QUANTITATIVE DETERMINATION OF GLYCEROL IN PHOSPHOLIPIDS BY GAS-LIQUID CHROMATOGRAPHY

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Since glycerol enters into the composition of the majority of natural neutral lipids and phospholipids, rapid and accurate methods for its quantitative determination are of great importance. An investigation of any glycerolipid necessarily comprises an estimate of the amount of bound glycerol and a determination of the ratio of the structural units of the lipid molecule: glycerol, fatty acids, phosphorus, and organic bases. The relevant results permit an evaluation of the homogeneity of the lipid fraction and also make it possible to draw some preliminary conclusions concerning the structure of the lipids.

The main difficulties in the quantitative determination of lipid glycerol arise principally in the preparation of the sample for analysis. High losses of glycerol are possible in the hydrolysis of lipids, and also in the extraction and evaporation of the hydrolysates, etc. In the acid or alkaline deacylation of neutral lipids, the glycerol is split out quantitatively, and therefore the accuracy of its subsequent determination depends on the completeness of the isolation of the glycerol from the hydrolysates and the accuracy of the method of analysis used.

It is considerably more difficult to obtain a quantitative yield of glycerol in the hydrolysis of phosphatides. The hydrolysis of the initial lipids takes place to completion only in the presence of concentrated mineral acids, which cause a considerable destruction of the glycerol liberated [1, 2]. In view of this, Holla et al. [3] proposed to perform the cleavage of phospholipids in two stages: dephosphorylation by heating with a mixture of acetic acid and acetic anhydride [4], and alkaline methanolysis of the resulting di- and monoglyceride acetates. The reaction products were acetylated and analyzed by gas-liquid chromatography (GLC) for their triacetin content. However, under these conditions a quantitative yield of glycerol could be obtained only for the cephalin fractions of the lipids of egg yolk. The lecithin fractions underwent only 30-50% dephosphorylation [3].

Later, the same authors [5] proposed to perform acetolysis under more severe conditions – by heating with a mixture of trifluoroacetic acid and acetic anhydride. In the presence of trifluoroacetic acid, the acetolysis of egg-yolk cephalin and lecithin took place quantitatively. After the performance of the appropriate operations, the yield of glycerol in both cases was 96-101% [5]. These authors give no information on the determination of the amount of glycerol in phospholipids isolated from other sources. The quantitative dephosphorylation of glycerophosphatides by acetolysis in the presence of trifluoroacetic acid leads to a considerable destruction of the unsaturated fatty acids, which must be determined on an aliquot part of the lipid sample. The presence of foreign peaks on the chromatogram in the determination of glycerol triacetate may lead to errors in quantitative calculations. The defects of the method given by Holla et al. [5] include the contamination of the metering device and the column of the chromatograph with phosphoric acid, phosphates and acetates of bases, and also products of the decomposition of the fatty acid. Furthermore, this method is very laborious: one determination occupies more than 20 h.

From the brief review considered it can be seen that the methods of determining the glycerol present in glycerophosphatides require further improvements. The present paper describes a new method of

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determining phospholipid glycerol free from the majority of deficiencies of the method of Holla et al. [3, 5].

The main stages of the new method of determining glycerol in phospholipids are shown in the scheme.

The total phospholipids or the phospholipid fractions are deacylated by brief heating with an aqueous methanolic solution of potassium hydroxide [14]. The reaction products are separated by partition in the chloroform–methanol–water (8:4:3) system. The fatty acids, their methyl esters, and the unsaponifiable phospholipids pass into the chloroform layer, and the potassium salts of the deacylated phosphatides (glycerylphosphocholine, glycerylphosphoethanolamine, etc.) pass into the aqueous methanol. Control experiments have shown that the acetolysis of the potassium salts of the phosphates takes place only to the extent of 20%, and therefore the latter are converted into the free phosphates by passage through a small column containing cation-exchange resin. The eluate is evaporated and acetolysis is performed [12]. The presence of a large amount of acetic acid in the reaction mixture interferes with the direct gas-chromatographic determination of the triacetin formed. Consequently, the acetolysis products are distributed between chloroform and aqueous methanol. At this stage, the triacetin passes into the chloroform, while the bulk of the acetic acid, the phosphoric acid, and the phosphates and acetates of bases and of inositol remain in the aqueous methanolic phase. The amount of glycerol triacetate in the chloroform is determined by GLC. According to the results of control experiments, the losses of triacetin in the distribution of the products of the acetolysis of phosphates are insignificant (see Table 1).

A check of the whole scheme on egg-yolk lecithin and cephalin showed that it is possible by this method to determine 96-97% of the glycerol entering into the composition of the glycerophosphatides mentioned (see Table 3).

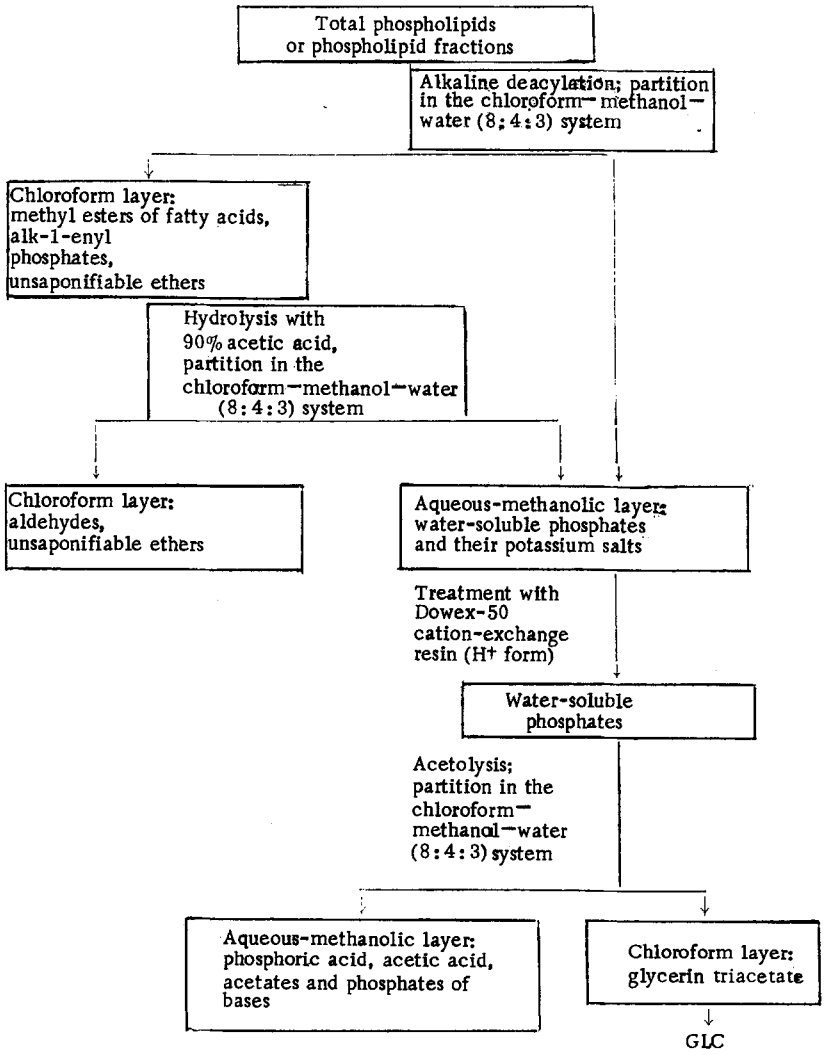

TABLE 1. Losses of Glycerol under Acetolysis Conditions

Experiment No.	Initial amount of glycerol, mg	Glyceryl triacetate found					
		in acetolysis products		in chloroform layer after partition of acetolysis products		in chloroform layer after neutralization	
		mg	%	mg	%	mg	%
1	5,00	11,78	100,0	11,58	98,5	11,10	94,5
2	5,02	11,70	99,5	11,40	97,0	11,20	94,8
3	5,10	11,95	99,5	11,65	97,0	11,40	95,0

TABLE 2. Acetolysis of Synthetic α-Glyceryl Phosphate

Experiment No.	Initial amount of glyceryl phosphate, mg	Amt. of triacetin found in chloroform layer after distribution of acetolysis products	
		mg	%
1	3,50	4,45	98,5
2	3,65	4,62	98,0
3	3,70	4,65	98,2

The method of analysis that we have proposed can be used to estimate the amount of bound glycerol of the plasmalogens entering into the composition of the phospholipid fractions. On mild alkaline deacylation of the phospholipids [14], the vinyl ethers of the acylalkenylphosphatides are not destroyed. They only split off a molecule of fatty acid, and on partition in the chloroform–methanol–water system they pass into the chloroform layer (see scheme). On mild acid hydrolysis [15] of the products present in the chloroform layer, with subsequent partition of the hydrolysate between chloroform and aqueous methanol, the water-soluble phosphates pass into the aqueous phase. An analysis of the aqueous methanolic layer according to the scheme shown permits the determination of the amount of glycerol in the acylalkenylphosphatides of the fractions under investigation. The use of a column of cation-exchange resin in this case is due to the fact that the alk-1-enyl phosphates liberated on acid hydrolysis are present partially in the form of salts the acetolysis of which does not take place quantitatively.

The possibility of the separate determination of glycerol in diacyl- and acylalkenylphosphatides was shown on the basis of an analysis of the lecithin and cephalin fractions of the cardiac muscle of large-horned cattle (see Table 3).

The method of analyzing phospholipids by the scheme given can be used successfully to determine the structure of natural polyglycerophosphatides. As an example, we have performed the analysis of the cardiolipin of the cardiac muscle of large-horned cattle.

The cardiolipin fraction isolated by column chromatography on silica gel was deacylated, and the reaction products were distributed between chloroform and aqueous methanol. The water-soluble phosphates were analyzed by the scheme given, and the amounts of phosphorus and glycerol in the acetolysis products were determined (see Table 3). The chloroform layer was treated with diazomethane to convert the free fatty acids into their methyl esters, and these were analyzed by the GLC method with methyl margarate as internal standard. A calculation of the chromatograms obtained showed that the mixture contained 11.2 mg (0.036 meq) of methyl linoleate, and also traces of methyl palmitate, stearate, oleate, and linolenate. The molar ratio of fatty acids to phosphorus to glycerol of 4 : 2 : 3 shows that the cardiolipin of the cardiac muscle is in fact a 1,3-diphosphatidyl-sn-glycerol.

The determination of the phospholipid glycerol by the scheme given has a number of advantages over that of Holla et al. [3, 5]. The method that we have described opens up the possibility of the subsequent determination of glycerol in the diacyl- and acylalkenylphosphatides: the analysis of the aqueous methanolic layer after alkaline deacylation gives an idea of the amount of glycerol in the diacyl phosphatides, and mild acid hydrolysis of the chloroform layer with subsequent analysis of the water-soluble phosphate permits the determination of the glycerol of the acylalkenylphosphatides of the fractions studied.

Working by the given scheme eliminates the contamination of the metering device and the column of the chromatograph with phosphoric acid and phosphates of bases, since the latter are separated in the partition of the acetolysis products between chloroform and aqueous methanol.

The cleavage of the deacylated phosphatides takes place quantitatively under comparatively mild conditions (heating at 150°C for 5 h with a mixture of acetic acid and acetic anhydride). The sequence of operations used (alkaline methanolysis and acetolysis of the deacylated phosphatides) ensures the possibility of the simultaneous determination of the fatty-acid composition of the phosphatides studied.

The determination of phospholipid glycerol by the method that we have developed occupies a comparatively short time.

TABLE 3. Determination of Glycerol Triacetate in the Products of the Acetolysis of Deacylated Glycerophosphatides

Experiment No.	Phosphate fractions	Amount of phosphorus			Amount of triacetin in products of acetolysis of			
		in initial phosphatides	in deacylated phosphatides		plasmalogens		esters	
			chloroform layer	aq. methanolic layer				
		μg			mg	%	mg	%†
1	Egg cephalin	500	—	495	—	—	3,40	97,0
2	Egg cephalin	500	—	490	—	—	3,36	96,0
3	Egg cephalin	500	—	505	—	—	3,40	97,0
1	Egg lecithin	540	—	530	—	—	3,70	98,2
2	Egg lecithin	675	—	662	—	—	4,45	94,0
3	Egg lecithin	510	—	495	—	—	3,30	95,0
1	Cardiac-muscle cephalin	1000	310	640	2,07	95,5	4,37	97,0
2	Cardiac-muscle cephalin	980	287	675	1,90	94,0	4,70	99,0
3	Cardiac-muscle cephalin	1150	350	780	2,41	98,0	5,49	100,0
1	Cardiac-muscle lecithin	1100	330	770	2,25	97,0	5,46	101,0
2	Cardiac-muscle lecithin	1200	400	750	2,73	97,5	5,12	97,0
3	Cardiac-muscle lecithin	2200	635	1500	4,40	99,0	10,60	100,0
1	Cardiac-muscle cardiolipin	585	—	580	—	—	5,40	98,0

*Calculated on the phosphorus found in the chloroform layer of the deacylation products.

†Calculated on the phosphorus found in aqueous methanolic layer of the deacylation products.

EXPERIMENTAL

The solvents were purified and made absolute by standard methods [6]. Before use, the acetic acid and the acetic anhydride were fractionated through a column with a glass packing. The glycerol used for the control experiments was redistilled in vacuum. α-Glyceryl phosphate was isolated from its potassium salt in a column containing the cation-exchange resin Amberlite IR-120 (H^+ form) and was neutralized with 10% potassium hydroxide solution, and stored at 0-5°C. The phosphorus content was determined by the method of Gerlach and Deuticke [7].

Isolation of Lecithin and Cephalin of Egg Yolk.* Chromatographically homogeneous egg lecithin was obtained by the homogenization of yolks with chloroform–methanol (2:1) and chromatography of the lipids on a column of alumina [8]. To isolate the cephalin, an aliquot part of the chloroform–methanol extracts of egg yolks containing 2.5-2.7 mg of lipid phosphorus was evaporated and chromatographed on a column of silica gel under the conditions described previously [11], a fraction being collected with R_f 0.45 [13×18-cm plate with KSK silica gel; chloroform–methanol–water (65:25:4) system]. On chromatography on paper impregnated with silica in the dibutyl ether–acetic acid–chloroform–water (80:70:12:10) system [9], the isolated cephalin fraction gave a single ninhydrin-positive spot with R_f 0.57, corresponding to phosphatidylethanolamine.

Isolation of the Cardiolipin, Cephalin, and Lecithin Fractions from the Total Phospholipids of the Heart of Large-Horned Cattle. The total lipids were extracted from the heart tissue by the method of Folch et al. [10] and were chromatographed on silica gel under the conditions mentioned above [11]. Cardiolipin, cephalin, and lecithin fractions were isolated. The homogeneity of the cardiolipin fraction was checked by thin-layer chromatography (TLC) on silica gel [one spot with R_f 0.64 in the chloroform–methanol–water (65:25:4) system]. The cephalin fraction was analyzed by chromatography on paper impregnated with silicic acid [9]. In the dibutyl ether–acetic acid–chloroform–water (80:70:12:10) system, this fraction gave an intense ninhydrin-positive spot with R_f 0.57 (phosphatidylethanolamine + phosphatidalethanolamine) and also weak spots of phosphatidylserine (R_f 0.50) and of lysophosphatidylethanolamine (R_f 0.27). The cardiac lecithin fraction was investigated by TLC on silica gel. In the chloroform–methanol–water (65:25:4) system, it showed one Dragendorff-positive spot with R_f 0.25.

*Here and below, the terms "cephalin" and "lecithin" are used to denote the phosphatidylethanolamine and phosphatidylcholine fractions, respectively.

Control Experiments. A. Determination of the Loss of Glycerol under the Conditions of Acetolysis. A solution of 5.0-5.1 mg of glycerol in 1 ml of a mixture of acetic acid and acetic anhydride (3: 2) was heated in a sealed tube at 150°C for 5 h [12]. The tube was cooled and opened, 10 mg of hexamethylene diacetate was added as a standard and the mixture was analyzed by the GLC method on a column (2000×3 mm) containing 10% of poly(ethylene succinate) on silanized Chromosorb W at 160°C. The instrument used was a "Khrom-2" chromatograph with a flame-ionization detector. The chromatograms were interpreted with allowance for the empirical correction factors found previously for the quantitative analysis of polyol acetates [13]. Then the acetolysis products were distributed in the chloroform–methanol–water (4: 2: 1.5 ml) system, the chloroform layer was separated off and treated with pentamethylene diacetate (9.0 mg) as a standard, evaporated at 20°C/20 mm, and again analyzed by GLC. Finally, the chloroform solution of the polyol acetates was neutralized with Amberlite XE-52 (OH^- form), a third standard – tetramethylene diacetate (9.0 mg) – was added, and the chromatogram of the mixture of acetates was recorded for the third time.

The results of all three determinations of glycerol in three parallel experiments are given in Table 1.

B. Acetolysis of the Potassium Salt of Synthetic α-Glyceryl Phosphate. In sealed glass tubes, 3.0-3.5 mg of the potassium salt of α-glyceryl phosphate was heated with 1 ml of a mixture of acetic acid and acetic anhydride (3: 2) at 150°C for 5 h [12]. After cooling, the tubes were opened and the contents were distributed in the chloroform–methanol–water (4: 2: 1.5 ml) system. The chloroform layer was standardized with hexamethylene diacetate and analyzed by GLC. The amount of glyceryl triacetate formed did not exceed 20% of the theoretical amount.

C. Acetolysis of Free α-Glyceryl Phosphate. A solution of 3.5-3.7 mg of the potassium salt of α-glyceryl phosphate was filtered through a column containing 2.5 g of the cation-exchange resin Dowex-50 (H^+ form), and the latter was then washed with water until the reaction was neutral. The combined eluate was evaporated. The further treatment was similar to that described above for the potassium salt of the glyceryl phosphate. The results of a quantitative determination of the triacetin are given in Table 2.

Determination of the Amount of Glycerol in Acylphosphatides. A. Deacylation of the Phospholipids. A solution of 12-17 mg of a phospholipid fraction in 0.1 ml of chloroform was treated with 1 ml of a 0.1 M solution of potassium hydroxide in 98% methanol [14]. The reaction mixture was kept at 40°C for 30 min, after which the excess of alkali was neutralized, and then the mixture was heated with 0.1 ml of freshly distilled ethyl formate (40°C, 5 min). After cooling, 4 ml of chloroform, 1.0 ml of methanol, and 1.2 ml of water were added to the hydrolysate. The mixture was shaken vigorously, and the chloroform and aqueous methanolic layers were separated and analyzed for their phosphorus contents [8].

B. Acetolysis of the Deacylated Phospholipids. The aqueous methanolic solutions were passed through a column of Dowex-50 cation-exchange resin (H^+ form), and the resin was washed with water to neutrality. The combined eluates were analyzed for their phosphorus content [7], evaporated at 40-45° C/20 mm, and dissolved in 0.5-0.7 ml of methanol. The methanolic solution was transferred to thick-walled glass tubes. The solvent was evaporated by passing a current of argon at 50-55°C through the tubes. The further treatment of the phosphate was similar to that described above. The results of the determination of glycerol are given in Table 3.

Determination of the Amount of Glycerol in Alkenylphosphatides. The chloroform layer obtained after the distribution of the products of alkaline methanolysis of the phospholipid fractions containing plasmalogens was evaporated and the residue was treated with 2 ml of 90% acetic acid at 40°C for 20 min [15]. The acetic acid was evaporated off and the hydrolysis products were distributed in the chloroform–methanol–water (8: 4: 3) system. The aqueous methanolic layer was passed through a column of Dowex-50 cation-exchange resin (H^+ form). The eluate was treated and analyzed just as has been given for the acylphosphatides. The results of the analysis are given in Table 3.

Analysis of the Cardiolipin Fraction of the Cardiac Muscle of Large-Horned Cattle. The cardiolipin fraction (14.7 mg) was subjected to alkaline methanolysis; the reaction products were distributed in the chloroform–methanol–water system. The amount of glycerol in the aqueous methanolic phase was determined as described above. The results are given in Table 3.

The chloroform layer was treated with an excess of an ethereal solution of diazomethane and the products were analyzed by the GLC method for methyl esters of fatty acids. Methyl margarate was added to the sample as internal standard. The amount of methyl esters of fatty acids was about 11.2 mg.

SUMMARY

1. A new method for the quantitative determination of glycerol entering into the composition of natural glycerophosphatides has been developed.

2. The method described has been used for the analysis of egg-yolk cephalin and lecithin and also the cephalin, lecithin, and cardiolipin of the cardiac muscle of large-horned cattle.

3. Using heart phospholipids as an example, the possibility has been shown of the separate determination of the glycerol in diacyl- and acylalkylidenephosphatides.

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