Colowick, S. P. & Sutherland, E. W. (1942). *J. biol. Chem.* **144**, 423.
Connell, G. E., Dixon, G. H. & Hanes, C. S. (1955). *Canad. J. Biochem. Physiol.* **33**, 416.
Eggleston, L. V. & Hems, R. (1952). *Biochem. J.* **52**, 156.
Hansen, R. G., Rutter, W. J. & Krichevsky, P. (1955). *Biochem. Prep.* **4**, 1.
Horecker, B. L. & Kornberg, A. (1948). *J. biol. Chem.* **175**, 385.
Isselbacher, K. J. (1958). *J. biol. Chem.* **232**, 429.
Kalckar, H. M. (1943). *J. biol. Chem.* **148**, 127.
Kosterlitz, H. W. (1943). *Biochem. J.* **37**, 318.
Krahl, M. E. & Cori, C. F. (1949). *Biochem. Prep.* **1**, 33.
Leloir, L. F. & Trucco, R. E. (1955). In *Methods in Enzymology*, vol. 1, p. 290. Ed. by Colowick, S. P. & Kaplan, N. O. New York: Academic Press Inc.
Maxwell, E. S. (1957). *J. biol. Chem.* **229**, 139.
Morton, R. K. (1954). *Biochem. J.* **57**, 595.
Nelson, N. (1944). *J. biol. Chem.* **153**, 375.
Rafter, G. W. & Colowick, S. P. (1955). In *Methods in Enzymology*, vol. 3, p. 887. Ed. by Colowick, S. P. & Kaplan, N. O. New York: Academic Press Inc.
Robbins, E. A. & Boyer, P. D. (1957). *J. biol. Chem.* **224**, 121.
Slater, E. C. (1953). *Biochem. J.* **53**, 157.
Strominger, J. L. (1955). *Biochim. biophys. Acta*, **16**, 616.
Trucco, R. E., Caputto, R., Leloir, L. F. & Mittleman, N. (1948). *Arch. Biochem.* **18**, 137.
Vladimirov, G. E., Vlassova, V. G., Kolotilova, A. Y., Lyzlova, S. N. & Panteleyeva, N. S. (1957*a*). *Nature, Lond.*, **179**, 1350.
Vladimirov, G. E., Vlassova, V. G., Kolotilova, A. Y., Lyzlova, S. N. & Panteleyeva, N. S. (1957*b*). *Biokhimiya*, **22**, 963.
Wilkinson, J. F. (1949). *Biochem. J.* **44**, 460.

Biochem. J. (1961) **78**, 820

Haemolysins in Venoms of Australian Snakes

OBSERVATIONS ON THE HAEMOLYSINS OF THE VENOMS OF SOME AUSTRALIAN SNAKES AND THE SEPARATION OF PHOSPHOLIPASE A FROM THE VENOM OF *PSEUDECHIS PORPHYRIACUS*

BY HAZEL M. DOERY AND JOAN E. PEARSON
Commonwealth Serum Laboratories, Parkville, Victoria, Australia

(*Received* 27 *June* 1960)

A supply of phospholipase A which was free from other enzymes was required during a study of the antagonism between staphylococcal toxin and certain venoms (North & Doery, 1958). Phospholipase A had already been separated from venoms of the honey-bee (Habermann & Neumann, 1957) and *Crotalus terrificus terrificus* (Habermann, 1957). However, for our purpose venoms of Australian species of snakes were considered as a source of this enzyme. The haemolytic activities of a number of these venoms were first studied by Kellaway & Williams (1933), Holden (1934, 1935), Feldberg & Kellaway (1937, 1938) and Trethewie (1939). Holden (1934) showed that washed red cells of the rabbit were haemolysed by solutions of these venoms at 37°. He observed an increase of activity on the addition of lecithin. It was generally agreed at that time, on chemical and pharmacological evidence, that the haemolytic activities of the venoms of the Australian snakes studied were dependent on the formation of lysolecithin by phospholipase A present in the venoms (Feldberg & Kellaway, 1938; Trethewie, 1939).

It is known that there are two types of venom haemolysins, 'direct' and 'indirect' (Holden, 1935; Neumann & Habermann, 1952; Habermann, 1954). The direct haemolysin lyses washed red cells of certain species readily without added lecithin, whereas the indirect haemolysin lyses such cells only feebly, but haemolysis is markedly enhanced by the addition of lecithin, which is converted into the haemolytic agent, lysolecithin, by the phospholipase A.

Both types of haemolysins have been found among the common venomous species of Australian snakes, the venom of *Pseudechis porphyriacus* being particularly rich in both. This venom was readily available and was chosen as a source of phospholipase A. In this paper the concentrations of both types of haemolysins which have been found in venoms of some Australian snakes will be recorded. Certain other venoms considered to be strongly haemolytic were also included in this study. The separation of phospholipase A from the venom of *P. porphyriacus* will be described and discussed in relation to the occurrence of this enzyme in some other venoms.

MATERIALS AND METHODS

Dried venoms. The venoms of the Australian snakes were either purchased from E. Worrell, Wyoming, Gosford North, N.S.W., Australia, or collected at these Laboratories and prepared as already described (Doery, 1958). Other venoms were obtained from Ross Allen's Reptile Institute, Florida, U.S.A., and the Medical Research Institute, Johannesburg, South Africa.

Fractions of the venom of Notechis scutatus. These were prepared in the course of separating the neurotoxins of this venom (Doery, 1958) when they were called fraction 1 which was the haemolysin-rich fraction, fraction 1+ and fraction 2.

Crude lecithin. This was prepared from egg yolk by the method described by Saunders (1957).

Preparation of resin Amberlite IRC-50 (XE-64). The resin was prepared for chromatography as described by Doery (1958).

Paper electrophoresis and elution. A hanging-strip type of cell was used (Spinco Model R) with 3 cm. wide strips of Whatman 3 MM paper. The apparatus was operated at constant current (5–25 mA/cell) and at 0–4°. Samples (500 μg.) were examined as 5% (w/v) solutions and applied as a band across the centre of each strip. Dextran was used as an indicator of the extent of electro-osmosis. The strips were stained and eluted as described by Doery (1958).

Preparation of red cells. Blood cells (human) were stored in Rous & Turner (1916) suspending fluid, and washed three times in 0·9% sodium chloride soln.

Estimation of direct haemolysin. The procedure was essentially that of Neumann & Habermann (1954), all dilutions being made in Sörensen's buffer, pH 7·0, diluted 1/5 with 0·9% sodium chloride soln. Portions (0·5 ml.) of a 2% (w/v) suspension of red cells were added to 0·5 ml. lots of venom solutions in a suitable range of dilutions, incubated at 37° for 30 min., and then held in an ice bath until centrifuged. To each supernatant, one drop of aq. 1% (w/v) potassium ferricyanide soln. was added, and the extinction at 630 mμ was measured. From the curves relating venom concentration and extinction at 630 mμ, the haemolytic activity of the venom solution was expressed as a percentage of that of the appropriate control.

Estimation of phospholipase A. The method was essentially that of Neumann & Habermann (1955), all solutions and suspensions being made in 0·9% sodium chloride soln. Portions (2·0 ml.) of 40% (v/v) suspension of egg yolk were added to 0·2 ml. lots of venom solutions covering an appropriate range of dilutions, and incubated at 37° for 2 hr. The reaction mixtures were then held in an ice bath until 0·2 ml. portions were removed. These were incubated with 4 ml. lots of a 0·6% (v/v) suspension of washed red cells (human) at 37° for 30 min. At the completion of this reaction the mixtures were held in an ice bath until they were spun in a refrigerated centrifuge at 5°. The extinctions of the supernatants were measured at 576 and 620 mμ. The difference between these gave a measure of the extent of haemolysis. From the curves relating concentration and percentage of complete haemolysis, the phospholipase A activity was expressed as a percentage of that of the appropriate control measured over a tenfold concentration range. By using the same preparations of egg-yolk substrate and red-cell suspension the mean deviation of the results was 12%, increasing to 15% for day-to-day estimations, when fresh substrate and red-cell suspensions were used. The results presented below are within these limits. In some experiments crude lecithin from egg yolk was used as the substrate.

Comparison of the haemolytic activities of venoms. For the study of the haemolytic activities of the venoms of some Australian snakes and other selected venoms, one sample of each venom only was examined. The direct haemolytic and phospholipase A activities of the sample of venom of *P. porphyriacus* served as standards of comparison and were taken as 100%. Venom solutions were examined in 50% dilution steps over the appropriate range of concentrations, and the direct haemolytic and phospholipase A activities were expressed as percentages of the activity of the venom of *P. porphyriacus* to the nearest 50%.

Estimation of cholinesterase. This was determined colorimetrically by the method of McCosker & Daniel (1959).

Estimation of the coagulating factor, hyaluronidase and 5′-nucleotidase. The methods used were those described by Doery (1958). 5′-Nucleotidase activity was examined against one substrate only, namely adenosine 5′-phosphate.

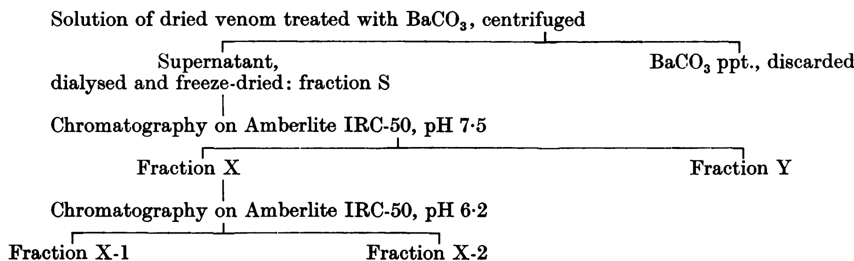
Procedures for fractionation of the phospholipase A in the venom of Pseudechis porphyriacus

These are outlined in Scheme 1.

Removal of the coagulating factor. This was carried out by adsorption on barium carbonate (Holden, 1933) as described by Doery (1958).

Ammonium acetate buffers. Aqueous solutions of ammonium acetate of the required molarity were adjusted to the selected pH with the addition of either aq. ammonia soln. or acetic acid.

Chromatography on Amberlite IRC-50 (XE-64) *at room temperature* (15–25°). The freeze-dried supernatant from the barium carbonate, fraction *S*, was examined by chromatography on this resin; 140 mg. were applied as a 10% (w/v) solution in 0·1 M-ammonium acetate, pH 7·5–7·8, to a



Scheme 1. Fractionation procedures

column of the resin (2·5 cm. × 18 cm.) which had been previously equilibrated with this buffer. Elution with the same buffer was followed by the measurement of the extinction at 278 mμ of successive eluates. After emergence of the first protein peak and when no further protein was eluted, the eluent was changed to M-ammonium acetate, pH 7·5, and a second peak emerged. Appropriate eluates within each protein peak respectively were combined and the products freeze-dried to give fractions X and Y (Fig. 1). Fraction X from each of three such columns was combined and subjected to chromatography again on the resin Amberlite IRC-50 equilibrated with 0·1 M-ammonium acetate at pH 6·2–6·3. With this buffer as the eluting fluid two protein peaks were eluted in succession. When no further protein was eluted, the eluent was replaced by M-ammonium acetate at the same pH, but this failed to elute more than 1% of additional protein. Appropriate eluates in each peak were combined, and, after freeze-drying, two fractions X-1 and X-2 were obtained (Fig. 2).

In early experiments fraction X was subjected to chromatography with the same system, namely the resin Amberlite IRC-50 equilibrated with 0·1 M-ammonium acetate, but at a lower pH, namely 6·0. Fraction X-1 was eluted at the elution volume, as was the case at the higher pH, but the remainder of fraction X was more strongly adsorbed and eluted only by changing the eluent to M-ammonium acetate at pH 6·0. The resultant fraction had a lower phospholipase A activity and was less homogeneous than fraction X-2 as subsequently prepared.

Dialysis of venom fractions. Aqueous solutions of the various fractions were dialysed in cellophan tubing against distilled water at 4° for 16 hr. The contents of the tubing were then analysed for phospholipase A.

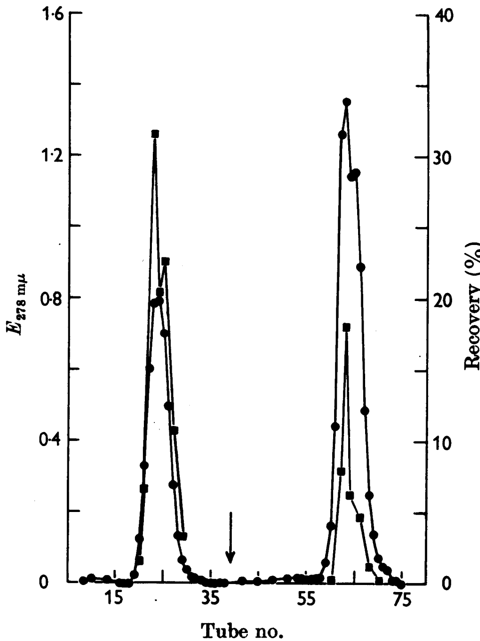


Fig. 1. Chromatography on Amberlite IRC-50 (XE-64) of 140 mg. of fraction S. Eluting buffer was 0·1 M-ammonium acetate, pH 7·7; at the arrow the eluent was changed to M-ammonium acetate, pH 7·5. ●, Extinction at 278 mμ; ■, % recovery of phospholipase A activity. Contents of tubes were pooled to provide the following fractions: tubes 20–29, fraction X; tubes 60–70, fraction Y.

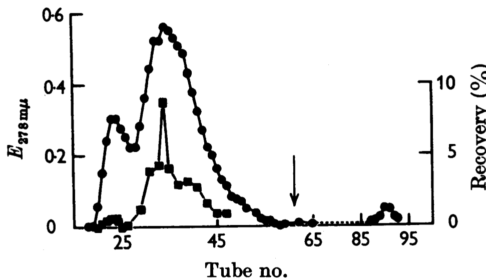


Fig. 2. Chromatography on Amberlite IRC-50 (XE-64) of 120 mg. of fraction X. Eluting buffer was 0·1 M-ammonium acetate, pH 6·2; at the arrow the eluent was changed to M-ammonium acetate, pH 6·2; ●, Extinction at 278 mμ; ■, % recovery of phospholipase A activity. Contents of tubes were pooled to provide the following fractions: tubes 20–26, fraction X-1; tubes 30–47, fraction X-2.

RESULTS

In preliminary studies it became evident that the method chosen for estimating phospholipase A was subject to considerable variation. This was decreased somewhat by holding samples at low temperatures wherever possible while sampling, as already indicated. Table 1 records the concentrations of phospholipase A and the direct haemolysin found in a number of venoms, expressed as percentages of those found in the venom of *P. porphyriacus*.

The haemolytic activity of the venom of *Notechis scutatus* was previously estimated by its weak activity as a direct haemolysin (Doery, 1958), and it was assumed to be phospholipase A because it was known to be enhanced by the addition of lecithin. Subsequent estimations of the phospholipase A activity by the method described in this

Table 1. *Haemolytic activity of venoms as percentages of that of the venom of* Pseudechis porphyriacus

Venom	Phospholipase A	Direct haemolysin
Pseudechis porphyriacus	100	100
P. australis	150	50
P. papuanus	150	100
Denisonia superba	50	50
Notechis scutatus scutatus	50	2–3
Oxyuranus scutellatus	100	<1
Acanthophis antarticus	50	<1
Demansia textilis textilis	<20	<12
Naja nigricollis	50	600
Vipera russelli	25	<10
Agkistrodon piscivoris	12	<3

paper confirmed this identification. In Table 2 the recovery of the haemolytic activity in three fractions of this venom by both methods of estimation are recorded. Fraction 1 is hereafter referred to as the phospholipase A-rich fraction of the venom of *Notechis scutatus*.

The separation of phospholipase A from the venom of *P. porphyriacus* was followed by the estimation of both this enzyme and the direct haemolysin at each step in the procedure outlined in Scheme 1. Table 3 records the recovery of the mass (dry wt. over P_2O_5 *in vacuo*), the phospholipase A and the direct haemolysin in each fraction, expressed as percentages of the original dried venom.

The barium carbonate treatment removed more than 90 % of the clotting factor and no less than 98 % of both the hyaluronidase and cholinesterase activities of the dried venom. However, 40, 51 and 98 % of the 5′-nucleotidase, the direct haemolysin and phospholipase A activities respectively, remained in the supernatant, i.e. fraction S. The complexity of this fraction S was also indicated by paper electrophoresis.

The separation of fraction S by chromatography on the resin Amberlite IRC-50 at pH 7·7 and 7·5 into two fractions X and Y, both of which contain phospholipase A activity, is illustrated by a typical elution curve in Fig. 1. An overall recovery was obtained of 82 % of the protein applied to the column. Fractions X and Y contained 29 and 49 % respectively of the protein and 54 and 30 % respectively of the phospholipase A applied to the column. The further separation of fraction X by chromatography at pH 6·2 into the two fractions X-1 and X-2, both of which again contained phospholipase A, is illustrated by the elution curve in Fig. 2. With an overall recovery of 81 % of the protein, fractions X-1 and X-2 contained 15 and 57 % respectively of the protein and 4 and 117 % respectively of the phospholipase A applied to the column, the latter figure being within the experimental error inherent in the method of analysis. These fractionation procedures finally resolved the venom of *P. porphyriacus* into the three fractions X-1, X-2 and Y which contained 3, 70 and 37 % respectively of the activity of the original dried venom (Table 3).

In the ultracentrifuge, X-2 [1·4 % (w/v) in 0·2 M-sodium chloride] moved as a symmetrical peak in a field of 250 000g with $S_{20,w}$ of 2·3. However, the spread was greater than one might expect for a single component.

Thus fraction X-2 is a preparation of phospholipase A with seven times the activity of the crude venom on the activity:mass ratio, whereas fractions X-1 and Y have about 0·6 and 0·8 of the activity on the same basis. Figs. 3–6 show paper-electrophoresis-elution curves of these three fractions of the venom of *P. porphyriacus* together with the phospholipase A-rich fraction of the venom of *Notechis scutatus* (Doery, 1958). The latter fraction, previously shown to be inhomogeneous, was resolved into two distinct components by paper electrophoresis in veronal buffer at pH 8·6 by the procedure used here (Fig. 3). Fraction X-1 was similarly resolved and showed a paper-electrophoresis pattern approximating to that of the fraction of the venom of *Notechis scutatus* in both veronal buffer at pH 8·6 and ammonium acetate buffer at pH 6·0 (Figs. 3 and 4). Fraction X-2 more closely followed the movement

Table 2. *Recovery of haemolytic activity of the venom of* Notechis scutatus *in fractions of this venom expressed as percentages of the dried venom*

Fraction	Method of estimation: Direct haemolysin*	Method of estimation: Phospholipase A
1	71	66
1+	4	4
2	3	2

* Doery (1958).

Table 3. *Recovery of mass, phospholipase A and direct haemolysin as percentages of dried venom of* Pseudechis porphyriacus

Fraction	Mass	Phospholipase A	Direct haemolysin
Dried venom	100	100	100
Supernatant from $BaCO_3$ ppt. freeze-dried, fraction S	78	98	51
Chromatography of fraction S on Amberlite IRC-50:			
Fraction X	23	66	3
Fraction Y	45	37	23
Chromatography of fraction X on Amberlite IRC-50:			
Fraction X-1	5	3	<1
Fraction X-2	10	70	<1

of dextran in ammonium acetate buffer at pH 6·0 than in veronal buffer at pH 8·6, indicating an iso-electric point in the region of pH 6 (Figs. 5 and 6). Fraction Y, shown to be inhomogeneous under both conditions of electrophoresis, contained components which overlapped with fractions X-1 or X-2 and included the most cationic components of the venom (Figs. 5 and 6).

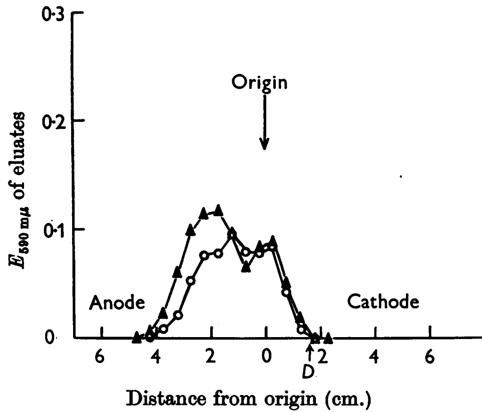


Fig. 3. Paper-electrophoresis-elution curves of venom fractions (500 µg.). ▲, Phospholipase A-rich fraction of venom of *Notechis scutatus*; ○, fraction X-1; *D*, position of dextran. Veronal buffer had *I* 0·05 and pH 8·6; electrophoresis was at 25 mA for 16 hr. and 3 v/cm. Protein eluted was estimated as described by Doery (1958).

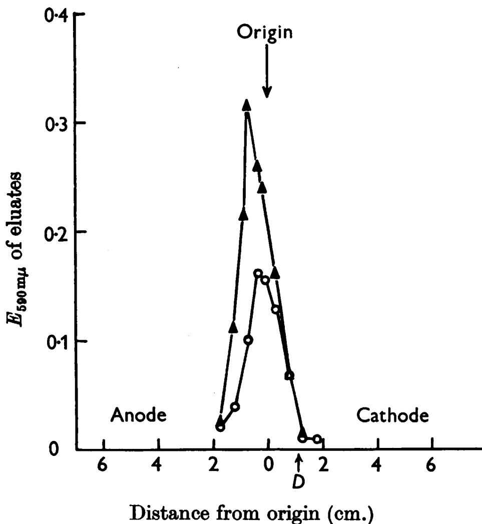


Fig. 4. Paper-electrophoresis-elution curves of venom fractions (500 µg.). ▲, Phospholipase A-rich fraction of venom of *Notechis scutatus*: ○, fraction X-1; *D*, position of dextran. The buffer was 0·2M-ammonium acetate, pH 6·0; electrophoresis was at 25 mA for 16 hr. and 3 v/cm. Protein eluted was estimated as described by Doery (1958).

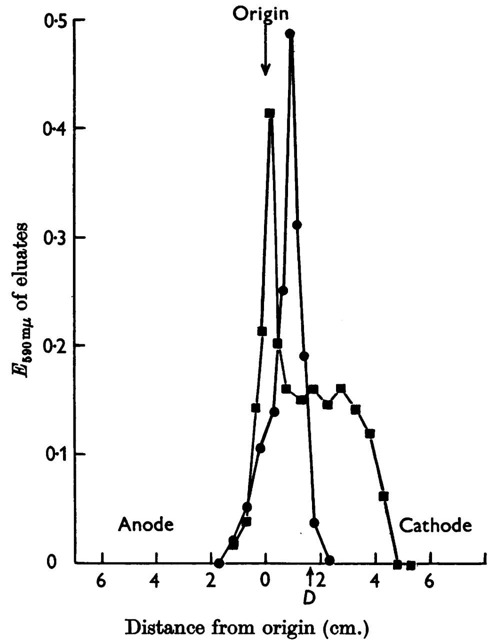


Fig. 5. Paper-electrophoresis-elution curves of venom fractions (500 µg.). ●, Fraction X-2; ■, fraction Y; *D*, position of dextran. Veronal buffer had *I* 0·05 and pH 8·6; electrophoresis was at 25 mA for 16 hr. and 3 v/cm. Protein eluted was estimated as described by Doery (1958).

All attempts to fractionate Y further have only added to the number of fractions with phospholipase A activity. In view of the possibility that the activity of fraction Y may have been due to incomplete separation from fraction X, fraction Y was re-examined by chromatography on the resin Amberlite IRC-50 at pH 6·2. However, although 30 % of the activity could then be eluted at pH 6·2 to give a preparation equal in activity to that of the crude venom on an activity: mass ratio, the paper electrophoresis pattern showed that it was still inhomogeneous and contained predominantly cationic components. Further attempts to separate the activity into a single component from fraction Y by means of precipitation with methanol at low temperatures over a range of pH values were equally unsuccessful. Phospholipase A activity was found in all portions of fraction Y which were separated.

Fraction S contained 40 % of the 5′-nucleotidase activity of the crude venom, but < 5 % of the original activity was found in each of the fractions X-1, X-2 and Y.

On dialysis against distilled water at 4° fraction X-1 lost 30 % of its activity with no detectable loss in protein. The phospholipase A-rich fraction of the venom of *Notechis scutatus* also showed a

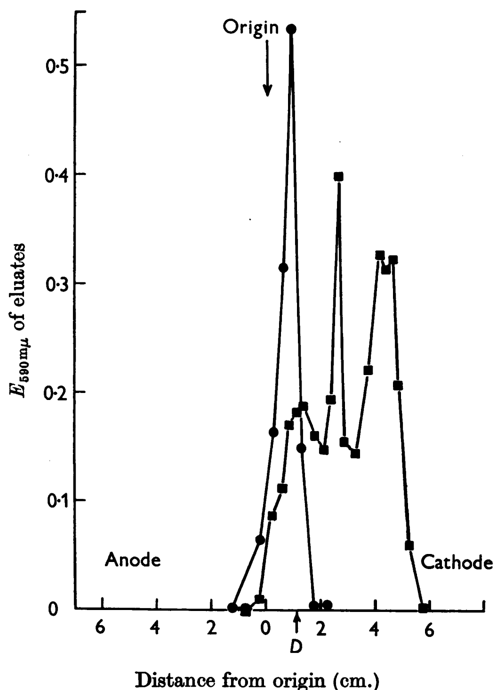


Fig. 6. Paper-electrophoresis-elution curves of venom fractions (500 µg.). ●, Fraction X-2; ■, fraction Y; *D*, position of dextran. Buffer was 0·2 M-ammonium acetate, pH 6·0; electrophoresis was at 25 mA for 16 hr. and 3 V/cm. Protein eluted was estimated as described by Doery (1958).

similar loss of activity on dialysis. Fractions X-2 and Y were dialysed without loss of activity.

A limited study of possible substrate specificity or activation was begun by comparing the activity of fractions against both crude lecithin and whole egg yolk as substrates. The ratio of the phospholipase A activity against these two substrates was compared for each of the three fractions X-1, X-2 and Y. Fraction X-2 showed a markedly lower ratio of activity against crude lecithin to activity against whole egg yolk than did either of the other two fractions. Sodium oleate when added to crude lecithin doubled the activity of fraction X-2 against this substrate, whereas no significant effect on the activities of fraction X-1 or Y could be detected.

When the distribution of the direct haemolysin is considered, Table 3 shows that, although fraction S contained about 50% of the direct haemolytic activity of the dried venom, only approximately 50% of this was recovered in fraction Y. Moreover, in attempts that were made to separate the various components of fraction Y we failed to find a component in which the direct haemolysin was specifically concentrated.

DISCUSSION

This study included the venom of eight of the common venomous species of Australian snakes. As found by earlier workers, phospholipase A is widely distributed. The venom of *Demansia textilis textilis* is an exception in this regard. The concentrations in which it occurs in the venoms studied are as high or higher than those found in samples of three venoms, from other than Australian species of snakes, namely *Naja nigricollis*, *Vipera russelli* and *Agkistrodon piscivoris*, which are frequently regarded as rich sources of phospholipase A.

The direct haemolysin is, however, less universally found within venoms of Australian species, the highest concentrations being found in those of the *Pseudechis* species of snakes studied. In the venoms of *Notechis scutatus scutatus*, *Oxyuranus scutellatus*, *Acanthophis antarticus* and *D. textilis textilis*, this haemolysin was either not detectable or present in a very low concentration relative to that in the venom of *P. porphyriacus*. This haemolysin is apparently less stable or possibly more strongly adsorbed to the resin than phospholipase A. This is borne out by the low overall recovery (23% in fraction Y) which was obtained in the course of separating phospholipase A from the latter venom. We have failed to identify this activity with a single component of the venom. This activity is associated with the most electropositive fraction of both the venom studied here, *P. porphyriacus*, and that of the honey-bee (Neumann & Habermann, 1954).

Reference was made earlier to the fact that phospholipase A may show weak activity as a direct haemolysin when added to washed red cells in the absence of added phospholipid. This activity has been attributed to the action of phospholipase A on that portion of the phospholipid of the red cell which is in a form loosely bound to the cell surface. Loosely bound phospholipid has been demonstrated by Lovelock (1955), who showed by successive washings with 'saline' that significant amounts of phospholipid could be readily removed from the red-cell membrane. The venom of *Notechis scutatus* has a markedly low activity as a direct haemolysin (Table 1). This fact and the agreement between the recovery of both the direct haemolytic and phospholipase A activity of this venom as shown in Table 2 lead us to believe that the venom of *Notechis scutatus* has no significant direct haemolytic activity other than that due to phospholipase A.

In the first step in the separation of phospholipase A from the venom of *Pseudechis porphyriacus*, namely adsorption on barium carbonate, the removal of cholinesterase, among other enzymes, is

of interest. This step removed less than 40 % of the cholinesterase from the venom of *Notechis scutatus* (Doery, 1958), in which the concentration is 60 times that found in the venom of *P. porphyriacus*. Thus, although barium carbonate removed specifically the clotting factor, a number of other enzymes occurring in small concentrations were also removed. It would seem possible that the extent to which they are removed depends on the concentration in which they occur in the venom. The only other enzyme of those studied which was present at this stage was 5′-nucleotidase. We have no indication whether the subsequent low recovery was due to instability or adsorption on the resin.

The separation of fraction X-2, with seven times the phospholipase A activity of the crude venom, provided a high concentration of this enzyme free from the other main enzymes of the venom. However, the distribution of phospholipase A activity in fraction X-2, as shown in Fig. 2, suggests that within this fraction itself there may be two phospholipase A components. These were not separated in the ultracentrifuge. From the results presented here, it is evident that phospholipase A activity is associated with still other fractions of the venom. Although the paper-electrophoresis patterns of fractions X-1 and Y overlap with that of fraction X-2 (Figs. 3–6), the differences which have been demonstrated by chromatography, dialysis and possible substrate or activation specificities lead us to believe that the venom of *P. porphyriacus* contains a number of components with phospholipase A activity. Attention has already been drawn to the occurrence of two or more enzymes of the same type within the one source (Doery, 1958).

The possible relationship between the phospholipase A-rich fractions of the venom of *P. porphyriacus* and those from other venoms was considered. Fraction X-1 is inhomogeneous, represents only 3 % of the activity of the crude venom, and might be considered of little significance but for the fact that it is consistently separated from this venom, and has similar properties to the phospholipase A-rich fraction of the venom of *Notechis scutatus*. The latter fraction was recently reexamined under the chromatographic conditions which were used here to separate fractions X-1 and X-2. It behaved entirely like fraction X-1 with no indication of a component like X-2. This fact, taken together with both the similarity in paper-electrophoresis pattern (Figs. 3 and 4) and similar loss of activity on dialysis, supports the belief that there is a close relationship between fraction X-1 and the phospholipase A-rich fraction of the venom of *Notechis scutatus*.

The phospholipase A prepared from the venom of *Crotalus terrificus* (Neumann & Habermann, 1955; Habermann, 1957) had an isoelectric point near pH 6·5, was strongly adsorbed to the resin Amberlite IRC-50 at pH 6·86 and lost activity on dialysis. No single fraction of the venom of *P. porphyriacus* can be identified with this preparation.

Possible fundamental differences between the three fractions of the venom of *P. porphyriacus* containing phospholipase A have been suggested from the experiments concerning substrate specificities. The lower activity of fraction X-2 against egg lecithin than against whole egg yolk as substrate could be explained by the presence of an activator in egg yolk that was specific for fraction X-2. A limited examination suggested that sodium oleate may contribute to such activation.

SUMMARY

1. Both phospholipase A and direct haemolysin are found among the common venomous species of Australian snakes, phospholipase A being more generally distributed. The venoms of three species of *Pseudechis* genus, namely *porphyriacus*, *papuanus* and *australis*, are rich in both haemolysins.

2. In the venoms of *Notechis scutatus scutatus*, *Oxyuranus scutellatus*, *Acanthophis antarticus* and *Demansia textilis textilis*, the direct haemolysin was either not detected or present in small concentrations compared with that of the venom of *Pseudechis porphyriacus*.

3. A number of fractions containing phospholipase A activity were separated from the venom of *P. porphyriacus*. One of these contained sevenfold the activity of the crude venom. A family of enzymes is indicated, with possible substrate or activation specificities.

4. The relationship of these preparations to phospholipase A prepared from other venoms is discussed.

We wish to thank the Director of Ross Allen's Reptile Institute, Florida, U.S.A., and the Director of the Medical Research Institute, Johannesburg, South Africa, for gifts of venoms. We thank Miss M. Gilbo for carrying out the paper electrophoresis, Dr I. J. O'Donnell for the ultracentrifuge analysis and Miss F. Bates and Miss S. Marklein for skilled technical assistance. This work was reported in part to the 4th Annual Meeting of the Australian Biochemical Society, Perth (1959).

REFERENCES

Doery, H. M. (1958). *Biochem. J.* **70**, 535.
Feldberg, W. & Kellaway, C. H. (1937). *J. Physiol.* **90**, 257.
Feldberg, W. & Kellaway, C. H. (1938). *J. Physiol.* **94**, 187.
Habermann, E. (1954). *Z. phys. Chem.* **297**, 104.
Habermann, E. (1957). *Biochem. Z.* **329**, 405.
Habermann, E. & Neumann, W. P. (1957). *Biochem. Z.* **328**, 465.
Holden, H. F. (1933). *Aust. J. exp. Biol. med. Sci.* **11**, 1.

Holden, H. F. (1934). *Aust. J. exp. Biol. med. Sci.* **12**, 55.
Holden, H. F. (1935). *Aust. J. exp. Biol. med. Sci.* **13**, 103.
Kellaway, C. H. & Williams, F. C. (1933). *Aust. J. exp. Biol. med. Sci.* **11**, 75, 81.
Lovelock, J. E. (1955). *Biochem. J.* **60**, 692.
McCosker, D. E. & Daniel, L. J. (1959). *Arch. Biochem. Biophys.* **79**, 1.
Neumann, W. P. & Habermann, E. (1952). *Naturwissenschaften*, **39**, 286.
Neumann, W. P. & Habermann, E. (1954). *Arch. exp. Path. Pharmak.* **222**, 367.
Neumann, W. P. & Habermann, E. (1955). *Biochem. Z.* **327**, 170.
North, E. A. & Doery, H. M. (1958). *Nature, Lond.*, **182**, 1374.
Rous, P. & Turner, J. R. (1916). *J. exp. Med.* **23**, 219.
Saunders, L. (1957). *J. Pharm., Lond.*, **9**, 834.
Trethewie, E. R. (1939). *Aust. J. exp. Biol. med. Sci.* **17**, 145.

Biochem. J. (1960) **78**, 827

The Partition of Solutes between Buffer Solutions and Solutions Containing Hyaluronic Acid

BY A. G. OGSTON* AND C. F. PHELPS†
Department of Biochemistry, University of Oxford

(*Received* 24 *August* 1960)

This paper describes experiments which show that hyaluronic acid may affect markedly the partition of diffusible solutes between solutions that contain it and buffer solutions; this effect can be described as the exclusion of the solute from a part of the volume of solution occupied by the hyaluronic acid. Johnston (1955) suggested the occurrence of a large degree of exclusion to explain the effect of hyaluronic acid on the ultracentrifugal sedimentation of serum albumin; however, Blumberg & Ogston (1956) were able to find only a small effect, by observing the effect of albumin on the sedimentation of hyaluronic acid. Aldrich (1958) observed directly a small degree of exclusion of raffinose by hyaluronic acid.

In spite of Blumberg & Ogston's result, steric considerations suggested that with large solute molecules quite large degrees of exclusion might be expected; such exclusions would be of considerable interest in relation to the partition of solutes between blood and tissue spaces believed to contain hyaluronic acid (reviewed by Rogers, 1961). Moreover, the occurrence of mutual exclusion is familiar in the physical chemistry of high polymers (Flory, 1943; Scott, 1949). Accordingly, we decided to investigate the effect by the direct method of dialysing solute to equilibrium between buffer solutions and solutions containing hyaluronic acid.

* Present address: Australian National University, Canberra, A.C.T., Australia.
† Present address: Department of Physiology, The University, Bristol.

EXPERIMENTAL

Methods of dialysis

β-Dextrin. As this solute passes easily through cellophan, a method like that of Aldrich (1958) was used. Since β-dextrin could not be estimated accurately in the presence of hyaluronic acid (because the latter reacts to some extent with periodate), only the buffer fluid was analysed after equilibration. The amount of β-dextrin in the hyaluronic acid solution was obtained by subtracting from the total β-dextrin that remaining in the buffer. Since the volume of the hyaluronic acid solution might change a little during dialysis, this was estimated by mopping buffer off the sac which contained it (at the end of dialysis), weighing the sac and subtracting the weight of the wet cellophan. A reasonably accurate estimate of the concentration of β-dextrin in the hyaluronic acid solution was thus made.

Other solutes. Instead of cellophan, Millipore HA membrane, of average pore diamter 0·45 μ, was used (Millipore Filter Corp., Watertown, Mass., U.S.A.). The membrane separated two compartments each of 5 ml. capacity, in the Perspex apparatus shown in Fig. 1. All the

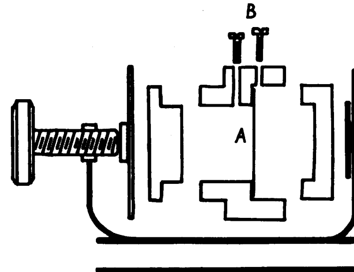


Fig. 1. Diagram of dialysis cell and clamp. *A*, Millipore membrane; *B*, filling plugs.