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# HYDROLYSIS OF LECITHINS BY VENOM PHOSPHOLIPASE A

## I. STRUCTURE OF THE ENZYMICALLY FORMED LYSOLECITHINS

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SUMMARY

Rat-liver [$^{32}$P]lecithin was hydrolyzed by snake venom phospholipase A to give quantitative yield of [$^{32}$P]lysolecithins. The lysolecithins were oxidized in buffered aqueous solutions in the pH range 2.8 to 7.0, with bromine or permanganate to yield a keto-lysolecithin derivative. The extent and rate of oxidation is pH dependent. Bromine oxidation is more rapid at pH 6.8 than at pH 5.5 or 2.8. Permanganate oxidation is rapid at pH 5.5 but very slow at pH 6.8. During the oxidation partial concomitant hydrolysis occurs.

The keto-lysolecithin can be separated and measured by paper chromatography and thus permits the determination of the α-lysolecithin isomer. The amount of this isomer was found to vary between 54–94 % depending on the pH at which the bromine oxidation is carried out. Definitive evidence for the formation of the lysolecithinic acid has not yet been obtained.

Passage of lysolecithin through silicic acid causes ester migration from the beta to the alpha position of glycerol. This is reflected by an increase in the keto-lysolecithin upon bromine oxidation.

A novel reaction occurs when lysolecithin is treated with bromine in chloroform or methanol. Brominolysis rather than oxidation takes place with the formation of lysophosphatidic acids and choline.

A discussion of these findings is presented.

## INTRODUCTION

In a previous paper[1] it was shown that permanganate oxidation of enzymically produced lysolecithins yielded two products, one of which was a ketone derivative. Acid hydrolysis of the oxidized lysolecithins gave rise to at least four water soluble phosphate compounds. These data provided evidence that the lysolecithins were a

mixture of alpha and beta isomers and consequently that the venom phospholipase A hydrolyzes both the alpha and beta fatty acid ester groups on lecithin. Supporting evidence that the phospholipase A can hydrolyze the beta fatty acid ester linkage was obtained by studies on the hydrolysis of choline-plasmalogens[2]. The lyso-plasmalogens which were formed were reduced with hydrogen and hydrolyzed to yield α-glycerol ethers.

Since permanganate oxidation did result in appreciable hydrolysis of the lysolecithins even in buffered solutions a search was made for another oxidant. Bromine was found to be effective[3]. Studies with this oxidant will be reported in this paper. By use of [$^{32}$P]lysolecithin the rate of oxidation at various pH values was investigated. Both bromine and permanganate oxidation were found to be pH dependent. The studies to date have shown that bromine oxidizes the secondary hydroxyl group on the lysolecithin to yield a keto-derivative. Evidence for the oxidation of the primary hydroxyl group to yield a lysolecithinic acid has not yet been obtained. As is the case with permanganate, during bromine oxidation some hydrolysis of the lysolecithins occurs.

In contrast to the oxidation which occurs in buffered aqueous solutions of bromine, in non-aqueous solvents a novel type of brominolysis takes place. Thus lysolecithin undergoes brominolysis in either chloroform or methanol to yield choline and lysophosphatidic acids[3,4].

The fatty acids which were liberated by hydrolysis by the venom phospholipase were also investigated. As shown by others[5,6] the liberated fatty acids are predominantly unsaturated. However, we have observed that the venom enzyme shows chain length preference. Hence when two different fatty acids are present on the lecithin, the phospholipase removes the longer chain acid. The latter experiments will be reported in another paper[7].

## METHODS AND REAGENTS

### *Preparation of lecithins and lysolecithins*

The lecithins of egg and the [$^{32}$P]lysolecithin of rat liver were prepared as described previously[1]. The lecithins of pig heart were made by the procedure reported elsewhere[8]. The snake venoms (*Crotalus adamanteus* and *Naja naja*) were obtained from the Ross Allen Reptile Institute, Silver Springs, Florida. The hydrolysis of the lecithins by the venoms was carried out in ether solution in a manner described previously[1].

The lecithins of egg and pig heart and the fatty acids liberated by venom hydrolysis of rat-liver lecithin were reduced with hydrogen over platinum oxide catalyst in ethanol at 50 lbs. pressure at room temperature.

### *Bromine and permanganate oxidation of the lysolecithins in buffered aqueous solution*

The oxidation of the lysolecithins was carried out as given in the legends to the figures.

### *Chromatography of the water soluble phosphate esters and choline*

The water soluble phosphate compounds which were liberated during the oxidation of the lysolecithins or which were liberated by 1 *N* HCl hydrolysis of the

oxidized and parent lysolecithins were qualitatively analyzed by paper chromatography as described previously[1]. Choline wasqualitatively analyzed by chromatography on Whatman No. 1 filter paper in the same solvents used for the phosphate compounds and in a solvent consisting of *n*-butanol–ethylene glycol–water (4:1:1). The phosphate esters were detected by the HANES AND ISHERWOOD reagent[9] or by autoradiography. Choline was detected by the phosphomolybdate-stannous chloride test[10].

## RESULTS

The autoradiograms in Fig. 1 demonstrate the qualitative behavior of the [$^{32}$P]rat-liver lysolecithin in various aqueous solutions. In all systems A–F the lysolecithin which was used had been passed through a column of silicic acid. The control system A represents lysolecithin dissolved in distilled water. Essentially no hydrolysis occurs over a period of 2 days at room temperature. The same is true for lysolecithin dissolved in acetate buffer pH 5.5 or in phosphate buffer pH 6.8 and 7.4. The lysolecithin appears as a single spot and practically no radioactivity is seen at the origin (spot 0).

The lysolecithin dissolved in 0.05 *N* HCl, shown in system B, has undergone appreciable hydrolysis. The origin spot No. 0 in this case represents either glycerol phosphate or/and glycerylphosphorylcholine. The extent of hydrolysis is 23 and 34 %

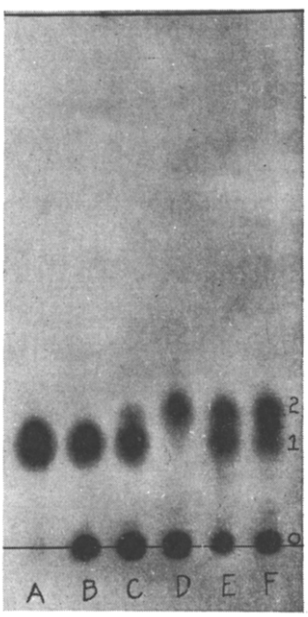


Fig. 1. Autoradiograms showing the behavior of [$^{32}$P]lysolecithin in various aqueous solutions with and without bromine. The lysolecithin was obtained by hydrolysis of [$^{32}$P]rat-liver lecithin with *Naja naja* venom. The lysolecithin in these experiments had been passed through silicic acid. The lysolecithin (4.2 mg) was dissolved in the following solutions: (A) 1.0 ml of distilled water (this served as the control); (B) 1.0 ml of 0.05 *N* HCl; (C) 1.0 ml of distilled water plus 4 $\mu$l of liquid bromine; (D) 1.0 ml of 0.2 *M* phosphate buffer pH 7.4 plus 4 $\mu$l of liquid bromine (the measured pH of this solution after addition of the bromine was 7.0); (E) 1.0 ml of 0.2 *M* acetate buffer pH 5.5 plus 4 $\mu$l of liquid bromine (the measured pH of this solution after addition of bromine was pH 5.1); and (F) 1.0 ml of 0.2 *M* phosphate buffer pH 6.8 plus 4 $\mu$l of liquid bromine (the measured pH of this solution after addition of bromine was 6.4). After 24 h 15 $\mu$l of each solution were pipetted on silicic acid impregnated paper and chromatography was carried out in a solvent consisting of diisobutyl ketone–acetic acid–water (40:25:5)[11]. The length of the chromatographic run was 26 h at 23°. The autoradiograms were done on No-Screen X-Ray film and required 5 days exposure. Spot 0 = water soluble phosphate esters which remain at the origin and form as a result of hydrolysis of the lysolecithins. Spot 1 = the parent lysolecithin and Spot 2 = keto-lysolecithin which forms as a result of bromine oxidation. Both spots 1 and 2 gave a positive test for choline[10,11] but only spot 2 gave a positive ketone test with dinitrophenylhydrazine[11]*.

* The test for ketone is carried out as follows. The chromatogram is washed in distilled water for 15 min and then dried. The dry chromatogram is passed several times through a solution of 2,4-dinitrophenylhydrazine in 3 *N* HCl[11]. The chromatogram is hung for 3 min and then immersed in distilled water to wash out the excess reagent. Three 10-min washes are generally sufficient. A positive test is indicated by a yellow or yellow orange spot which shows up as a dark absorbing spot under u.v. light (366 m$\mu$). This latter property is essential since some lipids may adsorb the dinitrophenylhydrazine and appeared yellow but do not absorb in the u.v. region.

after 24 and 48 h respectively. In system C is shown the behavior of lysolecithin in unbuffered bromine water (pH about 2.8). The extent of oxidation under these conditions is small (6–10 %) as evidenced by the small amount of keto-lysolecithin which forms (spot 2). The keto function is demonstrated by the dinitrophenylhydrazine test. However appreciable hydrolysis of the lysolecithin occurs due to the low pH. The extent of hydrolysis in system C is 29 and 38 % after 24 and 48 h respectively.

System D shows the bromine oxidation of lysolecithin in phosphate buffer pH 7.0*. It can be seen that the keto-lysolecithin, spot 2, is the major component, thus demonstrating that the oxidation of the lysolecithin was nearly complete and that the lysolecithin was predominantly the alpha-isomer. Hydrolysis also occurred during the oxidation since water soluble phosphate compounds appear at the origin. These latter compounds were not identified but at least three components were found to occur in this origin spot.

The bromine oxidation of the lysolecithin in acetate buffer pH 5.1* and in phosphate buffer pH 6.4* are depicted in systems E and F. In both cases the keto-lysolecithin is produced but the oxidation had not proceeded to completion. It is evident that in all cases where oxidation occurs in buffered solution hydrolysis also takes place, while in the buffer controls at about the same pH but not containing bromine no hydrolysis can be detected.

The chromatographic data in Fig. 2 show both the qualitative and quantitative aspects of the reaction of lysolecithin in buffered aqueous solutions containing bromine or permanganate. Also shown for sake of comparison is the behavior of lysolecithin in a non-aqueous solvent (chloroform) containing bromine (system G). The effect of silicic acid on the structure of the lysolecithin is demonstrated by the data. The NLL lysolecithin represents the normal lysolecithin which was not subjected to silicic acid treatment. The SLL lysolecithin represents the lysolecithin after passage through a column of silicic acid. The lysolecithin (25 mg) was dissolved in chloroform and put on a 10 g column of silicic acid and eluted with methanol. The total time that the lysolecithin was exposed to the silicic acid was 6 h.

It should be pointed out that in order to obtain quantitative data from the chromatograms, the origin spots must be cut off before the chromatograms are dipped in the Rhodamine 6G solution. Immersion of the chromatograms in the aqueous dye for 1–3 min does not effect an appreciable loss of material from the lysolecithin or the keto-lysolecithin spots but does effect an 80–90 % loss of material from the origin spots. The numbers in each spot in Fig. 2 represent the amount of radioactivity in these components. These activities are calculated as the per cent of total radioactivity applied to the paper in each strip. The extent of oxidation by bromine as measured by the appearance of the keto-lysolecithin (spot 2) was greater at pH 6.4* (systems C and D) than at pH 5.1* (systems A and B). The data show that after silicic acid treatment the amount of keto-lysolecithin is increased, in particular at pH 6.4 (and more so at pH 7.0* as seen in Fig. 1 system D). Therefore during chromatography on silicic acid beta to alpha acyl migration of the fatty acid occurs. A similar finding has been reported for monoglycerides[12,13].

* The pH of the buffers which were used was 5.5, 6.8 and 7.4. However the pH after addition of bromine to these buffers (and hence the pH at which the reaction occurred) was 5.1, 6.4 and 7.0 respectively. Permanganate had no measurable effect on the pH of the buffers.

In Table I are given data showing the amount of keto-lysolecithin and the lysolecithin which resists oxidation when egg lysolecithin is oxidized with bromine at pH 5.1 and 6.4. This experiment confirms the work on rat-liver lysolecithin since in both cases after treatment of the lysolecithins with silicic acid the amount of keto-lysolecithin increases. Therefore acyl migration of the fatty acid occurs with both egg and rat-liver lysophosphatides when chromatographed on silicic acid.

The oxidation of lysolecithin by permanganate at pH 5.5* is shown in system E.

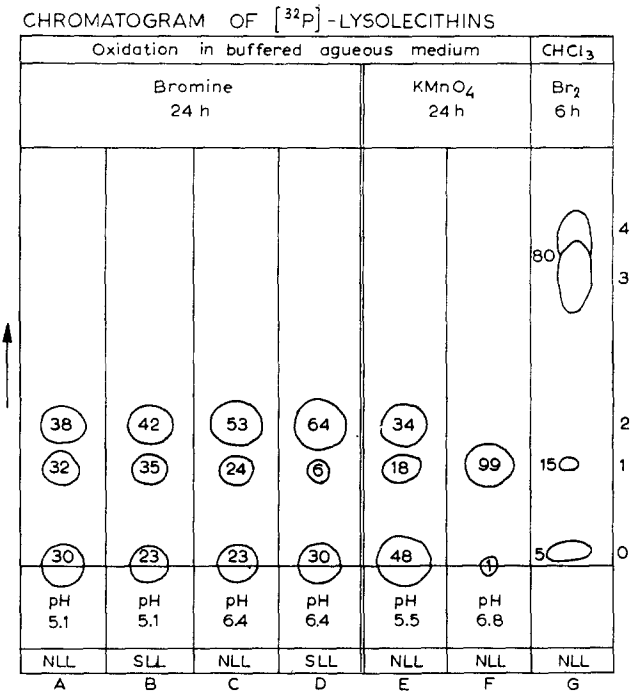


Fig. 2. Chromatograms showing the behavior of [32P]lysolecithin in various buffered solutions toward bromine and permanganate and in chloroform solution toward bromine. NLL = the normal lysolecithin which had not been exposed to silicic acid and SLL = the same lysolecithin which had been passed through a column of silicic acid as explained in the text. 2 mg of either the NLL or SLL lysolecithin were dissolved in the following solutions: (A) and (B) – 0.35 ml of water plus 0.35 ml of 0.2 *M* acetate buffer pH 5.5* plus 5 microliters of bromine; (C) and (D) 0.35 ml of distilled water plus 0.35 ml of 0.2 *M* phosphate buffer pH 6.8* plus 5 $\mu$l of bromine; (E) 0.35 ml of distilled water plus 0.35 ml of 0.2 *M* acetate buffer pH 5.5* plus 2 mg of solid $KMnO_4$; (F) 0.35 ml of distilled water plus 0.35 ml of 0.2 *M* phosphate buffer pH 6.8* plus 2 mg of $KMnO_4$; and (G) 0.5 ml of chloroform plus 3 $\mu$l of bromine. After 24 h at room temperature for systems (A)–(F) and after 6 h for system (G) 20-$\mu$l aliquots were taken for chromatographic analysis as described in Fig. 1. Spot 0 = water soluble phosphate esters, spot 1 = lysolecithin, spot 2 = keto-lysolecithin, and spot 3 plus 4 = lysophosphatidic acids. The origin spots were cut off and the radioactivity in each was determined. The remaining part of the chromatogram was stained with Rhodamine 6G[11] and the lipid spots observed and marked under u.v. light. Spots 1 and 2 stained yellow with Rhodamine 6G and gave a positive test for choline[10,11] whereas spots 3 and 4 stained blue with Rhodamine 6G and gave a negative test for choline. Only spot 2 gave a positive ketone test with 2,4-dinitrophenylhydrazine[11]**. After the chromatograms were dry the spots were cut off and the radioactivity in each was determined. The numbers in or by each spot refer to the per cent of total radioactivity in each component (*i.e.* the per cent of the total radioactivity in the 20-$\mu$l aliquot which was used for chromatography).

* See footnote on page 527.

** See footnote on page 526.

TABLE I

EFFECT OF SILICIC ACID ON THE STRUCTURE OF EGG LYSOLECITHIN

These analyses were performed on the bromine oxidation products of reduced lysolecithins obtained by venom hydrolysis of reduced egg lecithin. The bromine oxidation was carried out at pH 5.1 and 6.4 in the same manner as described in Fig. 2. Aliquots of the reaction mixture were taken for paper chromatographic analysis[11]. The unreacted lysolecithin spot (spot 1) and the keto-lysolecithin spot (spot 2) were combined from several chromatograms, eluted with methanolic-HCl and the lipid P was done on the eluates in a manner described previously[11]. The origin spots were not analyzed in this experiment. Hence the data are given as the fraction of the total lipid P which was found in the lysolecithin and keto-lysolecithin.

| *Sample* | *Percent of total lipid P* | | *pH* |
|---|---|---|---|
| | *Keto-lysolecithin* | *Lysolecithin** | |
| Egg lysolecithin | | | |
| Untreated | 72 | 28 | 5.1 |
| Passed through silicic acid | 80 | 20 | |
| Untreated | 84 | 16 | 6.4 |
| Passed through silicic acid | 91 | 9 | |

* The lysolecithin corresponds to spot 1 in Figs. 1 and 2. This lysolecithin resists oxidation.

We have reported details of this oxidation previously[1]. In the case of permanganate however, the extent of hydrolysis is much higher than when bromine is used at the same pH*. The unexpected observation was made that the permanganate oxidation of lysolecithin is completely abolished by raising the pH to 6.8 (system F). No keto-lysolecithin is formed after 24 h at room temperature. The buffer in this case contained phosphate rather than acetate.

In contrast to the oxidation of lysolecithin in aqueous solution, in non-aqueous solvents a novel reaction occurs. Thus in chloroform or methanol the lysolecithin undergoes brominolysis to yield choline and lysophosphatidic acids (spots 3 and 4 of system G). Details of this reaction will be reported elsewhere[4].

The use of [$^{32}$P]lysolecithin coupled with the quantitative paper chromatographic method permitted a study of the rate of bromine oxidation at various pH levels. In Fig. 3 are shown the results of an experiment at pH 5.1. The rate of disappearance of lysolecithin (curve 3), the rate of appearance of the keto-derivative (curve 2) and the rate of formation of water soluble phosphate compounds at the origin (curve 1) are given. The oxidation is complete after 24 h under these conditions. There is a slow increase in the production of water soluble phosphate compounds with a concomitant decrease in the amount of keto-lysolecithin after this latter compound has attained a maximum value.

In Fig. 4 are shown the curves for the rate of disappearance of lysolecithin at 3 different pH levels. The rate and hence the extent of oxidation of lysolecithin increases with increasing pH.

The rate curves for the concomitant production of the keto-lysolecithin are given in Fig. 5. It is again evident that the rate of oxidation is most rapid at pH 7.0 but that the keto-lysolecithin which is formed undergoes hydrolysis. An analysis of the water soluble phosphate compounds at the origin demonstrated a parallel increase

* In our previous work[1] the failure to observe appreciable radioactivity at the origin was due to this phenomenon.

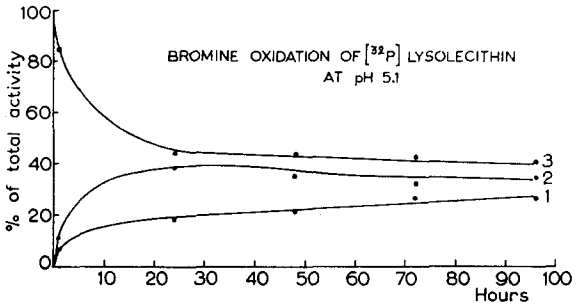


Fig. 3. Bromine oxidation of [$^{32}$P]lysolecithin in acetate buffer pH 5.1. The oxidation was carried out as given in system (E) in Fig. 1. At the time intervals indicated 15 $\mu$l aliquots were removed for paper chromatographic analysis. Curve 1 represents the water soluble phosphate compounds at the origin, curve 2 represents the keto-lysolecithin (spot 2) and curve 3 represents the lysolecithin (spot 1). The acetate buffer which was added was pH 5.5 but the actual pH of the reacting solution was pH 5.1.

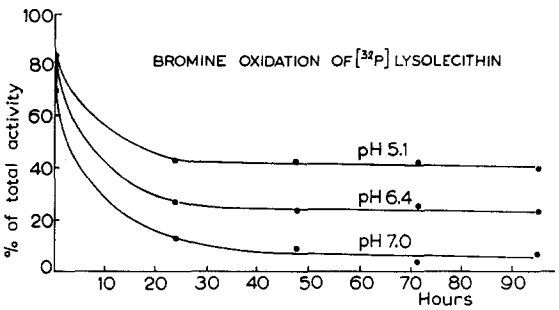


Fig. 4. Bromine oxidation of [$^{32}$P]lysolecithin at various pH. The oxidation was carried out as given in systems (D), (E) and (F) in Fig. 1. At the time intervals indicated 15-$\mu$l aliquots of the solution were removed for paper chromatographic analysis. The curves show the rate of disappearance of lysolecithin (spot 1). In Fig. 5 are given the curves for the rate of formation of the keto-lysolecithin (spot 2).

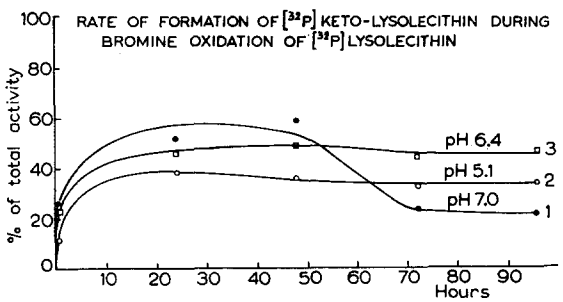


Fig. 5. Rate of formation of keto-lysolecithin during bromine oxidation of [$^{32}$P]lysolecithin a) various pH. The oxidation of lysolecithin was carried out as given in systems (D), (E) and (Ft in Fig. 1. The spots were taken from the same chromatograms used for the data shown in Fig. 4. In this case the radioactivity in the keto-lysolecithin (spot 2) was measured with time.

which accompanied the decrease in the keto-derivative. It is also evident that the rate of formation of the keto-lysolecithin parallels the rate of disappearance of lysolecithin (compare the curves in Figs. 4 and 5).

The products formed during the reaction of lysolecithin with bromine were also investigated. The lysolecithin and keto-lysolecithin were identified by paper chromatography and chemical analysis. These data are summarized in Table II. Both the lysolecithin and keto-lysolecithin contain P and choline and give a yellow staining reaction with Rhodamine 6G[11]. The keto-lysolecithin, however, has a higher $R_F$ value than lysolecithin and it alone gives a positive test with dinitrophenylhydrazine[11].

TABLE II

CHARACTERIZATION OF THE PRODUCTS OBTAINED AFTER BROMINE OXIDATION OF [$^{32}$P]RAT-LIVER LYSOLECITHIN

The spot tests were done on chromatograms similar to those shown in Figs. 1 and 2. These spots were combined from 18 chromatograms. The phosphate esters were determined by hydrolysis of the material in spots 1 and 2 with 1 *N* HCl for 1 h at 100° and chromatography in several solvents[1]. The choline released was determined qualitatively by paper chromatographic analysis of the same acid hydrolysate used for the phosphate esters. Choline was chromatographed on Whatman No 1 filter paper in a solvent consisting of *n*-butanol–ethylene glycol–water (4:1:1) or *n*-propanol–ammonia–water (60:30:10).

| *Compound** | *Spot tests** | | | | *Phosphate ester** | *Choline released** |
|---|---|---|---|---|---|---|
| | *Choline* | *DNPH* | *Rh.6G* | *$^{32}$P* | | |
| Spot-1 lysolecithin | + | — | Yellow | + | GP | ++++ |
| Spot-2 keto-lysolecithin | + | + | Yellow | + | PC + unid. | + |
| Spots 3 + 4 lysophosphatidic acid | — | — | Blue | + | GP | — |

* GP = glycerol phosphate, PC = phosphorylcholine, unid. = unidentified phosphate compound. DNPH = test with 2,4-dinitrophenylhydrazine, Rh.6G = Rhodamine 6G, $^{32}$P = radioactivity of labeled phosphate as determined by autoradiography. (See legend to Figs. 1 and 2).

Furthermore, the keto-lysolecithin yields a different phosphate compound on acid hydrolysis than does lysolecithin. The major phosphate ester obtained by acid hydrolysis of the keto-derivative has the chromatographic mobility of phosphorylcholine in three different solvent systems. The minor phosphate compound which is obtained from the keto-compound has not been identified. The phosphate ester obtained by acid hydrolysis of the lysolecithin (spot 1) which resists oxidation has the identical mobility as glycerolphosphate in three different solvents. Furthermore, free choline is liberated by acid hydrolysis of spot 1 but only a very small amount of free choline is obtained by acid hydrolysis of the keto-compound spot 2. We originally considered[1] the possibility that the slower spot 1 which resists oxidation might be lysolecithinic acid. This, however, is not the case since the evidence clearly shows that this material is the parent lysolecithin. It is noteworthy that lysolecithinic acid would not be expected to have the same mobility as lysolecithin, should stain blue with Rhodamine 6G (as do other acidic phosphatides such as inositol phosphatide, phosphatidylserine, and phosphatidic acids[11]) and would yield phosphoglyceric acids on acid hydrolysis.

The behavior of lysolecithin in 0.05 *N* HCl at room temperature is of further interest. As previously noted by HANAHAN[14] we have consistently found that when lysolecithin is dissolved in acid solution of this normality the solution slowly gels.

In our studies the gelling required between 10–24 h. However, appreciable hydrolysis (23–34 %) of the lysolecithin occurs (Fig. 1, system B). Subsequent bromine oxidation of these solutions (after the pH had been brought up to 5.1 to 7.0) demonstrated that acyl migration had not occurred, since the yield of keto-lysolecithin was not increased. Indeed, the yield of keto-lysolecithin showed a small decrease. Hence the gelling phenomenon does not appear to be due to beta to alpha acyl isomerization as HANAHAN proposes. We feel that the gelling may be due to one or more of the following: (a) a physical effect due to the liberation of fatty acids and water soluble phosphate compounds (b) an effect due to the cationic form of lysolecithin which would predominate in acid solution and (c) phosphate migration from the alpha to the beta position of glycerol to form lysolecithins having phosphorylcholine attached to the C-2 position. With regard to point (c) the properties of such synthetic "beta" lysolecithins (*i.e.* with respect to the position of attachment of the phosphorylcholine) reported by BAYLIS *et al.* is noteworthy[15]. These workers state that aqueous solutions of their synthetic "beta" lysolecithins form gelatinous solutions.

In order to test the effect of enzyme concentration and time of exposure of the lipides to the venom, egg lecithin was treated with a much smaller amount of venom (3 μg of *Naja naja* venom per mg of egg lecithin) for 40, 80, 120, and 180 min. The lysolecithins obtained in each case were oxidized by bromine in phosphate buffer pH 6.4. In all cases the keto-lysolecithin was obtained in essentially the same yield. These experiments make it unlikely that snake venom contains an active acyl isomerase which causes a migration of the fatty acid from the beta to the alpha position of glycerol.

## DISCUSSION

Some of the presented data are not in accord with the work of others[16–18]. The apparent discrepancy might be attributed to a number of factors some of which are as follows: (a) difference in enzyme preparation (b) difference in experimental conditions (c) acyl migration either during enzyme hydrolysis, during the oxidation of the lysolecithins or during the isolation and purification of the lysolecithins, (d) hydrolysis of the lysolecithins during oxidation. Some of these considerations have been pointed out previously[1].

The venom (cobra and rattlesnake) used in these experiments were the same as those employed by HANAHAN[16]. The venom used by GRAY[18] was obtained from water moccasin. Isomerase activity may conceivably play a role in this study.

Acyl migration during oxidation of the lysolecithins may occur but this possibility is unlikely in the case of bromine at pH 5.1 to 7.0 since the rate of lysolecithin disappearance levels off even in the presence of a large excess of oxidant. Furthermore, after passage of the lysolecithin through silicic acid a significant change in the amount of keto-lysolecithin was detected. These results would not be obtained if acyl migration occurred during the oxidation.

The experimental data demonstrate that both permanganate and bromine are capable of oxidizing lysolecithin but that the oxidation is pH dependent. It is important to note that there is a concomitant hydrolysis during the oxidation and that this hydrolysis is greater with permanganate than with bromine at pH 5.1–5.5. Hence it was concluded that the solution of this problem, in particular with respect

to the structure of the lysolecithins, is most easily accomplished by the direct measurement of the rate of formation of the keto-lysolecithin or the rate of disappearance of the lysolecithin rather than by the analysis of the water soluble phosphate esters or other degradation products which result by subsequent acid hydrolysis.

Although the yield of keto-lysolecithin is pH dependent it can be inferred that the amount of α-lysolecithin in the mixture of lysolecithins which are produced by venom hydrolysis is between 54–92 %. The reason why the remaining lysolecithin (spot 1) resists hydrolysis is not clear. The existence of beta lysolecithins (*i.e.* lysolecithins with the fatty acid on the C-2 position of glycerol) is supported by the finding that silicic and treatment results in acyl migration and increases the content of the alpha-isomer. The evidence therefore demonstrates that the phospholipase can hydrolyze the beta-linked fatty acid. Confirmatory evidence for this finding comes from our previous work with heart muscle choline plasmalogens where it was demonstrated that reduction and subsequent hydrolysis of the enzymically formed lysoplasmalogens yielded α-glycerol ethers[2*].

The reason why the yield of keto-lysolecithin increases with increasing pH is not understood. This effect may be due to the nature of the oxidant. Hence at lower pH levels the oxidant may be molecular bromine whereas at the higher pH level the oxidant may be the hypobromite ion or possibly molecular oxygen which forms as a result of decomposition of hypobromite. Oxidation at pH higher than 7.4 was not investigated because of the lability of ester groups in alkaline solution.

Since this study demonstrates that passage of lysolecithin through silicic acid brings about acyl migration, any work on the structure of these lipids must take this finding into account. The apparent discrepancy between our work and that of GRAY may in part be due to this phenomenon. Isomerization of lipids on silicic acid has also been demonstrated by BORGSTROM[12] and HIRSCH AND AHRENS[13]. These workers were able to show the production of 1-monoglycerides when 2-monoglycerides were passed through silicic acid.

The analysis of the water soluble phosphate compounds obtained by acid hydrolysis of the reaction mixture after oxidation of the lysolecithins presents a difficult problem since appreciable hydrolysis occurs during the oxidation and hence several phosphate compounds are formed. The number of water soluble phosphate esters produced in this manner would be decreased if the intermediate oxidized lysolecithins were first isolated and separated, and then hydrolyzed. In this case, however, the yield of each product must be considered in the evaluation of the quantitative significance of such findings. In our hands it has been simpler and more direct to analyze the oxidation product (keto-lysolecithin). These considerations may help explain the apparent discrepancy between our work and the work reported by others[16–18].

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* Drs. A. TATTRIE and M. KATES at the National Research Council, Ottawa, Canada have obtained further data by an independent method that the snake venom phospholipase A liberates preferentially the β-linked fatty acid of egg lecithin (personal communication). This work is now in press (J. Lipid Research).

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# HYDROLYSIS OF LECITHINS BY VENOM PHOSPHOLIPASE A

## II. FATTY ACID CHAIN LENGTH PREFERENCE OF THE ENZYME

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SUMMARY

Phospholipase A of snake venom shows chain length preference with respect to the hydrolysis of lecithin fatty acids. With pig heart, egg, and rat-liver lecithins the C-22, C-20 and C-18 acids are hydrolyzed in preference to the C-16 acids. It also appears that the C-22 and C-20 acids are hydrolyzed in preference to both the C-16 and C-18 acids.

## INTRODUCTION

In a previous paper[1] the structure of the enzymically produced lysolecithins was investigated. Evidence was provided that snake venom phospholipase A hydrolyzes either the alpha or beta linked fatty acid on lecithin to yield a mixture of alpha and beta lysolecithins. However, the beta linked fatty acid is preferentially removed in