Hydrolysis of Lecithin with Sodium Methoxide*

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Received August 1, 1961

The hydrolysis of egg lecithin with methanolic sodium methoxide has been studied at 0° and at room temperature. Both fatty acids are hydrolyzed at room temperature within 1 minute to yield glycerylphosphorylcholine and fatty acid methyl esters. However, at 0° the fatty acids are released slowly enough so that the intermediate lysolecithin can be isolated. The time course of hydrolysis of lecithin was therefore studied at 0°. The maximum yield of lysolecithin is attained after 4 minutes and represents a 35% yield. The time course of fatty acid release was also studied by gas chromatography. These analyses showed that both the α- and β-linked fatty acids are removed to form α- and β-lysolecithins, but there is a small preference to hydrolyze the α-linked fatty acid. The methoxide hydrolysis at 0° therefore allows for the ready chemical formation of lysolecithins and is useful for the identification of naturally occurring phosphatides.

The preparation of lysolecithin by controlled chemical hydrolysis of lecithin with alkali has not been achieved; nor has the stepwise hydrolysis of lecithin been investigated. Dawson (1954, 1960) has studied the mild alkaline hydrolysis of lecithin (and other phosphatides) at 37°, under which conditions the products are glycerylphosphorylcholine and fatty acid methyl esters. The experimental conditions used by Dawson do not permit the isolation of the intermediate lysolecithin. Hubscher *et al.* (1960) have observed that in methanolic NaOH the ester groups of the phosphatides undergo methanolysis to yield the methyl esters rather than sodium salts of these acids. These workers, however, did not report the production of lysophosphatides by their method. Their experimental conditions would not permit the isolation of these lysophosphatides, since the temperature of the reaction was too high.

In this study the hydrolysis of lecithin with methanolic sodium methoxide will be reported. This reaction occurs very rapidly at room temperature, so that within a few minutes both of the fatty acids of lecithin are released as their methyl ester derivatives. Glycerylphosphorylcholine is also formed. However, when the reaction was run at 0° the intermediate lysolecithin was isolated. By use of column and paper chromatography the rate of hydrolysis of lecithin, the rate of formation of the fatty acid esters, the rate of formation of lysolecithin, the rate of formation of glycerylphosphorylcholine, and the subsequent rate of hydrolysis of lysolecithin were determined.

Results

The sequence of hydrolysis of purified egg lecithin at 0° with methanolic sodium methoxide is shown in Figure 1. This paper chromatogram clearly depicts the hydrolysis of lecithin and the formation and subsequent hydrolysis of lysolecithin. The paper chromatographic method proved to be a simple but very effective way to follow the reaction from a qualitative standpoint. In the system used (Fig. 1), glycerylphosphorylcholine remains at the origin and the fatty acid methyl esters move near the solvent front. Hence all the products and the reactant are completely resolved. A proposed mechanism for the hydrolysis of lecithin is given in Figure 2. The reaction is depicted as a nucleophilic attack (S_{N2}) of the methoxide anion on the partially positive carbonyl carbon of the ester functional group. This mechanism is supported by the fact that the fatty acids are released as methyl esters. The very rapid rate of the reaction also favors this ionic mechanism. Since we have found that free fatty acids are not esterfied in this system, the possibility that these free acids are first liberated and then esterfied is ruled out.

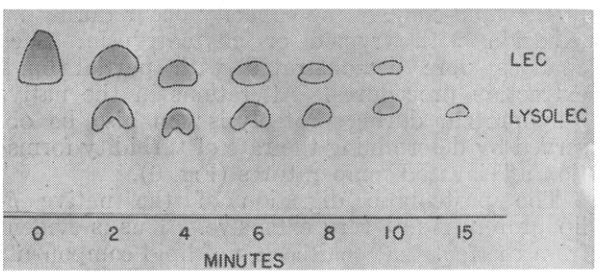

FIG. 1.—Paper chromatographic analysis of the hydrolysis of egg lecithin by sodium methoxide at 0°. Details of the procedure are given in the Experimental section, part B.

The rate curves for the reaction are shown in Figure 3. It can be seen that under these conditions the maximum yield of lysolecithin is attained after 4 minutes and represents about 35% of the original lecithin. One can therefore obtain this lysophosphatide in appreciable yield by this method.

To show the complete analysis of all the products, the rate curves for the formation of glycerylphosphorylcholine (free choline was not detected in the hydrolysate) and fatty acid methyl esters are given

* This work was supported in part by a grant H2063-7 from the U. S. Public Health Service, National Heart Institute, National Institutes of Health.

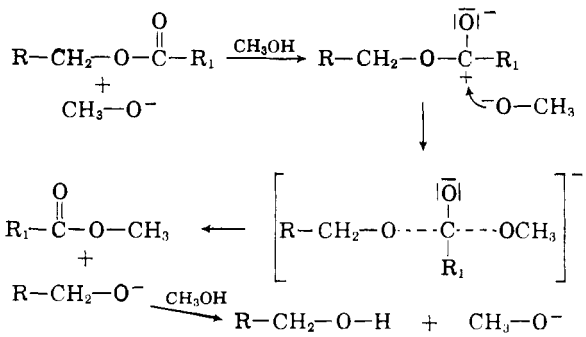

FIG. 2.—Proposed mechanism of hydrolysis of the ester group in lecithin by methoxide ion.

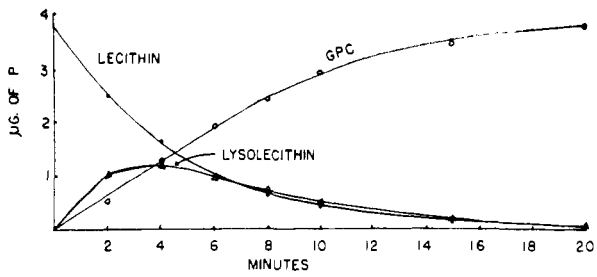

FIG. 3.—Rate curves showing the hydrolysis of egg lecithin by sodium methoxide at 0°. Details are given in the experimental section, part B. GPC = glycerylphosphorylcholine.

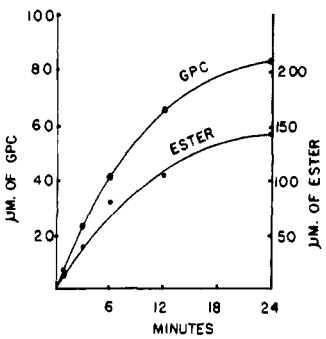

FIG. 4.—Rate curves showing the release of fatty acid methyl esters and glycerylphosphorylcholine (GPC) during the hydrolysis of lecithin by sodium methoxide at 0°. Details are given in the Experimental section, part B.

in Figure 4. The data in Figures 3 and 4 show that at 0° the reaction is essentially complete after 20-24 minutes.

The question arises whether there is a preferential hydrolysis of the fatty acid attached to the primary alcohol group (α position) or to the secondary alcohol group (β position) of glycerol or whether the α and β fatty acids are hydrolyzed at the same rate. This problem was solved in two ways. First, it is now known that snake venom phospholipase A cleaves off the β-linked fatty acid of lecithin to yield lysolecithins which contain predominantly saturated fatty acids which are esterfied to the α position of glycerol (de Haas *et al.*, 1960, 1961; Hanahan *et al.*, 1960; Marinetti *et al.*, 1960b; Tattrie, 1959). Naturally occurring lecithins therefore contain unsaturated fatty acids on the β position (saturated fatty acids also may be positioned here) and saturated fatty acids on the α position (very little unsaturated fatty acid is found on the α position). The lysolecithins formed by venom hydrolysis are thus predominantly saturated derivatives. However, the lysolecithins formed by sodium methoxide hydrolysis contain both saturated and unsaturated fatty acids. The lysolecithins formed by venom hydrolysis of egg lecithin do not react with permanganate, whereas the lysolecithins formed by methoxide hydrolysis react quite strongly with permanganate. This was observed by running spot tests on chromatograms containing these components. Unsaturated lysolecithins yield a brown spot, whereas saturated lysolecithins are not affected.

The second and more decisive method used to elucidate the above question was a careful analysis by gas chromatography of the fatty acids liberated after hydrolysis of lecithin with sodium methoxide at 0° for 3, 6, and 24 minutes. These data are given in Table I. It can be seen that both saturated and unsaturated fatty acids are released by methoxide hydrolysis but that there is a small preference for hydrolysis of the saturated fatty acids first (in particular palmitic acid, which is esterfied primarily to the α position). The percentage of palmitic acid is higher after 3 minutes of hydrolysis and slowly decreases with time, whereas the percentage of oleic and linoleic acids is lowest at 3 minutes and slowly increases with time. The small changes in myristic acid and stearic acid are not considered significant. The change in palmitoleic acid is just within experimental limitations of the analytical method. Hence the significance of this change is doubtful. The isomeric lecithins which are believed to occur in egg are shown in Figure 5. Isomers of the type I, II, VII, and VIII are probably the most abundant.

In a previous paper (Marinetti *et al.*, 1960a) we concluded that snake venom phospholipase A showed a preference for the hydrolysis of longer chain fatty acids, since these acids were released by enzyme hydrolysis, and that the lysolecithins which formed contained a high content of the shorter chain fatty acids, especially palmitic acid. However, in light of newer work it must be concluded that the longer chain acids are released because they are attached primarily to the β position of glycerol and that this is the position acted upon by the enzyme.

The controlled hydrolysis of lecithin by methoxide allows for the chemical production of mixed lysolecithins (α and β isomers). Since β to α ester migration of the fatty acids can be effected by silicic acid treatment (Borgstrom, 1954; Hirsch and Ahrens, 1958), the mixed lysolecithins can be converted to α-lysolecithins containing both saturated and unsaturated fatty acids. In contrast, the hydrolysis of lecithin by snake venom yields pre-

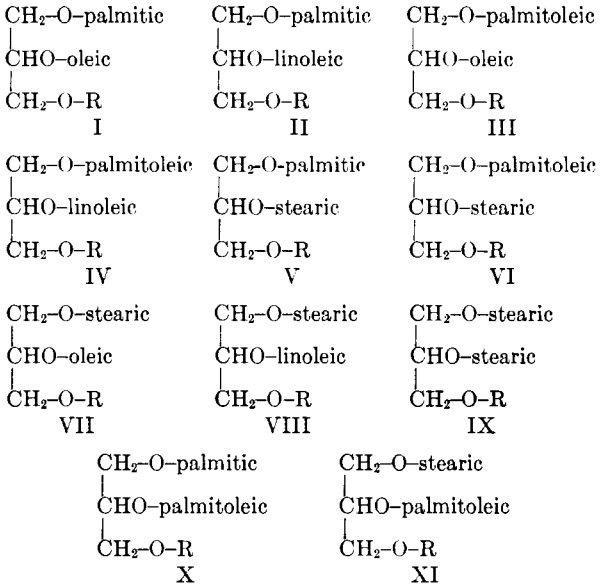

FIG. 5.—Various possible isomeric lecithins of egg yolk. The R group designates phosphorylcholine.

dominantly saturated α-lysolecithins, whereas the hydrolysis of choline-plasmalogens with acetic acid yields primarily β-lysolecithins which contain both saturated and unsaturated fatty acids. The present chemical method has the advantage of being simple and rapid and produces lysolecithins free from contaminating venom; this would be an important advantage if this lipid is to be used for biological testing on humans.

Sphingomyelin and cerebrosides are stable to methoxide hydrolysis at room temperature for at least several hours. Plasmalogens are converted to lysoplasmalogens. This method is therefore very useful for the identification of naturally occurring phosphatides and extends the hydrolytic procedure of Dawson (1954, 1960). Indeed, this present method is faster and has the advantage that the various hydrolysis products can be chromatographed directly from the reaction mixture, there by eliminating the need for further purification steps.

The controlled methoxide hydrolysis of other ester phosphatides and glycerides seems very feasible in light of the data reported in this paper. Thus this method should allow for the ready formation of other lysophosphatides such as lysophosphatidylethanolamine and lysophosphatidylserine. The chemical synthesis of these compounds has not yet been reported, although they may be produced in small yield as by-products in the synthesis of phosphatidylethanolamine (Baer and Buchnea, 1959; Baer *et al.*, 1952) and phosphatidylserine (Baer and Maurukas, 1955).

EXPERIMENTAL

A. Purification of Egg Lecithin.—Egg lecithin was purified by column chromatography on silicic acid as described previously (Marinetti *et al.*, 1959, 1960b). This phosphatide showed one component by paper chromatography in the systems of Marinetti *et al.* (1957). It had an ester/P ratio of 2.04 and a P content of 3.76 %. The lecithin was completely degraded by snake venom phospholipase A to yield lysolecithin and fatty acids. Hence by chemical, enzymatic, and chromatographic criteria this product was a highly purified sample of lecithin.

B. Hydrolysis of Lecithin with Methanolic Sodium Methoxide at 0°.

EXPERIMENT I.—Eight mg (9.6 μmoles) of lecithin was dissolved in 1 ml of chloroform and placed in an ice bath. To this solution was added 0.5 ml of ice-cold 0.5 N sodium methoxide in methanol. At 2, 4, 6, 8, 10, 15, and 20 minutes 20-μl aliquots (representing 3.82 μg of P) were immediately spotted on silicic acid–impregnated filter paper. The spots were rapidly dried by means of a special chromatographic applicator and drying unit. The components were separated by paper chromatography in 2-qt Mason jars in a solvent consisting of diisobutyl ketone–acetic acid–water, 40:20:3 (Marinetti *et al.*, 1957). The spots on the origin of the dried chromatogram (these origin spots contained glycerylphosphorylcholine) were cut off and eluted with distilled water and each was analyzed for P. The remaining part of the chromatogram was stained with rhodamine 6G in order to locate the lecithin, lysolecithin, and fatty acid esters. The lecithin and lysolecithin spots were eluted with 1.0 N methanolic–HCl. After evaporation of the methanol the amount of P in each spot was determined. The fatty acids were not analyzed in this experiment.

A duplicate chromatogram was also run and the lecithin and lysolecithin spots detected by the phosphomolybdic acid–stannous chloride test (Marinetti *et al.*, 1957). A photograph of this chromatogram is shown in Figure 1. The rate curves for the disappearance of lecithin, the formation of glycerylphosphorylcholine and the rate of appearance and subsequent hydrolysis of lysolecithin are shown in Figure 3.

EXPERIMENT II.—Eighty-one mg (96 μmoles) of lecithin were dissolved in 45 ml of chloroform–methanol, 1:1. The solution was placed on ice and then 5.0 ml of ice-cold 0.5 N methanolic sodium methoxide was added. At 1, 3, 6, 12, and 24 minutes 10.0 ml of the reaction mixture was rapidly removed and pipetted into a flask containing 1.0 ml of 1.0 N methanolic–HCl. This was done to stop the methoxide hydrolysis. The resulting solution was mixed and immediately poured into a separatory funnel containing water and ethyl ether. The ethyl ether phase was washed with water and evaporated to dryness. The residue representing fatty acid methyl esters and smaller amounts of lecithin and lysolecithin was dissolved in ethyl ether and passed through a column of silicic acid. The column was eluted with ethyl ether to yield fatty acid esters free from lecithin and lysolecithin. The fatty acid esters were ana-

lyzed by the hydroxamic acid–ferric chloride procedure (Rapport and Alonzo, 1955).

The aqueous phase representing the formed glycerylphosphorylcholine was analyzed for P. The rate curves showing the appearance of glycerylphosphorylcholine and fatty acid esters are shown in Figure 4.

The combined experiments I and II have thus given analytical data of the reactant and all the products of this system. In order to extend these analyses and to learn more about which ester group on the lecithin molecule was hydrolyzed, the individual fatty acid methyl esters were analyzed by gas chromatography.

C. Gas Chromatographic Analysis of the Fatty Acid Methyl Esters.—The fatty acid methyl esters liberated after 3, 6, and 24 minutes of hydrolysis in experiment II were analyzed by gas chromatography with a Perkin-Elmer Model 154D Vapor Fractometer. Thirty μl of an *n*-heptane solution of the fatty acid esters (3 to 5 mg of fatty acid esters) was injected into the machine by means of a Hamilton syringe. Fractionation was carried out by use of a Perkin-Elmer "P" column consisting of polyethyleneglycol succinate on chromosorb. The temperature of the run was 207°. The carrier gas was helium (at 30 lb pressure, flow rate of about 145 cc per minute). A thermistor detector was employed, and the areas of the peaks were measured by means of an automatic printing integrator. The data from these analyses are shown in Table I.

TABLE I

GAS CHROMATOGRAPHIC ANALYSIS OF THE FATTY ACID METHYL ESTERS RELEASED BY SODIUM METHOXIDE HYDROLYSIS OF EGG LECITHIN AT 0°

Hydrolysis Time (min.)	% of Total Fatty Acids[a]					
	Myristic	Palmitic	Palmitoleic	Stearic	Oleic	Linoleic
3	0.24	41.4	2.51	14.7	28.4	12.9
6	0.44	38.1	1.94	14.5	30.0	15.2
24[b]	0.12	35.1	1.71	14.5	32.1	16.1

[a] The details of the analytical method are given in the experimental section. A very small amount of longer chain fatty acids was also detected, but these emerged as a broad peak which was difficult to quantitate; hence these fatty acids are not included in the above data. [b] The reaction is complete after this time, and hence the analysis represents the total fatty acids of the egg lecithin.

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16α-Hydroxysteroids. XIII.* Carbonyl Reduction by *Streptomyces roseochromogenus*

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Received September 15, 1961

Streptomyces roseochromogenus fermentations of 9α-fluorohydrocortisone result in a small amount of C_{20}-carbonyl reduction, the 20β-dihydro derivatives of both 9α-fluorohydrocortisone and 16α-hydroxy-9α-fluorohydrocortisone being formed. Fermentations of 9α-fluorohydrocortisone-21-aldehyde result in rapid and complete reduction of the aldehyde to 9α-fluorohydrocortisone, which is then altered characteristically by the microorganism.

In our studies of the 16α-hydroxylation of 9α-fluorohydrocortisone (I) by *Streptomyces roseochromogenus* a variety of reducing (Smith *et al.*, 1960b; Smith *et al.*, 1961) and non-reducing (Smith *et al.*, 1962) steroids have been isolated and identified. Among the non-reducing steroids regularly detected in such fermentations is a polar steroid with paper chromatographic behavior indistinguishable from 20β-dihydro-9α-fluorohydrocortisone (II) and a still more polar component with the mobility of 16α-hydroxy-20β-dihydro-9α-fluorohydrocortisone (III).

* Paper XII of this series: Smith *et al.*, 1962.

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