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Breakdown of phospholipids in mild alkaline hydrolysis

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SUMMARY

Deacylation of phospholipids by mild alkaline hydrolysis is usually accompanied by some hydrolysis of phosphate ester bonds. This undesired side reaction is suppressed by polar solvents. Deacylation with lithium hydroxide in chloroform–methanol 2:8 will cause the breakdown of not more than 0.1% of egg lecithin.

Mild alkaline hydrolysis of phospholipids, a method introduced by Dawson (1), is based on the following principle. The carboxyl ester groups of a phospholipid are much more susceptible to nucleophilic attack by hydroxy or alcoholate ions than the phosphate group. Thus, by choosing appropriate concentrations of base, phospholipids can be deacylated (i.e., the fatty acid esters can be preferentially hydrolyzed), but the water-soluble phosphate diesters produced are left intact. However, such a clear-cut reaction has not been achieved in practice, there is always some attack on the phosphate group. In the best known deacylation procedure (2), there is a 6% breakdown of glyceryl phosphorylcholine and perhaps also some breakdown of glyceryl phosphorylinositol (2, 3). Seemingly milder methods (4, 5), employing lower temperature and shorter reaction time than Dawson's, have been reported to yield "unidentified compounds" (4, 5, 6), and it will be shown in the following that the use of chloroform as a solvent in these "mild hydrolysis" procedures is responsible for the formation of several breakdown products from lecithin.

Some of the factors governing the breakdown of phosphate esters, as it accompanies and follows the deacylation of phospholipids, have been investigated in this study, with the aim of establishing optimal conditions for deacylation with minimal breakdown of the phosphate esters.

MATERIALS AND METHODS

Lecithin. Egg yolk phospholipid was freed of acidic phospholipids by passage through basic aluminum oxide (Woelm, activity grade 1)[1] in chloroform–methanol 1:1 (7). The product, lecithin with a few per cent of sphingomyelin and lysolecithin, was completely free of phosphatidyl ethanolamine.

Lysolecithin was prepared according to Hanahan (8).

Phosphatidyl inositol was prepared from beef liver according to Hanahan *et al.* (9, 10).

Beef Brain Phosphoinositide. Crude beef brain inositide (11) was freed of water-soluble phosphates by distribution in chloroform–methanol–water (12).

Beef Liver Lipid, Cod Flesh Lipid, Cod Brain Lipid. These were extracted and purified according to Bligh and Dyer (12).

Beef liver cephalin, a mixture of phosphatidyl ethanolamine and phosphatidyl serine, was prepared according to Hanahan *et al.* (9).

Tetraethylammonium Hydroxide Solution. Tetraethylammonium bromide, 0.5 M in methanol, was passed through Dowex 1 [OH^-]. The solution was free of bromide.

Lithium Hydroxide Solution. The hydrate $LiOH \cdot H_2O$, was dissolved in methanol.

Triethylammonium-EDTA Solution. To 2.9 g EDTA (acid, technical) in 95 ml water, triethylamine was added with stirring until the acid was dissolved and the solution neutral.

Deacylation and Determination of Cyclic Ester Formation. All reactions were carried out in heavy wall, 12-ml Servall centrifuge tubes with tight-fitting polyethylene stoppers. The reaction volume was 1 ml. Reaction conditions are given in Results. Reactions

[1] Alupharm Chemicals, New Orleans, La.

were stopped by addition of 10 ml 0.01 N aqueous HCl. After centrifuging, the aqueous layer was passed through a column, 1 x 3 cm, of 2 g of Dowex 1 [Cl^-] ion exchange resin, 200–400 mesh. The column was then washed with 5 ml ethanol–water 1:1 and 20 ml water. The acidic breakdown products of glyceryl phosphorylcholine (i.e., cyclic glycerol phosphate, methyl glyceryl phosphates, and glyceryl phosphoric acids) were then eluted with 10 ml aqueous 0.2 M LiCl. Phosphate determinations (13) showed the amount of breakdown of glyceryl phosphorylcholine. The total acidic phosphate formed gave a measure of the cyclic phosphate ester formation during the time of reaction (see Discussion).

The progress of deacylation can be followed conveniently by thin-layer chromatography (14). After addition of the base, part of the reaction mixture is taken up in a pipette, from which drops are applied to the silicic acid plate at the desired time intervals.

Alkali-Labile Phosphorus. To determine the completeness of deacylation in the procedure finally adopted, 2 M methanolic NaOH was added to the residual chloroform layer, to give a concentration of 0.5 M NaOH. After 30 min, the mixture was extracted with water and the water-soluble phosphate was determined. Longer treatment with methanolic NaOH in no case yielded additional water-soluble phosphate. The yields of "alkali-labile phosphorus" given in Results are based on the total water-soluble phosphorus so obtained.

RESULTS

Effect of Base Concentration on Phosphate Ester Breakdown. The experiments represented in Fig. 1 show that the breakdown of lysolecithin increases rapidly with increasing concentration of sodium hydroxide. Lysolecithin (i.e., α-acyl glyceryl phosphorylcholine) was found to break down to a much larger extent than lecithin under identical conditions. A possible explanation is that lecithin is deacylated in the α-position first, so that not the common lysolecithin, but the isomer, β-acyl glyceryl phosphoryl choline, is the predominant intermediate. This compound presumably cannot form a cyclic phosphate ester (see Discussion). The common end product, glyceryl phosphorylcholine, appeared to be more stable than the α-acyl derivative in the solvent employed, methanol.

Effect of Different Bases. Lecithin, 30 μmoles, was dissolved in 1 ml chloroform–methanol 4:6 and incubated for 10 min at room temperature with various hydroxides and alcoholates. At 0.1 M concentrations of base, the following results were obtained: KOH

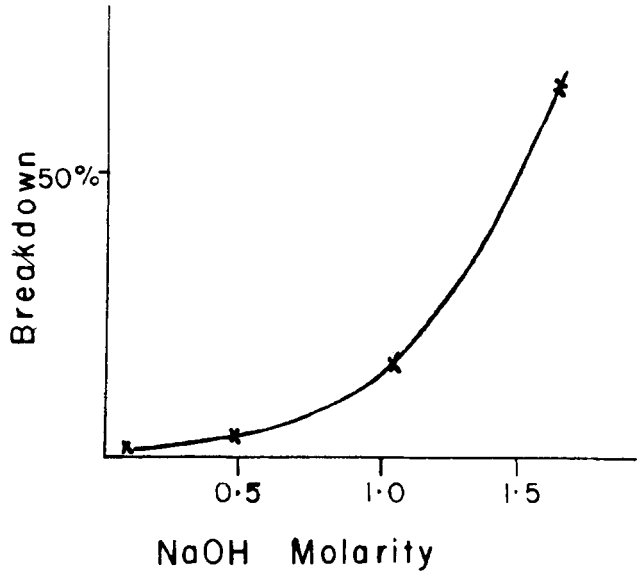


FIG. 1. Cyclic phosphate ester formation (breakdown) from lysolecithin with increasing hydroxyl ion concentration. Thirty μmoles lysolecithin in 1 ml methanol, reaction time 10 min, at room temperature.

caused 1.0% breakdown of phosphate ester; NaOH, 1.6%; LiOH, 0.6%; $NaOCH_3$ (15), 1.2%; $LiOCH_3$, 0.6%; and Et_4NOH, 0.1%. (In the last case, the incubation time was 30 min, and deacylation was incomplete.) Triethanolamine yielded only a trace of water-soluble phosphate after 1 hr incubation; triethylamine, none at all.

Time of Reaction. Prolonged action of base on the deacylated lipids caused in all cases additional breakdown of glyceryl phosphorylcholine. However, the conclusion was drawn from many experiments that slow deacylation at low concentration of base will cause less breakdown than short time incubation at a higher base concentration.

Influence of the Solvent. Fig. 2 demonstrates the effect of chloroform–methanol mixtures on the breakdown of lecithin and lysolecithin. It can be seen that the polar solvent methanol protects the glyceryl phosphorylcholine formed on deacylation from further degradation. When the deacylation of lysolecithin is carried out in various other solvent mixtures (Table 1), the results obtained suggest that the breakdown of phosphate ester is governed by the polarity of the solvent, among other factors. Dielectric constants are included in Table 1 as a measure of solvent polarity.

Deacylation Procedure. The combined experimental evidence shows that, for minimal breakdown of the desired water-soluble phosphates, the deacylation of phospholipids should be carried out at moderate base concentration in a highly polar solvent. Considering

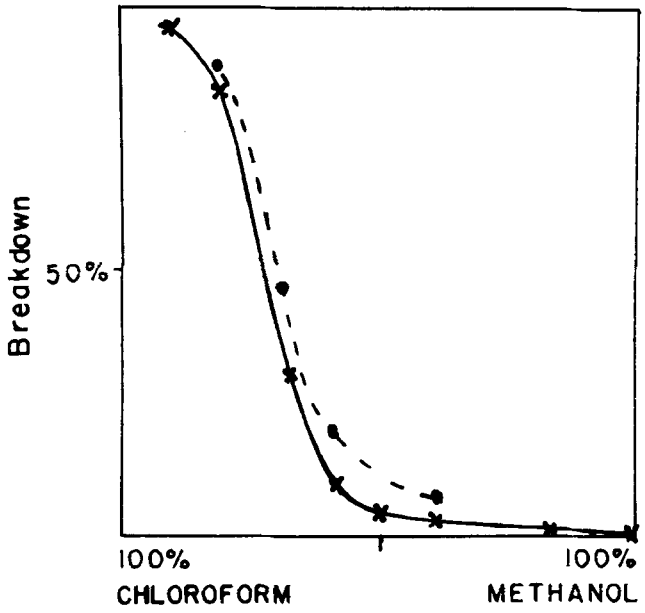


FIG. 2. Cyclic phosphate ester formation (breakdown) from lecithin (——) and lysolecithin (---) in chloroform–methanol mixtures. Thirty μmoles phospholipid in 1 ml solvent, 0.1 M NaOH, 10 min at room temperature.

these points, as well as the limits set by the solubility properties for lipids and the need for quantitative deacylation, the procedure described below was adopted. It gives almost quantitative results and less than 0.1% of breakdown products with most lipid preparations.

Methanolic 0.125 M $LiOH \cdot H_2O$, 0.8 ml, was added to 0.2 ml chloroform containing 20–30 μmoles of lipid phosphorus. After 15 min, 3 ml water, 2 ml ethanol, and 4 ml chloroform were added, in this sequence. The mixture was vigorously shaken, then separated by centrifugation. The upper layer was removed with a pipette and passed through a column, 1 x 3 cm, of ion exchange resin Dowex 50, pyridinium form, 50–100 mesh. Transfer was completed by rinsing the centrifuge tube twice with 1 ml each of water–methanol 1:1 from a pipette, without disturbing the lower layer. The washings were passed through the column, which was finally washed with 5 ml water–methanol 1:1. The collected eluate, containing the water-soluble phosphates, can immediately be applied to an ion exchange column for separation. For paper chromatography (2, 3), the eluate should be washed with ether and, after addition of a drop of concentrated ammonia, be concentrated at 40° *in vacuo*.

TABLE 1. INFLUENCE OF SOLVENTS ON CYCLIC PHOSPHATE ESTER FORMATION FROM LYSOLECITHIN*

Solvent		Breakdown
A	B	%
Methanol (33)	Water (81)	0.2
Methanol (33)	Methanol (33)	0.5
Pyridine (12)	Water (81)	2.0
Pyridine (12)	Methanol (33)	6.2
Chloroform (5)	Methanol (33)	19.3
Chloroform (5)	Ethanol (24)	84
Chloroform (5)	Butanol (18)	67
Octanol (10)	Chloroform (5)	63

* Thirty μmoles lysolecithin in a mixture of 0.6 ml of solvent A and 0.4 ml of B, at 0.1 M NaOH, were incubated for 10 min at room temperature. Dielectric constants are shown in parentheses.

The extraction that follows the deacylation will leave any intact phospholipid in the lower chloroform layer. Of α-acyl-glyceryl phosphorylcholine (lysolecithin), 4% was found in the aqueous phase in a control experiment.

The method was tested with the following lipids. Lecithin: yield of alkali-labile phosphorus, 98.5%; breakdown of glyceryl phosphorylcholine, <0.1%. Lysolecithin: yield, 99%; breakdown, 1.3%. Beef liver lipid: yield, 98.5%. Cod flesh lipid: yield, 98.5%.

If the residual chloroform layer is to be analyzed for plasmalogens and sphingolipids (2), it should first be subjected to further alkaline hydrolysis, as described under Methods.

As there are conflicting reports on the stability of phosphatidyl inositol (2, 3), this phosphatide was subjected to deacylation and the resulting water-soluble phosphates were subjected to paper chromatography according to Dawson (2). No cyclic glycerol phosphate or methyl glyceryl phosphate were detected.

Preparation of Acidic Phospholipids for Deacylation. With some lipid mixtures, especially those from brain, a turbidity will develop shortly after the methanolic LiOH solution has been added. This is caused by methanol-insoluble calcium, magnesium, and alkali salts of the acidic phospholipids. In these cases, only 80% or 90% of the alkali-labile lipid phosphorus will be liberated. The difficulty is resolved by converting the phospholipids into the triethylammonium salts as follows.

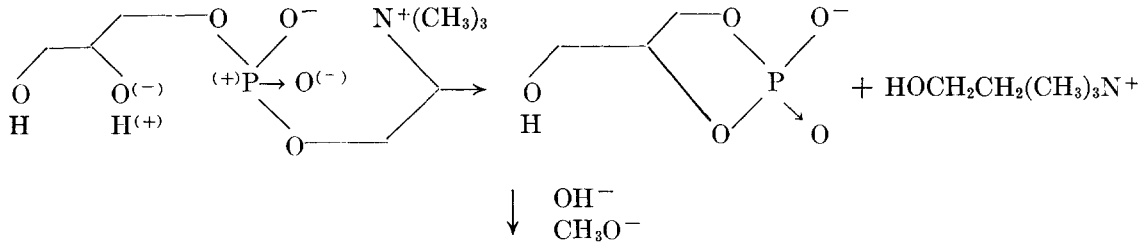
In the centrifuge tube, 3 ml of 0.1 M aqueous $CaCl_2$ and 1 ml methanol are shaken with the solution of the lipid (20–30 μmoles of P) in 2 ml chloroform. After centrifugation and removal of the upper phase, the process is repeated with 3 ml 0.1 M triethylammonium-EDTA solution and 1 ml methanol. The chloroform layer, containing all of the lipid now in methanol-soluble form, is evaporated in a stream of nitrogen, and the lipid is redissolved in 0.2 ml chloroform. Deacylation is now carried out as described. The yield of alkali-labile P for beef liver cephalin is 98.5%;

for cod brain lipid, 99%.

Deacylation of Beef Brain Inositide. This phospholipid mixture was treated with triethylammonium-EDTA solution as described. Washing with $CaCl_2$ was omitted, as these lipids are usually isolated as Ca-Mg salts. Since lithium hydroxide precipitates the triphosphinositide, it was replaced with tetraethylammonium hydroxide to a final 0.15 M concentration in chloroform–methanol 2:8. After 30 min at room temperature, the water-soluble phosphates were extracted with ethanol–water, and passed through pyridinium resin as described above. Of the "alkali-labile phosphorus," 96% was recovered.

DISCUSSION

The breakdown of glyceryl phosphorylcholine in alkaline hydrolysis proceeds through a cyclic phosphate (3):



glyceryl phosphates, methyl glyceryl phosphates

The initial step is attack on the phosphorus by the nucleophilic oxygen of the secondary hydroxyl group. The finding (3) that lecithin breaks down much more easily on deacylation than the acidic phospholipids might be best explained as due to the influence of the quaternary ammonium group. This strongly positive group can balance in part the negative charges of the phosphate oxygens and thus facilitate the approach of the nucleophilic agent toward the phosphorus. However, additional factors are perhaps involved.

Cyclic ester formation can be expected to be a function of the strength of the nucleophilic agent, among other factors. To be precise, breakdown will depend on the degree of dissociation of the secondary hydroxy group. An increase in hydroxyl, or alcoholate, ion concentration, which will cause an increasing number of secondary hydroxyl groups to dissociate ($>$C—OH + $OH^- \rightleftarrows$ $>$C—O^- + HOH), will therefore lead to an increase in breakdown (Fig. 1). The same qualitative considerations explain also the effects of different bases on phosphate ester breakdown (see Results). Tetraethylammonium hydroxide is a slightly weaker base than the alkali metal hydroxides (i.e., it furnishes less hydroxyl, and alcoholate, ions at equal molar concentration), and triethanolamine (pB 10.5) produces too little alcoholate ion in methanol even to attack the carboxyl ester groups of lecithin.

As an explanation of the effect of solvents on phosphate ester breakdown, the following hypothesis is offered. Cyclic ester formation will be a function of the attractive force between the secondary alcoholate ion and the electrophilic phosphorus. These ions can be shielded from each other by molecules (or one molecule) of a polar solvent. Displacement with a nonpolar solvent thus promotes cyclic ester formation. Additional factors are undoubtedly involved. The steep slopes in Fig. 2 and the sharp increase in breakdown on replacing methanol with ethanol suggest sudden changes in the solvation and conformation of the phosphate ester molecule.

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