# Phospholipase B in Snake Venoms and Bee Venom

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During a study of the action of phospholipase A on phospholipids in ox-brain homogenate by quantitative paper chromatography, we have observed that the lyso compounds formed accounted for only 65% of the phosphorus of the phospholipids hydrolysed. The remainder of the phosphorus-containing material was found close to the origin [solvent: di-isobutyl ketone–acetic acid–water (8:5:1, by vol.); Marinetti, Albrecht, Ford & Stotz (1959)]. A compound that gave a positive stain for choline (Chargaff, Levine & Green, 1948) was found in this region in the position of a control of glycerophosphorylcholine. Such a product could have resulted from the action of the phospholipase B, which has been found in low concentrations in brain tissue (Dawson, 1956; Marples & Thompson, 1960), or from the action of a similar enzyme in the venom of *Pseudechis porphyriacus* (black snake) from which the phospholipase A had been prepared (Doery & Pearson, 1961). Since no significant phospholipase B activity was detected in the brain homogenate in the absence of venom under our experimental conditions, the possibility that the venom of *P. porphyriacus* contained phospholipase B was considered. The enzyme was subsequently identified in this venom and a number of other venoms.

The present paper describes the identification, properties and distribution of phospholipase B in certain snake venoms and bee venom in detail.

## EXPERIMENTAL

*Venoms.* Snake venoms were obtained either from E. Worrell, Wyoming, Gosford North, N.S.W., Australia, or from Ross Allen's Reptile Institute, Fla., U.S.A. Bee venom was a freeze-dried preparation of bee stings.

*Lecithin.* Dimyristoyl-lecithin (synthetic) was obtained from La Motte Chemical Products Co., Chesterton, Md., U.S.A. Egg lecithin was prepared from egg yolk by the method described by Saunders (1957). Emulsions of lecithins (6 mM) which were stable at 37° were prepared by mixing dimyristoyl-lecithin in water at room temperature and by shaking egg lecithin in water at 37°. Portions of these were used as substrates as required.

*Lysolecithin.* Crystalline lysolecithin was prepared from egg lecithin as described by Saunders (1957).

*Buffer.* Unless otherwise stated, hydrolysis of lecithin and lysolecithin was carried out in 0·1 M-ammonium acetate buffer, obtained by the addition of the appropriate amount of 0·3 M-ammonium acetate buffer.

*Hydrolysis of lysolecithin by venom.* Mixtures of lysolecithin (2·5 $\mu$moles) and crude venom (25 $\mu$g.) were held at 37° for 1 hr. in 0·65 ml. of buffer (pH 4–11) containing $CaCl_2$ (4 mM). The reaction was followed by ester group analysis (Shapiro, 1953). In reaction mixtures at and above pH 9, interference with the subsequent ester group analysis was overcome by dilution of the mixture with 0·65 ml. of water after the addition of ethanol–ether (3:1, v/v) to stop the reaction.

Hydrolysis of lysolecithin in the absence of venom was determined over the pH range 3–13. For hydrolysis above pH 11, the reaction was carried out in NaOH solutions, and the pH determined with a Radiometer type M4b meter.

*Hydrolysis of lecithin by venom.* The action of both phospholipase A and B of venom was demonstrated by the hydrolysis of dimyristoyl-lecithin (1·5 $\mu$moles) by venom (0·5–50 $\mu$g.) in 0·65 ml. of buffer, in the pH range 5·5–9·5, containing $CaCl_2$ (4 mM). Hydrolysis was followed, as described above, by ester group analysis. For the hydrolysis of egg lecithin in ether, or dimyristoyl-lecithin in ether containing 10% (v/v) of ethanol, 20 $\mu$l. of a solution of venom in 0·3 M-ammonium acetate buffer containing $CaCl_2$ (12 mM) was added to 0·75 ml. of ether containing 2 $\mu$moles of lecithin. The mixture was shaken well for 10 sec. and then kept at 25° for 15 min. After the addition of 2·25 ml. of ethanol to stop the reaction, hydrolysis was followed as described above. The hydrolysis of lecithin indicated phospholipase A activity, and, when this exceeded 50%, phospholipase B activity was indicated also.

*Identification of the reaction products of the hydrolysis of lysolecithin.* At the end of the incubation, the reaction mixture at pH 9·5 was freeze-dried and extracted three times with chloroform (1 ml.), and the residue was dispersed in water. The aqueous solution was examined by paper chromatography (descending) in the solvent ethyl methyl ketone–*n*-butanol saturated with water–propan-1-ol–(98–100%) formic acid (9:5:1:4, by vol.) (Gerlach, Weber & Döring, 1955), and also by paper electrophoresis with pyridine–acetic acid–water buffer (25:1:225, by vol.). Glycerophosphorylcholine, sodium glycerophosphate and phosphorylcholine were used as markers.

In other experiments the reaction mixture was acidified with HCl at the end of the incubation, then freeze-dried and extracted with light petroleum (b.p. 40–60°). This was dialysed in a rubber membrane against light petroleum (van Beers, de Iongh & Boldingh, 1958) and the diffusate titrated with 2·5 mN-NaOH.

## RESULTS

*Hydrolysis of lysolecithin by the venom of* Pseudechis porphyriacus. In the aqueous extract of the reaction mixture a phosphorus-containing compound was found on paper chromatography to be

indistinguishable from glycerophosphorylcholine or sodium glycerophosphate, but to separate from phosphorylcholine. On paper electrophoresis the product migrated slowly along with glycerophosphorylcholine but separated from faster-migrating sodium glycerophosphate. Free fatty acid was found in the light petroleum diffusate from the reaction product.

These findings were consistent with the action of a phospholipase B.

Further studies of the enzyme were carried out at temperatures ranging from 25° to 80°; hydrolysis increased with temperature up to 55°, where the rate of hydrolysis was 20–25 % greater than at 37°.

*Effect of* pH *on the phospholipase B activity of the venoms of* Pseudechis porphyriacus, Naia naia *and* Agkistrodon piscivorus. Fig. 1 shows that, with 25 μg. of the venom of *Pseudechis porphyriacus*, hydrolysis of lysolecithin occurred in ammonium acetate buffer over the whole pH range 5·5–11. On the other hand, 25 μg. of the venoms of *Agkistrodon piscivorus* and *Naia naia* hydrolysed lysolecithin only above pH 8, and to a smaller extent than the venom of *Pseudechis porphyriacus*. In other experiments, with as much as 100 μg. of the venom of *Naia naia*, only 10 % of the lysolecithin was hydrolysed at pH 8. With each venom maximum hydrolysis occurred between pH 9·5 and 10.

In the pH range where ammonium acetate has the least buffering effect, namely pH 6–8, hydrolysis of lysolecithin by the venom of *Pseudechis porphyriacus* was examined in 0·05 M-collidine–hydrochloric acid buffer also. Between pH 6·5 and 8 hydrolysis in the latter buffer was greater than that found in the ammonium acetate buffer. Magee & Thompson (1960) found that 0·05 M-collidine–hydrochloric acid buffer, pH 6·5, enhanced the activity of phospholipase A above that found in a variety of other buffers at the same pH. However, at pH 8·4, in the buffer containing the highest concentration of free collidine, the activity of phospholipase B was depressed below that found in ammonium acetate at the same pH.

The hydrolysis of lysolecithin by other venoms showed maxima in the alkaline range between pH 8·5 and 10. Such hydrolysis was not due to the effect of pH alone since, in the absence of venom, hydrolysis occurred only below pH 4 and above pH 11 (Fig. 1).

The activity of phospholipase B was examined in the presence of phospholipase A, in the venoms of *Pseudechis porphyriacus* and *Naia naia*, by using aqueous emulsions of lecithin as substrate. Synthetic dimyristoyl-lecithin was used to eliminate any variation in the rate at which different fatty acids were hydrolysed by these enzymes. It was found that, with both venoms, phospholipase A was active over the pH range 5·5–9·5 (Figs. 2 and 3). Further, at pH 5·5 when the concentration of the venom of *Pseudechis porphyriacus* was increased from 1 to 50 μg., hydrolysis remained at a constant level near 50 % (Fig. 2). Though less than 50 % hydrolysis does not preclude the action of phospholipase B, since hydrolysis remained constant over the 50-fold concentration range it is unlikely that any significant phospholipase B activity had occurred under these conditions. However, between pH 6·5 and 9·5 more than 50 % hydrolysis occurred, indicating the action of phospholipase B in addition to that of phospholipase A. In contrast, with increasing concentra-

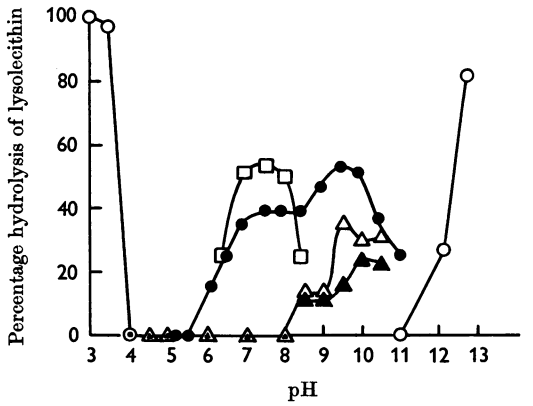


Fig. 1. Effect of pH on the hydrolysis of lysolecithin (2·5 μmoles) by venom (25 μg.) at pH 4–11 at 37° for 1 hr. ●, *Pseudechis porphyriacus*; □, *P. porphyriacus* in 0·1 M-collidine–HCl buffer; △, *Agkistrodon piscivorus*; ▲, *Naia naia*; ○, no venom added.

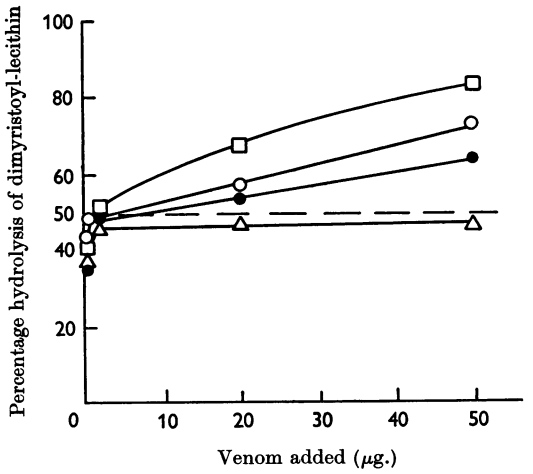


Fig. 2. Effect of concentration of the venom of *Pseudechis porphyriacus* (0·5–50 μg.) on the hydrolysis of dimyristoyl-lecithin (1·5 μmoles) in the range pH 5·5–9·5 at 37° for ½ hr. △, pH 5·5; ●, pH 6·5; ○, pH 7·5; □, pH 9·5.

tions of the venom of *Naia naia*, hydrolysis did not significantly exceed 50 % until the pH of the mixture was greater than pH 8 (Fig. 3). Thus the range of pH over which lysolecithin was hydrolysed by these two last-named venoms was unaltered by the presence of lecithin.

On the other hand, when dimyristoyl-lecithin was hydrolysed in ether by the venom of *Pseudechis porphyriacus*, under the conditions described above, there was no indication of phospholipase B activity, since hydrolysis remained constant at approx. 50 % over a wide range of both venom concentration and time of hydrolysis.

With egg lecithin as substrate similar results but lower activities were observed. The lower activities could be explained by the fact that unsaturated fatty acids, which occupy the $\beta$-position in egg lecithin, are less readily hydrolysed than saturated fatty acids by phospholipase A (Hanahan, Rodbell & Turner, 1954).

*Effect of concentration of phospholipase B of the venom of* Pseudechis porphyriacus *and the time of incubation at* 37° *on the hydrolysis of lysolecithin at* pH 9·5. A linear relationship was found between the hydrolysis of lysolecithin (2·5 $\mu$moles) and the concentration of venom between 5 $\mu$g. and 30 $\mu$g., measured at 37° and pH 9·5. A typical enzymic relationship was found between the extent of hydrolysis of lysolecithin and the time of incubation at 37° (Fig. 4).

*Stability of phospholipase B to heat.* The enzyme in the venom of *Pseudechis porphyriacus* was very resistant to heat. Even after the venom (0·02 %) in 0·15 M-sodium chloride had been kept at 100° at pH 7 for periods of 1 and 2 hr., 35 and 21 % respectively of the original activity remained.

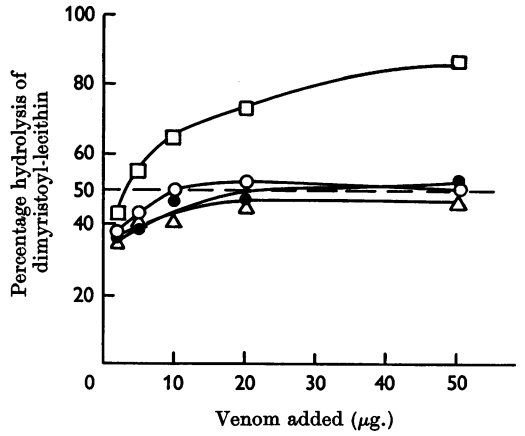


Fig. 3. Effect of concentration of the venom of *Naia naia* (2–50 $\mu$g.) on the hydrolysis of dimyristoyl-lecithin (1·5 $\mu$-moles) in the pH range 5·5–9·5 at 37° for $\frac{1}{2}$ hr. △, pH 5·5; ●, pH 6·5; ○, pH 7·5; □, pH 9·5.

*Effect of* $Ca^{2+}$ *ions,* $Mg^{2+}$ *ions and ethylenediaminetetra-acetate on the activity of phospholipase B.* The activating effect of both $Ca^{2+}$ and $Mg^{2+}$ ions and the inhibiting effect of EDTA on the hydrolysis of lysolecithin by venom is demonstrated in Fig. 5. The $Ca^{2+}$ ions were more effective than $Mg^{2+}$ ions as an activator.

*Relative phospholipase B activity of venoms.* Table 1 shows the phospholipase B activities of venoms at pH 9·5, relative to that of the venom of *Pseudechis porphyriacus*. All the snake venoms we examined, as well as bee venom, contained phospholipase B.

The venoms of the Australian snakes, with the exception of the venom of *Acanthophis antarticus*, proved to be the most active sources of phospholipase B, and activity in each case was detected at

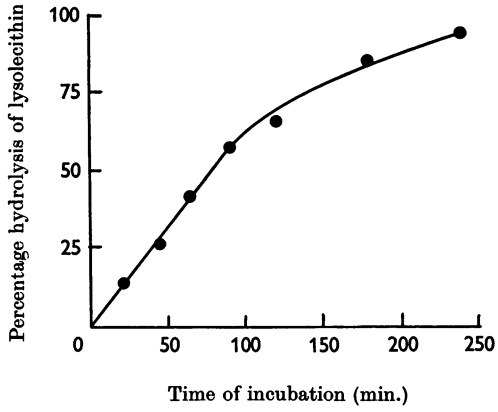


Fig. 4. Effect of time of incubation on the hydrolysis of lysolecithin (2·5 $\mu$moles) by the venom of *Pseudechis porphyriacus* (20 $\mu$g.) at pH 9·5 at 37°.

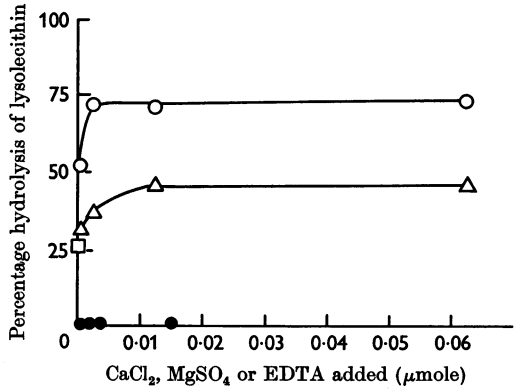


Fig. 5. Effect of $Ca^{2+}$ ions, $Mg^{2+}$ ions and EDTA on the hydrolysis of lysolecithin (2·5 $\mu$moles) by the venom of *Pseudechis porphyriacus* (20 $\mu$g.) at pH 9·5 at 37° for 1 hr. ○, $CaCl_2$; △, $MgSO_4$; □, no activator added; ●, EDTA.

Table 1. *Phospholipase B activity of venoms, expressed as a percentage of that of the venom of* Pseudechis porphyriacus

Experimental details are given in the text.

| Venom | Phospholipase B activity (%) |
|---|---|
| *Pseudechis porphyriacus* | 100 |
| *P. australis* | 112 |
| *Denisonia superba* | 112 |
| *Oxyuranus scutellatus* | 64 |
| *Notechis scutatus* | 57 |
| *Agkistrodon piscivorus* | 55 |
| *Naia naia* | 52 |
| *Vipera russelli* | 29 |
| *Acanthophis antarticus* | 21 |
| Bee venom | 13 |
| *Crotalus adamanteus* | 10 |

and above pH 6. The activities of the venoms of *Agkistrodon piscivorus*, *Naia naia*, *Vipera russelli*, *Acanthophis antarticus*, *Crotalus adamanteus* and bee were detected only at and above pH 8, and ranged from 55 to 10 % of the activity of the venom of *Pseudechis porphyriacus*.

## DISCUSSION

Enzymes with phospholipase B activity are known to occur in certain moulds and animal tissues with the maximum activity at pH 3·5–4·4 and at pH 6·0–6·5 respectively (Kates, 1960). In contrast, the enzyme identified in snake venoms and bee venom showed maximum activity in the alkaline region pH 8·5–10. The phospholipase B of venoms, like phospholipase A from the same source, is extremely stable to heat, and is activated by both $Ca^{2+}$ and $Mg^{2+}$ ions. These properties contrast with those of phospholipase B of rat liver, which is less stable to heat and inactivated by $Ca^{2+}$ and $Mg^{2+}$ ions (Dawson, 1956).

Venoms have been commonly used as sources of phospholipase A for the hydrolysis of phospholipids in aqueous solutions at a pH near neutrality. Since the venoms most frequently used, namely those of *Naia naia*, *Agkistrodon piscivorus* and *Crotalus adamanteus*, have little or no phospholipase B activity at this pH, it is not surprising that this enzyme has not been reported previously in these venoms. Nevertheless, phospholipase B activity has been detected in certain venoms of Australian snakes at a pH as low as 5·5, and activity at pH 7 is certainly significant. Further, since the demonstration that phospholipase A of venoms was activated by ether, studies of the positional specificity of this enzyme for the fatty acids of lecithin (Tattrie, 1959) have been chiefly carried out in ether, and with the assumption that phospholipase B was not present. This assumption seems justified for the venom of *Pseudechis porphyriacus* under the conditions of hydrolysis used. The inactivity of phospholipase B of the venom in ether is in keeping with the insolubility of lysolecithin in this solvent, and the inhibition of phospholipase B activity of liver by ether (Dawson, 1956).

The relatively high phospholipase A activity of a number of venoms of Australian snakes has already been reported (Doery & Pearson, 1961). It is now apparent that these venoms have phospholipase B activity also. In the earlier study of the haemolysins of venoms, phospholipase A was separated from the venom of *Pseudechis porphyriacus* in three fractions. All three fractions have now been shown to contain phospholipase B activity also. No attempt has yet been made to separate these activities.

## SUMMARY

1. Phospholipase B has been identified and its activity determined in the venom of a variety of snakes and in bee venom.

2. The pH of optimum activity of this enzyme in the venoms examined was between pH 8·5 and 10.

3. The enzyme showed considerable stability at 100° at pH 7 and was activated by both $Ca^{2+}$ and $Mg^{2+}$ ions.

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