Preparation of Schiff Base Adducts of Phosphatidylcholine Core Aldehydes and Aminophospholipids, Amino Acids, and Myoglobin[1]

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ABSTRACT: We have prepared Schiff base adducts of the core aldehydes of phosphatidylcholine and aminophospholipids, free amino acids, and myoglobin. The Schiff bases of the ethanolamine and serine glycerophospholipids were obtained by reacting *sn*-1-palmitoyl(stearoyl)-2-[9-oxo]nonanoyl-glycerophosphocholine (PC-Ald) with a twofold excess of the aminophospholipid in chloroform/methanol 2:1 (vol/vol) for 18 h at room temperature. The Schiff bases of the amino acids and myoglobin were obtained by reacting the aldehyde with an excess of isoleucine, valine, lysine, methyl ester lysine and myoglobin in aqueous methanol for 18 h at room temperature. Prior to isolation, the Schiff bases were reduced with sodium cyanoborohydride in methanol for 30 min at 4°C. The reaction products were characterized by normal-phase high-performance liquid chromatography and on-line mass spectrometry with electrospray ionization. The amino acids and aminophospholipids yielded single adducts. A double adduct was obtained for myoglobin, which theoretically could have accepted up to 23 PC-Ald groups. The yields of the products ranged from 12 to 44% for the aminophospholipids and from 15–57% for the amino acids, while the Schiff base of the myoglobin was estimated at 5% level. The new compounds are used as reference standards for the detection of high molecular weight Schiff bases in lipid extracts of natural products.

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The primary products of lipid peroxidation (e.g., hydroperoxides) decompose to form secondary products, which include low and high molecular weight aldehydes (1). The aldehydes may react with cellular components or may be metabolized to inactive tertiary products (alcohols and acids). The low molecular weight bifunctional aldehydes, malonaldehyde and 4-hydroxynonenal, have been extensively investigated for their reactivity with various amines, amino acids and proteins, with which they yield relatively stable covalently bound products. Specifically, malonaldehyde has been shown to undergo 1,4-addition with amino acids to form the eneamines (2), *N*-substituted 3-iminopropenals (3) and *N*,*N*′-disubstituted 1-amino-3-iminopropenes (4). Malonaldehyde also reacts with the amino groups in proteins (4,5) as well as with deoxynucleosides *in vitro* to produce a variety of adducts (6). The early lysine and histidine adduction chemistry of 4-hydroxynonenal has now been elucidated (7,8). It has been shown (7) that a 1:1 Michael adduct predominates in homogeneous aqueous solution and a 1:2 Michael–Schiff base adduct predominates under two-phase aqueous-organic conditions. These findings are in general agreement with recent conclusions regarding the interaction of 4-hydroxynonenal with proteins (9–11). The latter products differ from the amino acid and protein adducts formed with saturated monofunctional lipid ester core aldehydes about to be described. These adducts are more easily reversed than those resulting from the bifunctional aldehydes. Nevertheless, the formation of a Schiff base has been reported between *sn*-1-[9-oxo]nonanyl-2-acetylglycerophosphocholine and bovine thyroglobulin (12), which, following reduction with sodium cyanoborohydride, was suitable for the generation of antibodies that bound specifically to tritiated platelet-activating factor (PAF) and cross-reacted minimally with lyso-PAF, plasmalogens, and other phospholipids. The nature of the amino groups involved in the adduct formation was not established. We have previously identified the lipid ester core aldehydes among the secondary peroxidation products of glycerophospholipids and cholesteryl esters of low density lipoprotein and have noted their partial retention by the apoprotein (13,14). In the following study we have used liquid chromatography/mass spectrometry (LC/MS) with electrospray ionization (ESI) to demonstrate that lipid ester core aldehydes readily form Schiff bases with aminophospholipids, amino acids, and myoglobin, which can be stabilized by reduction with sodium cyanoborohydride before isolation and characterization.

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Abbreviations: GPC, glycerophosphocholine; GPE, glycerophosphoethanolamine; GPS, glycerophosphoserine; HPLC, high-performance liquid chromatography; LC/ESI/MS, liquid chromatography/electrospray ionization/mass spectrometry; PAF, platelet-activating factor; PC, phosphatidylcholine; PC-ALD, 1-palmitoyl(stearoyl)-2-[9-oxo]nonanoyl GPC; PE, phosphatidylethanolamine; PS, phosphatidylserine; TLC, thin-layer chromatography.

MATERIALS AND METHODS

Materials. Egg yolk phosphatidylcholine (PC), phosphatidylethanolamine (PE), sodium cyanoborohydride, horse skeletal muscle apomyoglobin, MW 16,950 (15), and the amino acids (valine, isoleucine, lysine, and lysine methyl ester) were obtained from Sigma Chemical Co. (St. Louis, MO). Since cyanoborohydride is a potential source of HCN, it should be used in a fume hood and acidification avoided. All solvents were of chromatographic purity, while all chemicals were of reagent grade or better quality and were obtained from local suppliers (Caledon Chemicals, Toronto, Canada).

Preparation of aldehydes. The core aldehydes were prepared from egg yolk PC by ozonolysis and triphenylphosphine reduction as previously described (16). The aldehydes were recovered from the reaction mixture with chloroform and were purified by preparative thin-layer chromatography (TLC) on Silica gel H (250 μm thick layer, 20 × 20-cm glass plate) using a phospholipid solvent system made up of chloroform/ethanol/acetic acid/water 75:45:12:6 (by vol) (16). The aldehyde-containing bands were located by spraying the plate with a Schiff base reagent, which gave a purple color (17). The product (5–10 mg from 10–20 mg egg yolk PC) was made up of 70% 1-palmitoyl-2-[9-oxo]nonanoyl and 30% 1-stearoyl-2-[9-oxo]nonanoyl glycerophosphocholine (GPC).

Preparation of reduced Schiff bases of aminophospholipids. Dioleoyl glycerophosphoethanolamine (GPE) (2 mg) and the 1-palmitoyl(stearoyl)-2-[9-oxo]nonanoyl GPC (PC-Ald) (1 mg) were dissolved in chloroform/methanol 2:1 and the mixture was kept at room temperature for 1 h. After this time freshly prepared $NaCNBH_3$ in methanol solution was added to a final concentration of 70 mM and the reaction mixture was kept at 4°C for 30 min. At end of reaction, the excess reagent was removed by washing with water. The reduced Schiff base of PE (**I**) was identified by normal-phase LC/ESI/MS as shown below. An identical procedure was used for the preparation and identification of the reduced Schiff base of phosphatidylserine (PS) (**II**). The yields of the Schiff bases were estimated by LC/ESI/MS to range from 30% for the PS adduct to 60% for the PE adduct.

Preparation of reduced Schiff bases of amino acids. The PC-Ald (1 mg) was dissolved in methanol (2 mL), and a twofold molar excess of the amino acid was added as saturated solution in water (2 mL). The reaction mixture was shaken at room temperature for 1 h, and then reduced with $NaCNBH_3$ as described above. The Schiff bases and the residual PC core aldehydes were recovered by extraction with chloroform/methanol 2:1 and were isolated by normal-phase high-performance liquid chromatography (HPLC) and identified by LC/ESI/MS as described below. The yields of the Schiff bases were 15% for lysine (**III**), 30% for isoleucine, 47% for valine (**IV**), and 57% for lysine methyl ester (**V**). Structures **I** to **V** are shown in Scheme 1.

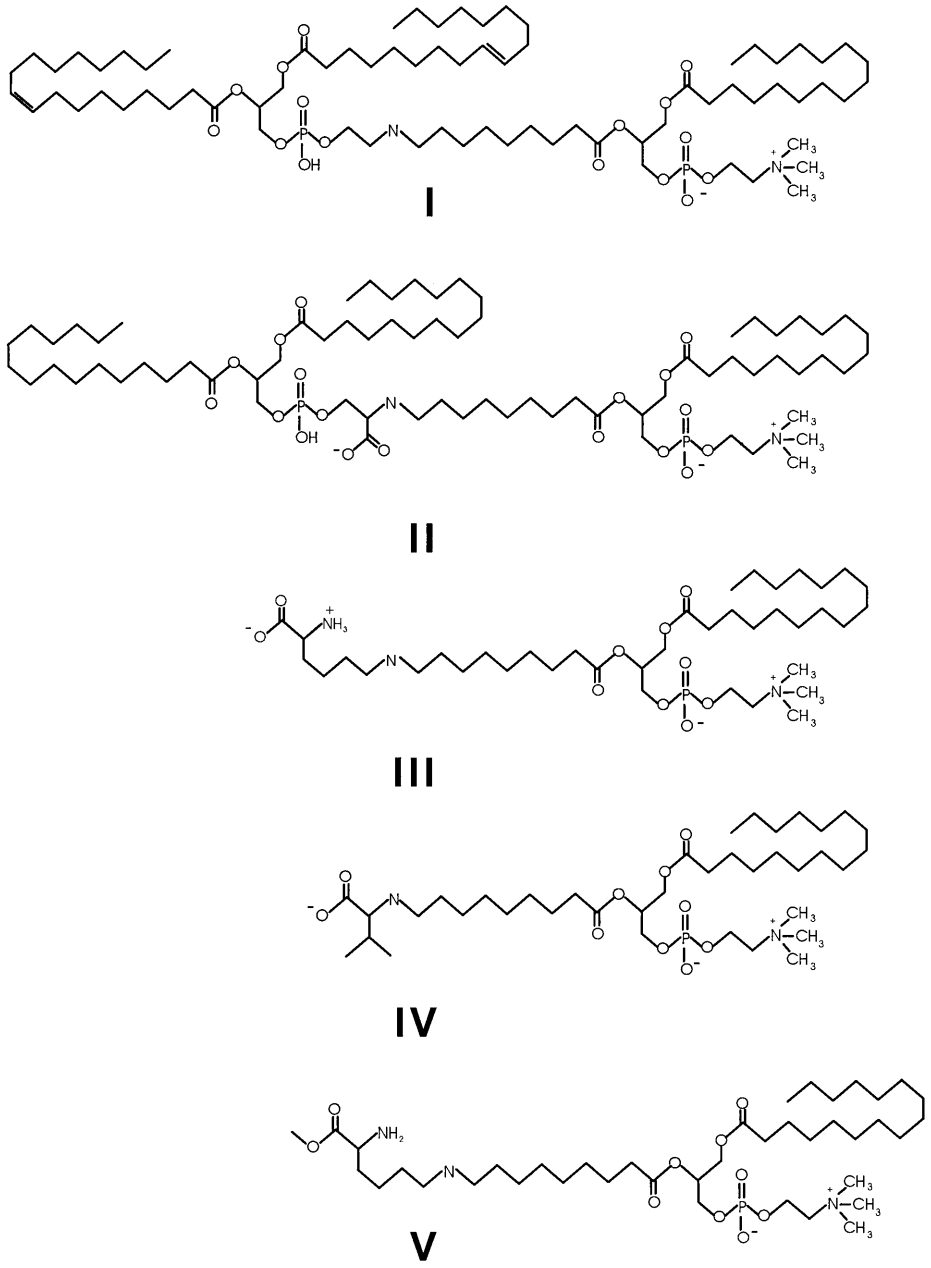
Preparation of reduced Schiff base of the myoglobin . The myoglobin (0.5 mg) was dissolved in distilled water (1 mL). To this solution was added the PC-Ald (2 mg) in ethanol (2 mL) (12) to give an approximate 100:1 ratio of aldehyde to protein. The reaction mixture was kept at room temperature for 1 h. Then $NaCNBH_3$ was added to a final concentration of 70 mM and the mixture kept at 4°C for 30 min. The reaction mixture was dialyzed against distilled water for 24 h with five changes of the solvent in order to remove excess reducing agent. The dialyzed sample was lyophilized and kept at –20°C until further analysis.

HPLC and LC/ESI/MS. Chromatographic analysis of the reduced reaction products of the aminophospholipids or amino acids with PC-Ald were performed on a silica column (Spherisorb, 3 μm, 100 × 4.6 mm i.d., Alltech, Guelph, Ontario) installed in a Hewlett-Packard (Palo Alto, CA) Model 1050 Liquid Chromatograph connected to a Hewlett-Packard Model 5988B quadrupole mass spectrometer equipped with a nebulizer-assisted ESI interface (18). The column was eluted with a linear gradient of 100% A (chloroform/methanol/30% ammonium hydroxide 80:19.5:0.5, by vol) to 100% B (chloroform/methanol/water/30% ammonium hydroxide 60:34:5.5:0.5, by vol) in 14 min, then at 100% B for 10 min (20). Reversed phase LC/ESI/MS was done as described by Kim *et al.* (20). Positive ionization spectra were taken in the *m/z* range 400–1200. Selected-ion mass chromatograms were retrieved from the LC/ESI/MS data. The molecular species of the various Schiff bases were identified from the molecular masses provided by the mass spectrometer, the knowledge of the composition of the reaction mixture, and the relative order of elution (less polar species emerging ahead of more polar species) of the anticipated products from the normal-phase column.

Flow ESI/MS. The Schiff bases of PC-Ald and myoglobin were analyzed by the Hewlett-Packard Model 5988B quadrupole mass spectrometer equipped with the ESI interface using the flow injection mode (19). The lyophilized sample was dissolved in 1 mL methanol/water/acetic acid (50:50:1, by vol), and 50 μL of the sample representing 1.5 nmol protein was injected into the ESI interface at 100 μL/min. Positive ion spectra were taken in the *m/z* range 300–2,000. Similarly the nonreduced Schiff bases of PC-Ald and amino acids were analyzed by flow ESI/MS to indicate that they could be detected as the primary product.

RESULTS

Schiff bases of aminophospholipids. Figure 1 shows the total positive ion current chromatogram (A) of the sodium cyanoborohydride reduced reaction products obtained for dioleoyl GPE along with the reconstructed single-ion chromatograms (B and C) for the $[M + H]^+$ of 16:0-9:0 Ald GPC (*m/z* 1378) and 18:0-9:0 Ald GPC (*m/z* 1406) derivatives, respectively, and the mass spectrum (D) averaged over the entire reduced Schiff base peak (PE + PC Ald). The PE–PC Schiff base is clearly resolved from the unreacted PE and the excess reagent, which has been converted to its hydroxy derivative by the reducing agent, and the small amounts of the azelaoyl GPC and lysoGPC present in the original reagent. On the basis of the peak area proportions, it was estimated



SCHEME 1

that about 40% of the PC-Ald had reacted with the PE. Reconstructed single-ion chromatograms for *m/z* 1378 (B) and 1406 (C), corresponding to $[M + H]^+$ of the reduced Schiff base of the dioleoyl GPE with 16:0-9:0 Ald GPC and 18:0-9:0 Ald GPC, respectively, document coelution of these two products. The mass spectrum averaged over the range of this peak (13.495–14.275 min) shows that the only other high mass ions are due to $[M + Na]^+$ at *m/z* 1400 and 1428, respectively.

Figure 2 shows the fragmentation spectra of the Schiff base of dioleoyl GPE and palmitoyl/[9-oxo]nonanoyl GPC as obtained by increasing the Cap Ex voltage to –300 V in the negative ion mode (A) and to +300 V in the positive ion mode (B). All the major fragment ions detected in the negative and positive ion mode can be assigned to the plausible cleavage products shown in A and B, respectively. The minor ions at *m/z* 125 and *m/z* 86 are due to loss of trimethylamine and phosphoric acid, respectively, from phosphorylcholine (*m/z* 184).

Figure 3 shows the total positive ion current chromatogram (A) of the sodium cyanoborohydride reduced reaction products obtained for dipalmitoyl glycerophosphoserine (GPS) along with the reconstructed single-ion chromatograms (B and C) for 16:0-9:0 Ald GPC (*m/z* 1370) and 18:0-9:0 Ald GPC (*m/z* 1398), respectively, along with the mass spectrum aver-

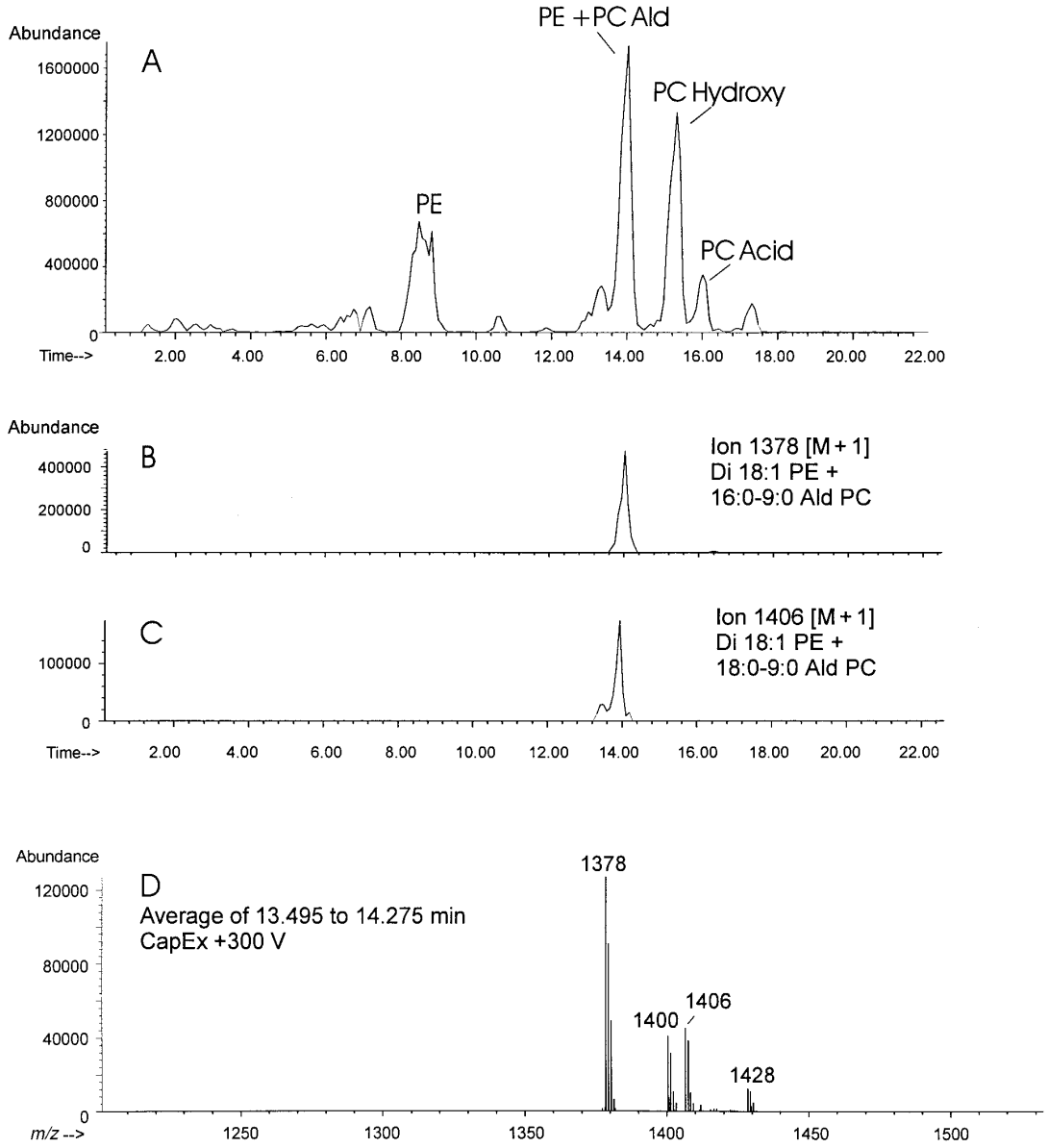


FIG. 1. Normal-phase liquid chromatography/electrospray ionization/mass spectrometry (LC/ESI/MS) of sodium cyanoborohydride-reduced reaction products of dioleoyl glycerophosphoethanolamine (GPE) and 1-palmitoyl(stearoyl)-2-[9-oxo)nonanoyl-*sn*-glycerophosphocholine (GPC). (A), total positive ion current chromatogram; (B) and (C), reconstructed single-ion chromatograms for the 16:0-9:0 Ald GPC (*m/z* 1378) and 16:0-9:0 Ald GPC (*m/z* 1406) derivatives, respectively; (D), total mass spectrum averaged over the entire Schiff base peak in (A). LC/ESI/MS equipment and operating conditions are found in the Materials and Methods section. PE, phosphatidylethanolamine; PC-Ald, phosphatidylcholine core aldehyde; PC Hydroxy, reduction product of PC core aldehyde. Major ions are identified in figure; other ions are described in text.

aged over the entire peak of the Schiff base (D). The reduced PS–PC Schiff base is only partially resolved from the PC 9-hydroxynonanoates [*m/z* 652 and *m/z* 680, not shown in (D)], formed by reduction of the unreacted PC-Ald. The reduced PS–PC Ald Schiff base is preceded by a peak containing a mixture of unidentified PC-Ald condensation products with *m/z* values ranging from 664 to 852. On the basis of the peak area proportions, it was estimated that about 20% of the PC-Ald had reacted with the PS. Reconstructed single-ion chromatograms for the *m/z* 1370 and 1398, corresponding to $[M + H]^+$ of the reduced Schiff base of the dipalmitoyl GPS with 16:0-9:0 Ald GPC and the 18:0-9:0 Ald GPC, respectively, again document coelution of the two products. Only the molecular ions for the PS–PC Ald Schiff bases are seen, with no other ions being detected in the high mass range. Clearly absent are the sodium adducts that were so prominent for the PE–PC Ald Schiff base adducts. The other peaks in Figure 3A were identified as the carboxy (*m/z* 666 and *m/z* 694) and hydroxy (*m/z* 652 and *m/z* 680) derivatives of the 16:0 and 18:0 GPC-Ald. Ionization of the PS–PC Ald Schiff base at Cap Ex of 300 V resulted in fragment ions, which closely resembled the pattern just established for the PE adduct (ion chromatograms not shown).

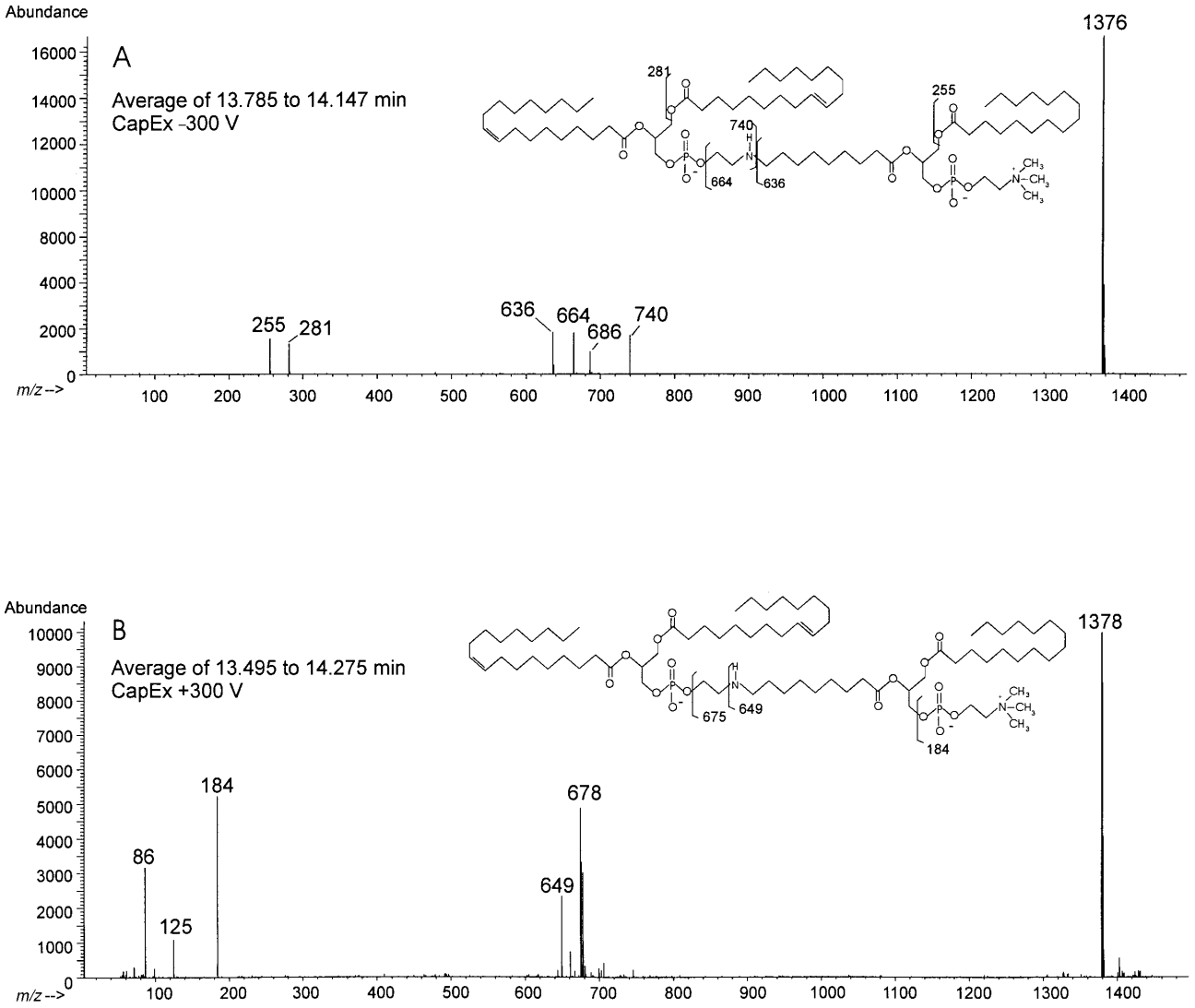


FIG. 2. Normal-phase LC/ESI/MS fragmentation spectra of the reduced Schiff base of dioleoyl GPE and 16:0-9:O Ald GPC at negative Cap Ex voltage of –300 V (A) and of positive Cap Ex voltage of +300 V (B). LC/ESI/MS conditions are as given in Figure 1. The generated ions correspond to the plausible bond cleavages depicted in the sketches accompanying the figures. Other chromatographic and mass spectrometric conditions are as given in the Materials and Methods section. The double bond in the *sn*-1 fatty chains are *cis* and not *trans* as would appear from the artwork. See Figure 1 for abbreviations.

Schiff bases of amino acids. Figure 4 shows the total ion current chromatogram as obtained by reversed-phase LC/ESI/MS for the reduced reaction products of free lysine and 16:0-[9-oxo]nonanoyl GPC (A) and the full mass spectra averaged over the entire peak of the Schiff base (Lys + PC Ald) as obtained by the use of Cap Ex –300 V (B) and Cap Ex +300 V (C), respectively. In both (A) and (B), all the major ions are accounted for by the characteristic fragmentation of the PC moiety indicated in the structural formulae given above. The low molecular weight ion at *m/z* 86 is due to loss of phosphoric acid from phosphocholine, while *m/z* at 71 is due to a cleavage of the α-β carbon bond of bound lysine. The insert in Figure 4A demonstrates that the nonreduced free lysine/PC core aldehyde Schiff base is stable under the conditions of flow injection, yielding the molecular ion (*m/z* 778). The ions at *m/z* 650 and *m/z* 666 are due to the PC acid and aldehyde present in the reaction mixture. It is known that the ε-amino group is more reactive towards Schiff base formation with saturated aldehydes (21).

Figure 5 shows the reconstructed single-ion chromatograms (A and B) corresponding to $[M + H]^+$ ions for the reduced valine-16:0-9:0 Ald GPC (*m/z* 751) and reduced valine-18:0-9:0 Ald GPC (*m/z* 779) adducts, respectively, along with the full mass spectrum (C) averaged over the entire peak of the Schiff base. The mass spectrum shows major ions corresponding to reduced Schiff bases of 16:0-9:0 Ald GPC (*m/z* 751) and of 18:0-9:0 Ald GPC (*m/z* 779) with valine. The ions at *m/z* 773 and *m/z* 801 correspond to the monosodium adducts of the two Schiff bases, respectively.

Figure 6 shows the total positive ion current chromatogram (A) of the reaction mixture of the reduced isoleucine and the

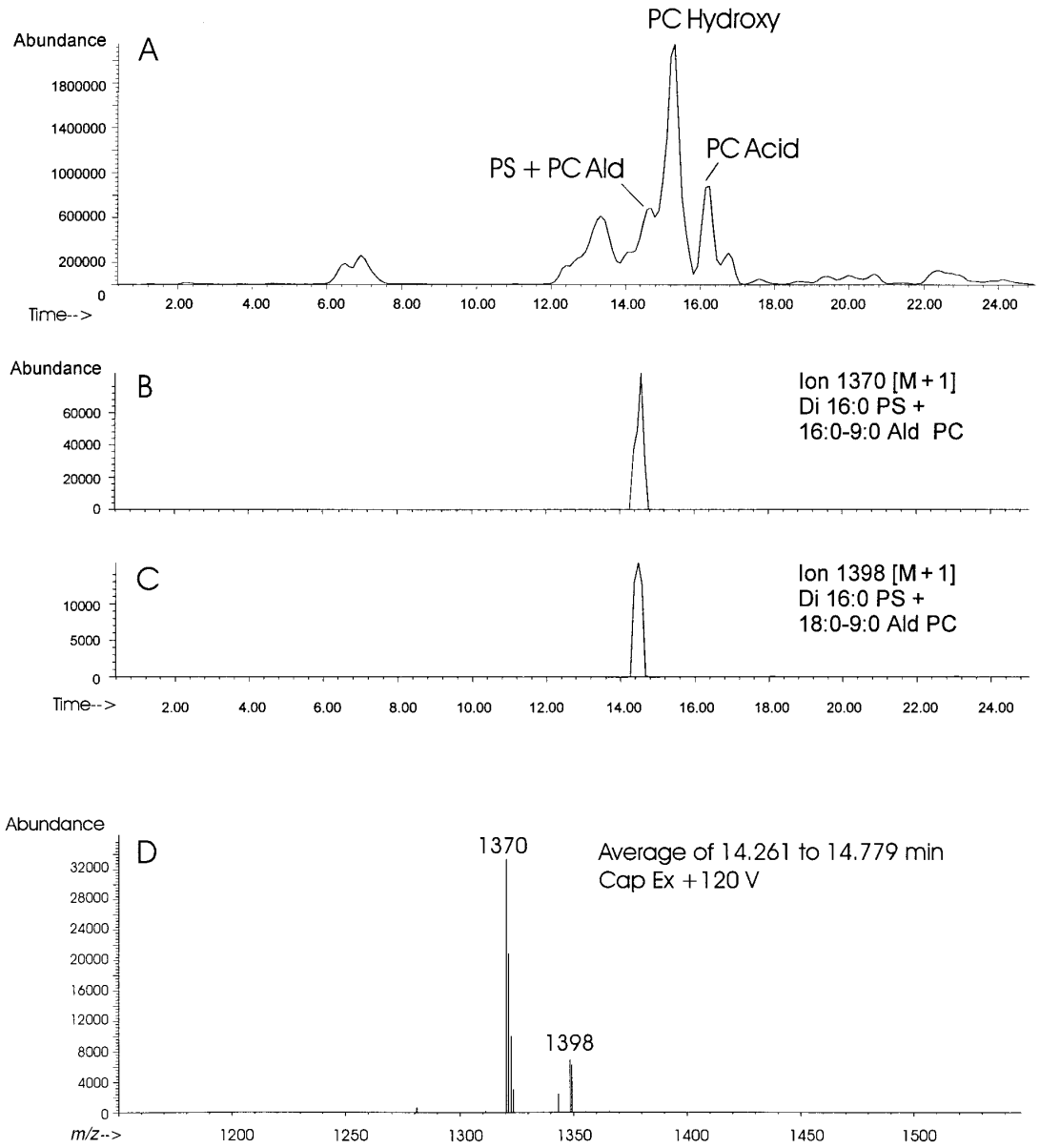


FIG. 3. Normal-phase LC/ESI/MS of sodium cyanoborohydride-reduced reaction products of dipalmitoyl glycerophosphoserine (GPS) and 1-palmitoyl(stearoyl)-2-[9-oxo]nonanoyl-*sn*-GPC. (A), total positive ion current chromatogram; (B) and (C), reconstructed single-ion chromatograms for the 16:0-9:0 Ald (*m/z* 1370) and 18:0-9:0 Ald (*m/z* 1398) derivatives, respectively; (D), total mass spectrum averaged over the Schiff base peak in (A). LC/ESI/MS equipment and operating conditions are found in the Materials and Methods section. PS, phosphatidylserine; PC Acid, oxidation product of PC core aldehyde. See Figure 1 for other abbreviations.

PC-Ald along with the reconstructed single-ion chromatograms (B and C) of the isoleucine-16:0-9:0 Ald (*m/z* 765) and isoleucine-18:0-9:0 Ald (*m/z* 793) GPC Schiff base adduct, respectively, with the full mass spectrum (D) averaged over the entire adduct peak (14.464–14.954 min). The mass spectrum of the reduced Schiff base gives major ions corresponding to the adduct of isoleucine and the 16:0-9:0 Ald (*m/z* 765) and the 18:0-9:0 Ald (*m/z* 793) GPC. The ions at *m/z* 787 and *m/z* 815 are due to the monosodium adducts, respectively.

Figure 7 shows the total positive ion current chromatogram (A) of the reaction mixture of the PC-Ald with the methyl ester of lysine along with the single-ion chromatograms (B and C) for the 16:0-9:0 Ald (*m/z* 794) and 18:0-9:0 Ald GPC (*m/z* 822) of the lysine methyl ester Schiff bases, as well as the full mass spectrum (D) averaged over the entire adduct peak. The ions at *m/z* 794 and *m/z* 822 correspond to the reduced Schiff bases of the C_{16} and the C_{18} homologs of the PC-Ald, respectively, while the ions at *m/z* 816 and *m/z* 844 corresponded to the monosodium adducts of the two reduced Schiff bases. The structures of the reduced homologous Schiff bases were confirmed by fragmentation at Cap Ex 300 V.

Schiff bases of myoglobin. Figure 8 shows the flow/

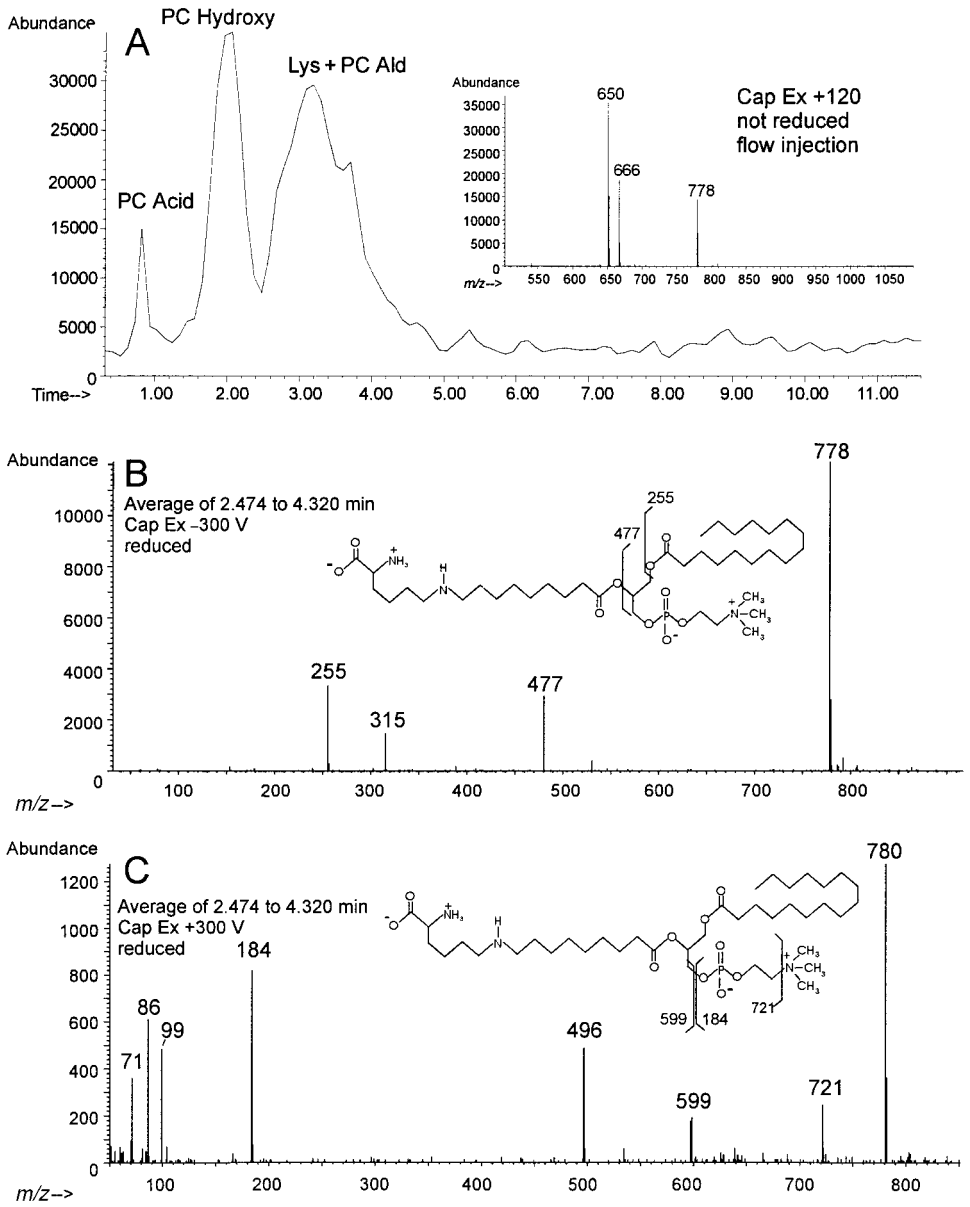


FIG. 4. Reversed phase LC/ESI/MS of the reduced reaction products of free lysine and 16:0/9:0 Ald GPC (A) and fragmentation spectra of the reduced Schiff base of free lysine and 16:0-9:0 Ald PC at negative Cap Ex voltage of –300 V (B) and at positive Cap Ex voltage of +300 V (C). Chromatographic and mass spectrometric conditions are found in the Materials and Methods section. The generated ions correspond to the plausible bond cleavage products depicted in the sketches accompanying the figures. Other ions are as described in text. See Figure 1 for abbreviations.

ESI/MS spectra and the deconvoluted molecular weights of the horse skeletal muscle apomyoglobin (A), its sodium cyanoborohydride reduction product (B), and the product of PC-Ald-apomyoglobin interaction and sodium cyanoborohydride reduction (C). The original apomyoglobin gives a multicharged ion spectrum, which can be deconvoluted to give a MW of 16,948.73. This MW corresponds to the value of 16,950 reported in the literature (17). Following reduction with sodium cyanoborohydride, the apomyoglobin mass spectrum shows two series of multicharged ions, one of which deconvolutes to the original horse skeletal muscle apomyoglobin (major peak) and another one, which deconvolutes to a MW 80 mass units higher (minor peak). The myglobin treated with PC Ald shows the presence of three series of multicharged ions, one corresponding to the original horse skeletal muscle apomyoglobin (MW 16,949.89), a second corresponding to the sodium cyanoborohydride treatment product (MW 17,030.74), and a third corresponding to the reduced PC Ald Schiff base adduct containing two molecules of the 16:0-9:0 Ald (MW 18,218.14). Other incubations gave evidence of the formation of Schiff bases with the mono-16:0-9:0 Ald and mono-18:0-9:0 Ald, and the di-16:0-9:0 Ald and the mixed 16:0-9:0 Ald and 18:0-9:0 Ald adduct. The determination of the actual sites of Schiff base complexing in the apomyoglobin molecules requires further work.

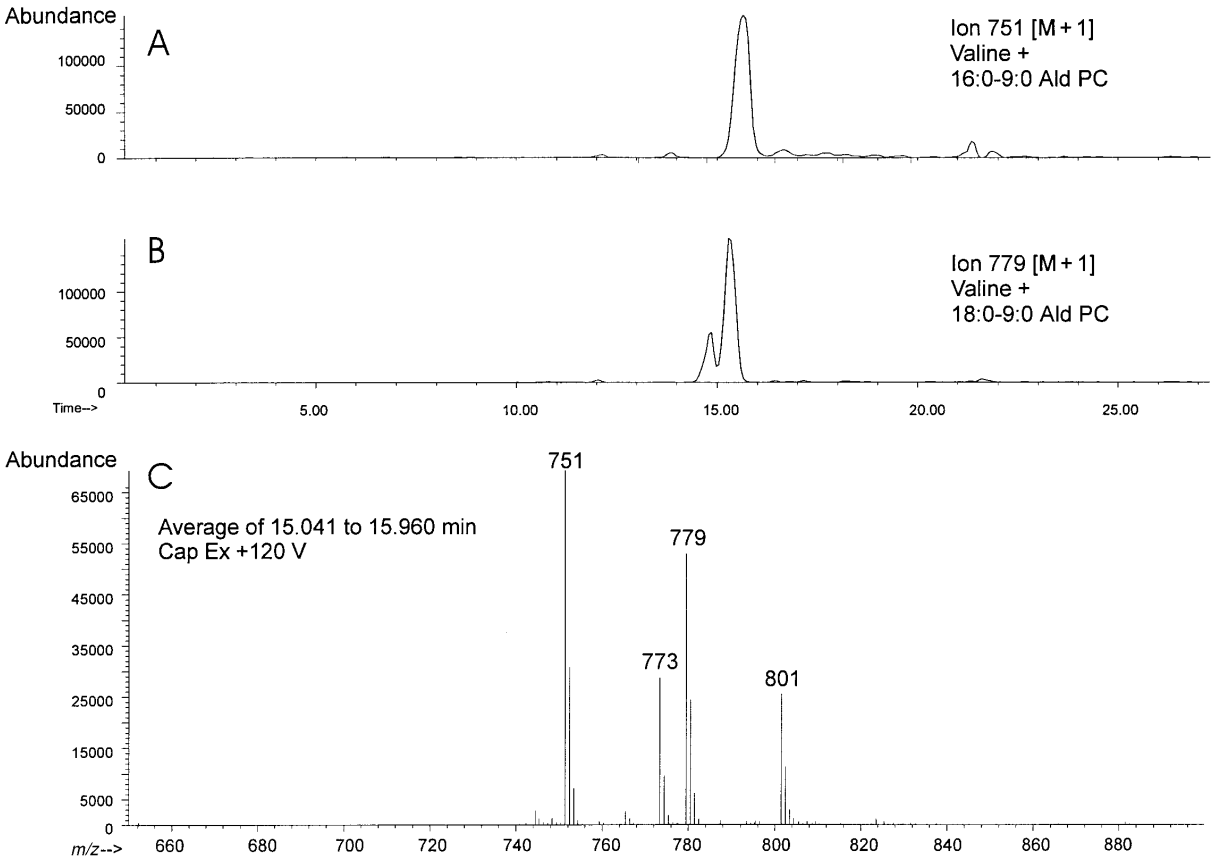


FIG. 5. Normal-phase LC/ESI/MS of sodium cyanoborohydride reduced reaction products of valine and 1-palmitoyl(stearoyl)-2-[9-oxo]nonanoyl-*sn*-GPC. (A) and (B), reconstructed single-ion chromatograms for the 16:0-9:0 Ald (*m/z* 751) and 18:0-9:0 Ald (*m/z* 779) derivatives, respectively; (C), total mass spectrum averaged over the entire reduced Schiff base peak. LC/ESI/MS instrumentation and operating conditions are found in the Materials and Methods section. Major ions are identified in figure; other ions are described in text. See Figure 1 for abbreviations.

DISCUSSION

The present study establishes that PC Ald can react with the amino groups of aminophospholipids, amino acids, and polypeptides. Aminophospholipids gave Schiff bases, which could be reduced to yield adducts of characteristic chromatographic and mass spectrometric behavior. There had been no previous demonstration of Schiff base formation between the phospholipid core aldehydes and the amino groups of aminophospholipids. Previous work had shown that the amino groups of the aminophospholipids react with simple aliphatic aldehydes, including malonaldehyde (22), but the exact structure of the products has not been established. Recently, evidence has been obtained for the glucosylation of aminophospholipids (23,24), which represent a type of Schiff base formation. The present study shows that PC Ald also reacts readily with the α-amino group of free amino acids and with the ε-amino group of free and peptide-bound lysine. In a mixed-phase system, the liposomal lipid ester core aldehyde reacts rapidly with the amino compounds to yield a yellow tinge, which deepens with time, resulting in an extensive conversion of the aldehyde into a Schiff's base when incubated with an excess of free amino acid or polypeptide. The Schiff bases could be isolated by TLC and HPLC and could be shown to give molecular ions by flow ESI/MS of concentrated samples in methanol/water solution. This indicated that the Schiff base was the primary reaction product in each instance. Addition of 0.5% ammonia to the HPLC mobile phase resulted in extensive dissociation of the Schiff base and loss of sensitivity of detection. In order to increase the stability of the bases and to permit the use of stronger ionizing solutions, the Schiff bases were reduced with sodium cyanoborohydride. The reduction increased the mass of the Schiff bases by two mass units without any significant effect on their TLC or HPLC migration when compared to the unreduced parent molecules.

Theoretically, free lysine could form Schiff bases either *via* the α- or ε- or both amino groups. Furthermore, the greater reactivity of the primary in comparison to the secondary amino group would suggest that the main product would be the Schiff base of the ε-amino group. This was confirmed by an examination of the Schiff base formed from free lysine and pure 1-palmitoyl 2-[9-oxo]nonanoyl GPC. Both flow injection and reversed-phase LC/ESI/MS with fragmentation of the reduced adduct gave the anticipated ε-amino derivative as the sole or major product. The possibility of for-

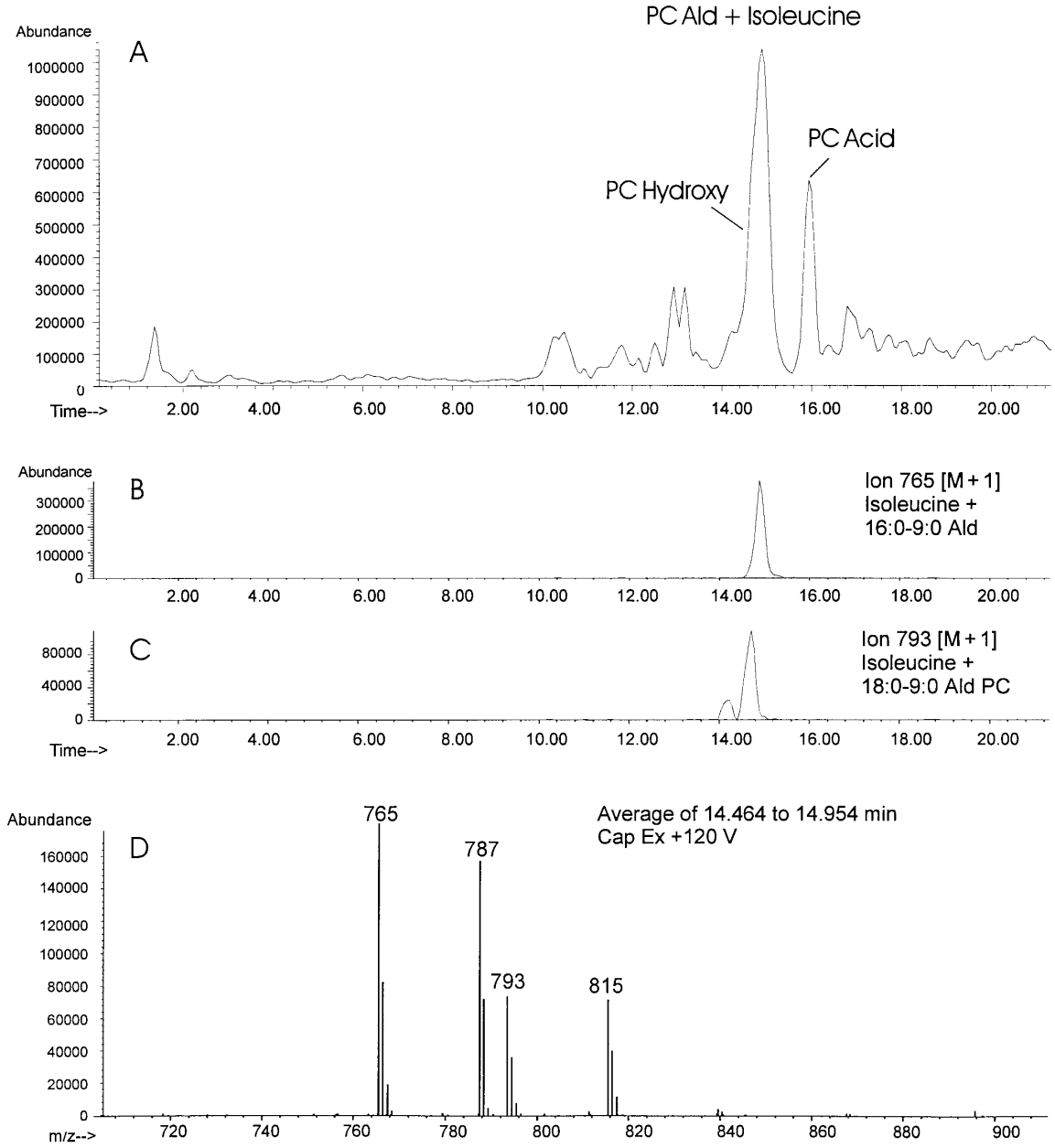


FIG. 6. Normal-phase LC/ESI/MS of sodium cyanoborohydride-reduced reaction products of isoleucine and 1-palmitoyl(stearoyl)-2-[9-oxo]nonanoyl-*sn*-GPC. (A), total positive ion current chromatogram; (B) and (C), reconstructed single-ion chromatograms for the 16:0-9:0 Ald (*m/z* 765) and 18:0-9:0 Ald (*m/z* 793) derivatives, respectively; (D), total mass spectrum averaged over the entire reduced Schiff base peak. LC/ESI/MS instrumentation and operating conditions are found in the Materials and Methods section. Major ions are identified in figure; other ions are described in text. See Figure 1 for abbreviations.

mation of an α-amino derivative could not be excluded. The reaction products, if both present, probably would not be separated by the chromatographic methods employed before or after reduction, and would both give the same MW in the mass spectrometer. The matter was not pursued further at this time.

The Schiff bases of the amino acids, including lysine, previously have been prepared using the low MW aldehydes, e.g., malonaldehyde (2-4) and 4-hydroxynonenal (7,8). In these instances, the reaction products are stabilized by secondary reactions, although the structures have not been completely established in all instances. The simple aliphatic aldehydes yield Schiff bases that are more easily dissociated than those of the bifunctional aldehydes and require chemical reduction for stabilization (12,25).

In case of myoglobin, the Schiff base formation would be expected to occur with the N-terminal amino group of glycine and any or all of the ε-amino groups of the internal lysines. Horse skeletal muscle apomyoglobin has a total of 153 amino acid residues with 1 α-amino group and 19 ε-amino groups

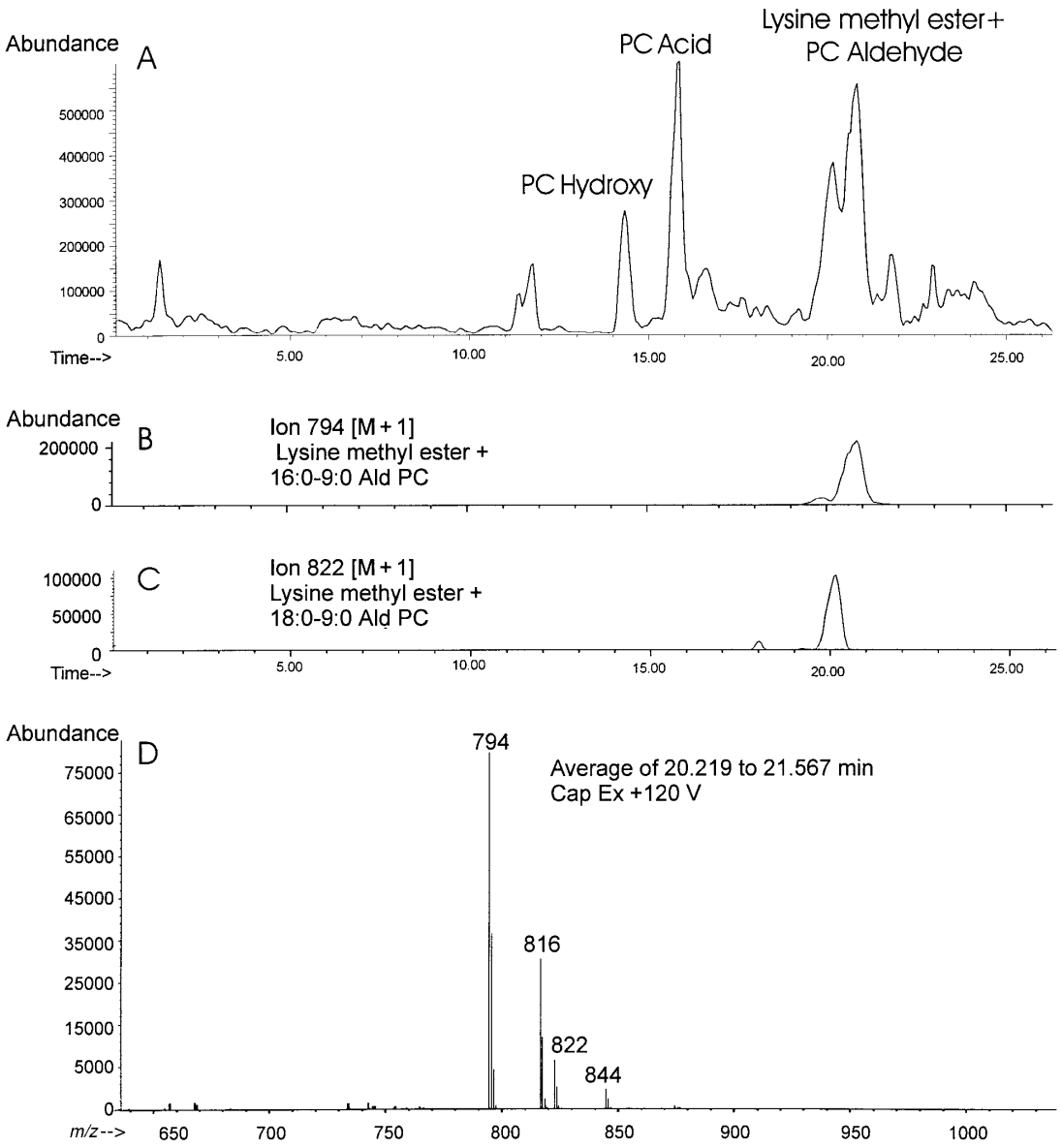


FIG. 7. Normal-phase LC/ESI/MS spectra of sodium cyanoborohydride-reduced reaction products of lysine methyl ester and 1-palmitoyl(stearoyl)-2-[9-oxo]nonanoyl-*sn*-GPC. (A), total positive ion current chromatogram; (B) and (C), reconstructed single-ion chromatograms for the 16:0-9:0 Ald (*m/z* 794) and 18:0-9:0 Ald (*m/z* 822) derivatives, respectively; (D), total mass spectrum averaged over the Schiff base peak. LC/ESI/MS instrumentation and operating conditions are found in the Materials and Methods section. Major ions are identified in figure; other ions are described in text. See Figure 1 for abbreviations.

that can react with carbonyl compounds. In the present experiments, however, only two and no more than four PC Ald appeared to be involved in Schiff base formation with horse skeletal muscle apomyoglobin. It is possible that the apomyoglobin molecule contains two particularly reactive sites susceptible to Schiff base formation, which could explain the presence of only minor amounts of the single Schiff base adduct. The formation of a bis-Schiff base adduct corresponding only to the 1-palmitoyl species is probably due to the predominance of this species (80%) compared to the 1-stearoyl species (20%) in the reaction mixture. The reduced bis-Schiff base adduct of the dipalmitoyl derivative was selected for illustration.

In other studies LC/MS with electrospray has been employed to demonstrate the Schiff base formation between acetone and the ε-amino groups of horse myoglobin lysine (26). The identified protein species contained from 1 to 6 adducts of methyl isobutyl ketone or acetone. In the present experiments, two to four residues of the core aldehyde were bound to horse apomyoglobin. The specific amino acid residues involved in the Schiff base formation were not determined. Since the amino acid sequence of horse myoglobin is known, the exact location of the Schiff base forming lysines could be established in the future by trypsin digestion and reversed-phase LC/ESI/MS of the released peptides, the MW of which

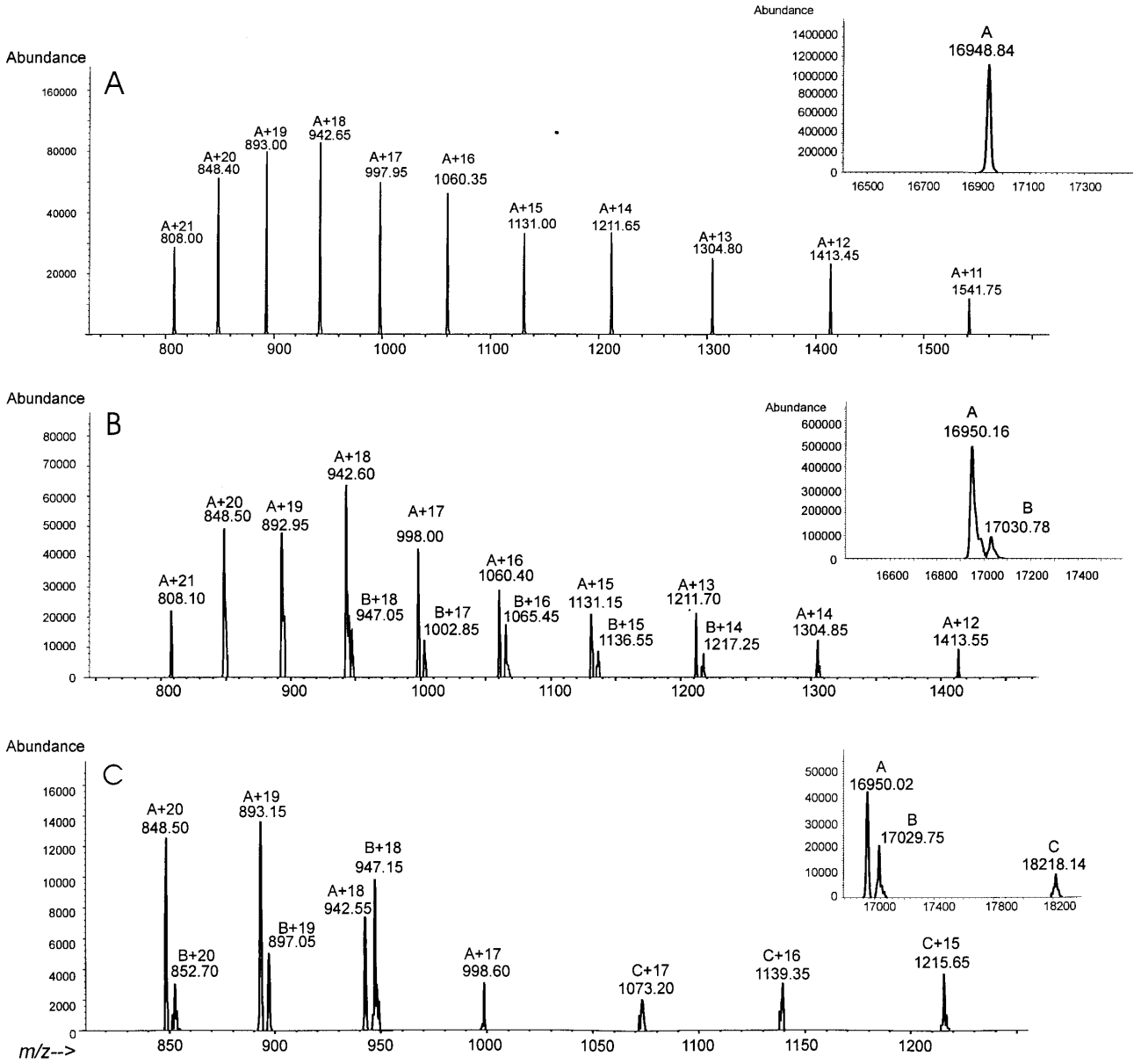


FIG. 8. Flow ESI/MS spectra and the deconvoluted MW of the horse skeletal muscle apomyoglobin (MW 16,948.84) (A), its sodium cyanoborohydride reduction product (MW 17,030.78) (B), and the product of interaction of myoglobin with two 16:0-9:0 Ald GPC (MW 18,218.14) (C). Flow injection ES/MS instrumentation and operating conditions are found in the Materials and Methods section. Major ions and charge distribution are given in figure. See Figure 1 for abbreviations.

could be calculated. The peptides bearing the phospholipid moieties would be expected to be retained much longer on reversed-phase columns than the corresponding peptides without the phospholipid moiety (8).

The previously prepared Schiff base of C_9 core aldehyde of 2-acetylglycerophosphocholine and thyroglobulin (12) was not characterized beyond the demonstration that an antibody could be raised to the reduced adduct to recognize in tissue extracts the PAF, which the C_9 core aldehyde resembles structurally. Uncharacterized have also remained the radioactive complexes formed between apoprotein B and oxidized 2-[1-^{14}C]arachidonoyl PC (27), which must have involved the C_5 core aldehyde, because the malonaldehyde and 4-hydroxynonenal would not be labeled.

The present findings are of interest because the lipid ester core aldehydes like the short-chain aldehydes would be expected to form covalently-bound complexes with proteins. Malonaldehyde and 4-hydroxynonenal-modified lipoproteins have been found entrapped in aortic walls of both humans and animals (28,29), while lysine modification of low density lipoprotein or lipoprotein (a) by 4-hydroxynonenal or malonaldehyde decreases platelet serotonin secretion (30). The generation of reactive aldehyde species next to membrane components possessing active amino groups, which may lead to

Schiff base formation, could lead to membrane damage, loss of enzyme activity, and protein–protein or protein–lipid cross-linking (31). Proteins in the red blood cell membrane show increase in MW, cross-linking, when exposed to lipid-oxidizing conditions (32). The hydrophobicity acquired from complexing with the lipid ester core aldehydes would promote greatly the membrane association of the proteins and contribute to their resistance to proteolytic digestion and *trans*-membrane transport among other effects. Recent work (33) with monoclonal antiphospholipid antibodies has shown that they are directed against epitopes of oxidized phospholipids. A variety of structures that could occur in peroxidized tissues have been suggested, but only a few have been experimentally demonstrated. The present work shows that some of these compounds can be synthesized as well as records the LC/ESI/MS characteristics of these compounds that will be required for the isolation and identification of the Schiff base adducts from natural sources.

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