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THE USE OF THE ACETOLYSIS REACTION FOR THE INVESTIGATION OF NATURAL PHOSPHOLIPIDS

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The dephosphorylation of glycerophosphatides by heating with a mixture of acetic acids and acetic anhydride leads to acetates of the corresponding diglycerides [1-7]. The acetolysis reaction has been used repeatedly for determining glycerol in phospholipids [4, 5] and for studying their molecular composition on the basis of a structural analysis of the diglycerides formed [3-5, 8, 9]. Nutter and Privett [10] have questioned the suitability of this method for determining molecular types of phospholipids, since they found that acetolysis is accompanied by a redistribution of the acyl residues. At the same time, other authors [3, 8] have not observed acyl migration and have used acetolysis to determine the molecular types of the lecithins isolated from egg yolk.

The exhaustive identification of the products of the acetolysis of phospholipids has not hitherto been performed. Such an attempt is described in the present paper. The results obtained have shown that the acetolysis of glycerophosphatides isolated from various mammalian tissues followed by the methanolysis of the acetolysis products is accompanied by the formation of acylals of fatty aldehydes and of some dihydric alcohols, which complicates the interpretation of gas–liquid chromatograms (GLC) and distorts the results of the determination of glycerol.

Since literature information relating to the optimum temperature and time of acetolysis is self-contradictory (see [3-5, 7]), we first studied the quantitative side of this reaction. The total phospholipids isolated from various mammalian tissues was subjected to acetolysis at 150°C [3] for 1-10 h. The acetolysis products were evaporated, the residue was distributed in the chloroform–methanol–water (8 : 4 : 3) system [3], and the completeness of the reaction was judged from the amount of phosphorus passing into the aqueous methanolic layer. The results of the dephosphorylation of natural phospholipids with a mixture of acetic acid and acetic anhydride [1 ml of a mixture of acid and anhydride (2 : 3) to 25 mg of phospholipids] showed that the process is practically complete after 5 h.

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Source of phospholipids	Time of heating, hours	Percentage dephosphorylation (average of three determinations)
Rat liver	1,0	22
	2,5	80
	5,0	98
	10,0	98
Cattle brain	5,0	92
Cattle heart	5,0	91
Rat lungs	5,0	95
Rabbit blood serum	5,0	93

Consequently, the conditions of acetolysis proposed by Renkonen [3] do actually ensure the dephosphorylation of glycerophosphatides to the extent of more than 90%. The use of more severe conditions (boiling the phospholipids with a mixture of trifluoroacetic acid and acetic anhydride [5]) is unjustified.

Gas–liquid chromatography showed that in the majority of cases in addition to the acetates of diglycerides, at an elution temperature T_R of 280-290°C the acetolysis products contained substances with T_R 190-200°C, which is close to the T_R values of diacetates of monoglycerides (see Fig. 1a and b). However, these substances are not diacetates of monoglycerides, since they are separated from the latter on chromatography on a thin layer of silica gel (TLC), and on being heated with a methanolic solution of hydrogen chloride are converted into a mixture of aldehyde dimethyl acetals. Apparently, the substances with T_R 190-200°C are acylals of higher fatty aldehydes formed under the conditions of acetolysis through the cleavage of natural plasmalogens.

To check this hypothesis, we performed two control experiments. In the first of them, a mixture of stearaldehyde and palmitaldehyde was heated under the conditions of acetolysis. The TLC and GLC of the reaction products (see Fig. 1c) showed that the acylals obtained from the aldehydes, $CH_3(CH_2)_nCH(OCOCH_3)_2$

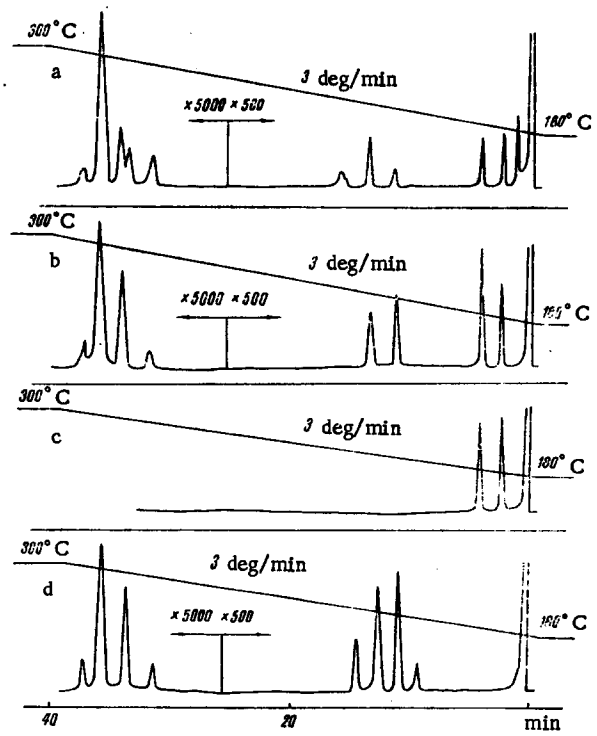

Fig. 1. Gas–liquid chromatograms of acetolysis products: a) of the total phospholipids of rat liver; b) of the phospholipids of bovine cardiac muscle; c) of a model mixture of palmitaldehyde and stearaldehyde; d) of the total phospholipids of bovine cardiac muscle previously freed from plasmalogens.

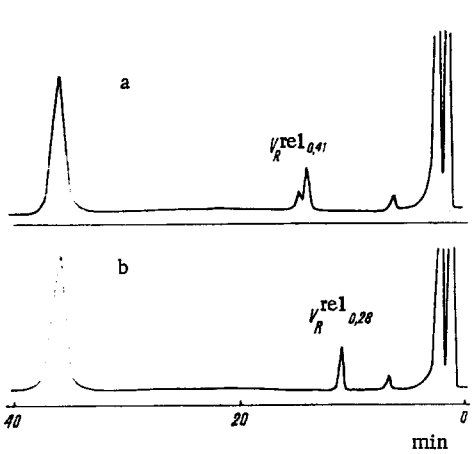

Fig. 2. Gas–liquid chromatograms of acetates of polyols obtained after the acetolysis and acid (a) or alkaline (b) methanolysis of the total phospholipids of rat liver.

(n = 14, 16), coincide in their R_f and T_R values with the substances detected in the products of the acetolysis of natural phosphatides. In the second experiment, the total phospholipids of cattle heart, which, after acetolysis, gave a particularly large amount of substances with T_R 190-200°C, was treated with a solution of mercuric chloride in dilute hydrochloric acid [11], rechromatographed on silica gel, and again subjected to acetolysis. The GLC of the acetolysis products (see Fig. 1d) showed that they contained only the acetates of mono- and diglycerides. Thus, not only control experiments but also the result of the acid ethanolysis of the substances with T_R 190–200°C confirmed the hypothesis that they consisted of acylals of fatty aldehydes.

The products of the acetolysis of the total phospholipids were subjected to acid or alkaline methanolysis, and the polyols liberated in the form of the corresponding acetates were analyzed by GLC. It was found that glycerol is not the only alcohol present in the hydrolyzates: In addition to glycerol the products of alkaline methanolysis contained 5–15% of an alcohol (Fig. 2b) the acetate of which had a relative retention volume V_R^{rel} of 0.28 (referred to triacetin). The products of the acid methanolysis of the same diglyceride acetates contained glycerol and another alcohol the acetate of which had V_R^{rel} 0.41 (see Fig. 2a). When this acetate was boiled with an ethanolic solution of potassium methoxide and the reaction products were then acetylated, an acetate with V_R^{rel} 0.28 was formed. Under the conditions of acid and alkaline methanolysis, both acetates (with V_R^{rel} of 0.41 and 0.28) gave a vicinal diol with an R_f value close to that of ethylene glycol.

To determine the nature of the substance with V_R^{rel} 0.41, the acetolysis products from the total phospholipids of cattle heart were subjected to acid methanolysis. The methanolysis products, which passed into the methanol layer on distribution in the hexane–methanol (1 : 1) system, were separated by chromatography on a column of silica gel. The fraction corresponding to the dihydric alcohols was acetylated and purified by preparative GLC. The acetate with V_R^{rel} 0.41 that was isolated contained halogen and had the same V_R^{rel} value as 1-chloropropane-2,3-diol diacetate. The NMR spectra of the substance with V_R^{rel} 0.41 and of synthetic 1-chloropropane-2,3-diol diacetate proved to be completely identical (Fig. 3).

The acetate with V_R^{rel} 0.28 was also isolated by preparative GLC and was investigated by NMR and mass spectrometry. The NMR spectrum had the signals of the protons of two acetyl groups (δ 2.02 and 2.05 ppm), the signals of the protons of methyl groups in an ether (3.34 ppm), and a doublet of the protons of a methylene group attached to a methoxy group (3.45 ppm). A group of peaks with its center at 4.2 ppm represented an AB multiplet of the protons of a CH_2 group connected with an acetyl group, and signals at 5.07 ppm corresponded to the proton of a CH group to which an acetyl group is attached (Fig. 4). On this basis, the acetate with V_R^{rel} 0.28 can be ascribed the structure of 1-methoxypropane-2,3-diol diacetate. The mass spectrum of synthetic 1-methoxypropane-2,3-diol diacetate and that of the natural sample coincided completely (Fig. 5).

Thus, the substance with V_R^{rel} 0.28 is 1-methoxypropane-2,3-diol diacetate, which can be formed in the alkaline methanolysis of 1-chloropropane-2,3-diol (V_R^{rel} 0.41) or its esters present in the products of the acetolysis of natural phosphatides.

The formation of derivatives of 1-chloropropane-2,3-diol when phospholipids are heated with a mixture of acetic acid and acetic anhydride shows that the reaction mixture contained inorganic chlorides. The latter were possibly "trapped" by the phospholipid micelles and were not separated during the subsequent washing [12] and chromatography of the extracts. It is also not excluded that the contact of natural phosphatides with chloroform causes the formation of hydrochlorides of aminophosphatides. To check this, an experiment was performed in which a solution of the total phospholipids of cattle heart in a mixture of ethanol and ether (3 : 2) was dialyzed for two days against an excess of water. In another experiment, an equimolar amount of ethanol-

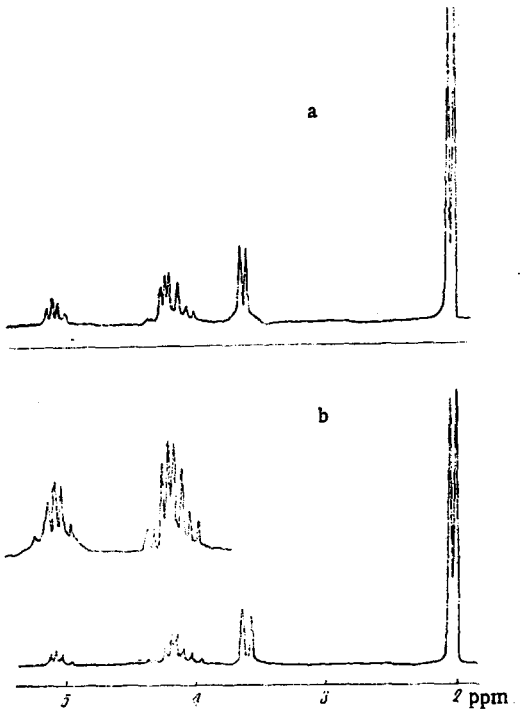

Fig. 3. NMR spectra of synthetic 1-chloropropane-2,3-diol diacetate and that formed as a by-product (b).

amine hydrochloride or choline hydrochloride was added to the same phospholipids. Both samples were heated with a mixture of acetic acid and acetic anhydride, and the reaction products were subjected to acid or alkaline methanolysis. The polyols isolated from the hydrolyzates were acetylated and analyzed by the GLC method. The chromatograms obtained show that after dialysis of the phospholipids, 1-chloropropane-2,3-diol and 1-methoxypropane-2,3-diol were absent from the hydrolyzate, while additions of ethanolamine hydrochloride to the initial phospholipids formed increased the amounts of these substances.

Thus, in the use of the acetolysis reaction for the study of molecular types of natural phospholipids or for determining the bound glycerol, one must take into account the possibility of a distortion of the results of the analysis through the formation of by-products. To prevent their formation, the phospholipids undergoing analysis must be freed from plasmalogens and from "trapped" chloride ions.

EXPERIMENTAL

Acetolysis of the Phospholipids. A solution of phospholipids containing about 1 mg of lipid phosphorus was placed in a thin-walled glass tube. The solvent was eliminated by blowing dry argon through the tube. The evaporated phospholipids were treated with 1 ml of a mixture of freshly distilled acetic anhydride and glacial acetic acid (2:3). Then the tube was sealed and it was heated at 150°C for 5 h. After cooling, the tube was opened and the contents were distributed in the chloroform–methanol–water (4:2:1.5) system. The chloroform layer was separated off and evaporated, and the phosphorus in the aqueous ethanolic layer was determined. The products that had passed into the chloroform layer were analyzed by the GLC method.

GLC of the Acetolysis Products. This was performed on a Pye-Unicam Series 104 Model 24 chromatograph using a 1000 × 4 mm column containing 3% of QF-1 polysiloxane. From the moment of injection of the sample, the temperature of the thermostat was raised from 180 to 300°C at the rate of 3 deg/min. The rate of flow of carrier gas was 60 ml/min. The GLC of the acylals of fatty aldehydes was performed under the same conditions.

GLC of the Dimethyl Acetyls of the Aldehydes. This was performed on a 1200 × 4 mm column containing 10% of Reoplex-400 at 140°C; the rate of flow of carrier gas was 50 ml/min, the instrument being a Pye argon chromatograph.

Mild Acid Hydrolysis of the Natural Phospholipids. About 25 ml of the total phospholipids were treated with a mixture consisting of 5 ml of methanol, 3.5 ml of water, and 1 ml of a 1% solution of

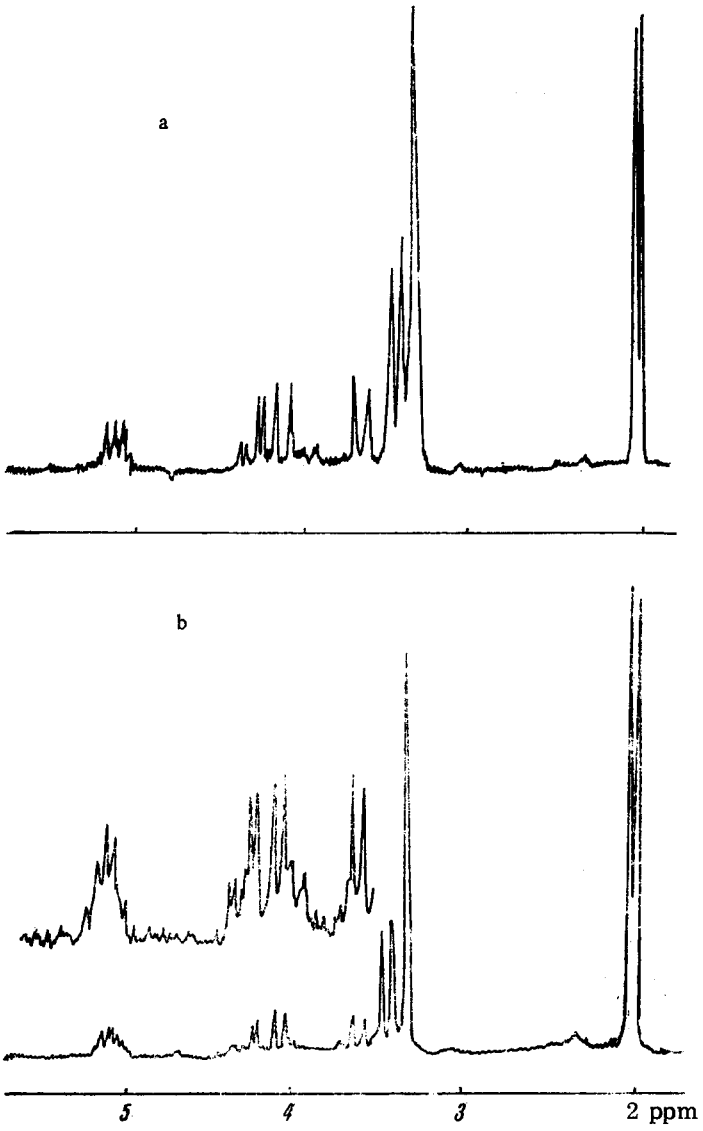

Fig. 4. NMR spectra of synthetic I-methoxypropane-2,3-diol (a) and that formed as a by-product (b).

mercuric chloride in 0.1 N hydrochloric acid. The reaction mixture was stirred for 2-3 min, and then 9 ml of chloroform was added. The layers were separated and the products that had passed into the chloroform layer were rechromatographed on a column of silica gel to eliminate aldehydes.

Investigation of the Polyol Composition of Natural Phospholipids. The total phospholipids extracted by method [12] (25 mg) was subjected to mild acid hydrolysis, rechromatographed on a column of silica gel, and kept under acetolysis conditions. The acetolysis products passing after separation into the chloroform layer were subjected to alkaline or acid methanolysis.

Alkaline Methanolysis. This was performed with a 2.5% solution of potassium methoxide in methanol (2 ml). The reaction mixture was boiled for 1 h and it was then neutralized with a 0.1 N solution of sulfuric acid in methanol. The potassium sulfate was separated off by centrifuging, the solvent was evaporated, and the residue was dissolved in 1 ml of a 1 : 1 mixture of ether and methanol. The polyols were separated from the methyl esters of fatty acids by TLC.

Acid Methanolysis. This was performed with a 2.5% solution of hydrogen chloride in abs. methanol (1 ml) at the boil for 1 h. The reaction mixture was evaporated, and the residue was dissolved in 1 ml of a 1 : 1 mixture of ether and methanol and was chromatographed on a preparative plate of silica gel. The polyols obtained by alkaline and acid methanolysis of the dephosphorylated phosphatides were acetylated [13] and investigated by GLC.

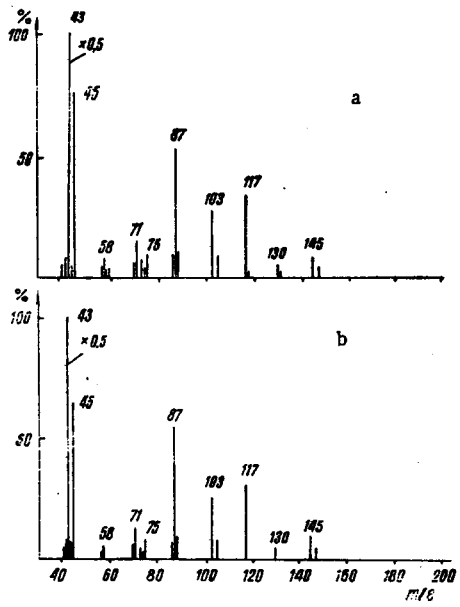

Fig. 5. Mass spectra of synthetic 1-methoxypropane-2,3-diol diacetate (a) and of 1-methoxypropane-2,3-diol diacetate formed as a by-product (b).

Isolation of 1-Chloropropane-2,3-diol Diacetate from the Products of the Acetolysis and Acid Methanolysis of the Total Phospholipids of Cattle Heart. About 1.5 g of the total lipids extracted by method [12] from cardiac muscle tissue was separated into neutral lipids and phospholipids on a 400 × 40 mm column containing 200 g of KSK silica gel (100-150 mesh). The total neutral lipids was eluted with chloroform (250 ml) and the total phospholipids successively with 150 ml of chloroform–methanol (5 : 95), 150 ml of methanol, and 150 ml of a 4% solution of methanol in water. The last three eluates were combined and evaporated and the residue was dissolved in 10 ml of a mixture of acetic acid and acetic anhydride (3 : 2) and kept under acetolysis conditions. After cooling, the tube was opened and the contents was evaporated at 40°C/20 mm, the residue being distributed in chloroform–methanol–water (16 : 8 : 6). The chloroform layer was evaporated in vacuum and the residue was subjected to acid methanolysis in the presence of 10 ml of a 2.5% solution of hydrogen chloride in absolute methanol. The methanolysis products were evaporated and the methanol was distributed in the hexane–methanol (2 : 1) system. The polyols that had passed into the methanol layer were isolated by chromatography on a 100 × 20 column containing 12 g of KSK silica gel (100-150 mesh) in the chloroform–methanol (8 : 1) system, the completeness of separation being checked by the TLC method. Fractions containing a substance with R_f 0.53 were combined and evaporated, and the residue was acetylated [12]. This gave about 20 mg of 1-chloropropane-2,3-diol diacetate containing 15-20% of triacetin as impurity. Pure 1-chloropropane-2,3-diol diacetate was isolated from this mixture by preparative GLC.

Isolation of 1-Methoxypropane-2,3-diol Diacetate from the Products of the Acetolysis and Alkaline Methanolysis of the Total Phospholipids of Cattle Heart. About 750 mg of the total phospholipids isolated by extraction [12] and column chromatography on silica gel was kept under acetolysis conditions. The acetolysis products were subjected to alkaline methanolysis by being heated with 10 ml of a 0.4 N solution of potassium methoxide in methanol. The reaction mixture was neutralized with a 0.4 N solution of sulfuric acid in methanol and it was distributed in the hexane–methanol (2 : 1) system. The methanol layer was evaporated and the residue was chromatographed on a layer of silica gel under the conditions described above, a fraction with R_f 0.53 being isolated, and this was subjected to acetylation and then to preparative GLC. This gave about 10 mg of 1-methoxypropane-2,3-diol diacetate.

Preparative GLC of 1-Methoxypropane-2,3-diol and 1-Chloropropane-2,3-diol Diacetates. The acetates were isolated preparatively on a Pye "Panchromatograph" using a 2700 × 10 column filled with 10% of PEGA on Chromosorb W (60-80 mesh). The temperature of the thermostat was 160°C and the rate of flow of the carrier gas 50-70 ml/min. The separated components were trapped in U-shaped glass traps at 0°C. Samples were selected visually with the aid of a Hamilton preparative collector.

Mass Spectrometry of 1-Methoxypropane-2,3-diol Diacetate. The mass spectrum was recorded on a standard MKh-1303 instrument with a system for the introduction of the samples close to the ionization chamber. The energy of the ionizing electrons was 30 eV, and the temperature 40-55°C.

Dialysis of the Total Phospholipids. The total phospholipids extracted by the method of Folch et al. [12] (25-30 mg) was dissolved in 10 ml of ethanol ether (2 : 1) and dialyzed in a cellophane film

against an excess of water at 20°C for 24 h. After the end of dialysis, the solvent was evaporated off and the residue was dissolved in 5 ml of benzene.

Acetolysis of the Total Heart Phospholipids with the Addition of Choline Hydrochloride. A mixture of 25 mg of the total heart phospholipids and 3 mg of choline hydrochloride was kept under acetolysis conditions. The products were subjected to acid methanolysis. The polyols were isolated by comparative TLC, acetylated, and analyzed by the GLC method.

SUMMARY

1. When the acetolysis reaction is used to study the molecular types of natural phospholipids or to determine the bound glycerol, the possibility of a distortion of the results of analysis through the formation of by-products must be taken into account.

2. In order to prevent the formation of aldehyde acylals, the phospholipids undergoing analysis must first be freed from plasmalogens.

3. To prevent the formation of derivatives of propane-1,2-diol in the hydrolyzates of the acetolysis products of the phospholipids, the initial lipids must be freed from chloride ions.

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