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ISOELECTRIC ANALYSIS OF SOME AUSTRALIAN ELAPID SNAKE VENOMS WITH SPECIAL REFERENCE TO PHOSPHOLIPASE B AND HEMOLYSIS

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A. W. BERNHEIMER, S. A. WEINSTEIN, and R. LINDER. Isolectric analysis of some Australian elapid snake venoms with special reference to phospholipase B and hemolysis. *Toxicon* **24** 841 – 849, 1986. —Venoms of the Australian elapid snakes *Austrelaps superbus* and *Pseudechis colletti* were analyzed in an electrofocusing column. *A. superbus* venom, little studied in the past, was found to have a mouse i.p. lethal potency of 0.48 mg/kg and to contain at least four lethal components. Venoms of both species had relatively high direct hemolytic activity for washed rabbit erythrocytes, as contrasted with venoms from 23 other species of snakes that were not hemolytic under the conditions used. Among venoms of the same 25 species, those of *A. superbus* and *P. colletti* produced turbidity in diluted egg yolk, those of *Bungarus caeruleus* and *Bungarus multicinctus* were quantitatively less active on egg yolk, whereas venoms of the 21 remaining species were negative. The component of the venoms responsible for egg yolk reactivity was partially purified and the preparations obtained were strongly active when tested with diluted egg yolk or with erythrocytes. Thin layer and paper chromatographic studies showed that these preparations possessed phospholipase B activity for phosphatidylcholine, lysophosphatidylcholine, phosphatidylethanolamine and phosphatidylserine, but sphingomyelin was not degraded. The results suggest that hydrolysis of phosphatidylcholine is responsible for both egg yolk reactivity and hemolysis.

INTRODUCTION

THE VENOM of the common Australian elapid snake *Austrelaps superbus* (COGGER, 1956), formerly *Denisonia superba* (RAWLINSON, 1965), has received very little attention. We observed, as did DOERY and PEARSON (1964), that the venom of this snake is directly lytic for washed rabbit erythrocytes, and we also observed that hemolysis is of the 'hot–cold' type (for discussion of the mechanism of this phenomenon see AVIGAD, 1976). This suggested that the venom component responsible for hemolysis could be a phospholipase, an inference supported by finding that the venom produced turbidity after addition to a dilute solution of egg yolk. The latter observation suggested that the venom component producing these effects is a phospholipase B or C acting on phosphatidylcholine, and not phospholipase A which causes clearing of egg yolk (MARINETTI, 1965). Further experiments indicated that hemolysis and egg yolk turbidity may result from the same initial biochemical reaction, namely the hydrolysis by phospholipase B of phosphatidylcholine to fatty acids and glycerophosphorylcholine.

Using the egg yolk reaction as a detector of phospholipase B (PLB) activity, we surveyed venom samples from 24 additional species of snakes. An appreciable amount of enzyme(s) was found in the venom of *Pseudechis colletti*, and relatively small amounts in the venoms of *Bungarus multicinctis* and *Bungarus caeruleus*.

MATERIALS AND METHODS

Sources of venoms

Dried venoms of *A. superbus* and *P. colletti* were purchased from Sigma Chemical Co., St. Louis, MO. Samples of *A. superbus* venoms from specific localities were the generous gift of Sherman A. Minton. Other venoms were obtained from captive specimens in our own collection, as well as from other sources, and were either air dried or lyophilized.

Lethality determinations

The i.p. LD_{50} of crude *A. superbus* venom was determined by injection of male white mice (20 – 22 g) in groups of 4 mice each. Dosages were obtained from a 1 mg/ml crude venom solution dissolved in phosphate buffered saline (0.05 M, pH 7.2) which was injected into the lower abdominal quadrant. Animals were observed for 24 hr. The LD_{50} was calculated by the Spearman – Karber method (WORLD HEALTH ORGANISATION, 1981) programmed into an Apple II computer in Apple Pascal. The 95% fiducial limits ascribed for the LD_{50} (stop) were determined.

Assay of hemolytic activity

To estimate hemolytic activity test solutions were diluted in 0.15 M NaCl buffered at pH 7.2 with 0.01 M Tris – HCl, 0.2% gelatin, 0.01 $MgCl_2$ and 0.01 M $CaCl_2$ (buffer I). Volumes of test dilutions each decreasing by about 25% as compared to the one before were delivered into tubes (12 x 75 mm) and the volume in all tubes was brought to 0.5 ml by addition of buffer I. To each tube was added 0.5 ml of washed rabbit erythrocytes suspended in 0.15 M NaCl buffered at pH 7.2 with 0.01 M Tris – HCl (buffer II). The density of the erythrocyte suspension was adjusted to give an absorbance of 0.8 at 545 nm when complete lysis occurred. After mixing, the tubes were incubated at 37°C for 90 min, with mixing at 30 min intervals, followed by immersion in ice-water for 10 min, followed by brief centrifugation. The per cent hemolysis was estimated from the color of the hemoglobin compared with that of standards. One hemolytic unit (HU) is that amount of test material needed to release the hemoglobin from 50% of the cells. Results were generally reproducible to within 20%. Calcium ions were needed for maximal hemolysis, as illustrated by the finding that a solution of partially purified *A. superbus* venom gave a hemolytic activity of 60 HU/ml in the presence of Ca^{2+} as compared to 21 HU/ml when neither Mg^{2+} nor Ca^{2+} was present and 23 HU/ml when only Mg^{2+} was present.

Chromatographic demonstration of PLB activity

Reaction mixtures used to demonstrate enzymatic changes in phospholipids by thin layer chromatography utilized either rabbit erythrocytes or chromatographically pure phospholipids (Supelco, Inc., Bellefonte, PA) as substrates. The former consisted of 10 ml of a 0.7% (v/v) suspension of washed rabbit erythrocytes centrifuged and resuspended in 5 ml of buffer I. For phospholipid substrates 400 μg of the indicated lipid was dried in test tubes and then suspended by bath sonication and vortex mixing in 1 ml of buffer I. Substrates were incubated with enzyme preparations at 37°C for 2 hr. Incubation mixtures were extracted by the method of BLIGH and DYER (1959) and spotted on thin layer plates of silica gel G. Development, together with appropriate reference standards, was in either solvent system I [chloroform – methanol – acetic acid – water (150 : 90 : 24 : 11.4)] (SKIPSKI and BARCLAY, 1969) or solvent system II [petroleum ether – diethyl ether – acetic acid (180 : 20 : 2)] (THOMAS and HARPER, 1978). Spots were detected by spraying with 55% (v/v) H_2SO_4 and heating at 175°C.

Glycerophosphorylcholine (GPC), the water-soluble product of PLB action on phosphatidylcholine, was isolated from reaction mixtures containing 25 mg of phosphatidylcholine and the indicated quantities of enzyme preparations. Incubation was for 2 hr at 37°C, after which the entire reaction mixtures were spotted on Whatman SG81 silica gel impregnated paper. Development was by descending chromatography in methanol – 98% formic acid-water [(80 : 13 : 7) (solvent III)] (RENKONEN, 1974). The product, as well as authentic GPC (Sigma Chemical Co., St. Louis, MO), was located by spraying with phosphate reagent (RENKONEN, 1974), modified by inclusion of 2 ml freshly prepared 10% ascorbic acid for each 40 ml of reagent. Papers were heated at 50°C for 30 min to detect the blue spots representing loci of orthophosphate.

Assay of PLB activity using diluted egg yolk

To estimate PLB activity test solutions were diluted in 0.05 M dimethylglutaric acid – NaOH buffer (pH 6.5) containing 0.1% bovine serum albumin (buffer III). Volumes of test solutions each decreasing by about 25% as compared to the one before were delivered into tubes (12 x 75 mm) and the volume in all tubes was brought to 0.5 ml by addition of buffer III. To each tube was added 0.5 ml of diluted egg yolk prepared according to the method of VAN HEYNINGEN (1941), but modified by centrifugation at 12,000 *g* for 10 min instead of filtering and by adding calcium acetate to 0.005 M and sodium azide to 0.02%. The tubes were placed in a 37°C bath for 30 min and then allowed to stand at 22° for about 16 hr. After mixing the turbidity was read against a blank in a Zeiss PMQ spectrophotometer at 520 nm. Turbidity was a linear function of concentration of test material. One unit of egg yolk activity is defined as that quantity of test solution giving an absorbance of 1.0 at 520 nm under the conditions stated. Turbidity estimations were reproducible to within 20%.

Estimation of protein

Protein was estimated optically by the method of WHITAKER and GRANUM (1980).

Electrofocusing

Isoelectric focusing was carried out in a 110 ml electrofocusing column (LKB Instruments, Rockville, MD). The gradient was prepared from a less dense solution consisting of 51 ml of water, 4 ml of 8% (w/v) ampholine (pH 3 – 10) and a more dense solution consisting of dialyzed test material, 8.5 ml of 8% (w/v) ampholine (pH 3 – 10) and 25 g of sucrose in a final volume of 55 ml. Focusing was done at about 4°C for 24 hr with a final potential of 1000 V. Four milliliter fractions were collected at constant time intervals, using a pump to produce a constant flow rate.

RESULTS

Turbidity in egg yolk by venoms

Six samples of venoms of *A. superbus* and two of *P. colletti* were assayed for egg yolk activity, with the results shown in Table 1. In addition to these, venoms of 23 other species of snakes were examined for capacity to produce turbidity in dilute egg yolk, as well as for direct hemolytic activity. Venoms of two species only were weakly positive for the former, namely that of *Bungarus caeruleus*, which had 4 egg yolk units/mg, and that of *Bungarus multicinctis*, which had 1 egg yolk unit /mg. Both had less than 1 hemolytic unit/mg. Venoms of the following elapid snakes were negative for both activities: *Naja nigricollis*, *Naja naja*, *Naja naja atra*, *Naja naja philippinensis*, *Naja nivea*, *Naja melanoleuca* and *Ophiophagus hannah*. Venoms of the following crotaline viperids were negative for both activities: *Trimeresurus wagleri*, *Calloselasma rhodostoma*, *Agkistrodon bilineatus*, *Agkistrodon brevicauda*, *Agkistrodon piscivorus*, *Agkistrodon contortrix mokasen*, *Deinagkistrodon acutus*, *Crotalus adamanteus*, *Crotalus scutulatus*, *Crotalus atrox* and *Bothrops nasuta*. Venoms of the viperine viperid *Bitis nasicornis* and of the hydrophiids *Aipysurus laevis* and *Laticauda colubrina* were also negative for both activities.

Electrophoretic analysis of A. superbus *venom*

Forty milligrams of venom (Sigma, Lot A) containing a total of 420 egg yolk units and 90 hemolytic units were subjected to isoelectric focusing with the results shown in Fig. 1. Fractions representative of the major peaks of 280 nm absorbance were tested for mouse lethality. Lethal activity was detected in the five peaks marked by stars. A substantial amount of egg yolk activity was found in fraction 7 (pH 4.0) and in fraction 8 (pH 4.4) and traces in fractions 9, 27 and 28. The total egg yolk activity recovered in fractions 7 and 8 was 62% of the input activity. Fractions 7 and 8 were also strongly

TABLE 1. CAPACITY OF *A. superbus* AND *P. colletti* VENOMS TO PRODUCE TURBIDITY IN DILUTE EGG YOLK

Species	Source	Egg yolk units/mg
A. superbus	Commercial, Lot A	21
	Commercial, Lot B	50
	New England (northeastern New South Wales)	35
	Mittagong (southwest of Sydney)	59
	Flinders Island (off of northeast Tasmania)	50
	Mt. Lofty (near Adelaide)	79
P. colletti	Commercial	60
	New South Wales	76

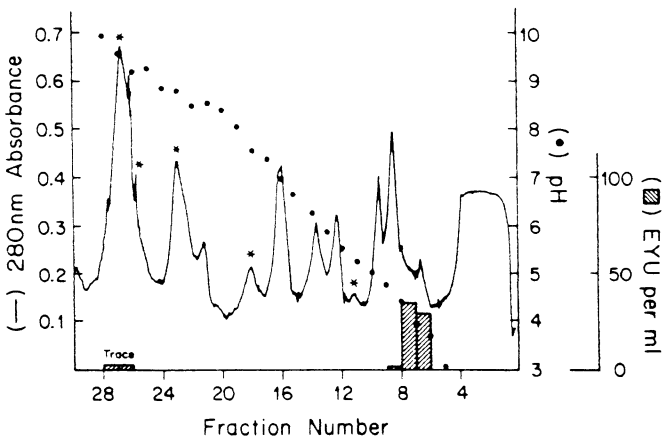

FIG. 1. ELECTROFOCUSING OF *A. superbus* VENOM.
Four milliliter fractions were measured for pH (●) and egg yolk units (EYU) (cross-hatched bars). The continuous line is 280 nm absorbance. The starred peaks were lethal for mice, as determined by injection of 0.1 ml of appropriate fractions into the caudal veins of 20 – 22 g male white mice.

hemolytic, both before and after adjusting the pH to neutrality. With the exception of acid induced hemolysis in fractions 1 – 6, no other fraction tested was hemolytic. The low pH of some of the fractions did not affect the assay results, because the assay system was well buffered. However, the possibility that there occurred some loss of egg yolk activity prior to assay has not been excluded.

In order to obtain a larger amount of the agent producing turbidity in egg yolk, 97 mg of venom (Sigma, Lot B) were subjected to electrofocusing under the conditions described (see Materials and Methods), but with reversed electrodes, i.e. with the anode at the top of the column and the cathode at bottom. The electrodes were reversed in order to cause the active material to focus at a low rather than a high sucrose concentration. The four most active fractions were pooled and the resulting 16 ml were dialyzed overnight at 4°C against 200 ml of saturated ammonium sulfate (enzyme grade). The resulting precipitate was dissolved in 1 ml of 20% glycerol in buffer II and dialyzed for two days at 4°C against 200 ml of 20% glycerol in buffer II. The final solution having a volume of 1 ml was designated '*Austrelaps* egg yolk factor'. It contained 1900 egg yolk units (39% of the starting activity), 7.9 mg protein, and its specific activity was five times that of the starting material. It retained full activity after storage at 4°C for five weeks.

Electrophoretic analysis of P. colletti *venom*

Eighty milligrams of venom (Sigma) containing a total of 4800 egg yolk units and 480 hemolytic units were subjected to electrofocusing as described in the preceding paragraph. The most active fractions (Fig. 2) were pooled and processed in the same way as for *A. superbus*. The final product, designated '*Pseudechis* egg yolk factor', had a volume of 1 ml, contained 3300 egg yolk units (69% recovery) and 5.0 mg protein. Like *Austrelaps* egg yolk factor it was strongly hemolytic. It retained 95% of its egg yolk activity after two weeks at 4°C. When electrofocusing was done with normally positioned electrode the egg yolk activity focused at pH 5.2, a full unit higher than did *Austrelaps* egg yolk factor. The specific activity of *Pseudechis* egg yolk factor was 660 egg yolk units/mg, as compared to 240 egg yolk units/mg for *Austrelaps* egg yolk factor. Whereas the unfractionated venoms

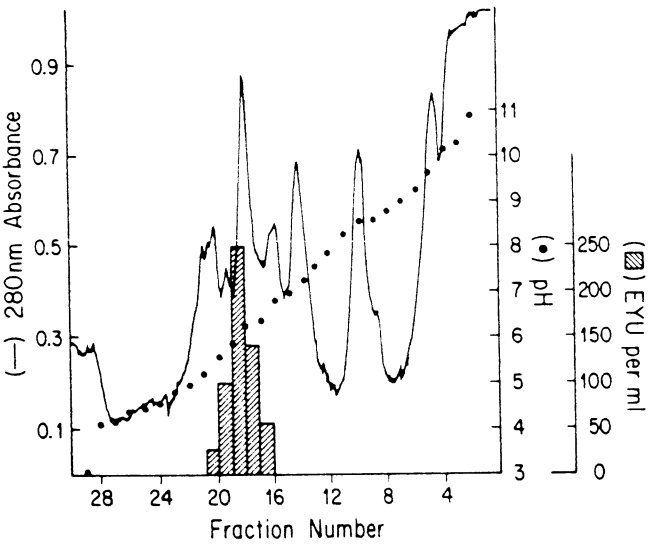

FIG. 2. ELECTROFOCUSING OF *P. colletti* VENOM.
Conditions were as for Fig. 1, except the electrofocusing was with electrodes reversed. Four milliliter fractions were measured for pH (●) and egg yolk units (EYU) (cross-hatched bars). The continuous line is 280 nm absorbance.

exhibited the phenomenon of hot–cold hemolysis, the effect of cold was less when the fractions were assayed.

Probable identity of egg yolk factor with PLB

We first considered the possibility that the venom component responsible for turbidity in egg yolk is a phospholipase C (PLC). However, to our knowledge, with but one possible exception (see Discussion), no snake venom has ever been shown to have PLC activity where phosphatidylcholine (PC) is the substrate. BRAGANCA and KHANDEPARKAR (1966) demonstrated that a fraction obtained from *N. naja* has PLC activity for phosphatidylethanolamine (PE), phosphatidylserine (PS) and their lyso-derivatives, but they did not demonstrate activity for PC.

Second, PLB is known to produce turbidity in egg yolk agar plates cleared by phospholipase A (HABERMANN and HARDT, 1972) suggesting that PLB-produced turbidity in tubes is not without some precedent. Finally, PLB activity is known to be present in the venoms of a number of snakes, the most potent in respect to this enzyme activity being the venoms of *A. superbus*, *Pseudechis porphyriacus* and *Pseudechis australis* (DOERY and PEARSON, 1964), as well as *Notechis scutatus*, *Bungarus caeruleus* and *Acanthophis antarcticus* (FLETCHER *et al.*, 1979). In view of this information we sought to identify the products of the reaction where PC is the substrate for the two egg yolk factors and further to examine their substrate specificity.

The major phospholipids of the erythrocyte membrane, PC, PE, PS and sphingomyelin (SM), can be detected by their migration characteristics in thin layer chromatography (TLC) using solvent I. When rabbit erythrocytes were treated with 30 μl of *Austrelaps* egg yolk factor or 20 μl of *Pseudechis* egg yolk factor (see Materials and Methods) the PC, PS and PE spots were found to disappear and new spots were seen at the solvent front.

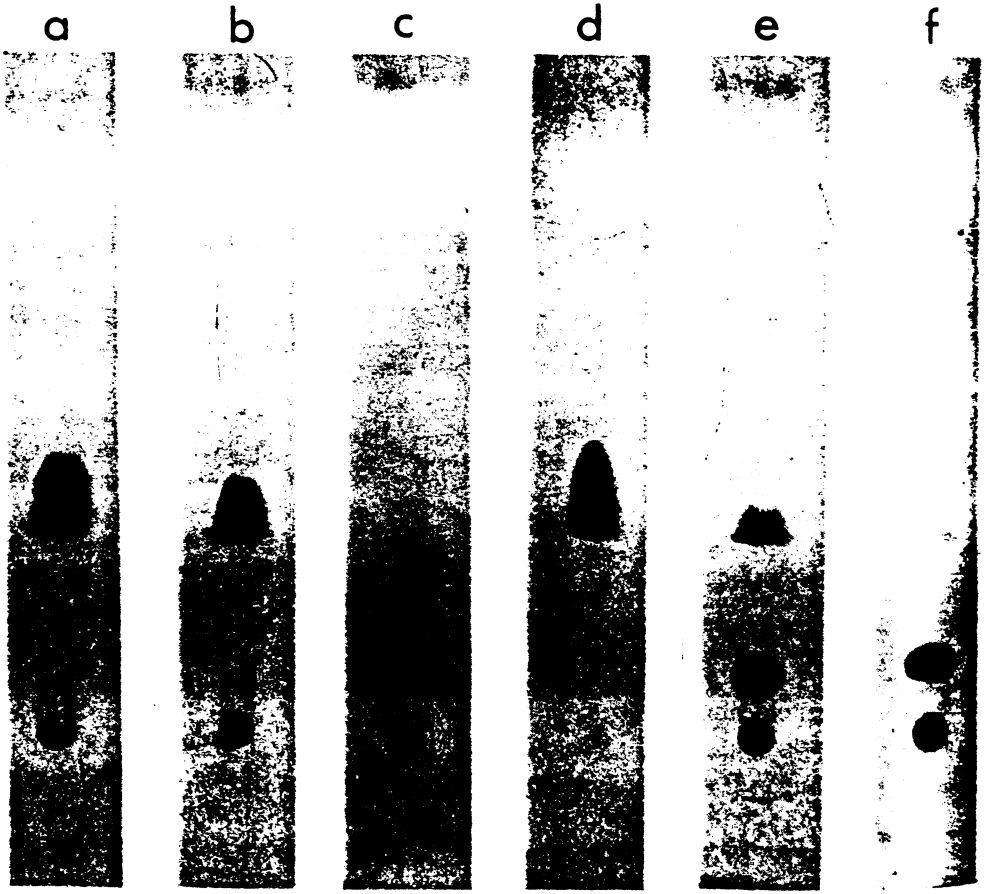

FIG. 3. THIN-LAYER CHROMATOGRAPHIC EVIDENCE FOR PLB ACTIVITY OF EGG YOLK FACTORS. Lanes *a* and *b* show fatty acid spots produced by treating phosphatidylcholine (PC) with *Austrelaps* and *Pseudechis* egg yolk factors, as compared to lane *c*, which is untreated PC, and to lane *d*, which is authentic fatty (oleic) acid as a reference standard. Lanes *a* and *b* also show nearly total disappearance of PC spot. Lane *e* shows fatty acid generated from erythrocytes treated with *Pseudechis* egg yolk factor, as compared to the near absence of fatty acid for untreated control erythrocytes (lane *f*). The dark spot just above the origin in lanes *e* and *f* is cholesterol, while the light spot between the origin and cholesterol is unknown. See text for further details.

Authentic PC was also hydrolyzed with production of a solvent-front spot. SM was unchanged, as shown by comparison with an untreated control.

In order to determine the nature of the enzymatic change, similar reactions were carried out and extracts therefrom examined by TLC in solvent II. In this system phospholipids remain at the origin, while other classes of lipids migrate with characteristic R_f values. Figure 3 shows the results of incubation of PC with 30 μl of *Austrelaps* egg yolk factor (lane *a*) and 20 μl of *Pseudechis* egg yolk factor (lane *b*). The origin spot corresponding to unhydrolyzed PC (lane *c*) disappeared and there developed spots (lanes *a* and *b*) co-chromatographing with oleic acid reference standard (lane *d*).

Rabbit erythrocytes treated with 20 μl of *Pseudechis* egg yolk factor produced a fatty acid spot as well (lane *e*), in contrast to untreated erythrocytes (lane *f*). In the former

instance complete hemolysis had occurred. The spot running just beyond the origin in extracts from both treated and untreated erythrocytes is cholesterol (THOMAS and HARPER, 1978). Identical results were obtained when the *Austrelaps* preparation was used. Lysophosphatidylcholine, PE and PS were also substrates for the enzyme(s), showing patterns identical with that for treated PC. SM was not a substrate.

The generation of a product co-chromatographing with fatty acid suggested that PLB activity was involved. Paper chromatography (solvent III) of incubation mixtures containing PC and 30 μl of each of the two egg yolk factor preparations revealed spots co-chromatographing with authentic GPC, the expected water soluble product of PLB action. PC in this solvent system remained at the origin (data not shown).

Morphology of ghosts resulting from treatment of erythrocytes with egg yolk factors

Phase contrast microscopic examination of preparations resulting from treatment of washed rabbit erythrocytes with *Austrelaps* and *Pseudechis* egg yolk factors revealed many ghosts of reduced diameter, containing many vesicles of various sizes. The vesicles appeared to result from internalization of portions of the plasma membrane. In contrast to phase-dense masses seen in ghosts resulting from hemolysis by phospholipases C (BERNHEIMER *et al.*, 1974), such entities were inconspicuous or missing altogether. Enzymotically prepared ghosts showed few or no internal vesicles comparable to those seen with egg yolk factor lysed cells.

Lethal potencies

BROAD *et al.* (1979) reported a subcutaneous mouse LD_{50} of 0.5 mg/kg for *A. superbus* venom. Since this is the only published LD_{50} for this species we determined the i.p. LD_{50} using a specimen of venom obtained commercially (Sigma). A value of 0.48 mg/kg was found, nearly identical with the s.c. value reported earlier. For the venom of *P. colletti* LD_{50} values of 1.2 (i.p.) and 2.38 (s.c.) mg/kg have been reported by FISCHER and CABARA (1967) and by BROAD *et al.* (1979), respectively. *Pseudechis* egg yolk factor proved lethal for mice, but its lethal potency was not quantitatively determined.

DISCUSSION

One aim of this study was to identify the component in the venoms of *A. superbus* and *P. colletti* responsible for direct lysis of rabbit erythrocytes. Although rigorous proof is lacking, our observations are consistent with the thesis that the component is PLB. Considerable significance can be attached to the finding that among the venoms of 25 species only the two mentioned showed direct hemolytic activity measurable by the method used. These same two species were the only ones that exhibited relatively high egg yolk activity. Parenthetically, a related species, *P. porphyriacus*, was observed nearly a century ago to be lytic for frog erythrocytes (MARTIN, 1894).

The kinetics of hemolysis (not described) caused by partially purified PLB (*Austrelaps* and *Pseudechis* egg yolk factors) differed from that of whole venoms in two respects. Tests done with whole venom showed a 'hot–cold' effect, whereas this effect was somewhat reduced when partially purified PLB preparations were used. In addition, it was observed that per cent hemolysis caused by whole venom decreased sharply with decreasing venom concentration, whereas per cent hemolysis caused by partially purified PLB decreased very gradually with decreasing enzyme concentration. Presumably whole venom contains one or more components, phospholipase A_2 for example, that may be

missing from the partially purified PLB. This (these) factor(s) could influence the course of hemolysis and give rise to differing kinetics. A careful analysis of the effects of various venom components on lysis by PLB would be needed to further clarify the mechanism of these effects.

DOERY and PEARSON (1964) detected PLB activity in venoms of 10 species of snakes among which the most potent were the venoms of *A. superbus* and *P. porphyriacus*. Their enzyme assays were routinely carried out at pH 9.5, whereas our tests were done at the more physiological pH of 6.8. Doery and Pearson also pointed out that in the venoms of a number of species PLB activity was detected only at and above pH 8. PLB activity has also been detected in the venoms of a number of Egyptian snakes (MOHAMED *et al.*, 1969), as well as in that of *Vipera palaestinae* (SHILOAH *et al.*, 1973). PLC activity on PC has been reported in the venom of *Bothrops alternatus* (VIDAL BREARD and ELIAS, 1950). In our view, confirmation of this finding would be desirable.

A. superbus ranges from the New England district of New South Wales to Victoria, the southeast corner of South Australia, Tasmania and the Bass Strait islands, whereas *P. colletti* ranges through Queensland. It may be significant that STORR (1982) considered *Austrelaps* to be a member of the genus *Notechis*. FLETCHER *et al.* (1979) found that *Notechis scutatus* venom displayed the highest PLB activity of 16 snake venoms surveyed. While only one member of the genus *Pseudechis* was examined for venom PLB activity, the report of DOERY and PEARSON (1964) indicates high PLB activity in the venom of *P. porphyriacus*. It may be significant that *P. colletti*, *P. porphyriacus*, *P. guttatus* and *P. australis* all are sympatric and have similar prey preferences. The venoms of *B. multicinctis* and *B. caeruleus* displayed low levels of PLB activity. Both species possess highly toxic venom [i.p. LD_{50}, 0.07 – 0.09 mg/kg (MINTON, 1974)]. Peninsular India is considered to be the locality of origin for *B. caeruleus*, while *B. multicinctis* is known to range throughout China and Laos. Most of these elapids display close similarity in habitat.

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