SUMMARY

1. Reduced haemoglobin combines successively with four molecules of nitrosobenzene and the nitrosotoluenes.

2. The rate constants for the combination of the first molecule of ligand at pH 9·1 and 20° are (M^{-1} sec.$^{-1}$): nitrosobenzene, $9·2 \times 10^4$; *o*-nitrosotoluene, $1·7 \times 10^4$; *m*-nitrosotoluene, $10·5 \times 10^4$; *p*-nitrosotoluene, $10·5 \times 10^4$.

3. The fourth molecule to combine does so more rapidly than the first, the average increase in rate is sevenfold. The increase compared with the first molecule is about the same for all four compounds.

4. The rate of dissociation of the first molecule of the same compounds from fully saturated haemoglobin has been measured. For nitrosobenzene, and *o*- and *m*-nitrosotoluene, the rates lie within the range 0·016–0·032 sec.$^{-1}$ at 20°. The value for *p*-nitrosotoluene is 0·2 sec.$^{-1}$.

5. The activation energies for the combination of the first molecule of nitrosobenzene and the dissociation of the first molecule of nitrosobenzene give a normal value to the frequency factor in both cases.

6. The rates of combination of the four nitroso compounds with myoglobin are numerically equal to those for the combination of the first molecule with reduced haemoglobin, with the exception of *o*-nitrosotoluene which combines with myoglobin almost as fast as do the other compounds.

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The Estimation of Phospholipase A Activity in Aqueous Systems

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The action of snake-venom phospholipase A on phospholipid fractions was first investigated in aqueous reaction systems. Levene & Rolf (1923) and Levene, Rolf & Simms (1924) carried out the reaction in phosphate buffer, which has been used extensively since that time (Chargaff & Cohen, 1939; Fairbairn, 1945; Rimon & Shapiro, 1959). In phosphate buffer, however, the conversion of lecithin into lysolecithin by venom preparations is slow and incomplete. For example, Chargaff & Cohen (1939) reported a lysolecithin yield of from 15 to 50% of the original lecithin employed in a reaction system maintained at 38° for a period of 24 hr.

The finding that phospholipase A can split lecithin in ether solution (Hanahan, 1952; Hanahan, Rodbell & Turner, 1954) has been of great practical importance. The ethereal reaction system was further studied and improved by Long & Penny (1957), and is used generally at the present time for the preparation of lysolecithin. The system is somewhat inflexible, however, as some diacyl phospholipids are relatively insoluble in ether, and pH adjustment is difficult.

The rate at which phospholipase A attacks lecithin or other phospholipids has been followed by a

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variety of different analytical methods. The release of free fatty acid during the reaction has been determined both by direct titration (Fairbairn, 1945; Hanahan, 1952; Hanahan *et al.* 1954) and by manometric techniques (Zamecnik, Brewster & Lipmann, 1947; Habermann, 1957). Long & Penny (1957), Rapport & Franzl (1957) and Rimon & Shapiro (1959) followed the course of lecithin hydrolysis by measuring the decrease in acyl ester bonds taking place. This is a relatively simple procedure, is very reproducible and permits the use of less substrate in the assay system than other methods.

The present work was undertaken in an attempt to devise an aqueous medium which would be suitable for the determination of phospholipase A activity in crude enzyme preparations or tissue homogenates. A 2:4:6-collidine-buffered system containing ether is described in which the hydrolysis of ovolecithin by phospholipase A goes rapidly to completion. Enzyme activity was measured by the decrease in acyl ester bonds during the reaction. The system has been used to investigate some of the properties of snake-venom phospholipase A. A modification of the basic method is described which permits its use with commercial Pancreatin or other crude tissue preparations.

EXPERIMENTAL

Materials

Ovolecithin. Ovolecithin was prepared from fresh egg yolks, and was purified by chromatography on an alumina column according to the method of Rhodes & Lea (1957). It was stored in brown glass bottles at $-15°$ in chloroform solution. For certain experiments, the ovolecithin was washed by the procedure described by Folch, Lees & Sloane-Stanley (1957) to remove Ca^{2+} ions.

Sources of phospholipase A. The dried venom of the cotton-mouth moccasin (*Agkistrodon piscivorus piscivorus*) was purchased from Ross Allen's Reptile Institute, Silver Springs, Florida, U.S.A. Pancreatin was obtained from British Drug Houses Ltd.

Buffers. Phosphate buffer (0·067 M) was prepared from KH_2PO_4 and Na_2HPO_4 stock solutions. 2-Hydroxymethyl-propane-1:3-diol (tris)–maleate buffer (0·05 M) was made up as described by Gomori (1955). The preparations of diethyl barbiturate (veronal)–HCl (0·04 M), triethanolamine–HCl (0·05 M) and 2:4:6-collidine–HCl (0·05 M) buffers are described by Dawson & Elliott (1959). Final pH adjustment was made by using a glass electrode. Triethanolamine and 2:4:6-collidine were redistilled before use. Other chemicals were commercial samples of the purest grade available.

Methods

Measurement of enzyme activity. Phospholipase A activity was measured by following the decrease in acyl ester bonds occurring during the conversion of lecithin into lysolecithin. Ester content was determined by a hydroxamic acid method, essentially as described by Stern & Shapiro (1953). The hydroxylamine reagent was prepared as required by dissolving 2·09 g. of recrystallized hydroxylamine hydrochloride in 15 ml. of water and adding 15 ml. of 3·5 N-NaOH.

Some difficulty was experienced in the selection of a suitable standard for the ester determination, as different substances produced slightly different colour yields/acyl ester bond. It was finally decided to use the ovolecithin itself as a standard. This was made possible by the fact that both ovolecithin and lysolecithin produced coloured solutions obeying Beer's law over a concentration range of 0·13–2·00 μg./sample. The colour yield of lysolecithin/μmole was half that of ovolecithin. Lysolecithin was not attacked by the phospholipase A, or any other enzyme, in the reaction systems described.

The extinctions of individual samples were calculated as a percentage of the extinction of the zero-time controls. The values thus obtained were used to calculate the amount of hydrolysis (%) that had taken place, and hence the amount (μg.) of ovolecithin hydrolysed. A correction was applied to compensate for the lysolecithin content of the original substrate.

Snake-venom reaction system. An appropriate portion of the ovolecithin stock solution was dried *in vacuo*. Buffer was added and the lipid emulsified by vigorous shaking before final volume adjustment. In most experiments, 1 ml. of lipid emulsion contained 1 mg. of ovolecithin. Samples (1 ml.) of the ovolecithin emulsion were pipetted into glass-stoppered test tubes (110 mm. × 17 mm.). The tubes were tightly stoppered after the addition of a quantity of diethyl ether (usually 0·1 ml.), and placed in a rack. The tubes were immersed in a water bath at 30°, and shaken continuously during temperature equilibration and the subsequent reaction period.

Immediately before use, an aqueous solution of the moccasin venom was prepared, and brought to a temperature of 30°. In a typical experiment, 2 mg. of dried venom were dissolved in 1 ml. of 5 mM-$CaCl_2$ solution. At fixed time intervals, 0·05 ml. of the venom solution was added to each reaction tube. After the appropriate reaction period, enzymic hydrolysis was stopped by the addition of 2 ml. of ethanol and 1 ml. of hydroxylamine reagent (ethanol alone did not stop the reaction immediately).

All samples were examined in duplicate. In the zero-time samples containing ovolecithin, the ethanol and hydroxylamine reagent were added before the enzyme. After the addition of the hydroxylamine reagent, the tubes were left on the bench for a minimum of 20 min.

Colour development was carried out by the addition of 0·6 ml. of 3·3 N-HCl, followed, after thorough mixing, by the further addition of 0·5 ml. of $FeCl_3$ reagent (Stern & Shapiro, 1953). The samples were read against a reagent blank (zero-time, no ovolecithin) in a spectrophotometer at a wavelength of 525 mμ.

In a few instances it was noticed that a slight turbidity or outright precipitate appeared in the sample after the addition of the $FeCl_3$ reagent. This sometimes occurred when the substrate concentration was high. It has also been observed during the analysis of high concentrations of lysolecithin and other phospholipids, in the presence of excessive free fatty acid, and with certain other enzyme sources. The addition of 1 ml. of diethyl ether to such samples (including all others in the set) cleared them completely.

Pancreatin reaction system. To each test tube was added 0·5 ml. of ovolecithin emulsion (2 mg. of ovolecithin/ml. of

buffer), 0·5 ml. of pancreatin syspension (50 mg. of pancreatin/ml. of buffer), 0·05 ml. of 5 mM-$CaCl_2$ solution, and 0·1 ml. of ether. The tubes were tightly stoppered and maintained at 30° with constant shaking as above. The reaction was stopped by adding 4 ml. of chloroform–methanol (2:1, v/v) to each tube and shaking vigorously for 30 sec. After centrifuging, the upper aqueous phase and the solid material at the boundary between the two phases were aspirated off. A 2 ml. sample of the lower phase was transferred to another glass-stoppered test tube. The solvents were carefully evaporated off under reduced pressure. The tubes were dipped periodically into a bath of hot water during the evaporation procedure. Once the solvents had been removed, each tube had added to it 3 ml. of 67% aq. ethanol and 1 ml. of hydroxylamine reagent.

All samples were prepared in duplicate, and colour development was carried out as described above.

RESULTS

In preliminary experiments, ovolecithin was emulsified in a variety of different buffers, and calcium-containing moccasin-venom solutions were added. Although the nature of the buffering agent made some difference to the yield of lysolecithin, hydrolysis was always incomplete. In an experiment with phosphate buffer (0·067 M, pH 6·5) 68% of the ovolecithin was hydrolysed after 2 hr.; contact with the enzyme for a further period of 18 hr. had no additional effect. This result is in agreement with the findings of other authors. For example, Fairbairn (1945) calculated a yield of fatty acids from a mixed lipid substrate that was 72% of the theoretical, in a reaction carried out in a phosphate-buffered (0·05 M, pH 7·0) medium with moccasin venom as the source of phospholipase A.

The addition of ether to buffered reaction media increased both the rate at which lecithin was converted into lysolecithin and the final amount of lysolecithin produced. The nature of the buffering agent used in such an ether-containing system had a considerable influence on the rate of the reaction. Fig. 1 shows that the hydrolysis of ovolecithin by moccasin-venom phospholipase A was relatively slow in phosphate, succinate, malonate, tris–maleate and triethanolamine buffers, more rapid in diethyl barbiturate buffer, and most rapid in 2:4:6-collidine buffer. The time courses of hydrolysis in phosphate, succinate, malonate and tris–maleate buffers were very similar, and are represented in the figure by mean values only. The curve for 2:4:6-collidine buffer was plotted with mean values obtained in eight experiments, and the standard errors of the means are indicated. All the buffers were of approximately the same molar strength; the pH was adjusted to 6·5 in them all.

The nature of the effect of the buffering agent on the reaction rate has not been further investigated.

The beneficial effect of 2:4:6-collidine when it was used as a buffering agent does not appear to be due to an effect similar to that produced by ether, as is shown in the next section.

Effect of ether. The effect of the addition of various volumes of diethyl ether to the collidine-buffered reaction system is shown in Fig. 2. Without added ether the reaction proceeds slowly and only 16% of the substrate was hydrolysed at the end of a 1 hr. reaction period. The maximum rate of hydrolysis was attained with 0·1 ml. of ether in the system, an amount almost sufficient to saturate the buffer.

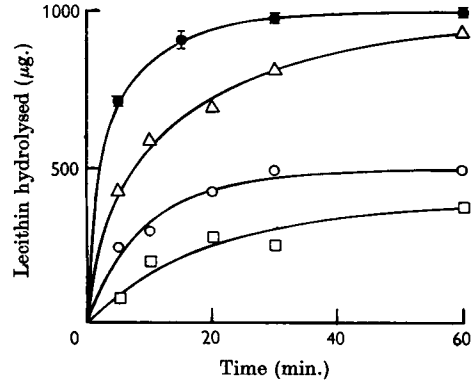

Fig. 1. Time course of hydrolysis of ovolecithin by moccasin-venom phospholipase A in different buffers at pH 6·5. Reaction mixtures contained: 1 mg. of ovolecithin in 1 ml. of buffer, 0·1 ml. of ether and 100 μg. of venom added in 0·05 ml. of 5 mM-$CaCl_2$. Temp. 30°. ●, 2:4:6-Collidine buffer, 0·05 M (mean ±S.E.M. for eight experiments); △, diethyl barbiturate buffer, 0·04 M; ○, triethanolamine buffer, 0·05 M; □, mean values obtained with phosphate (0·067 M), succinate (0·05 M), malonate (0·05 M) and tris–maleate (0·05 M) buffer.

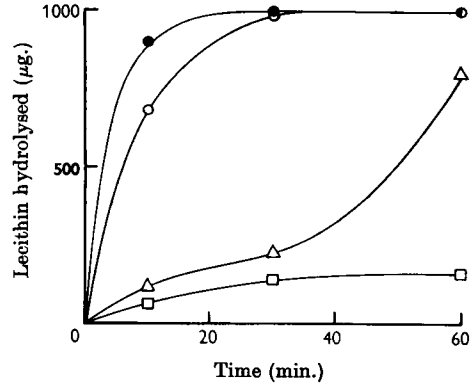

Fig. 2. Effect of added ether on the hydrolysis of ovolecithin by moccasin-venom phospholipase A. Reaction mixtures contained: 1 mg. of ovolecithin in 1 ml. of 2:4:6-collidine buffer (0·05 M, pH 6·5), 100 μg. of venom was added in 0·05 ml. of 5 mM-$CaCl_2$. □, No added ether; ○, with 0·05 ml. of ether; ●, with 0·10 ml. of ether; △, with 1·0 ml. of ether. Temp. 30°.

With 1·0 ml. of added ether the reaction velocity was initially low, but became more rapid after about 30 min. This was a biphasic system, with a layer of ether above the buffer. The peculiar nature of the curve can be explained largely on the basis of pH, as in such biphasic systems the ether gradually extracted the 2:4:6-collidine from the underlying buffer. The result was that the buffer became more acidic, finally stabilizing at about pH 5·5. Curves, similar to the one shown with 1·0 ml. of ether and 100 μg. of venom in the system, have been observed in experiments carried out in buffers at pH values slightly below 6. In an experiment with only 10 μg. of venom/sample, it was observed that hydrolysis in the biphasic system during the initial 30 min. reaction period was less than, but similar to, that in the curve shown (Fig. 2). However, during the 30–60 min. time period the hydrolysis stopped completely. This phenomenon also occurs in experiments conducted at pH values below 6 with 10 μg. of venom/sample.

The distribution of ovolecithin, venom and Ca^{2+} ions in a biphasic system is undoubtedly very complicated. Hanahan (1952) found that lecithin was extracted from buffer by ether in the form of a complex with the enzyme, in which the Ca^{2+} ions may also be bound (Long & Penny, 1957). The venom itself is not ether-soluble (Hanahan *et al.* 1954).

Some attempts were made to replace the ether with other reagents, all of which, however, interfered with the ester linkage determination. Deoxycholate, saponin and Tweens (Atlas Powder Co., U.S.A.) at a final concentration of 1 % in the buffer, or replacing the ether with dioxan gave extremely high blank readings. The addition of 0·1 ml. of either light petroleum (b.p. 40–60°) or 4-methylpentan-2-one to a suspension of ovolecithin (1 mg./ml.) in 2:4:6-collidine buffer decreased the final colour yield in the ester method by 25 %. 4-Methylpentan-2-one had earlier been found to be a good solvent in which to prepare lysolecithin from ovolecithin. Under comparable conditions the reaction velocity in the ketone was equal to that in ether; the reaction could be carried out at a temperature of 37°.

The mechanism by which ether, in appropriate concentration in the buffer, stimulates phospholipase A activity is obscure. It may be related, on an enzyme–substrate level, to the coalescence observed by Kates & Gorham (1957) of lecithin micelles and plant plastids in the presence of ether and certain other organic solvents.

Effect of pH. The time course of hydrolysis of ovolecithin by moccasin-venom lecithinase A at pH 6·0, 6·5 and 8·0 is shown in Fig. 3. At pH 6·0 there appeared to be a short lag period in the reaction between the 3 and 6 min. time intervals (observed in all experiments at this pH), after which the conversion of lecithin into lysolecithin progressed more rapidly. At pH 6·5 the reaction went smoothly to completion. The initial reaction velocity at pH 8·0 was greater than that at pH 6·5, but the rate of hydrolysis fell off more quickly, being only 85 % complete after 1 hr., with little or no change after a further 1 hr. incubation. At pH 6·5 there is no initial time lag in the hydrolysis, as there is in the ethereal reaction system (Long & Penny, 1957).

Fig. 4 shows that the initial reaction velocity was decreased at pH 5·5 and 6·0, but was almost constant from pH 6·5 to 8·5. This curve is very similar to that found with cholinesterase acting on acetylcholine (Laidler, 1955). It does not appear to agree with previous reports dealing with the effect of pH on snake-venom phospholipase A activity. Levene *et al.* (1924) stated that cobra venom was active between pH 6·5 and 7·5 and completely inactivated at pH 8·0. Hughes (1935) reported that the rate at which snake venoms attacked surface films of lecithin was optimum at pH 7·3. Hanahan (1952) suggested that the optimum range lay between

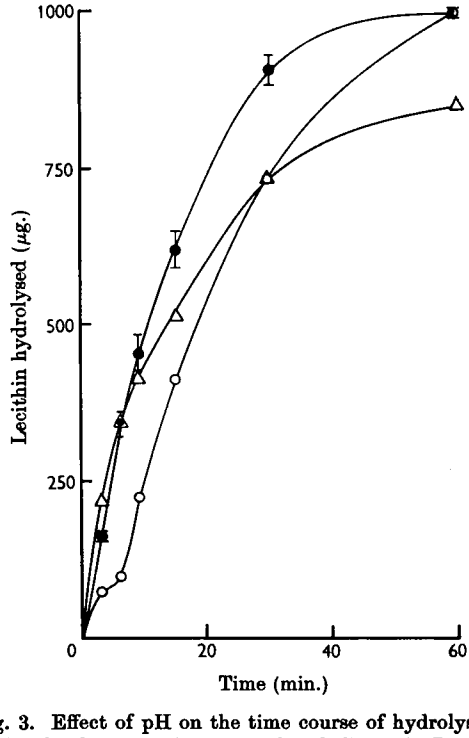

Fig. 3. Effect of pH on the time course of hydrolysis of ovolecithin by moccasin-venom phospholipase A. Reaction mixtures contained: 1 mg. of ovolecithin in 1 ml. of 2:4:6-collidine buffer (0·05 M), 0·1 ml. of ether and 10 μg. of venom added in 0·05 ml. of 5 mM-$CaCl_2$. Temp. 30°. ○, pH 6·0; ●, pH 6·5 (mean ±S.E.M. for eight experiments); △, pH 8·0.

pH 6·5 and 7·0. In all the previous work, however, initial reaction velocities were not determined and relatively long incubation periods were employed. Fig. 4 demonstrates that an apparent optimum at pH 6·5–7·0 is observed when the values obtained after a 30 min. reaction period are plotted. This is explained by the fact that the moccasin-venom phospholipase A is gradually degraded in solutions with a pH higher than 7·0, as is clearly shown in the curve for the preincubated enzyme preparations. Snake-venom phospholipase A is apparently quite stable in slightly acid solutions, but not in alkaline solution, confirming the early observations of Levene *et al.* (1924). Hughes (1935) reported that snake-venom phospholipase A is stable to prolonged boiling at pH 5·9, but is rapidly destroyed in solutions more alkaline than pH 7·0.

The high initial reaction velocities observed at the higher pH values are not the result of spontaneous hydrolysis of the substrate under these conditions. Emulsions of ovolecithin in 0·05 M-2:4:6-collidine buffer at pH 8·5 were quite stable during prolonged incubation at 30°.

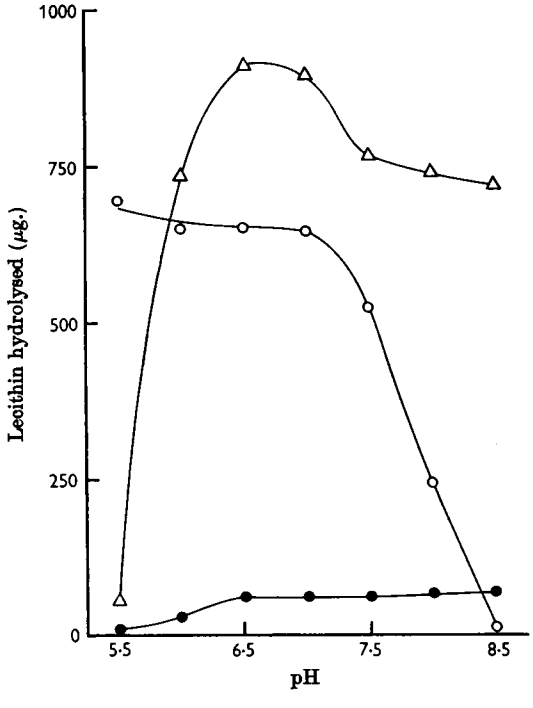

Fig. 4. Effect of pH on moccasin-venom phospholipase A. Reaction mixtures and conditions were as described in Fig. 3. ●, Effect of pH on initial reaction velocity (μg. of ovolecithin hydrolysed/min.); △, μg. of ovolecithin hydrolysed/30 min. at pH values given; ○, in these experiments, venom samples were preincubated in 0·05 M-2:4:6-collidine buffer for 4 hr. at the pH values given (temp. 30°), after which their activity was determined at pH 6·5; values shown are μg. of ovolecithin hydrolysed/15 min.

Effect of venom and substrate concentrations. With increasing venom concentrations in the reaction system the initial reaction velocity increased linearly at first (Fig. 5), fell off rather abruptly at a venom concentration of between 25 and 50 μg./sample, and then decreased gradually up to the highest concentration used (100 μg./sample). The non-linearity of the rate with increasing venom concentration may be due to some physical limitation in the system, for example availability of substrate to enzyme. With the lowest concentration of venom studied (1·25 μg./sample), the reaction did not go to completion, even after prolonged incubation.

The effect of substrate concentration on the initial reaction velocity is shown in Fig. 6. When the mean experimental values shown were replotted

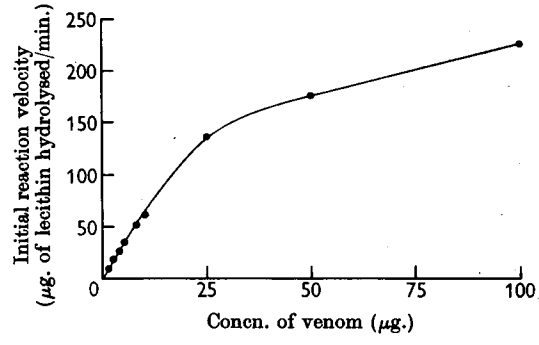

Fig. 5. Effect of venom concentration on initial reaction velocity. Reaction mixtures contained: 1 mg. of ovolecithin in 1 ml. of 2:4:6-collidine buffer (0·05 M, pH 6·5), and 0·1 ml. of ether. Venom was added in 0·05 ml. of 5 mM-$CaCl_2$. Temp. 30°.

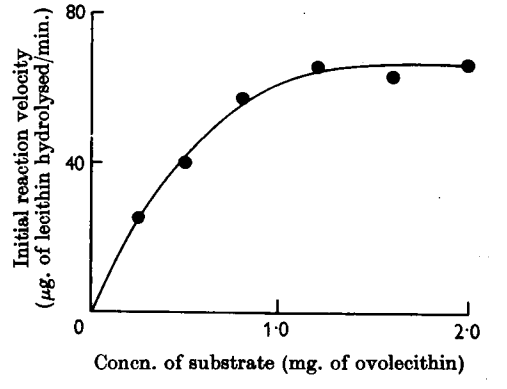

Fig. 6. Effect of substrate concentration on initial reaction velocity. Reaction mixtures contained: 1 ml. of ovolecithin emulsion (in 0·05 M-2:4:6-collidine buffer, pH 6·5), 0·1 ml. of ether and 7·5 μg. of venom added in 0·05 ml. of 5 mM-$CaCl_2$. Temp. 30°.

according to the method of Lineweaver & Burk (1934) a straight line was obtained. A Michaelis constant (K_m) of $1{\cdot}1 \times 10^{-3}$ M was calculated from the data.

Effect of Ca^{2+} *ions.* Fig. 7 shows that when $CaCl_2$ solution was not added to the reaction mixture, the rate of conversion of ovolecithin into lysolecithin was less rapid than that in a medium containing added Ca^{2+} ions. The addition of oxalate (final concentration 0·5 mM) to the $CaCl_2$-free samples lengthened the initial lag phase. The effect was more pronounced in the presence of 0·2 mM- and 0·5 mM-citrate. Ethylenediaminetetra-acetic acid (EDTA) produced a marked inhibition of the enzymic reaction at a concentration of 0·05 mM.

It was clear from the results that the system contained more Ca^{2+} ions than had been added in the $CaCl_2$ solution. Oxalate, at a concentration of 0·2 mM (not shown in Fig. 7) did not inhibit the enzymic reaction at all. The much greater inhibition produced by citrate as compared with that produced by an equivalent concentration of oxalate cannot be explained entirely on the basis of a higher calcium-binding capacity. This suggests that citrate has some other inhibitory action. It was noted above that certain buffering agents were much less satisfactory than others as reaction media. The ionic strength of the medium may play some part here, as 0·1 M-NaCl in the collidine-buffered reaction system produced an inhibition of over 50 %.

Assuming that the Ca^{2+} ion concentration in the reaction system is of the order of 0·5 mM, it is impossible to explain the marked inhibition produced by 0·05 mM-EDTA on the basis of its calcium-binding capacity alone. Possibly some other ion is required as a cofactor, or else EDTA has a direct inhibitory effect on the enzyme.

Washing the ovolecithin by the method of Folch *et al.* (1957) greatly increased the sensitivity of the assay system to added Ca^{2+} ions. Fig. 8 illustrates the dependence of initial reaction velocity (μg. of ovolecithin hydrolysed/min.) on the amount of $CaCl_2$ added to the samples. The optimum concentration of added Ca^{2+} ions in the system was 0·5 mM. Higher concentrations brought about a considerable inhibition. For example, the initial reaction velocity was decreased to 19 μg. of ovolecithin hydrolysed/min. in the presence of 150 mM-$CaCl_2$.

The calcium content (if any) of the washed ovolecithin preparation was not determined, but

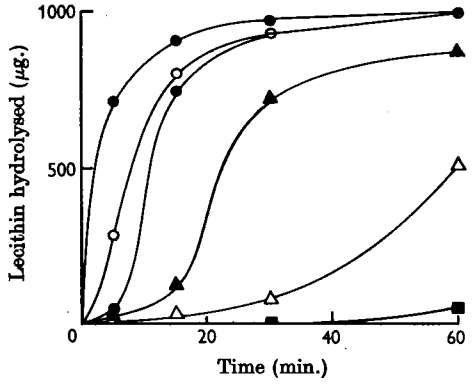

Fig. 7. Effect of Ca^{2+} ions and Ca^{2+}-complexing agents on the enzymic hydrolysis of ovolecithin by moccasin-venom phospholipase A. Reaction mixtures contained: 1 mg. of ovolecithin in 1 ml. of 2:4:6-collidine buffer (0·05 M, pH 6·5), 0·1 ml. of ether and 100 μg. of venom added in 0·05 ml. of either water or $CaCl_2$ solution. Other reagents were added individually in a volume of 0·02 ml. ●, 0·22 mM-$CaCl_2$; ○, no added $CaCl_2$; ⊕, no added $CaCl_2$, 0·5 mM-oxalate; ▲, no added $CaCl_2$, 0·2 mM-citrate; △, no added $CaCl_2$, 0·5 mM-citrate; ■, no added $CaCl_2$, 0·05 mM-EDTA. Temp. 30°.

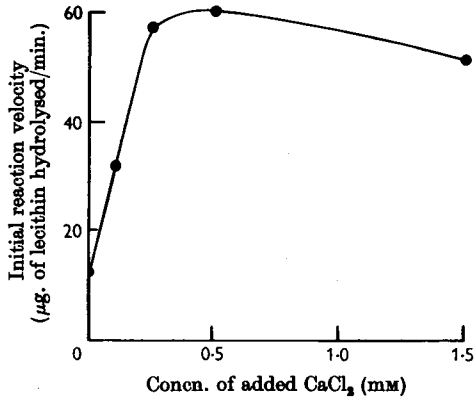

Fig. 8. Dependence of initial reaction velocity on the concentration of Ca^{2+} ions. Reaction mixtures contained: 1 mg. of ovolecithin (washed by the method of Folch, Lees & Sloane-Stanley, 1957) in 1 ml. of 2:4:6-collidine buffer (0·05 M, pH 6·5), 0·1 ml. of ether and 10 μg. of venom added in 0·05 ml. of either water or $CaCl_2$ solution. Temp. 30°.

Table 1. *Effect of certain metallic cations on the hydrolysis of ovolecithin by moccasin-venom phospholipase* A

Reaction mixtures contained: 1 mg. of washed ovolecithin in 1 ml. of 2:4:6-collidine buffer (0·05 M, pH 6·5), 0·1 ml. of ether and 10 μg. of venom. Other reagents were added in a volume of 0·02 ml. Incubation was at 30° for 30 min.

Concn. in medium of added cation (mM)	Lecithin hydrolysed (μg.)
0	47
0·5 Ca^{2+}	846
0·5 Mg^{2+}	0
0·5 Ca^{2+} + 0·5 Cu^{2+}	38
0·5 Ca^{2+} + 0·5 Zn^{2+}	173

Table 2. *Effectiveness of chloroform–methanol treatment in the extraction of ovolecithin from protein-containing mixtures*

Sample contents (in 1 ml. of collidine buffer)			Colour yield ($E_{525\,m\mu}$) Expt.		
Ovolecithin (mg.)	Pancreatin (mg.)	Assay procedure	A	B	C
0	25	Direct	0·996	1·004	1·069
0	25	Chloroform–methanol	0·031	0·033	0·034
1	0	Direct	0·462	0·477	0·452
1	0	Chloroform–methanol	0·464	0·472	0·451
1	25	Chloroform–methanol	0·439	0·460	0·438

certainly it would appear from the results to be lower than that in the untreated substrate. The concentration of calcium in the moccasin venom is not known. Under the conditions described for Fig. 8, only 15 % (150 μg.) of the ovolecithin in each sample was converted into lysolecithin after a 1 hr. incubation period when no $CaCl_2$ solution was added. The effect of added $CaCl_2$ on the washed ovolecithin preparation is also shown in Table 1.

$MgCl_2$ (0·5 mM) not only did not replace 0·5 mM-$CaCl_2$, but brought about a definite inhibition (Table 1). Confirming the results of Long & Penny (1957) both $CuCl_2$ and $ZnCl_2$ inhibited phospholipase A. The above authors reported that NaCl, KCl, $BaCl_2$, $SrCl_2$, $MgCl_2$ and $CdCl_2$ were unable to replace $CaCl_2$ in the ether reaction system.

Phospholipase A activity of commercial pancreatin. Commercial pancreatin was selected as a convenient source of phospholipase A which could be used to adapt the method to the estimation of enzyme activity in crude preparations. However, a preliminary experiment soon showed that the presence of large amounts of pancreatin in the reaction system interfered markedly with the subsequent ester linkage determination. Attempts were made with various organic solvents to extract the ovolecithin or lysolecithin or both, and not the pancreatin, from the reaction system. Finally the addition of 4 vol. of chloroform–methanol (2:1, v/v) proved satisfactory. This results in a biphasic system in which both ovolecithin and lysolecithin are quantitatively extracted into the organic phase; the pancreatin remains in the upper phase or at the boundary between the two phases. The addition of the organic solvents stops the enzymic reaction immediately.

The effectiveness of the chloroform–methanol treatment can be assessed from Table 2. In these experiments, the colour yield was determined by the ester linkage method either directly in the original samples (direct assay) or after chloroform–methanol treatment as described fully under Methods. Pancreatin alone gave a very high colour yield by the direct assay method which was decreased to a reasonable level after chloroform–methanol partitioning. Table 2 also shows that ovolecithin standards, either with or without added pancreatin, gave approximately the same colour yield (after appropriate blank and sampling corrections) after partitioning as did ovolecithin standards in which the colour was developed directly. The presence of 25 mg. of pancreatin in the samples decreased the colour yield of ovolecithin slightly, but as this effect was reasonably consistent in any one set of determinations with mixtures of lecithin and lysolecithin it was not considered to be a serious fault. With less pancreatin in the samples this effect became negligible. It was possible to use the partitioning method successfully with as much as 100 mg. of pancreatin in each sample.

Table 3. *Time course of hydrolysis of ovolecithin by pancreatin suspensions*

Reaction mixtures contained: 1 mg. of ovolecithin plus 25 mg. of pancreatin in 1 ml. of 2:4:6-collidine buffer (0·05 M, pH 6·5), 0·1 ml. of ether and 0·05 ml. of 5 mM-$CaCl_2$. Samples were incubated at 30° with constant shaking.

Reaction period (min.)	Lecithin hydrolysed (μg.) Expt. A	Expt. B
15	150	115
30	275	230
60	500	440
90	650	625
120	763	785

The time course of hydrolysis of ovolecithin by pancreatin suspensions is shown in Table 3. A plot of mean values was used to determine an initial reaction velocity of 8·9 μg. of lecithin hydrolysed/min. Comparing the phospholipase A content of moccasin venom with that of pancreatin on a specific activity basis (μg. of ovolecithin hydrolysed/hr./mg. dry wt.), it was calculated that the venom is 17 000 times as active as the pancreatin.

DISCUSSION

The method outlined, in which phospholipase A activity is determined in a reaction system containing ovolecithin in ether containing 2:4:6-collidine buffer, has been shown to be of practical use. In the presence of an active source of the enzyme, such as

the venom of the cottonmouth moccasin, the conversion of lecithin into lysolecithin proceeds rapidly and smoothly to completion under appropriate conditions. The method is simple and sensitive, and requires only small amounts of substrate. There is no lag phase in the reaction, which simplifies the determination of initial reaction velocities for kinetic purposes. This is an advantage which the buffer system has over the ether reaction system, as Long & Penny (1957) found in their experiments that there was an appreciable time lag at the start of the enzymic reaction in ether. The adjustment of pH in the aqueous buffer system is not a problem.

The degree of reproducibility of the acyl ester determination can be assessed from the fact that after the reaction is complete (e.g. after an incubation period of 1 hr. with 1 mg. of ovolecithin and 100 μg. of venom in 2:4:6-collidine buffer and other conditions as described in Fig. 1), the amount of ovolecithin hydrolysed was 1000 ± 10 μg. or 100 ± 1% (mean $\pm$ S.E.M. for eight experiments).

A comparison of the reaction velocity in the system described with that obtained by other workers is complicated by the multiplicity of different reaction conditions and assay techniques used. The slow rate of hydrolysis in ether-free buffers has already been discussed. The results of Long & Penny (1957) show that in a reaction system containing 1·5–2·0 μmoles of lecithin in 1 ml. of ether and 10 μg. of moccasin venom added in 0·01 ml. of 5 mM-calcium chloride the substrate was hydrolysed to the extent of approx. 70% after 30 min. at a temperature of 18°. With 1·2 μmoles of ovolecithin in 1 ml. of 2:4:6-collidine buffer (0·05 M, pH 6·5), 10 μg. of moccasin venom added in 0·05 ml. 5 mM-calcium chloride and 0·1 ml. of ether, 91% of the ovolecithin was converted into lysolecithin after 30 min. at a temperature of 30° (Fig. 3). Reaction rates in the two systems thus would appear to be of the same order of magnitude.

Increasing the venom concentration increased the rate of the reaction. The effect was linear with venom concentrations of from 1·25 to almost 25 μg./sample, but fell off at higher concentrations (Fig. 5). The method is applicable, therefore, to the assay of phospholipase A activity over a useful range.

With a venom concentration of 7·5 μg. and substrate concentrations ranging from 0·25 to 2·00 mg./sample a Michaelis constant of 1·1 mM was determined. Hanahan *et al.* (1954) found a K_m of 10 mM for *Naia naia* venom, but as the experimental conditions and enzyme source were different, it is not correct to compare the two values. Long & Penny (1957) noted that the venom of the cottonmouth moccasin showed a significantly higher phospholipase A activity than did *Naia naia* venom.

A requirement for Ca^{2+} ions by phospholipase A preparations from various sources has been noted previously (e.g. see Rezek, 1945; Zamecnik *et al.* 1947; Zeller, 1950; Hayaishi & Kornberg, 1954; Long & Penny, 1957; Rimon & Shapiro, 1959). The stimulation of moccasin-venom phospholipase A by Ca^{2+} ions was first studied in detail by Long & Penny (1957), who reported that the requirement for Ca^{2+} ions depended on the concentration of lecithin employed. They calculated that each μmole of ovolecithin in an ether reaction system required about 0·03 μmole of Ca^{2+} ions for maximal enzymic hydrolysis. The optimum concentration ratio of added calcium chloride in the system described in this paper was about 0·4 μmole of Ca^{2+} ions/μmole of lecithin. That more calcium chloride had to be added to the aqueous buffer system than to the ether system for maximum activity is not, perhaps, surprising, as it is unlikely that the distribution of Ca^{2+} ions in the two systems is comparable. In the ether system Long & Penny (1957) suggested that the calcium takes part in the binding of the enzyme with the substrate, and they observed that no calcium remained in the ether supernatant after the reaction was complete.

Commercial pancreatin, which on a dry-weight basis has only 0·007% of the phospholipase A activity of moccasin venom, was used to test the applicability of the method to the detection of phospholipase A activity in crude enzyme preparations. It is hoped that the method described can be used as a basis for a tissue survey of phospholipase A.

SUMMARY

1. A study has been made of the enzymic degradation of ovolecithin by snake-venom phospholipase A in a number of different buffered media.

2. A system containing 2:4:6-collidine buffer and ether is described in which the conversion of lecithin into lysolecithin proceeds rapidly to completion.

3. The course of the reaction was followed by determining the decrease in acyl ester bonds. The substrate itself was used as a standard in the method described.

4. The effect of pH, venom concentration and substrate concentration on the activity of snake-venom phospholipase A has been investigated. A Michaelis constant for the enzymic reaction was determined.

5. The optimum concentration of added Ca^{2+} ions in the reaction system was 0·5 mM.

6. The aqueous assay system has been adapted to the detection and estimation of phospholipase A activity in a crude tissue preparation (commercial pancreatin). It was calculated on a dry weight basis that the phospholipase A activity of the venom of the cottonmouth moccasin was 17 000 times as great as that of pancreatin.

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The Fractionation of Phosphate Esters on Ion-Exchange Resin by a new System of pH-Gradient Elution

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During a study of division lag in bacterial cultures an attempt was made to compare the acid-soluble phosphates from resting cells of *Escherichia coli* with those from rapidly dividing cells and also to compare the processes of ribonucleic acid degradation. A scheme of analysis on filter paper was devised for this purpose (Wade & Morgan, 1955) but, upon applying it to acid-soluble extracts, the constituents were found to be too numerous to fractionate and isolate in this way. Ion-exchange techniques were considered as an alternative method of achieving this.

The technique most commonly used is essentially that applied by Cohn (1950) to the fractionation of ribonucleoside 2′(3′)-phosphates. It consists of passing a solution of the phosphates through an anion-exchange resin at a pH which strongly favours adsorption and then to elute them, in order of increasing affinity for the resin, with a solution (eluent) of lower pH. It is usual to employ several eluents with progressively higher salt concentration and lower pH (Cohn & Volkin, 1953). The separation of very dissimilar phosphates is greatly simplified by changing the composition of the eluent continuously, e.g. Hurlbert, Schmitz, Brumm & Potter, 1954; Pontis & Blumson, 1958. A disadvantage of such systems is that the useful range of conditions is usually very limited and sometimes necessitates a discontinuous change in the elution programme (Hurlbert *et al.* 1954).

The system of pH-gradient elution described in this paper avoids these limitations by providing a uniform fall in eluent pH between wide limits of pH and rate of pH change. This has enabled the effects of changing the rate of pH change and the eluent salt concentration upon the fractionation of a standard mixture of ribonucleoside 2′(3′)-phosphates to be examined. The practical information gained in this way has been summarized and used to select the best conditions for the fractionations of nucleoside polyphosphates and nucleoside 2′:3′-cyclic phosphates.