Mass Spectral Analysis of Glycerophospholipids*

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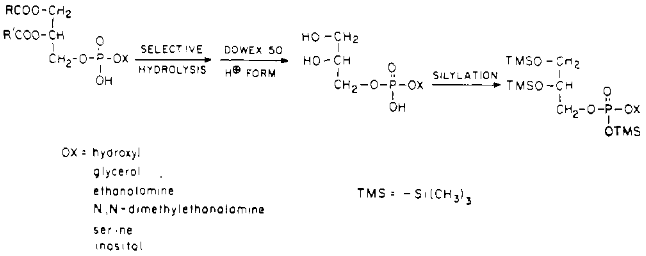
ABSTRACT: Mass spectrometry and combined gas chromatography–mass spectrometry have been applied to the determination of the "backbone" components of glycerophospholipids. By chemical modification of the parent phospholipids, the characteristic glycerophosphate esters have been isolated and analyzed as their trimethylsilyl derivatives.

The technique of mass spectrometry for proof and elucidation of organic structures is a well established and widely employed method of analysis. With the more recent development of high-resolution instrumentation, computerized acquisition and reduction of data, and combined gas chromatography–mass spectrometry has come the realization that such a method is a valuable asset to the study of complex biochemical systems. One area of interest in this field of biological mass spectrometry is the development of a suitable vapor phase separation and identification of phospholipid mixtures, and more precisely the analysis of the backbone structure of the fatty diacyl phosphoglycerides. The unique structure of these glycerophospholipids, that of possessing both long chain fatty acids and polar head groups, leads to a marked affinity for both nonpolar solvents and water. At the present time there is a great deal of interest in not only the structure but also the function of such compounds, particularly in the area of membrane chemistry since these phospholipids compose 25–50% of the weight of all cell membranes.

Basically two types of gas phase procedures have been previously applied in studying these diacyl phosphoglycerides: analysis of the intact, underivatized phospholipids (Perkins and Johnston, 1969) and analysis of the trimethylsilyl derivatives of the intact compounds (Horning *et al.*, 1969). In both instances the only products detected were the nonphosphorus-containing diglycerides or diglyceride trimethylsilyl ethers, respectively. The products were proposed to arise *via* ortho acid (ester) intermediates. These results, then, provided only information pertaining to the fatty acids present and not to the backbone components of these lipids.

Because the glycerophospholipids themselves were unsuitable for determination of the polar headgroups present, it was decided to investigate the possibility of analyzing only a portion of these molecules. The method chosen involved removal of the fatty acids and analysis of the glycerophosphate ester "backbones" that resulted.[1] This approach to the backbone determinations is summarized in Scheme I where -OX represents: hydroxyl, glycerol, ethanolamine, *N*,*N*-dimethylethanolamine, serine, and inositol groups which are common in both mammalian and bacterial membranes. The procedure consists of an initial selective hydrolysis using methanolic sodium hydroxide which preferentially removes the fatty acids leaving the phosphate ester linkages intact (Dittmer and Wells, 1969). The resulting sodium salts of the phosphodiesters are then passed through a column of Dowex 50 hydrogen ion exchange resin using deionized water.[2] The aqueous glycerophosphate ester solutions recovered from the resin are then concentrated, dried, and derivatized as shown in the final step of this sequence. The increased volatility of these trimethylsilyl glycerophosphate esters makes them suitable for analysis by mass spectrometry and combined gas chromatography–mass spectrometry at relatively low temperatures.

SCHEME I



Results and Discussion

In Figure 1 is presented the mass spectrum of the parent compound of this series, tetrakis(trimethylsilyl)-α-glycerophosphate. Although the molecular ion of this derivative, *m/e* 460, is found in only trace amount, several characteristic ions formed from $M\cdot^+$ by loss of $CH_3\cdot$ (M − 15), $Me_3Si\cdot$ (M − 73), trimethylsilanol (M − 90), and $Me_3SiOCH_2\cdot$ (M − 103) serve to confirm its molecular weight.

With the known rearrangement behavior of trialkyl phosphates (Quayle, 1959) and the trimethylsilyl derivatives of nucleotides (McCloskey *et al.*, 1968), dinucleotides (Hunt *et al.*, 1968), and sugar phosphates (Zinbo and Sherman, 1970) one expected fragmentation sequence for the trimethylsilyl derivative of glycerophosphate consists in cleavage of the carbon and phosphate ester oxygen bond with charge retention by the oxygen accompanied by either double hydrogen

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[2] This ion-exchange process functions merely to regenerate the acid form of these compounds and is not designed as a separation procedure; however, it does serve to separate glycerophosphorylcholine from the phosphoglycerides studied here. This material is retained by the column and after elution of the other compounds may be recovered by elution with dilute acid and its presence determined by more conventional methods.

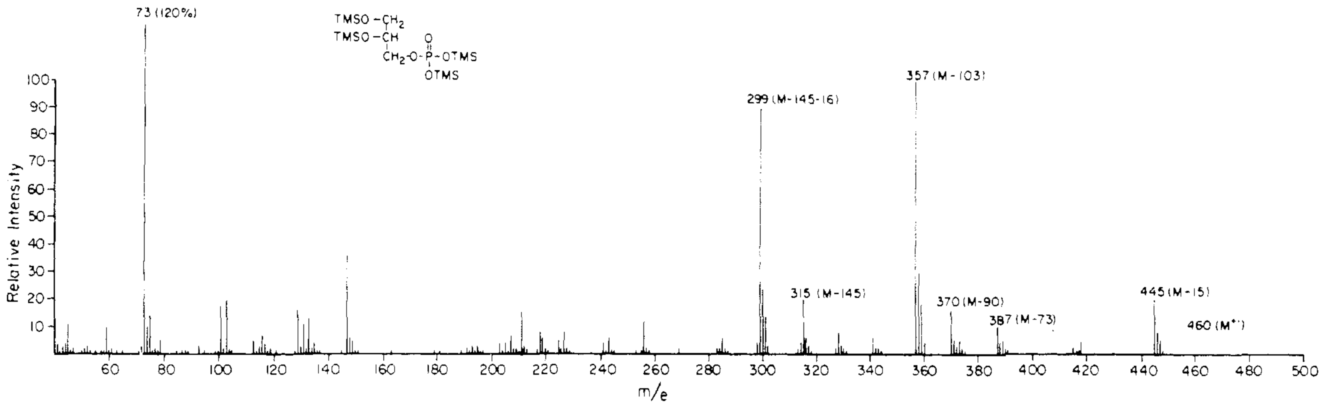


FIGURE 1: The 70-eV mass spectrum of tetrakis(trimethylsilyl)-α-glycerophosphate.

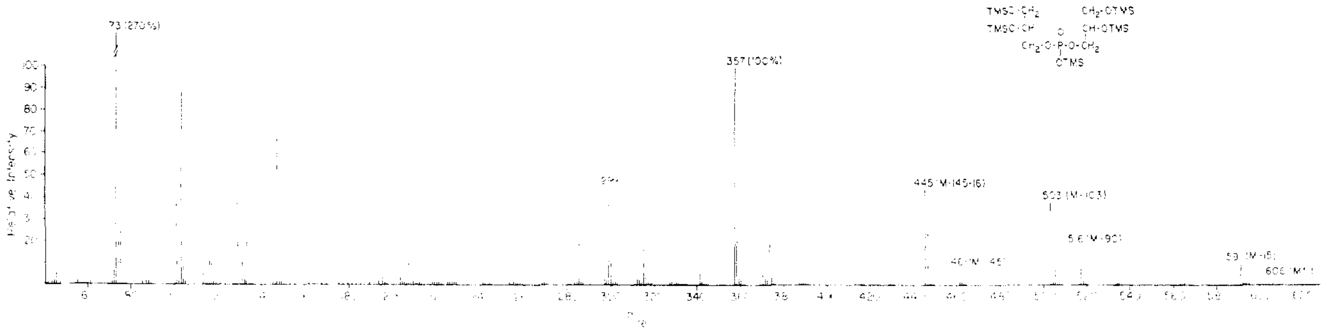
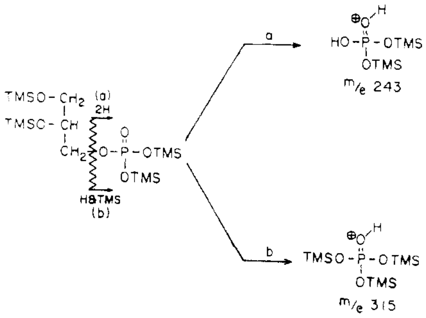


FIGURE 2: The 70-eV mass spectrum of pentakis(trimethylsilyl)-α-glycerophosphorylglycerol.

or hydrogen and trimethylsilyl back-transfer yielding a protonated phosphate ion as illustrated in Scheme II. Thus the presence of these two fragment ions, as well as products from their further decomposition, in the mass spectrum of this compound was not unexpected, and although no metastable or isotopic labeling evidence can be given in support of these structures, high-resolution mass measurements do confirm their empirical formulas (calcd 315.1032; found 315.1058; calcd 243.0638; found 243.0665). The intense signal at *m/e* 299 (calcd 299.0720; found 299.0771) most likely results from loss of methane by the rearrangement fragment ion at *m/e* 315 as previously noted by McCloskey and coworkers (McCloskey *et al.*, 1968) and by Zinbo and Sherman (Zinbo and Sherman, 1970).

One further point of interest concerning this trimethylsilyl glycerophosphate derivative is the comparison of its behavior upon electron impact with that of its positional isomer, tetrakis(trimethylsilyl)-β-glycerophosphate. Under the gas chromatographic conditions employed these two isomers can be successfully separated and their mass spectra compared. Although there are no differences in the particular fragment ions formed, distinct variations in relative ion abundancies do result. The most striking difference is in the intensity of two signals, *m/e* 243 (Scheme II) and *m/e* 357 (M − ·CH_2OTMS). As can be seen in Figure 1, the α-isomer exhibits an abundant *m/e* 357 ion with *m/e* 243 found in only minor abundance. In the case of the β-isomer the pattern is reversed, *m/e* 357 being less than 10% of the relative intensity of the signal at *m/e* 243.

SCHEME II



In Figures 2–6 are presented the mass spectral–bar graph representations of the other glycerophosphate ester derivatives.[3]

In each case the molecular ions are found in only trace amounts but the M − 15 fragment ions are abundant. In addition, varying amounts of the characteristic fragment ions at M − 73, M − 90, and M − 103 are produced. As in the case of the parent compound (Scheme II) these derivatives also exhibit double hydrogen and hydrogen and trimethylsilyl rearrangements. Here, however, two possible sets of ions can arise corresponding to either back-transfer from and loss of the X group or back-transfer from and loss of the glycerol moiety. The former pathway in all cases leads to the structurally uninformative ions at *m/e* 389 (double H transfer)

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[3] Figures 1–5 represent mass spectra obtained using the LKB-9000 combined gas chromatograph–mass spectrometer as explained in the experimental section of this report. The mass spectrum of Figure 6 was obtained by direct probe analysis employing the CEC-21-110 as explained in the Experimental Section.

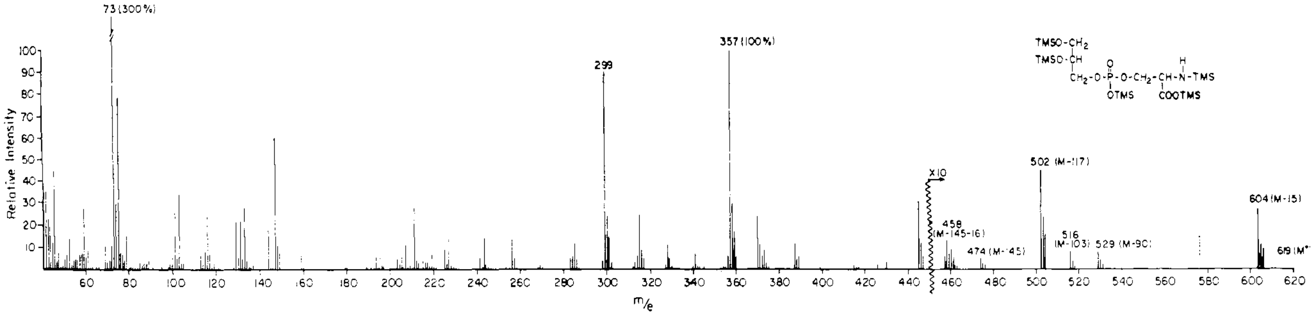


FIGURE 3: The 70-eV mass spectrum of tetrakis(trimethylsilyl)-*N*-trimethylsilyl-α-glycerophosphorylserine.

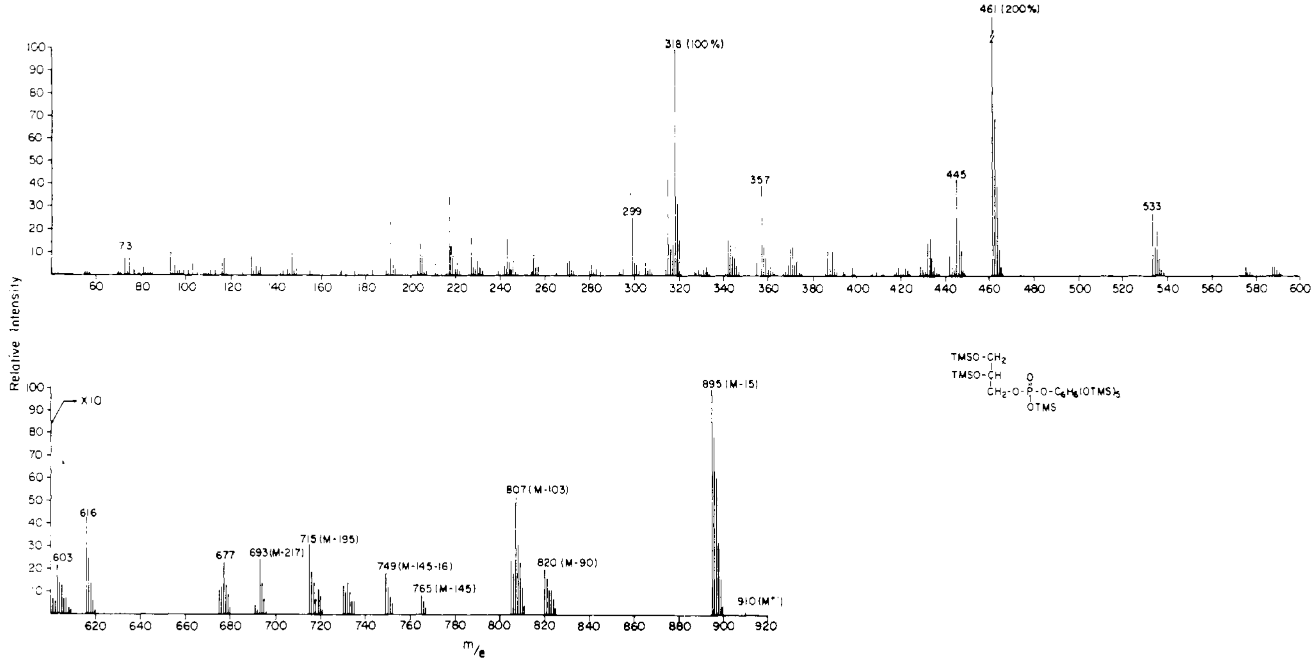


FIGURE 4: The 8-eV mass spectrum of octakis(trimethylsilyl)-α-glycerophosphorylinositol.

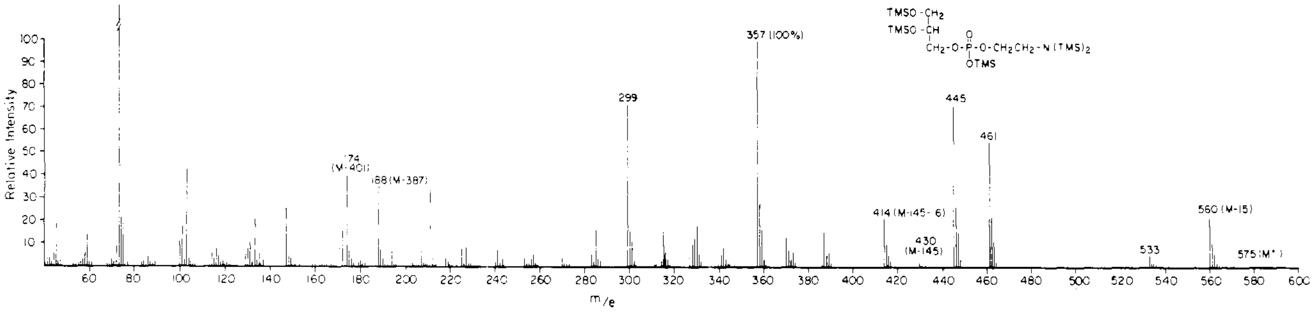


FIGURE 5: The 70-eV mass spectrum of tris(trimethylsilyl)-*N*,*N*-bis(trimethylsilyl)-α-glycerophosphorylethanolamine.

and 461 (H and TMS transfer) while the latter yields fragment ions diagnostic of the various X groups present at M − 217 (double H transfer) and M − 145 (H and TMS transfer). These fragmentation pathways are summarized in Schemes III and IV.

The last general type of fragmentation sequence which might be expected to provide structurally relevant information concerning the X portion of these molecules is that of simple single bond cleavage with charge retention on the X group as depicted in Scheme V. This pathway is of particular importance for the ethanolamine derivatives: tris(trimethylsilyl)-*N*,*N*-bis(trimethylsilyl)-α-glycerophosphorylethanolamine yields intense *m/e* 188 (calcd 188.1290; found 188.1304) and 174 fragments (calcd 174.1133; found 174.1147), whereas tris(trimethylsilyl)-*N*,*N*-dimethyl-α-glycerophosphorylethanolamine affords abundant *m/e* 72 (calcd 72.0813; found

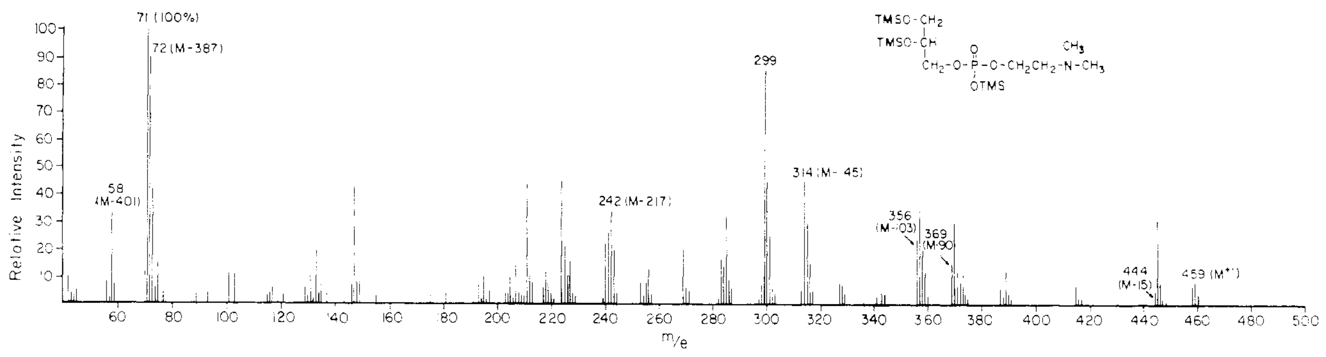
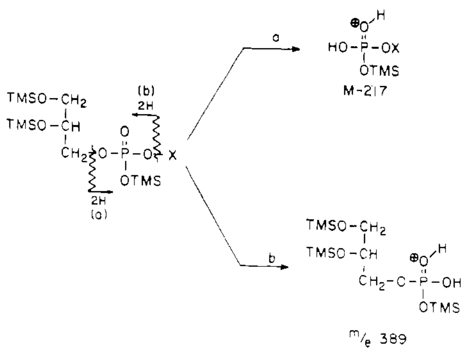
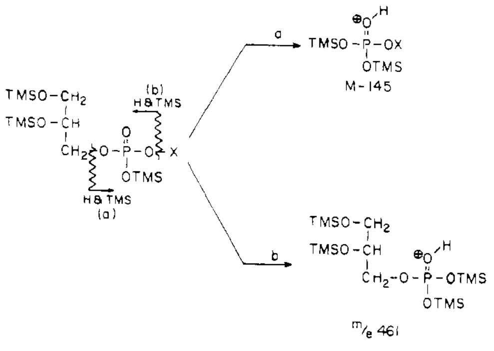


FIGURE 6: The 70-eV mass spectrum of tris(trimethylsilyl)-*N*,*N*-dimethyl-α-glycerophosphorylethanolamine.

Scheme III

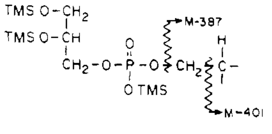


Scheme IV



72.0824) and 58 ions. At low ionizing energies these simple cleavages predominate to an even greater extent, the M − 387 in both cases resulting in the base peak of the spectrum (excluding *m*/*e* 71 for the *N*,*N*-dimethylethanolamine derivative).

Scheme V



In addition to the more general fragmentation schemes already presented for these trimethylsilyl derivatives, tetrakis(trimethylsilyl)-*N*-trimethylsilyl-α-glycerophosphorylserine and octakis(trimethylsilyl)-α-glycerophosphorylinositol exhibit unique fragment ions characteristic of their individual structures. For example, the trimethylsilyl derivative of α-glycerophosphorylserine exhibits an abundant loss of the carbotrimethylsiloxy radical (·COOTMS) from the molecular ion at M − 117 (calcd 502.2059; found 502.2110), a cleavage process noted previously for the trimethylsilyl derivatives of amino acids (Teeter, 1962, and Bergström *et al.*, 1970). Upon rearrangement as depicted in Scheme IV, the α-glycerophosphorylinositol trimethylsilyl derivative can form the protonated molecular ion of hexakis(trimethylsilyl)-inositol phosphate and thus exhibits numerous fragment ions which are analogous to those of the previously reported trimethylsilyl derivative of *myo*-inositol phosphate (Sherman *et al.*, 1969).

In this preliminary report it has been shown that indeed phospholipid components can be analyzed by gas-phase procedures and that definitive data can be obtained concerning the polar head groups present. Although the method presented does possess the capability of being used as a convenient, qualitative method of analysis, its use as a quantitative analysis procedure for these backbone components has not as yet been realized. For example, during gas chromatography varying amounts of α- and β-trimethylsilyl glycerophosphate have been detected in all of the glycerophosphate ester derivatives examined, and although the origin of these two derivatives has not yet been determined studies are currently under way to eliminate such occurrences.

The classical methods of analyzing glycerophospholipid mixtures consist of selective deacylation and analysis of the resulting glycerophosphate esters by either paper chromatography, electrophoresis, or anion-exchange column chromatography (Dittmer and Wells, 1969). It is believed that the method presented here will not only shorten the time required for analysis but will also increase the sensitivity and make detectability and identification of new phospholipids more feasible.

Experimental Section

Reagents. Purified phospholipids were purchased from Mann Research Laboratories, New York, N. Y., and Applied Science Laboratories (Lawshe Instrument Company, Bethesda, Md.). In addition samples of glycerophosphorylethanolamine, glycerophosphorylinositol, and glycerophosphorylserine were purchased from Supelco Inc., Bellefonte, Pa. Bis(trimethylsilyl)trifluoroacetamide was purchased from Regis Chemical Company, Chicago, Ill., and contained 1% trimethylchlorosilane. Pyridine was distilled from and stored over calcium hydride. Chloroform, methanol, isobutyl alcohol, sodium hydroxide, and acetic acid were reagent grade and were used without further purification.

Selective Hydrolysis and Ion Exchange. The glycerophospholipid (5–15 μmoles) contained in a 10–15 ml tube was dried at reduced pressure and dissolved or suspended in 1 ml of chloroform and methanol (1:4). To this mixture was added 0.1 ml of 1.0 N aqueous sodium hydroxide; the reaction mixture was then mixed well, heated at 37° for 15 min, and neutralized with 0.1 ml of 1.0 N aqueous acetic acid. The neutralized mixture was treated with 2 ml of chloroform and methanol (9:1), 1 ml of isobutyl alcohol, and 2 ml of water followed by thorough mixing and centrifugation. The upper aqueous layer containing the phosphoglyceride was isolated, washed twice with an equal volume of ether, and concentrated by rotary evaporation at or below 35°.

The concentrated aqueous phase was passed through a column of Dowex 50W-X4 hydrogen ion exchange resin (Sigma; dry mesh 100–200; 1–2 g) using water as eluent. Fractions of 5 ml were collected; elution of the glyceryl phosphate esters was monitored by the decrease in pH or by phosphate determination (Dittmer and Wells, 1969). Each phosphodiester was contained within the first two fractions of the collection except glycerylphosphorylcholine. This latter compound was removed from the column employing 10–15 ml of 0.01 M HCl.

Silylation. An aliquot of the phosphoglyceride fraction from the ion-exchange procedure, containing approximately 1 mg of the glycerophosphate ester, was concentrated and dried at reduced pressure in a 10–15 ml glass-stoppered centrifuge tube. To the dry residue 0.1 ml of pyridine and 0.1 ml of bis(trimethylsilyl)trifluoroacetamide containing 1% trimethylchlorosilane were added, and the reaction mixture was stirred magnetically at room temperature for 1 hr.

Upon occasion, silylation of the various glycerophosphate esters has resulted in compounds whose mass spectra, obtained by direct probe insertion, contain fragment ions corresponding to small amounts of material with less than the number of trimethylsilyl groups listed in Figures 1–6. However, combined gas chromatography–mass spectrometry has failed to confirm the presence of such material. It also appears that the use of bis(trimethylsilyl)acetamide for the chemical modification of glycerylphosphorylserine results in a higher overall yield of the pentasilyl product (Figure 3).

Mass Spectral Analysis. (a) Direct probe inlet system. The silylation reaction mixture (50 μl) was transferred to a melting point capillary tube and concentrated at reduced pressure (~0.1 mm). The sample contained in the capillary was then introduced through the vacuum lock into the ion source of a double focusing CEC-21-110 mass spectrometer. Spectra were obtained at ion source and insertion probe temperatures of 120–150°. Small amounts of α- and/or β-tetrakis(trimethylsilyl)glycerophosphate may have been present in the samples, but did not interfere with the characteristic ions used to identify each of the glycerophosphate ester derivatives.

(b) Combined gas chromatography–mass spectrometry. Combined gas chromatography–mass spectrometry was carried out on a LKB-9000. Direct, on-column injections of the silylation reaction mixture (1–5 μl) were employed utilizing a 1% OV-17 on Supelcoport (80–100 mesh) glass column (6 ft × 2 mm) with temperature programming from 150 to 250° at 5°/min and a helium flow rate of 25 cc/min. The flash heater of the gas chromatograph was maintained at 250°, the molecular separator at 250°, and the ion source of the mass spectrometer at 290°. Spectra were recorded at ionizing energies of 8 and 70 eV.

Difficulty has been experienced in obtaining consistent transfer of the gas chromatograph effluent from the column to the ion source of the mass spectrometer. Similar difficulties were noted by Quayle (Quayle, 1959) in obtaining the mass spectra of trialkyl phosphates and by Zinbo and Sherman (Zinbo and Sherman, 1970) during the combined gas chromatography–mass spectrometry of trimethylsilyl sugar phosphates. The latter workers, however, were able to overcome this difficulty through modification of their instrument.

The most serious problem of this nature has occurred in attempts to obtain mass spectra of the *N,N*-dimethylethanolamine derivative (Figure 6) by combined gas chromatography–mass spectrometry. Upon injection of this compound only one gas chromatographic peak other than those corresponding to α- and β-trimethylsilyl glycerophosphate was observed. However, the mass spectral results from this peak exhibited fragment ions characteristic of the *N,N*-dimethyl-α-glycerophosphorylethanolamine trimethylsilyl derivative only at *m/e* 58, 71, and 72, the remaining ions corresponding to those of the glycerophosphate trimethylsilyl derivative (Figure 1).

The remainder of the glyceryl phosphate esters exhibited the following gas chromatographic retention times as measured from point of injection: trimethylsilyl derivative of: β-glycerophosphate, 0.9 min; α-glycerophosphate, 1.3 min; α-glycerophosphorylglycerol, 5.8 min; α-glycerophosphorylethanolamine, 6.1 min; α-glycerophosphorylserine, 6.5 min; α-glycerophosphorylinositol, 12.5 min.

(c) High-resolution mass measurement data for tris(trimethylsilyl)-*N,N*-dimethyl-α-glycerophosphorylethanolamine. Because of the close proximity of the fragment ions of the glycerophosphate derivative (Figure 1) to those assigned to the *N,N*-dimethylethanolamine derivative (Figure 6), the following high-resolution data were determined for the latter: M − 90 (calcd 369.1555; found 369.1506); M − 103 (calcd 356.1477; found 356.1472); M − 145 (calcd 314.1372; found 314.1349); M − 217 (calcd 242.0976; found 242.0930); M − 217 − 18 and/or M − 145 − 90 (calcd 224.0871; found 224.0878); $C_4H_{10}N$ (calcd 72.0813; found 72.0824); C_4H_9N (calcd 71.0735; found 71.0736).

Acknowledgment

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Reversible and Irreversible Denaturation of Collagen Fibers*

Helmut Hörmann and Harald Schlebusch

ABSTRACT: The degree of denaturation of heat-shrunk rattail tendon collagen was determined by dissolving the tendons with pronase and then calculating the native portion in the resultant solution from optical rotation values. Re-formation of triple-helical structures during the dissolution process was largely prevented when the tendons were dissolved at +4°. In this way, it was possible to detect the reversibly denatured portion together with the more severely denatured collagen. The dependence of the entire denaturation process on temperature is shown in a sigmoid curve with a point of inflection coinciding with that of the curve for the temperature dependence of the shrinkage process of collagen fibers. Both methods of measurement thus indicated the same denaturation temperature. This method also permits measurement of the renaturation processes in denatured insoluble collagen. Considerable renaturation was observed up to 3 hr after denaturation. The degree of renaturation was dependent on the degree of previous denaturation. The optimal renaturation temperature was found to be 30°. Stretching the denatured fiber promoted the re-formation of native material considerably.

Native collagen fibers shrink to about one-third their original length when heated in water above 62° (Gustavson, 1956). This phenomenon is due to denaturation of collagen molecules which are covalently cross-linked within the fiber network. During denaturation the triple-helix structure of the monomers is transformed to randomly coiled peptide chains.

Many conclusive investigations have dealt with the denaturation of collagen in solution (Doty and Nishihara, 1958; Engel, 1962) and with the re-formation of dissolved triple-helix structures (Von Hippel and Harrington, 1959; Kühn *et al.*, 1964; Beier and Engel, 1966; Harrington and Rao, 1970). In contrast far less is known about the denaturation and renaturation of insoluble collagen fibers. Limited information is available here from shrinkage measurements (Weir, 1949; Verzár, 1963) and X-ray investigations (Wright and Wiederhorn, 1951). The latter show that during denaturation the low-angle pattern as well as the 2.89-Å arc disappear. After cooling and stretching only the 2.89-Å arc reappears, indicating some re-formation of triple-helix structures in a random orientation.

In order to determine the degree of denaturation of insoluble collagen fibers the principle may be useful that the triple helix of collagen is very resistant to most proteases, while denatured collagen is easily broken down (Grassmann, 1936; Grassmann *et al.*, 1937). Of special interest in this respect is pronase which dissolves all the insoluble collagen, the native as well as the denatured portion (Fujii, 1965, 1966; Drake *et al.*, 1966). At temperatures up to approximately 20°, the native portion goes into solution as collagen monomers with a triple-helix structure exhibiting a high optical rotation. Denatured collagen, on the other hand, is broken down into small peptides of low rotation. The proportion of native material thus can be measured in solution by polarimetric methods (Hörmann and Schlebusch, 1968a).

However, regarding the prolonged time required to dissolve insoluble collagen by pronase, this method yielded reproducible results only when the degree of denaturation changed little, if at all, over prolonged periods of time. Therefore, only predominantly irreversibly denatured collagen could be detected which was present after equilibrating the heat-denatured fibers at room temperature prior to pronase treatment (Hörmann and Schlebusch, 1968a).

On the other hand, there was evidence that, immediately after arresting the denaturation procedure, considerable re-formation of triple-helix structures took place. A complete description of the denaturation process thus requires the determination of reversibly denatured collagen as well. For this purpose, the enzymatic method seemed applicable only if one could prevent the re-formation of pronase-resistant triple-helix structures, at least during the dissolution process with pronase.

In this study, the problem outlined was overcome by lowering the temperature during pronase treatment to +4°. As a result, a method was developed for the determination of total denatured collagen. In addition the process of denaturation as well as that of renaturation immediately following the denaturation was recorded by measurements.

Materials and Methods

Tail tendons of 12–18-month-old rats with a 75–80% content of collagen insoluble during denaturation were used. The tendons, about 0.12 mm thick, were prepared as described previously (Hörmann and Schlebusch, 1968a). For denatura-

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