3. The significance of the relationship is discussed.

I am indebted to Professor A. Haddow of the Chester Beatty Research Institute for the supply of rat livers and to Mrs Wendy ap Rees for technical assistance.

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The Structure of the Naturally Occurring Phosphoglycerides

3. ACTION OF MOCCASIN-VENOM PHOSPHOLIPASE *A* ON OVOLECITHIN AND RELATED SUBSTANCES*

BY C. LONG† AND I. F. PENNY‡
Department of Biological Chemistry, University of Aberdeen

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The enzyme phospholipase *A*, which is present in the venom of several species of snake, removes a single fatty acid radical from the naturally occurring lecithins to give the corresponding lysolecithins. Lecithin, when isolated from biological materials by solvent-fractionation procedures, usually contains saturated and unsaturated fatty acid radicals in approximately equimolar proportions; on treatment with phospholipase *A*, the unsaturated fatty acid radicals are found to be preferentially removed (Levene & Rolf, 1923; Chargaff & Cohen, 1939; Fairbairn, 1945). However, the enzyme does not act specifically on unsaturated fatty acid ester linkages, since Zeller (1952) and Hanahan, Rodbell & Turner (1954) have demonstrated the removal by phospholipase *A* of a single fatty acid radical from synthetic dimyristoyl-L-α-lecithin and yeast dipalmitoleyl-lecithin respectively. In the first case, the lecithin contains two saturated fatty acid radicals; in the second, both are unsaturated. It has been shown by Hanahan (1954) and by ourselves (Long & Penny, 1954) that it is the fatty acid radical attached to the α′ position in the lecithin molecule which is selectively detached by phospholipase *A*.

In the present work, the phospholipase *A* of cottonmouth-moccasin venom has been studied with regard to its action on ovolecithin and other naturally occurring and synthetic phosphoglycerides. The enzyme has been found to require Ca^{2+} ions and to be specific for the L-α form of synthetic lecithins; phosphatidylethanolamine and probably certain other naturally occurring phosphoglycerides have also been found to act as substrates. Some of this work has been published in preliminary form (Davidson, Long & Penny, 1955).

* Part 2: Long & Maguire (1954).

† Present address: Department of Applied Physiology, Institute of Basic Medical Sciences, Royal College of Surgeons of England, London, W.C. 2.

‡ Present address: Ministries of Agriculture, Fisheries and Food, Experimental Factory, Torry, Aberdeen.

EXPERIMENTAL

Materials

Source of phospholipase A. Samples of the dried venom of the cottonmouth moccasin (*Agkistrodon piscivorus piscivorus*), rattlesnake (*Crotalus adamanteus*) and cobra (*Naia naia*) were purchased from Ross Allen's Reptile Institute, Silver Springs, Florida, U.S.A. Aqueous solutions (0·1%) were used either immediately or after storage for up to 24 hr. at 2°. Six samples of moccasin venom were examined; all showed a significantly higher phospholipase *A* activity than did the rattlesnake or cobra venom, and were therefore used in the experiments to be described.

Ovolecithin. The ovolecithin used in most experiments was prepared by the method of Pangborn (1951), which involves purification through the cadmium complex. The average I_2 val. was 54 and the ratio equivalents of ester:atoms of P (ester:P ratio) was about 1·89:1; such preparations contain about 10–15% of lysolecithin (Lea & Rhodes, 1954). Samples of ovolecithin free from lysolecithin were obtained by silica-gel chromatography according to Lea, Rhodes & Stoll (1955).

Egg phosphatidylethanolamine. A pure specimen was given to us by Dr C. H. Lea. Other samples were prepared according to Lea *et al.* (1955).

Ox-brain 'kephalin' fractions. (*a*) One of the samples used was prepared by Long & Maguire (1953) and corresponded with 'fractions III–V' of Folch (1942), i.e. it consisted largely of a mixture of phosphatidylethanolamine and phosphatidylserine, contaminated with plasmalogens. (*b*) Another sample was prepared from the acetone-insoluble ethanol-soluble fraction of ox-brain lipids, obtained by the procedure of Folch (1942). After removing the ethanol by evaporation under reduced pressure, only part of the residue dissolved in absolute ethanol. The ethanol-insoluble material was dissolved in $CHCl_3$–methanol (4:1, v/v) and chromatographed on silica gel according to Lea *et al.* (1955). The material eluted from the column was free from lecithin and occupied the position expected for phosphatidylethanolamine. However, it was heavily contaminated with plasmalogen and had an ester:P ratio of 1·58:1.

Other naturally occurring phosphoglycerides. The following preparations were given to us: 'phosphatidylinositol' (Hawthorne, 1955) from liver by Dr J. N. Hawthorne, diphosphoinositide (Folch, 1949) from brain by Dr J. Folch, and dipalmitoyl-lecithin from yeast (Hanahan *et al.* 1954) by Dr D. J. Hanahan. The latter had an ester:P ratio of 1·84:1.

Synthetic phosphoglycerides. The following compounds were given to us by Drs T. Malkin and T. H. Bevan: dipalmitoyl-DL-α-lecithin, distearoyl-DL-α-lecithin and dipalmitoyl-β-lecithin. Synthetic dimyristoyl-L-α-lecithin was a gift from Dr E. Baer.

Ether. The ether (British Drug Houses, AnalaR) was washed with a solution of $FeSO_4$, dried over $CaCl_2$ and distilled. It was free from peroxides and from esters (as determined by the hydroxamic acid method).

Analytical methods

Total phosphorus was converted into inorganic P by ashing an amount of phospholipid, containing not more than 0·2 μg. atom of P, with 0·3 ml. of 60% $HClO_4$; the inorganic P was determined by the method of Berenblum & Chain (1938), as modified by Long (1943). Ester content was measured by the hydroxamic acid method described by Shapiro (1953); a pure sample of ethyl palmitate, given by British Industrial Solvents (Distillers Co. Ltd.), was used as a standard.

All colorimetric determinations were made with a photoelectric colorimeter (Evans Electroselenium Ltd.).

RESULTS

Action of moccasin-venom phospholipase A on ovolecithin

All the experiments described in this section were carried out on samples of ovolecithin prepared by the procedure of Pangborn (1951). When the method of Hanahan *et al.* (1954) was used to follow the action of the various snake venoms on an ethereal solution of ovolecithin, consistent results were obtained only when Ca^{2+} ions were added to the reaction mixture. The following modification proved satisfactory. Into each of several matched test tubes (125 mm. × 15 mm.) was pipetted 1 ml. of dry peroxide-free ether containing 1·5–2·0 μmoles of ovolecithin. At fixed time intervals, 0·01 ml. of an aqueous venom solution (1 mg. of venom/ml. of 0·005 M-$CaCl_2$) was added to each tube. The contents were mixed and the tubes allowed to stand at room temperature (usually 18°). Lysolecithin, which is insoluble in ether, was slowly precipitated as the reaction proceeded. After 5–120 min., enzymic activity was stopped by adding 2 ml. of ester-free ethanol, which also dissolved the lysolecithin. Ester content was determined by the hydroxamic acid method, the red–brown colours being measured against a standard ethyl palmitate solution in ether–ethanol and an ester-free ether–ethanol blank. In the zero-time sample containing ovolecithin, the ethanol was added before the enzyme. With moccasin venom, the ester:P ratio decreased from 1·89 to 1·00 in about 2 hr. Fig. 1 shows the time course of the reaction, the mean values being

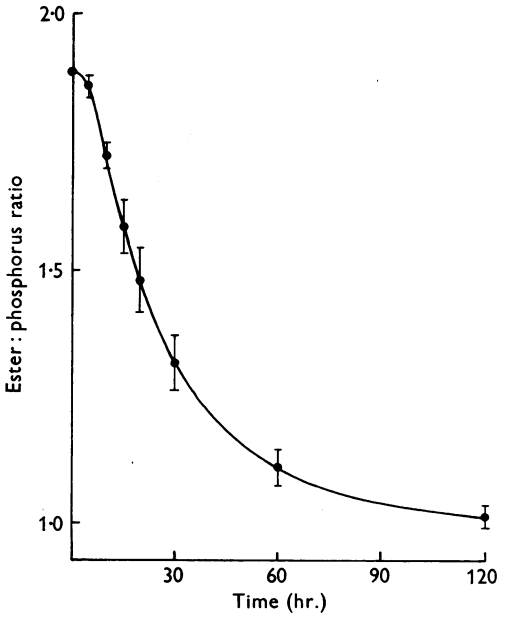

Fig. 1. Time course of enzymic hydrolysis of ovolecithin, prepared according to Pangborn (1951), by moccasin-venom phospholipase *A*. Reaction mixtures contained 1·5–2·0 μmoles of ovolecithin in 1 ml. of dry peroxide-free ether, and 10 μg. of venom in 0·01 ml. of 0·005 M-$CaCl_2$. Temp., 18°. Mean values ± S.E. based on twelve experiments.

based on 12 experiments, with the indicated standard error of the mean. It appears that there is a very noticeable time lag of about 5 min. in the enzymic degradation. Qualitatively similar results were obtained with rattlesnake and cobra venom, although their activities were somewhat lower than that of moccasin venom.

Effect of calcium ions. The presence of Ca^{2+} ions at certain concentrations increased the rate of degradation of ovolecithin by moccasin-venom phospholipase *A*. The optimum concentration of Ca^{2+} ions was found to be independent of the amount of venom used, but varied with the concentration of ovolecithin, as shown in Fig. 2. Optimum final concentrations of Ca^{2+} ions (in the ethereal solution) for 1·3, 2·0 and 3·3 μmoles of ovolecithin/ml. were about 40, 50 and 80 μM respectively. These concentrations are based on the assumption that the Ca^{2+} ions, introduced with the venom solution, are distributed throughout the moist ethereal phase. This seems to be so in the early stages of the reaction. However, it has been found in a large-scale experiment that at the end of the reaction the Ca^{2+} ions are no longer present in the ethereal solution. Ovolecithin (75 mg.), dissolved in ether (50 ml.), was treated with 0·5 ml. of 0·1% moccasin venom in 0·005 M-$CaCl_2$. After 2 hr. at room temperature, the precipitated lysolecithin was removed by centrifuging and the ethereal supernatant was evaporated to dryness and incinerated. No calcium could be detected in the residue, although 0·05 ml. of the venom solution, on analysis by an oxalate method, was found to contain 92 μg. of calcium (theoretical, 100 μg.).

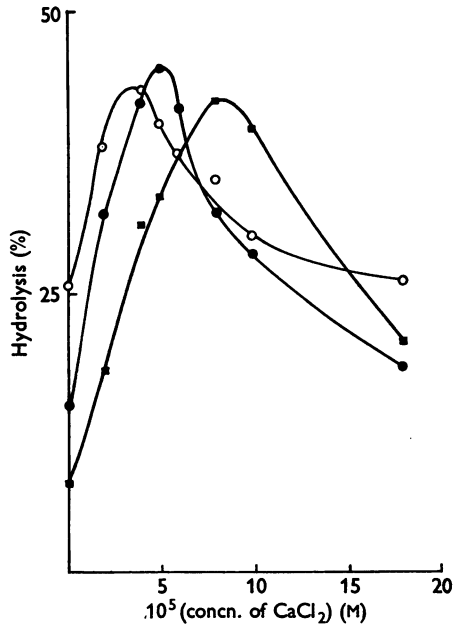

Fig. 2. Dependence of optimum concn. of Ca^{2+} ions on concn. of ovolecithin. Separate samples of venom (1 mg.) were dissolved in 1 ml. of aqueous $CaCl_2$, having 100 times the concn. shown on the abscissa, and 0·01 ml. portions were added to 1·0 ml. of dry peroxide-free ether containing ovolecithin. Concns. of ovolecithin (μmoles/ml.): ○, 1·3; ●, 2·0; ■, 3·3. Reaction stopped after 45 min. at room temp. (18°). Removal of one ester group/mole of ovolecithin is represented by 50% hydrolysis.

That Ca^{2+} ions are probably essential for phospholipase *A* activity is shown by the inhibition of the reaction by ethylenediaminetetraacetic acid (Table 1).

The following metal chlorides were tested for their ability to replace $CaCl_2$, but were found lacking in activity: NaCl, KCl, $BaCl_2$, $SrCl_2$, $MgCl_2$ and $CdCl_2$. Inhibition was observed with $CuCl_2$ and $ZnCl_2$.

Effect of inhibitors. Ethylenediaminetetraacetic acid inhibited moccasin-venom phospholipase *A* activity, presumably by its ability to form a complex with Ca^{2+} ions (Table 1). Inhibition (95%) by mM-Cu^{2+} ions suggested that the active centre of the enzyme might contain a sulphydryl group. However, at final concentration mM, neither sodium iodoacetate nor sodium *p*-chloromercuribenzoate had any inhibitory action and the enzyme was not activated by mM sodium mercaptoacetate (thioglycollate). With regard to inhibitors, this enzyme closely resembles the phospholipase *B* of rat liver (Dawson, 1956). There seems little evidence for the presence of a sulphydryl group in the active centre of phospholipase *A*.

Action of moccasin-venom phospholipase A *on saturated lecithins*

Since those lecithins which contain only saturated fatty acid radicals are sparingly soluble in pure ether, mixtures of other solvents with ether were tested for their effects on phospholipase *A* activity.

Table 1. *Effect of ethylenediaminetetraacetic acid on moccasin-venom phospholipase* A *activity*

To a series of tubes, each containing about 2·0 μmoles of ovolecithin in 1 ml. of ether, was added 0·01 ml. of 0·1% venom containing $CaCl_2$ and ethylenediaminetetraacetic acid (EDTA) in the concentrations shown. Incubation for 30 min. at 18°.

Concn. of $CaCl_2$ in venom (mM)	Concn. of EDTA in venom (mM)	Hydrolysis (%)
0	0	38
0	1	0
0	2	0
3·5	0	45
3·5	1	27
3·5	2	0

With ovolecithin as substrate, ether–dioxan (2:1, v/v), ether–$CHCl_3$ (19:1, v/v) and ether–ethanol (99:1, v/v) had virtually no inhibitory effect when compared with pure ether. Ether–ethanol (19:1, v/v), however, inhibited completely. The experiments with saturated lecithins were carried out either in ether–ethanol or ether–$CHCl_3$ (both 99:1, v/v), in which solvents they were soluble.

Fig. 3 shows the rates of enzymic degradation of some natural and synthetic saturated lecithins, compared with that of ovolecithin. The hydrolysis of natural dipalmitoyl-L-α-lecithin (and also of synthetic dimyristoyl-L-α-lecithin, not shown in Fig. 3) was more rapid than that of ovolecithin. The degradation of synthetic dipalmitoyl-DL-α-lecithin (and of distearoyl-DL-α-lecithin, not shown in Fig. 3) took place more slowly than that of ovolecithin and the reaction stopped when 0·5 μmole of ester/μg. atom of phosphorus had been removed. Synthetic dipalmitoyl-β-lecithin was not attacked.

Hydrogenated ovolecithin was also attacked by phospholipase *A* though less rapidly than was ovolecithin; it will be shown later (Fig. 7) that the rate of degradation of hydrogenated ovolecithin could be increased by raising the pH of the ethereal solution.

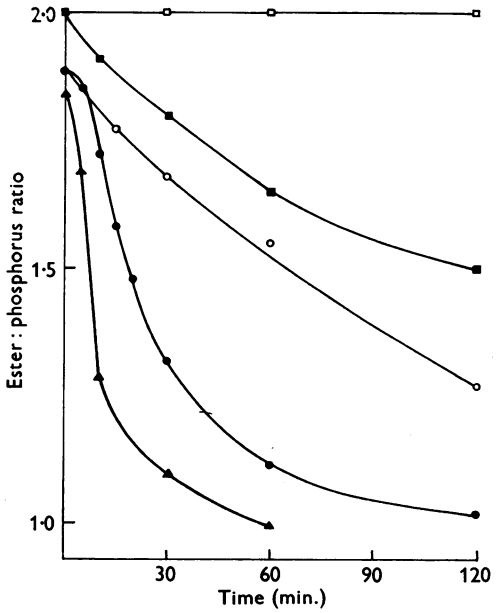

Fig. 3. Enzymic degradation of saturated lecithins. The substrates (about 20 μmoles) were dissolved in ethanol (0·1 ml.) and diluted to 10 ml. with ether. Samples (1 ml.) were treated with 0·01 ml. of 0·1% moccasin venom in 0·005 M-$CaCl_2$. □, Dipalmitoyl-β-lecithin (synthetic); ■, dipalmitoyl-DL-α-lecithin (synthetic); ▲, dipalmitoyl-L-α-lecithin (natural); ○, hydrogenated ovolecithin; ●, ovolecithin (for comparison).

The enzymic hydrolysis of dimyristoyl-L-α-lecithin was stimulated by Ca^{2+} ions.

Action of moccasin-venom phospholipase A on 'kephalins'

The literature contains conflicting statements concerning the action of snake-venom on 'kephalins'. Levene & Rolf (1923) showed that ethanolamine-containing phospholipids, present in a phosphate-buffered egg-yolk emulsion at pH 7·5, were attacked by snake venom, and Levene, Rolf & Simms (1924) isolated both lysolecithin and 'lysokephalin' from such a reaction mixture. However, Chargaff & Cohen (1939) reported that snake venom was without effect on aqueous emulsions of several partially purified kephalins.

In preliminary experiments, using the technique of Hanahan *et al.* (1954) modified by the addition of Ca^{2+} ions, we were able to demonstrate the breakdown in ethereal solution of crude ox-brain kephalin fractions by moccasin venom. These substrates were virtually free from choline-containing material, and consisted of a mixture of phosphatidylethanolamine and phosphatidylserine, grossly contaminated with plasmalogens. Their rates of enzymic degradation were rather slow compared with that of ovolecithin, as shown in Fig. 4. Nevertheless, after

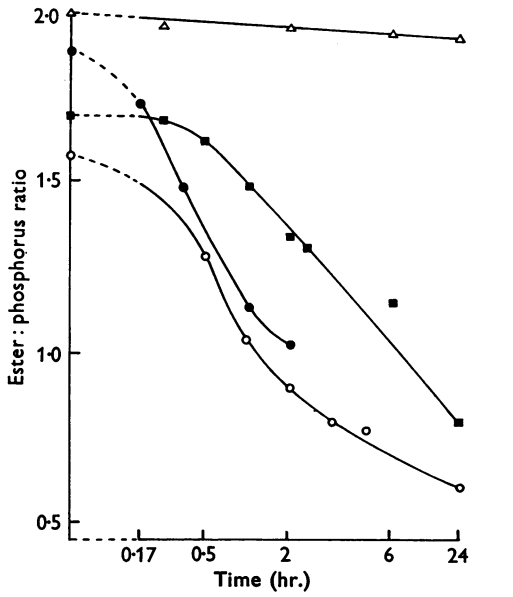

Fig. 4. Degradation of kephalins by moccasin venom. Conditions as in Fig. 3. ■, Ox-brain kephalin, 'fractions III–V' (Folch, 1949); ○, an ethanol-insoluble fraction of ox brain (see Methods section); △, pure egg phosphatidylethanolamine (Lea *et al.* 1955); ●, ovolecithin (for comparison). The abscissa has been drawn to a logarithmic scale.

24 hr. these substrates had lost almost exactly one ester group/atom of phosphorus.

When pure egg phosphatidylethanolamine, prepared by silica-gel chromatography (Lea *et al.* 1955), was used as substrate, however, no enzymic degradation could be detected (Fig. 4). Furthermore, addition of a small quantity of pure egg phosphatidylethanolamine to ovolecithin strongly inhibited the enzymic degradation of the latter (Fig. 5). The lag phase was also greatly prolonged, a finding which suggested that the short lag phase previously found with ovolecithin, prepared by the Pangborn (1951) procedure (Fig. 1), could have been due to the presence of a small amount of phosphatidylethanolamine as an impurity. However, when ovolecithin, free from phosphatidylethanolamine, prepared by the chromatographic method of Lea *et al.* (1955), was tested, its rate of enzymic degradation by moccasin venom was practically zero (see Fig. 6). An explanation of these results is given in the next section.

Effect of pH *on phospholipase* A *activity*

In the experiments of Levene & Rolf (1923) aqueous emulsions of egg yolk, buffered with phosphate to pH 7·5, were treated with snake venom. Hanahan (1952), when first reporting the increased activity of phospholipase *A* in ethereal solution, buffered his aqueous enzyme–lecithin reaction mixture to pH 7·0 with glycylglycine before extracting with ether. In subsequent work, however, Hanahan *et al.* (1954) did not buffer the reaction mixture, since the enzymic hydrolysis took place in ether–water (99:1, v/v) where pH was considered to have little significance. In the experiments so far described, no buffers were used.

The observation that small amounts of pure phosphatidylethanolamine strongly inhibited the degradation of ovolecithin by snake-venom phospholipase *A* (Fig. 5) raised the possibility that inhibition might also be obtained in the presence of ethanolamine itself. However, when 0·5 μmole of the free base was added to 1 ml. of an ethereal solution of ovolecithin (2 μmoles), the rate of degradation of the latter by moccasin venom was significantly increased. Ethanolamine acetate, by contrast, had no such stimulating effect. This suggested that the increased enzymic activity was caused by a rise in pH of the ethereal solution, support for which was obtained by using ammonia in place of ethanolamine (see Fig. 8). Other experiments, in which the enzyme was first dissolved in water-soluble buffers, in an attempt to control the pH of the ethereal reaction mixture, proved unsuccessful since the ether either did not dissolve the buffer at all or else preferentially dissolved one of

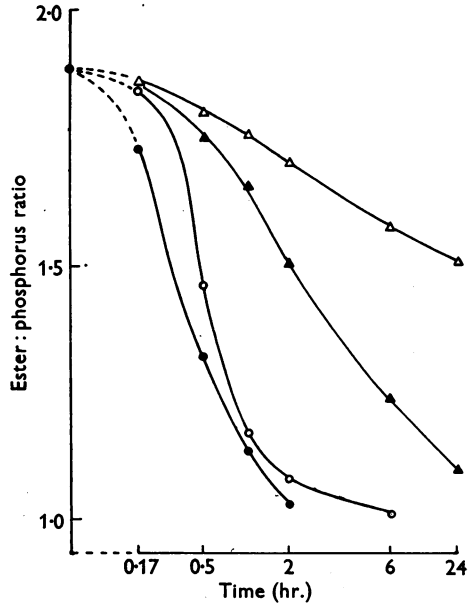

Fig. 5. Inhibition of enzymic degradation of ovolecithin by pure egg phosphatidylethanolamine. All ethereal reaction mixtures (1 ml.) contained 2 μmoles of ovolecithin. Amount of added phosphatidylethanolamine (μmoles): ●, Nil; ○, 0·01; ▲, 0·02; △, 0·10.

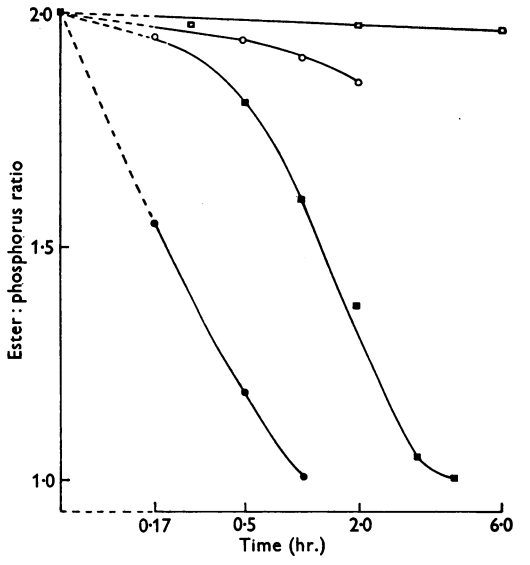

Fig. 6. Effect of ammonia on the degradation of ovolecithin and egg phosphatidylethanolamine, prepared chromatographically (Lea *et al.* 1955), by snake-venom phospholipase *A*. Substrates (2 μmoles) were dissolved in 1 ml. of ether; enzyme, moccasin venom (0·01 ml. of a 0·1% solution in aqueous 0·005 M-$CaCl_2$). With lecithin: ○, no addition; ●, 0·5 μmole of ammonia present. With phosphatidylethanolamine: □, no addition; ■, 0·5 μmole of ammonia present.

the buffer constituents, e.g. succinic acid from a succinate buffer. In subsequent experiments, therefore, ammonia, which is very soluble in ether, was used exclusively for adjusting the pH of the ethereal reaction mixture.

When 0·5 μmole of ammonia was added to an ethereal solution (1 ml.) of pure egg phosphatidylethanolamine (2 μmoles), the latter was also degraded by snake-venom phospholipase *A*, reaction being complete in less than 6 hr. at room temperature (Fig. 6). When 1·5 μmoles of ammonia were added, the stimulation of enzymic activity was rather less, presumably because the reaction mixture now had a pH higher than the optimum.

It is difficult to determine accurately the pH of the ethereal reaction mixtures by means of indicators. However, it has been found empirically that the optimum pH for enzymic activity could be obtained in the following way. One drop of an ethereal solution of bromocresol green (0·1 %) was added to an ethereal solution of the phosphoglyceride, and an ethereal solution of ammonia (50 μmoles/ml.) was added until the tint matched that of the same indicator in an aqueous buffer of pH 5·2. It has been observed, however, that the pH ranges of bromocresol green in aqueous solution and in ethereal solution are different, and that the value of 5·2 is only the apparent pH optimum. Thus, when the pH of such an ethereal solution was determined with a pH meter and glass electrode assembly, a stable reading of 7·2 was obtained. Further comparisons between the apparent pH of other ethereal solutions, as estimated by their colours with bromocresol green, and the true pH values, as determined with a pH meter, showed the latter to be consistently about 2 pH units higher. Thus it appears that the pH optimum for moccasin-venom phospholipase *A* activity in ethereal solution is about 7·2, a value which is within the range found for aqueous emulsions by Levene & Rolf (1923) and Hanahan (1952).

For convenience in estimating the pH of ethereal solutions of the phosphoglycerides, the colorimetric method using bromocresol green has been applied and the values obtained corrected by adding 2 pH units. In this way, an ethereal solution of ovolecithin, prepared by Pangborn's (1951) procedure, was found to have a pH of 6·6, while ovolecithin and egg phosphatidylethanolamine, prepared by silica-gel chromatography (Lea *et al.* 1955), had pH values below 6·0; addition of the required amount of ammonia to 2 μmoles of any one of these substrates in 1 ml. of ether in order to raise the pH to 7·0–7·2, caused a rapid hydrolysis by moccasin-venom

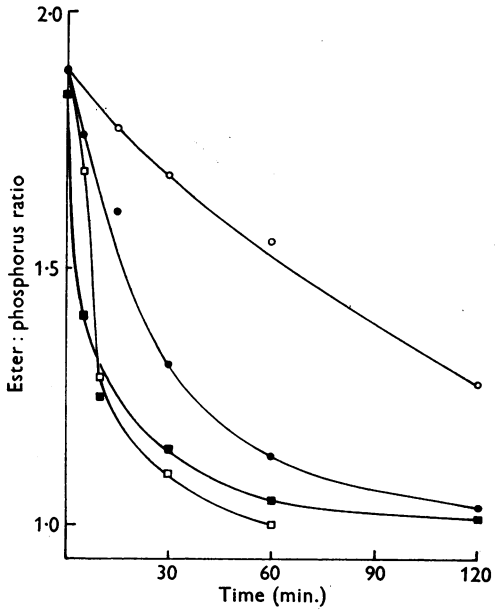

Fig. 7. Effect of ammonia on the degradation of hydrogenated natural lecithins by snake-venom phospholipase *A*. Experimental conditions as in Fig. 6. Hydrogenated ovolecithin (original pH 6·0–6·2): ○, no addition; ●, 0·5 μmole of ammonia present. Dipalmitoyl-L-α-lecithin (Hanahan *et al.* 1954; original pH 7·0–7·2): □, no addition; ■, 0·5 μmole of ammonia present.

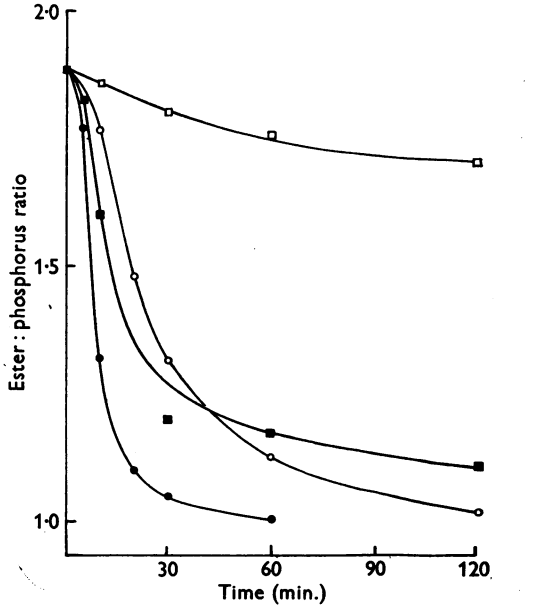

Fig. 8. Effect of ammonia on the inhibition of the enzymic degradation of ovolecithin by egg phosphatidylethanolamine. Experimental conditions as in Fig. 6. All reaction mixtures contained 2 μmoles of ovolecithin, prepared according to Pangborn (1951). ○, No addition; ●, 0·05 μmole of ammonia; □, phosphatidylethanolamine (0·1 μmole); ■, phosphatidylethanolamine and ammonia.

phospholipase *A* (Fig. 6). Hydrogenated ovolecithin, the slow breakdown of which has already been mentioned (Fig. 3), had a pH in ethereal solution of 6·0–6·2; its enzymic degradation was likewise stimulated by the addition of ammonia (Fig. 7), although even at the optimum pH it was not hydrolysed as rapidly as ovolecithin. On the other hand, the sample of dipalmitoyl-L-α-lecithin, prepared by catalytic hydrogenation of yeast dipalmitoleyl-L-α-lecithin (Hanahan & Jayko, 1952), which in turn had been eluted from an alumina column, had a pH of 7·0–7·2, and its rate of enzymic degradation was not increased by addition of ammonia (Fig. 7).

The inhibition of the enzymic degradation of ovolecithin by egg phosphatidylethanolamine may partly be attributed to its action in lowering the pH of the ethereal reaction mixture. This is shown by the experiment in Fig. 8, in which addition of ammonia almost abolishes this inhibition. The mixture of ovolecithin (2 μmoles) and phosphatidylethanolamine (0·1 μmole) had a pH in ethereal solution of 6·2, which was raised to 7·0 in the presence of 0·5 μmole of ammonia.

Neither diphosphoinositide (Folch, 1949) nor phosphatidylinositol (Hawthorne, 1955) was attacked by phospholipase *A*, even in the presence of ammonia.

DISCUSSION

Requirement for calcium ions

The stimulation of moccasin-venom phospholipase *A* activity by calcium ions (Fig. 2) has not previously been reported, so far as we are aware, although Zeller (1952) carried out the enzymic degradation of synthetic dimyristoyl-L-α-lecithin in the presence of 5×10^{-4} M-Ca^{2+} without commenting on this addition. It may also be significant that Ca^{2+} ions were found by Braganca & Quastel (1953) to increase the inhibition of glucose oxidation in rat-brain homogenate caused by cobra venom. Ca^{2+} ions stimulated the phospholipase *A* activity of all eight venom samples tested and increased the rate of enzymic degradation of all the substrates examined except the dipalmitoyl-L-α-lecithin of Hanahan *et al.* (1954).

It is of interest that the optimum requirement for Ca^{2+} ions should depend upon the concentration of lecithin used. It has been calculated from the data in Fig. 2 that each μmole of ovolecithin requires about 0·03 μmole of Ca^{2+} ions for maximal rate of hydrolysis. This might suggest that the Ca^{2+} ions acted by combining with some inhibitor present in the ovolecithin were it not for the fact that the synthetic lecithins also require Ca^{2+} ions for full activity. The absolute necessity for Ca^{2+} ions is also supported by the observed inhibitory effect of ethylenediaminetetraacetic acid on phospholipase *A* activity (Table 1) and the fact that Ca^{2+} ions could not be replaced by other bivalent cations. The possibility that Ca^{2+} ions act by combining with the liberated fatty acids can also be excluded by the observation that only 0·03 μmole of Ca^{2+} ions is required for each μmole of fatty acid liberated and that the Ca^{2+} ions are carried out of solution by the precipitated lysolecithin, whereas the fatty acids remain in the ethereal supernatant. The most likely explanation is that the Ca^{2+} ions take part in the binding of the enzyme with the substrate, since Hanahan *et al.* (1954) have shown that the enzyme is also carried out of solution by the precipitated lysolecithin and we have found that when reaction is incomplete, the precipitated lysolecithin adsorbs unchanged lecithin from the ethereal solution.

Substrate specificity of phospholipase A

The results of experiments with synthetic lecithins leave no room for doubt that phospholipase *A* is specific for the L-α-structure. Synthetic dimyristoyl-L-α-lecithin and the naturally occurring lecithins, which also possess the L-α-structure (Long & Maguire, 1953, 1954), are hydrolysed with the removal of one ester group per molecule. The nature of the fatty acid radicals does not seem to be very important, provided that a small quantity of ethanol or chloroform is present so as to bring the saturated lecithins into ethereal solution, and that the pH is in the region of 7·0. Synthetic DL-α-lecithins, on the other hand, lose one ester group/two molecules of substrate, which strongly suggests that the D-α form is not attacked. A synthetic β-lecithin was not degraded by phospholipase *A*.

Pure egg phosphatidylethanolamine has also been found to act as a substrate for phospholipase *A* when the pH of the ethereal solution was adjusted to about 7·0. It seems probable that phosphatidylethanolamine has a higher affinity than lecithin for the active centres of the enzyme, for otherwise it is difficult to account for the slower rate of attack on a mixture of ovolecithin and phosphatidylethanolamine (95:5) than on ovolecithin alone, even when the pH has been correctly adjusted (Fig. 8).

Whether other phosphoglycerides can act as substrates for phospholipase *A* must remain in doubt until pure samples become available. Present evidence indicates, however, that phosphatidylserine and ethanolamine plasmalogen are hydrolysed (Fig. 4), while Franzl & Rapport (1955) have stated in a preliminary report that choline plasmalogen (phosphatidalcholine) was degraded by snake-venom phospholipase *A*, although the ethanolamine analogue was not. Neither of the inositol phosphoglycerides was attacked by phospholipase *A*. Perhaps it is necessary that the substrate molecule should possess a positively charged group, as in lecithin and phosphatidylethanolamine.

SUMMARY

1. The enzymic degradation of ovolecithin and certain other phosphoglycerides in ethereal solution by snake-venom phospholipase *A* is stimulated by Ca^{2+} ions. The optimum Ca^{2+} ion concentration varies between 40 and 80 μM, when the lecithin concentration varies between 1·3 and 3·3 mM.

2. The phospholipase *A* activity of moccasin venom is inhibited by ethylenediaminetetraacetic acid and by Zn^{2+} and Cu^{2+} ions, but not by iodoacetate or *p*-chloromercuribenzoate.

3. All the natural and synthetic L-α-lecithins studied lost one ester group/molecule of substrate in the presence of the enzyme. Synthetic DL-α-lecithins lost only 0·5 mole of ester/molecule of substrate, whereas synthetic β-lecithins did not undergo any enzymic hydrolysis.

4. Egg phosphatidylethanolamine was degraded by phospholipase *A* when the ethereal solution was adjusted to pH 7·0, and there is some evidence for the breakdown of phosphatidylserine and ethanolamine plasmalogen.

5. Inositol phosphoglycerides were not attacked by moccasin-venom phospholipase *A*.

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Inhibition of Glycosidases by Aldonolactones of Corresponding Configuration

By J. CONCHIE and G. A. LEVVY
Rowett Research Institute, Bucksburn, Aberdeenshire

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There is no need to stress the potential value in many connexions of a class of selective inhibitors for the different glycosidases. Levvy (1952) discovered that mouse-liver β-glucuronidase is very powerfully inhibited by saccharo-1:4-lactone, and the presence of this lactone in traces in saccharate solutions explained the powerful inhibitory action of the latter on this enzyme (Karunairatnam & Levvy, 1949). Saccharo-1:4-lactone, as such, or in the form of a saccharate solution, has been shown to inhibit β-glucuronidase from every source that has been studied. Conchie (1954) found that β-glucosidase from rumen micro-organisms was inhibited by the 1:4- and 1:5-lactones of gluconic acid, but not by saccharo-1:4-lactone. These findings have been confirmed with β-glucosidase from other sources (Jermyn, 1955; Robinson, 1956). Although efficient inhibitors, the gluconolactones are not as powerful as saccharo-1:4-lactone.

It was decided to extend these studies to other types of glycosidase, and at the same time examine the specificity of response to a range of lactones. This work also served another purpose in that rumen liquor was screened for enzymes hydrolysing