation and purification of this novel product was required and this was accomplished through use of neutral aluminum oxide chromatography as outlined below.

Purification by aluminum oxide chromatography. A particularly effective approach to the purification of I involved passage of the chloroform-soluble extract through aluminum oxide using the following solvents: chloroform–methanol 1:1 (v/v/v); ethanol–chloroform–water 5:2:1 (v/v/v); and ethanol–chloroform–water 5:2:2 (v/v/v). As expected from the absence of a choline moiety in this lipid sample and its apparent acidic nature, no lipid phosphorus was recovered in the chloroform–methanol 1:1 (v/v) wash. The sequential use of ethanol–chloroform–water 5:2:1 (v/v/v) and ethanol–chloroform–water 5:2:2 (v/v/v) then allowed recovery of 85 to 90% of the applied lipid phosphorus (in at least six separate experiments), primarily in the 5:2:1 solvent mixture. The TLC behavior of the eluate is shown in Fig. 1B, lane C, which illustrates the apparent purity of this fraction. In a control experiment, when intact (non-treated) 18:0 *sn*-3 AGEPC was passed through the column under similar conditions, over 99% of the lipid phosphorus was recovered in the chloroform–methanol 1:1 (v/v) wash.

Sensitivity of various compounds to base-catalyzed methanolysis. It was important to establish the spectrum of susceptibility of various analogs and homologs of AGEPC to base-catalyzed methanolysis. Consequently, several derivatives were subjected to base treatment at 60°C as described above. When 18:0 (lyso) *sn*-3 GEPC was used, the pattern of attack was similar to that of 18:0 *sn*-3 AGEPC in that conversion of the lyso-GEPC derivative to the monomethyl ester derivatives was complete within 20 min at 60°C. As shown in **Table 1**, base-catalyzed methanolysis at 60°C

TABLE 1.   Differential susceptibility of various glycerophospholipids to 1 N NaOH in methanol at 60°C[a]

| | Phosphorus and Choline Distribution (in μg) | | | | | |
| | Starting Sample | | Chloroform-Rich Fraction | | Methanol–Water-Rich Fraction[b] | |
Compound	P	Choline	P	Choline	P	Choline
Sensitive						
18:0 *sn*-3-AGEPC	110	432	94	N.D.[c]	N.D.	429
18:0 *sn*-1-AGEPC	934	3675	890	N.D.	N.D.	3670
18:0 *sn*-2-AGEPC	228	896	226	N.D.	N.D.	897
18:0 lyso *sn*-3-GEPC	520	2028	512	N.D.	2	2025
Dimyristoyl *sn*-3 GPC	650	2535	N.D.	N.D.	658	2517
Insensitive						
18:0-2-O-ethyl *sn*-3 GEPC	159	620	157	605	N.D.	N.D.
18:0-2NAc-*sn*-3 GEPC	285	1118	279	1109	N.D.	N.D.

Each individual glycerophospholipid was treated, as described in Methods, for 20 min. Subsequent to acidification, the reaction mixture was separated into a chloroform-rich fraction and a water-methanol-rich fraction. These were analyzed for phosphorus and choline.

[a] The following compounds were also shown to be insensitive to treatment with methanolic 1 N NaOH at 60°C for periods up to 1 hr: 1-O-octadecyl-2-O-methyl-*rac*-glycero-3-phosphocholine and also two analogs of *rac*-18:0 AGEPC in which the *sn*-2 position was occupied by the following groups: – OCONHCH₃ and – CH₂COCH₃ (D. E. Ayer. unpublished procedures).

[b] The methanol–water-rich extract from the base-catalyzed methanolysis at 60°C of *sn*-3-AGEPC was examined by electron impact-mass spectrometry with sample introduction by means of a direct insertion probe heated at 200°C. The observation of prominent peak at m/z 58 ([CH₂ = N(CH₃)₂]⁺) and a peak at m/z 89 ([HOCH₂CH₂N-(CH₃)₂]⁺) provided excellent supporting proof that choline was being removed during this reaction.

[c] N.D., not detectable.

led to excellent recoveries of chloroform-soluble phosphorus for those compounds classified as sensitive. At the same time, nearly 100% of the choline was found in the free form. The reaction pattern was exactly the same for the sn-3, sn-2, and sn-1 stereoisomeric forms of AGEPC, and for those homologs containing various chain lengths of alkyl residue, i.e., 16:0, 18:0, and 18:1.

Interestingly, the reaction patterns of analogs of 18:0 sn-3 AGEPC in which the number of methylene units was increased between the phosphate and the quaternary nitrogen showed some decided differences in sensitivity. At the end of a 20-min reaction period (at 60°C), U66983 (methylene space of 3) showed approximately 50% conversion to the lyso form and 50% conversion to a fast moving component (R_f 0.49), while U66985 (methylene spacer of 6) showed over 80% of the starting lipid phosphorus still as the lyso derivative and the remainder as a fast moving (R_f 0.49) phosphorus-containing material. Even less reactive was U66982 (methylene space of 10) which was converted primarily to the lyso form (95%) with less than 5% as a compound migrating with an R_f 0.47. Further, 18:0-2-O-ethyl GEPC was resistant to base-catalyzed methanolysis at 60°C as shown in Fig. 1A, lane 6. Similarly, sphingomyelin was unaffected by the same treatment (Fig. 1A, lane 8) as was 18:0-2-N-acetamido-GEPC (see Table 1).

AGEPE, with an R_f 0.38 in chloroform–methanol–water 65:35:6 (v/v/v), upon treatment with 1 N NaOH in methanol at 60°C for 1 hr gave two spots on thin-layer chromatography, one with an R_f 0.37 and the other with an R_f 0.25. Both were ninhydrin- and phosphate-positive. Upon acetylation of this reaction mixture (acetic anhydride in presence of perchloric acid) only one component with an R_f 0.38 was found. This result indicated that AGEPE was not converted to the fast moving (R_f 0.48) derivative but rather underwent only a simple deacetylation. Whether there was retention of configuration was not explored.

DEPC under similar conditions did not undergo any alteration after reaction with 1 N NaOH in methanol at 60°C for 1 hr. This was expected on the basis of the resistance of the 1-O-alkyl-2-O-ethyl-sn-glycero-3-phosphocholine to a similar treatment (as shown in Table 1).

Gas–liquid chromatography–mass spectrometry

It has recently been reported that (lyso) phosphatidic acid (1-O-acyl-glycero-3-phosphate) can be analyzed by GLC–MS as a trimethylsilyl (TMS) derivative without any hydrolytic pretreatment (12). This technique has been found applicable to the identification of various analogs of (lyso) phosphatidic acid, such as the monomethyl and dimethyl esters (A. Tokumura, unpublished observations). We, therefore, utilized this procedure to characterize the major lipid phosphate derivative formed after base-catalyzed methanolysis of AGEPC at 60°C.

Fig. 2 shows the mass spectrum of the substance emerging at 5.8 min during the GLC–MS analysis of the TMS derivative of the base-catalyzed methanolysis product of 18:0 sn-3 AGEPC. For ions at m/z 567 and m/z 492, an assignment of [M – CH$_3$]$^+$ and [M-TMSOH]$^+$, respectively, is consistent with the structure being the monomethyl ester of 1-O-octadecyl-glycerophosphoric acid.
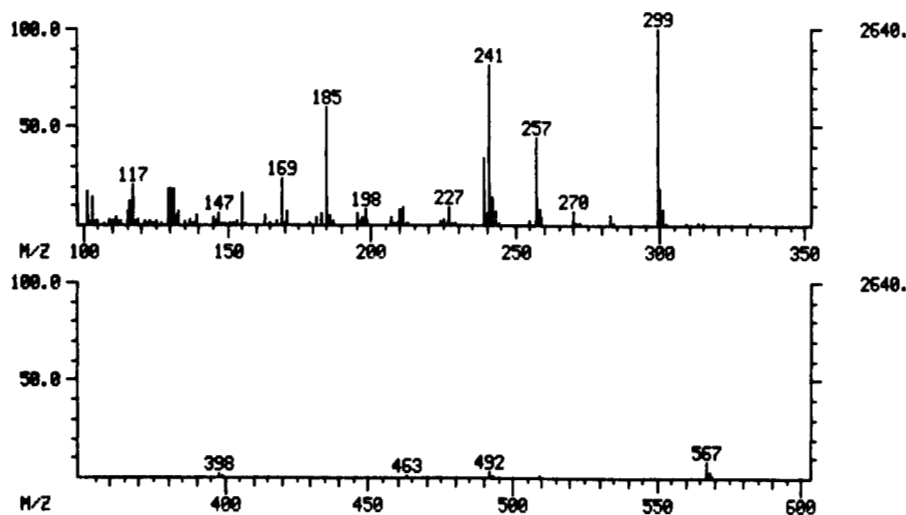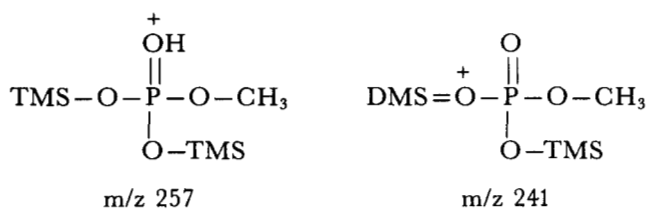

Fig. 2. Electron impact mass spectrum (70 eV) of the trimethylsilyl derivative of the monomethyl ester of 1-O-octadecyl-glycero-3-phosphoric acid.

For the TMS derivative of (lyso) phosphatidic acid, the predominant electron impact-induced fragments include the phosphate portion of the molecule (12). In a similar manner in the current study, the intense peak at m/z 299, seen in Fig. 2, is proposed to be formed after cleavage between carbons 1 and 2 of the glycerol backbone, resulting in the following ion:

$$TMS-O-\overset{+}{C}H \quad O$$
$$H_2C-O-\overset{|}{P}-O-CH_3$$
$$O-TMS$$

m/z 299
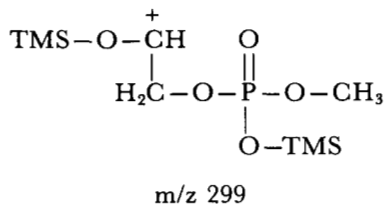
The relatively intense ions at m/z 257 and m/z 241 arise from a transfer of a TMS group to the phosphate moiety along with cleavage from the glycerol portion of the molecule. The proposed structures for these ions are given below.

$$\overset{+}{O}H$$
$$TMS-O-\overset{\|}{P}-O-CH_3$$
$$O-TMS$$

m/z 257

$$O$$
$$DMS=\overset{+}{O}-\overset{\|}{P}-O-CH_3$$
$$O-TMS$$

m/z 241

When this cleavage occurs in combination with a two hydrogen transfer, the ion at m/z 185, shown below, is formed.

$$\overset{+}{O}H$$
$$HO-\overset{\|}{P}-O-CH_3$$
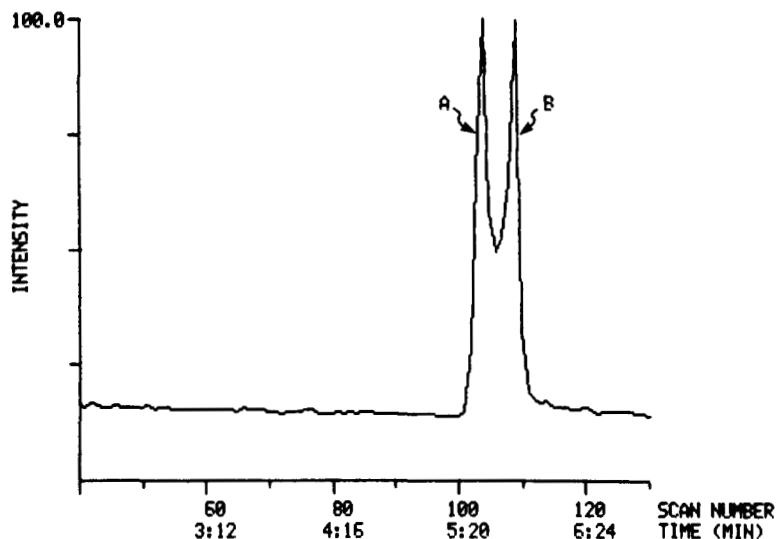$$O-TMS$$

m/z 185
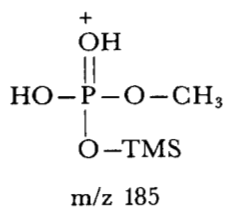
When a 15-meter, 0.32-mm i.d. BP-1 fused silica column (Scientific Glass Engineering, Inc., Austin, TX) was employed at a column temperature of 260°C for the GLC separation, two peaks were observed for the TMS derivative of each homolog of AGEPC after alkaline methanolysis at 60°C. In **Fig. 3** can be seen the reconstructed total ion current chromatogram for the TMS derivative of the methanolysis product of 18:0 sn-3 AGEPC. The mass spectrum of the peak eluting at 5.5 min (compound A) is shown in **Fig. 4**. While the ion profile is similar to that presented in Fig. 2 for the compound eluting at 5.8 min (compound B), the relative intensities of the major ions are different. A comparison of the current results with the mass spectra of the trimethylsilyl derivatives of α- and β-glycerophosphate (13) indicated that the identity of compound A was the sn-2 isomer of the monomethyl ester of 1-O-octadecyl-glycerophosphoric acid. In further support of this hypothesis for the identity of compound A, it was found that when synthetic 1-O-octadecyl-2-acetyl-sn-glycero-3-phosphocholine was subjected to methanolysis as described above, similar product formation was observed.

Significant differences were found in the fragmentation profile of the base-catalyzed methanolysis product from unsaturated AGEPC as compared to the saturated counterpart. The mass spectrum of the compound derived from 18:1 sn-3 AGEPC is shown in **Fig. 5**. Of particular importance is the presence of the molecular ion, seen at m/z 580, in direct contrast to the spectrum of the 18:0 derivative in which no molecular ion is observed. The [M−15]⁺ ion (m/z 565) is found as expected, but the fragment at m/z 463 ([M−117]⁺) does not appear to correspond to any analogous ion in the spectrum of the saturated species. This is noteworthy because evaluation of the mass spectrum of the presumed sn-2 isomer of 1-O-octadecenyl-glycerophosphate (**Fig. 6**) reveals that an [M−119]⁺ ion is found at m/z 461 which is comparable to the m/z 463 ion in the saturated compound, along with a similarly derived fragment at m/z 476 in which the loss of 104 occurs from the molecular ion. At present, the m/z 463 ion shown in Fig. 5 remains without interpretation.

In the current study it has been determined that the presence of the choline moiety is necessary for methyl ester formation to occur. When 1-O-hexadecyl-2-acetyl-sn-glycero-3-phosphate was subjected to alkaline methanolysis at 60°C, the major product formed was 1-O-hexadecyl-glycero-3-phosphate with less than 10% found as the sn-2 isomer (data not shown). Thus, without the choline ester on the phosphate, only hydrolysis of the acyl group on carbon 2 is seen after alkaline methanolysis at 60°C.

As an aid to clarification of the mechanism of esterification of 1-O-alkyl-glycerophosphate during base treatment, separate utilization was made of CH₃¹⁸OH and CD₃OH as solvents. **Fig. 7** shows the mass spectra of TMS derivatives produced from base treatment of 16:0 sn-3 AGEPC in CH₃OH (A) and CH₃¹⁸OH (B). It is important to note that both of these samples were characterized by GLC–MS using a well-conditioned 30-meter 0.25-mm i.d. SE-54 fused silica column (J & W Scientific, Inc., Rancho Cordova, CA) at 270°C. In this instance, no separation of the two isomers is obtained, and therefore, the spectra show a composite of the two isomers. As anticipated, for the labeled compound the [M−15]⁺ and [M−90]⁺ ions were found at m/z 541 and m/z 466, respectively, instead of m/z 539 and m/z 464 as observed in the non-labeled analog. Furthermore, all major phosphorus-containing fragments were shifted upwards by two mass units, for example, m/z

Fig. 3. Reconstructed ion chromatogram of the trimethylsilyl derivative of the alkaline methanolysis product (60°C) of 1-O-octadecyl-2-acetyl-*sn*-glycero-3-phosphocholine. Gas–liquid chromatographic separation was accomplished by means of a 15-meter, 0.32-mm i.d. BP-1 fused silica column (Scientific Glass Engineering, Austin, TX) with a column temperature of 260°C, an injector temperature of 250°C, a linear velocity of helium of 75 cm/sec, and a 40 ml/min split flow from the injector. The effluent from the column was introduced directly into the mass spectrometer by means of a transfer line maintained at 250°C.

299 to m/z 301, m/z 257 to m/z 259, and m/z 185 to m/z 187. In a similar fashion, all corresponding peaks were found three mass units higher in the deuterated analog formed with CD_3OH (not shown). Of interest, no monomethyl ester was formed when CD_3OD was used as the solvent. It is interesting to note that the ion at m/z 435 in

Fig. 7A was not shifted in the spectrum in Fig. 7B and also remained at m/z 435 following the CD_3OH reaction. This indicates that the formation of this ion involves loss of the OCH_3 moiety and most likely includes the combined loss of TMS and CH_3 along with the OCH_3. A mechanism for the formation of the m/z 435 ion
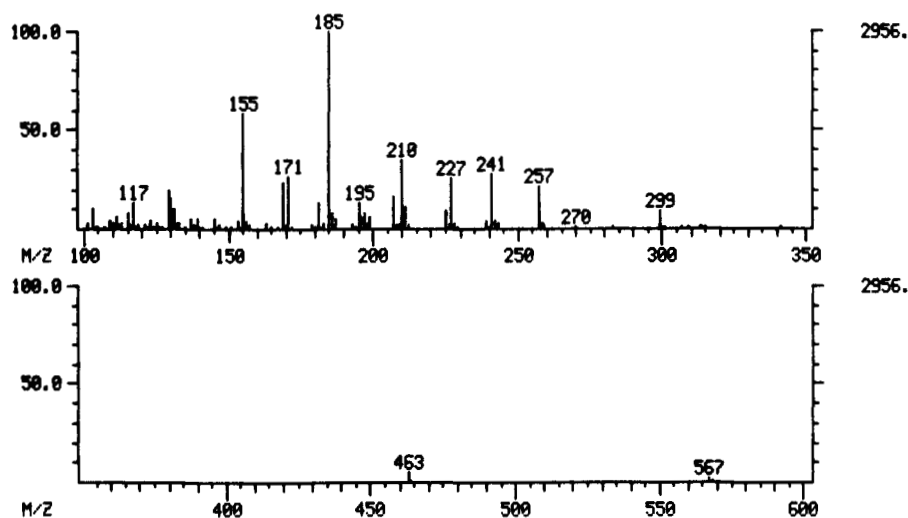
Fig. 4. Electron impact mass spectrum (70 eV) of the trimethylsilyl derivative of the monomethyl ester of 1-O-octadecyl-glycero-2-phosphate.
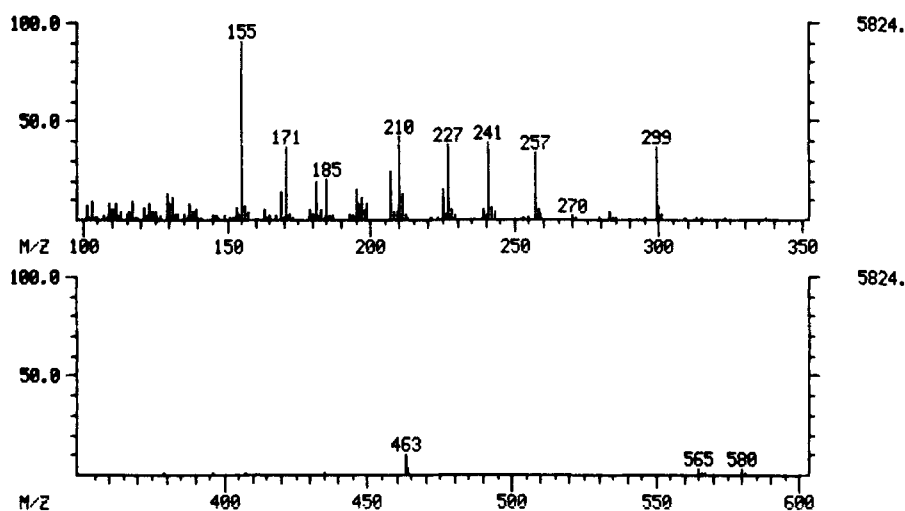
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Fig. 5. Electron impact spectrum (70 eV) of the trimethylsilyl derivative of the monomethyl ester of 1-O-octadec-*cis*-9-enyl-glycero-3-phosphate.

([M–119]$^+$) that accomodates the fact that this ion is observed mainly in the *sn*-2 isomer is not being proposed at this point. The results of the stable isotope studies support a mechanism in which the choline moiety of AGEPC is replaced by a methoxyl group during base-catalyzed methanolysis.

DISCUSSION

This study has clearly shown several unique features of the base-catalyzed methanolysis at 60°C of 1-O-alkyl-2-acetyl-*sn*-glycero-3-phosphocholine (AGEPC). Three facets of this reaction deserve comment at this point: first, the very rapid and quantitative release of choline under comparatively mild conditions; second, concomitant with the release of choline, the formation of a 1-O-alkyl-monomethyl phosphate derivative in excellent yield; and third, pronounced influence of the substituent on the C-2 position and the nature the C-3 polar head group on the course of the reaction. Each of these points will be discussed below.

The rapid and quantitative release of choline (within 15 min) from AGEPC under these conditions is rather sur-
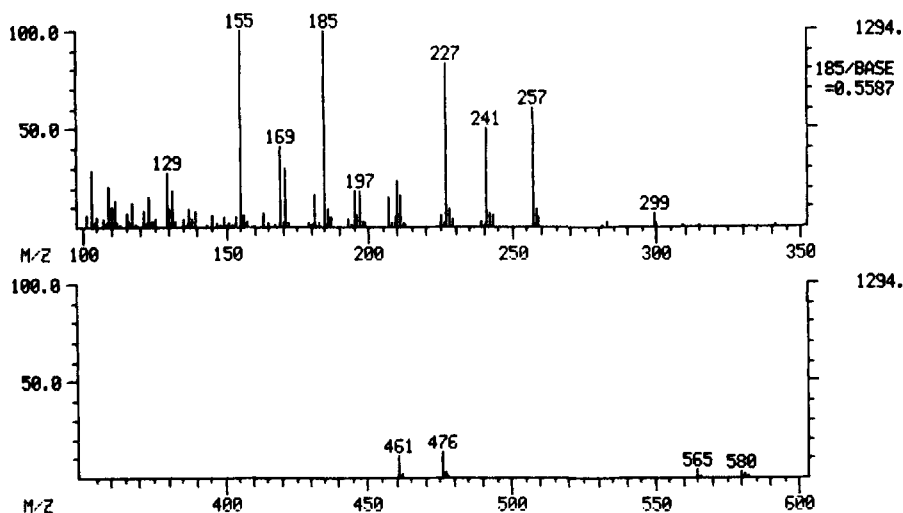

Fig. 6. Electron impact mass spectrum (70 eV) of the trimethylsilyl derivative of the monomethyl ester of 1-O-octadec-*cis*-9-enyl-glycero-2-phosphate.
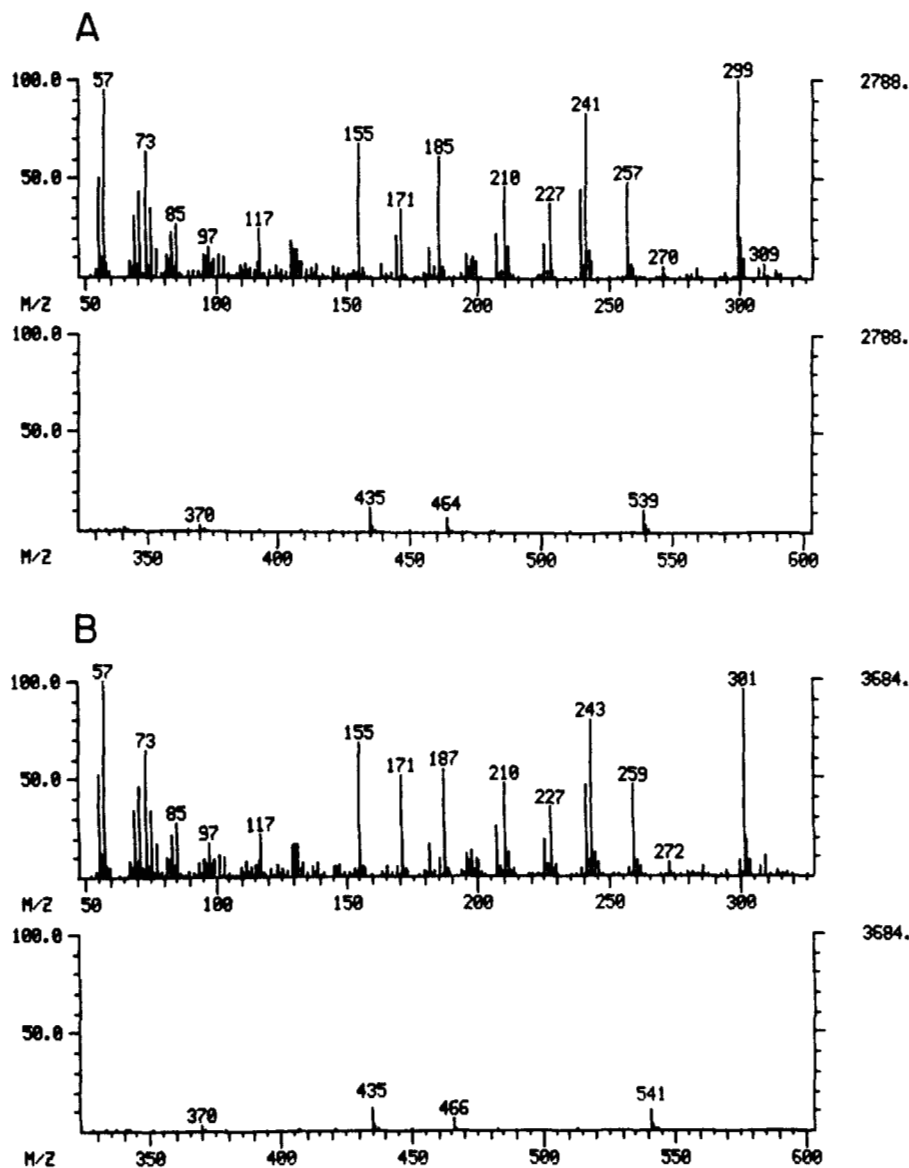
Fig. 7. Electron impact mass spectrum (70 eV) of the trimethylsilyl derivative of the monomethyl ester of 1-O-hexadecylglycerophosphate produced with: A, CH₃OH; B, CH₃¹⁸OH. Both spectra represent the composite of the glycero-2-phosphate and glycero-3-phosphate isomers.

prising. Usually a choline-containing glycerophospholipid (for example, egg lecithin) must be subjected to more drastic conditions, such as reflux for 1 to 2 hr either in methanolic acid or base, for quantitative release (9). The base-catalyzed methanolysis of dimyristoyl phosphatidylcholine at 60°C, led to the very rapid release (within 15 min) of fatty acid, as well as the complete liberation of choline; the water-soluble fraction from the latter reaction contained a glycerophosphoric acid monomethyl ester (data not shown). It is evident, then, that this type of procedure provides a convenient, very rapid route to the release of choline in a quantitative manner.

The formation of the monomethyl ester of 1-O-alkyl-glycerophosphoric acid was unexpected, but the data presented here showed conclusively that it was the predominant phosphorus-containing product. The reaction proceeded quite cleanly and this derivative could easily be isolated in high yields. Although the results showed that there was isomerization during this reaction, with the *sn*-3 and *sn*-2 forms both being detected, conditions for gas chromatographic examination (i.e., increase in column temperature) can be chosen such that both isomers elute together and, thus, allow a quantitative estimation of this glyceryl ether component. This approach could provide a

more facile route to determination of the glyceryl ether composition and characterization of naturally occurring platelet-activating factor or other glyceryl ether derivatives than that of using the more involved and rather tedious procedure of acetolysis or hydrogenolysis.

The mechanism of attack in the base-catalyzed methanolysis at 60°C of 18:0 sn-3 AGEPC can be described by the following scheme:
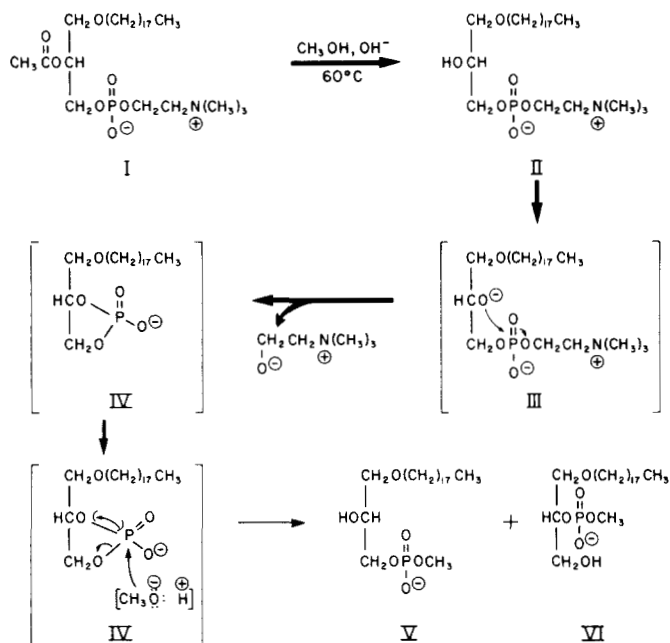
In this mechanism, it is proposed that a cyclic phosphate, IV, is formed and that this transient intermediate is the primary species attacked by methanol under our experimental conditions. Supporting arguments for the sequence shown above are as follows.

a. The earliest detectable product is 1-O-alkyl-2-(lyso)-glycero-3-phosphocholine, II. In fact, this is the only phosphorus-containing derivative observed in the first 5 min of the reaction. There is apparently complete conversion of I to II prior to the appearance of any other detectable products, such as V and VI.

b. Subsequently, it is proposed that II is converted to a transient intermediate, III. Then there is a rapid nucleophilic attack by this 2-alkoxide ion on the phosphate with displacement of choline, an excellent leaving group, and simultaneous formation of the cyclic phosphate, IV.

c. Finally, IV is considered to be the species under attack by methanol. There is a likelihood that a change in the stereochemical configuration of the phosphate occurs. Hence, the [CH$_3$O] moiety has an equal opportunity to attack on either side of the phosphate to yield the sn-2 and sn-3 methyl esters, V and VI, respectively.

Certain other features of the above mechanism deserve

comment. Ukita, Bates, and Carter (14) concluded, on the basis of an elemental analysis of a barium salt, that a monomethyl ester of glycerophosphate was formed during the alkaline hydrolysis of phosphatidylcholine in methanol. They proposed that a cyclic glycerophosphate could be formed during this hydrolytic procedure, but due to the instability of a synthetic cyclic glycerophosphate under similar conditions, doubted that such an intermediate could be detected. In the study of Ukita et al. (14) as well as our own investigation, it seems likely that a cyclic phosphate is formed, but the mechanism involves participation of the entire polar head group of the choline glycerophospholipid. The fact that 1-O-alkyl-2-(lyso)-sn-glycero-3-phosphoric acid did not appear to form any monomethyl ester even after a reaction period of 60 min at 60°C would tend to support this proposal. Finally, on the basis of results obtained with deuterium and ^{18}O-labeled methanol, it is evident that the [CH$_3$O] moiety of the methanol molecule is the attacking species and that this solvent may be represented by the following "ionic" formulation:

$$[CH_3\overset{..}{\underset{..}{O}}{:}^- \ H]^+$$
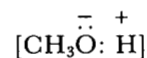
The charge separation in the resonance structure of this compound will be facilitated by base. Our conclusion that a cyclic intermediate is the major species under attack is strongly supported by the additional observation that the 2-O-ethyl analog of AGEPC is not attacked during incubation with methanolic 1 N NaOH at 60°C. Probably this is due to the chemical stability of the 2-O-ethyl linkage which would not lead to formation of a cyclic intermediate. Further supporting evidence derives from the fact that the U66985, which contains six methylene groups in the polar head group, does not lose any significant amount of choline under our experimental conditions. One might speculate that due to its more "hydrophobic" nature, the sn-3 substituent of U66985 might associate more readily with the alkyl ether side chain and hence the configuration of the molecule might be changed, reducing the potential for cyclization.

These reactions show the decided influence of the substituent on the C-2 (sn-2) position of AGEPC on its chemical reactivity and potentially could be construed as a factor in its biological activity. It is logical to propose that, due to the very polar nature of the C-2 and C-3 substituents of AGEPC, these components of the molecule might form either a quasi-cyclic structure or, at least these two groups, on interaction with a cell would be more readily oriented toward the polar phase of the outer lipid bilayer. The alkyl residue would be the anchor in the hydrophobic environment. Examination of a Corey Pauling model of AGEPC (as well as that of the propionyl derivative which has high biological activity) shows such orien-

tations are possible. The 2-butyryl derivative, which has a greatly reduced biological activity, might not react in the same manner due to the more hydrophobic nature of the sn-2 substituent. These hypotheses must stand the test of future experimental studies. ■

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