Toxicon, Vol. 30, No. 8, pp. 815–823, 1992.
Printed in Great Britain.

0041–0101/92 $5.00 + .00

AN ELECTROPHORETIC STUDY ON PHOSPHOLIPASE A_2 ISOENZYMES IN THE VENOMS OF CENTRAL AMERICAN CROTALINE SNAKES

CARMEN VALIENTE,[1] EDGARDO MORENO,[2,3] ANA SITTENFELD,[2] BRUNO LOMONTE[4] and JOSÉ MARÍA GUTIÉRREZ[4*]

[1]Facultad de Microbiología, Universidad de Costa Rica; [2]Centro de Investigación en Biología Celular y Molecular, Universidad de Costa Rica; [3]Escuela de Medicina Veterinaria, Universidad Nacional, Heredia; and [4]Instituto Clodomiro Picado, Facultad de Microbiología, Universidad de Costa Rica, San José, Costa Rica

(*Received* 12 *November* 1991; *accepted* 5 *March* 1992)

C. VALIENTE, E. MORENO, A. SITTENFELD, B. LOMONTE and J. M. GUTIÉRREZ. An electrophoretic study on phospholipase A_2 isoenzymes in the venoms of Central American crotaline snakes. *Toxicon* **30**, 815–823, 1992.—The number and isoelectric points of phospholipase A_2 isoenzymes were studied in the venoms of 12 Central American crotaline snakes of the genera *Bothrops*, *Crotalus*, *Lachesis* and *Agkistrodon*. The study was carried out by using a methodology based on electrophoretic separation of venoms, transfer to nitrocellulose and detection of activity of the bands by an indirect hemolytic assay in agarose–erythrocyte–egg yolk gels. All venoms tested had indirect hemolytic activity, although they varied in the number and isoelectric point of their phospholipases A_2. Most venoms had predominantly acidic isoenzymes, with the exception of *A. bilineatus* which had mainly basic isoenzymes and *B. schlegelii* which had both acidic and basic isoenzymes. Analysis of interindividual variability in *B. asper* venom demonstrated that two phospholipase A_2 isoenzymes are present in some venoms but absent in others. Polyvalent antivenom was effective in neutralizing phospholipase A_2 activity of the 12 venoms tested, when venoms and antivenom were incubated in the fluid phase. This work demonstrates a conspicuous interspecific variability in the number and isoelectric points of phospholipases A_2 present in Central American crotaline snake venoms.

INTRODUCTION

PHOSPHOLIPASES A_2 (E.C. 3.1.1.4.) are present in a large variety of venoms and pancreatic secretions (TU, 1977; KOCHVA, 1977; DUBOURDIEU *et al.*, 1987). In snake venoms these enzymes exert, besides their digestive role, various pharmacological activities such as neurotoxicity, myotoxicity, anticoagulant effect, hypotensive effect and cardiotoxicity (TU, 1977; MEBS, 1978; ROSENBERG, 1990).

* Author to whom correspondence should be addressed.

Usually venom from a single species contains several isoenzymes of phospholipase A_2, as has been shown by chromatographic and electrophoretic separation of a variety of snake venoms (SALACH *et al.*, 1971; SHILOA *et al.*, 1973; SHIER and TROTTER, 1978; DURKIN *et al.*, 1981; DUBOURDIEU *et al.*, 1987). A simple technique was described by MORENO *et al.* (1988) to detect phospholipase A_2 isoenzymes in a venom. It is based on the separation of venom components by isoelectric focusing followed by transfer of proteins to nitrocellulose paper and detection of phospholipase A_2 activity by an indirect hemolytic assay in agarose–erythrocyte–egg yolk gels.

In this work we have used this technique, together with other electrophoretic procedures, to study the phospholipase A_2 isoenzymes of 12 Central American species of the subfamily Crotalinae. In addition, the total phospholipase A_2 activity of these venoms and its neutralization by a polyvalent antivenom were assessed.

MATERIALS AND METHODS

Venoms and antivenoms

The venoms of adult specimens of the following species were studied: *Bothrops asper* (Atlantic and Pacific populations from Costa Rica), *B. schlegelii*, *B. godmani*, *B. lateralis*, *B. picadoi*, *B. nasutus*, *B. ophryomegas*, *B. nummifer*, *B. nigroviridis*, *Crotalus durissus durissus*, *Lachesis muta stenophrys* and *Agkistrodon bilineatus*. All of the specimens from which venoms were obtained were from Costa Rica, with the exception of those of *A. bilineatus* which were collected in Guatemala. The snakes were kept at the serpentarium of the Instituto Clodomiro Picado. Venom from each species was a pool obtained from more than 40 specimens, except in the case of *A. bilineatus*, obtained from four individuals. In order to study individual variability in the phospholipase A_2 isoenzyme pattern in *B. asper*, venom from eight adult specimens was obtained individually. Once collected, all venoms were lyophilized and stored at −20°C. The polyvalent antivenom produced at the Instituto Clodomiro Picado was used in neutralization studies. It was prepared from the plasma of horses immunized with a mixture of equal parts of the venoms of *B. asper*, *C. durissus*, and *L. muta* (BOLAÑOS and CERDAS, 1980).

Quantitation of phospholipase A_2 activity by radial indirect hemolysis in gels

Agarose gels containing washed human erythrocytes, $CaCl_2$ and egg yolk were prepared according to the method of GUTIÉRREZ *et al.* (1988). For each venom, solutions of various concentrations were prepared using phosphate-buffered saline solution, pH 7.2, as diluent. Ten microliters of each solution were applied to wells made in the gels. For controls, 10 µl of saline solution were tested. After 20 hr of incubation at 37°C, the diameters of hemolytic halos were measured and dose–response curves plotted. The minimum hemolytic dose (MHD) of each venom was defined as the amount of venom that induced a hemolytic halo of 15-mm diameter.

Phospholipase A_2 separation, blotting and detection

Venoms were separated by isoelectric focusing electrophoresis (IEF) in agarose gels (11.5 × 5 cm) containing ampholytes of pH range from 3–10, using a flat bed IEF apparatus (Pharmacia FBE 3000; Pharmacia, Sweden) with a cooling system (Coolnics CTR-220, Japan) at 4°C in combination with an electrophoresis power supply ECPE 3000/150 (Pharmacia, Sweden). In some experiments, IEF was carried out in a Phast System TM apparatus (Pharmacia, Sweden) for minigels (pH range 3–9), at 540 V-hr, according to the manufacturer's instructions. Electrophoresis for basic proteins was carried out on 10% polyacrylamide gels, pH 4.3, according to the method of REISFELD *et al.* (1962).

Blotting of polyacrylamide gels was performed as described by TOWBIN *et al.* (1979) with some modifications (LOMONTE and KAHAN, 1988). Blotting of isoelectric focusing gels was done as described by SITTENFELD and MORENO (1987). Blotting of IEF minigels was carried out by transferring the proteins for 20 min in a humid chamber onto nitrocellulose sheets, according to the manufacturer's (Pharmacia) instructions. Detection of phospholipase A_2 activity on nitrocellulose sheets after transfer was done on agarose gels containing washed human erythrocytes, egg yolk and $CaCl_2$, as described by MORENO *et al.* (1988).

In order to determine the detection limit of this hemolytic assay, known amounts of bovine pancreatic phospholipase A_2 (Sigma) were applied to nitrocellulose sheets which were then placed on agarose–erythrocyte–egg yolk gels as described above. After incubation, the minimum enzymatic activity giving a visible hemolytic reaction was determined and defined as the detection limit of the assay.

Neutralization of indirect hemolytic activity by antivenom

Mixtures of venoms and polyvalent antivenom were prepared in order to have various ratios of μl antivenom/mg venom. They were incubated for 30 min at 37°C, and then 10 μl of each mixture (containing one minimum hemolytic dose of venom) were tested for hemolytic activity as described. Hemolytic activity was expressed as a percentage, taking as 100% the diameter of hemolytic halos induced by the samples in which venom was incubated with no antivenom. Neutralizing ability of antivenom was expressed as effective dose 50% (ED_{50}), defined as the ratio of μl antivenom/mg venom where hemolytic activity of venom was reduced 50%.

RESULTS

Indirect hemolytic activity of venoms

All venoms tested had phospholipase A_2 activity, as evidenced by the formation of hemolytic halos in the agarose–erythrocyte–egg yolk gels. In contrast, saline solution and antivenom did not induce hemolysis. When egg yolk was not added to the gels there was no hemolysis, indicating that hemolysis was only of the indirect type, i.e. due to phospholipase A_2 activity in these venoms. There were quantitative differences in this activity between venoms of different species. Minimum hemolytic doses ranged from 0.59 $\pm$ 0.24 μg in the case of *B. asper* (Pacific) venom to 10.0 $\pm$ 0.02 μg in the case of *B. nigroviridis* venom (Table 1).

Phospholipase A_2 isoenzymes detected after isoelectric focusing

The detection limit of the indirect hemolytic assay used for the analysis of isoenzymes corresponded to an enzymatic activity of 0.3 μEq fatty acids released per min at 37°C. With the exception of *B. nigroviridis* venom, the venoms showed several electrophoretic variants of phospholipase A_2 (Figs 1–3). Fewer isoenzymes were detected when the Phast system was used (Fig. 3), although in this case bands were sharper. The isoenzymes varied among species, not only in number, but also in p*I* and intensity of the hemolytic reaction. The majority of the venoms had predominantly acidic isoenzymes, with p*I* values ranging from 4.0 to 5.0. However, the venom of *A. bilineatus* had mainly basic isoenzymes (Fig. 3) and that of *B. schlegelii* had abundant acidic and basic variants (Figs 1, 3). Since the range

TABLE 1. INDIRECT HEMOLYTIC ACTIVITY OF VENOMS FROM CENTRAL AMERICAN CROTALINE SNAKES ON AGAROSE–ERYTHROCYTE–EGG YOLK GELS

Venom	Minimum hemolytic dose (μg)*
Bothrops asper (Pacific)	0.59±0.24
Bothrops asper (Atlantic)	1.75±0.87
Bothrops godmani	0.70±0.09
Bothrops schlegelii	1.07±0.20
Bothrops lateralis	2.65±0.75
Bothrops nasutus	7.60±0.46
Bothrops nigroviridis	10.00±0.06
Bothrops nummifer	7.80±0.07
Bothrops picadoi	7.50±3.00
Bothrops ophryomegas	7.50±2.88
Lachesis muta stenophrys	0.76±0.16
Crotalus durissus durissus	4.75±0.29
Agkistrodon billineatus	1.09±0.53

*Minimum hemolytic dose (MHD): amount of venom (in μg) which induced a hemolytic halo of 15 mm diameter after 20 hr of incubation at 37°C. Results are presented as mean±S.E. (n = 4).

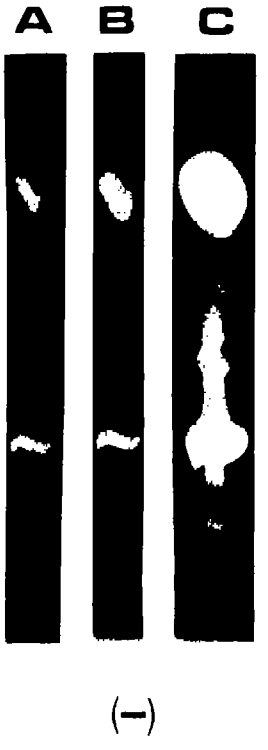

FIG. 1. PHOSPHOLIPASE A_2 ISOENZYMES IN *Bothrops schlegelii* VENOM.
Venom was electrophoretically separated, transferred onto nitrocellulose paper and then tested for indirect hemolytic activity on agarose–erythrocyte–egg yolk gels at 37°C for 1 hr (A), and then at 4°C for additional 4 hr (B) and 15 hr (C). The cathode is located at the bottom.

of ampholyte pH was from 3 to 10, the resolution of basic isoenzymes having p*I* higher than 9 was not adequate. These variants were analyzed by electrophoresis in polyacrylamide gels at pH 4.3.

Individual variability in the pattern of phospholipase A_2 isoenzymes in B. asper *venom*

In order to detect individual variability in *B. asper*, venom from eight adult specimens of the Pacific region of Costa Rica were separated by isoelectric focusing. Individual variability was evident, since there were two isoenzymes of p*I* 4.0 and 7.5 which were present in only two samples. The rest of the isoenzymes were observed in all samples, although there were quantitative variations among them, as judged by the intensity of hemolysis.

Phospholipase A_2 isoenzymes detected after polyacrylamide gel electrophoresis for basic proteins

When venoms were separated by electrophoresis on polyacrylamide gels, pH 4.3, several isoenzymes were readily resolved (Fig. 4). Hemolytic bands were observed in the venoms of *B. asper, B. schlegelii, B. nummifer, B. godmani, B. lateralis, B. picadoi, A. bilineatus, L. muta* and *C. durissus.*

FIG. 2. PHOSPHOLIPASE A_2 ISOENZYMES IN *Crotalus durissus durissus* VENOM.
After isoelectric focusing separation, proteins were transferred onto nitrocellulose paper and tested for activity as described in Fig. 1. Incubations in agarose–erythrocyte–egg yolk gels were carried out for 1 hr (A) at 37°C and then at 4°C for additional 6 hr (B).

Neutralization of indirect hemolytic activity of crotaline snake venoms by polyvalent antivenom

Polyvalent antivenom produced in Costa Rica effectively neutralized indirect hemolytic activity of all venoms on agarose–erythrocyte–egg yolk gels. There were quantitative differences in neutralization of different venoms, and ED_{50} ranged from 508 $\pm$ 47 μl antivenom/mg venom (for the venom of *C. durissus durissus*) to 4411 $\pm$ 276 μl antivenom/mg venom (for the venom of *B. schlegelii*) (Table 2).

DISCUSSION

The techniques used in this work offer several advantages for the study of phospholipase A_2 isoenzymes present in snake venoms. Since separation is based on the charge, i.e. isoelectric point, of the molecules, many variants can be resolved and detected. It has been shown that phospholipases A_2 present in snake venoms are of similar mol. wt but have a large variability in charge (TU, 1977; SHIER and TROTTER, 1978; DUBOURDIEU *et al.*, 1987; MORENO *et al.*, 1988; ROSENBERG, 1990). Highly basic isoenzymes were not adequately resolved by isoelectric focusing. In these cases venoms were also analyzed by polyacrylamide gel electrophoresis at pH 4.3, under non-denaturating conditions, and

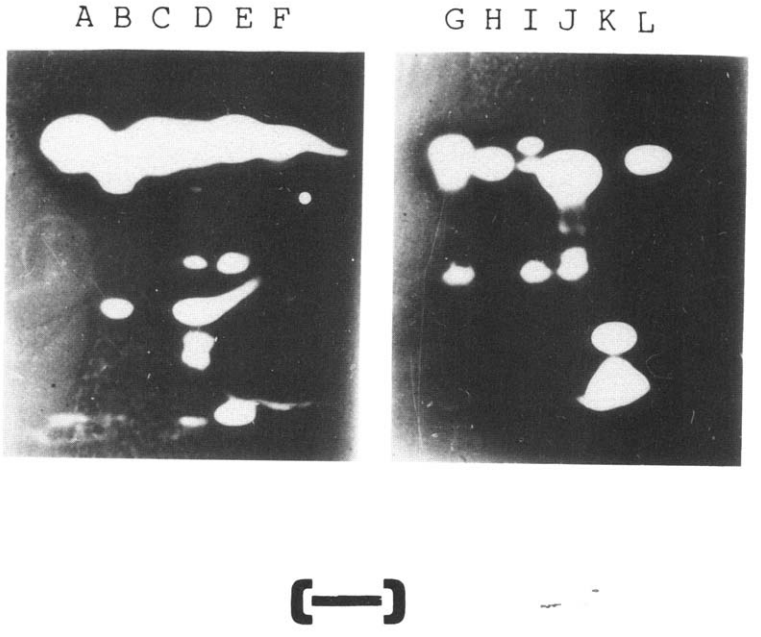

FIG. 3. PHOSPHOLIPASE A_2 ISOENZYMES IN CENTRAL AMERICAN CROTALINE SNAKE VENOMS. Separation of venom proteins was performed by isoelectric focusing using the Phast system. Then, proteins were transferred onto nitrocellulose paper and tested for indirect hemolytic activity as described in Fig. 1. Incubations on agarose–erythrocyte–egg yolk gels were carried out for 16 hr. A = *Bothrops lateralis*; B = *B. godmani*; C = *B. nasutus*; D = *B. schlegelii*; E = *B. asper* (Pacific population); F = *B. asper* (Atlantic population); G = *B. picadoi*; H = *B. nummifer*; I = *B. ophryomegas*; J = *Lachesis muta stenophrys*; K = *Agkistrodon bilineatus*; L = *Crotalus durissus durissus*.

isoenzymes having isoelectric points higher than 9.0 were readily resolved. Since different phospholipases A_2 have different activity and concentration, it was important to allow the hemolytic reaction to proceed for several hours. Highly active or abundant isoenzymes were detected during the first hour of incubation, whereas isoenzymes of low enzymatic activity or concentration were detected only after a prolonged incubation period. Thus, by taking pictures at several incubation intervals, all isoenzymes can be identified (MORENO *et al.*, 1988). Under the experimental conditions followed in this study, the detection limit of the hemolytic assay corresponded to 0.3 μEq fatty acid released per min at 37°C, indicating a high sensitivity of this assay.

In this work we compared two isoelectric focusing systems: the flat bed and the Phast systems. More phospholipase A_2 isoenzymes were detected when using the flat bed system, probably due to the larger sample load, although with the Phast system the bands were sharper. When working with snake venoms, this methodology would be highly useful in laboratories where venoms are stored for long periods of time, such as in antivenom production centers. A qualitative analysis for the identity of venoms can be readily performed by using these procedures, based on our finding that venoms from different species have strikingly different patterns of phospholipase A_2 variants. In addition, these techniques would be of use in the study of intra- and interspecific variability in snake venoms, as well as in designing fractionation protocols for the purification of phospholipases A_2.

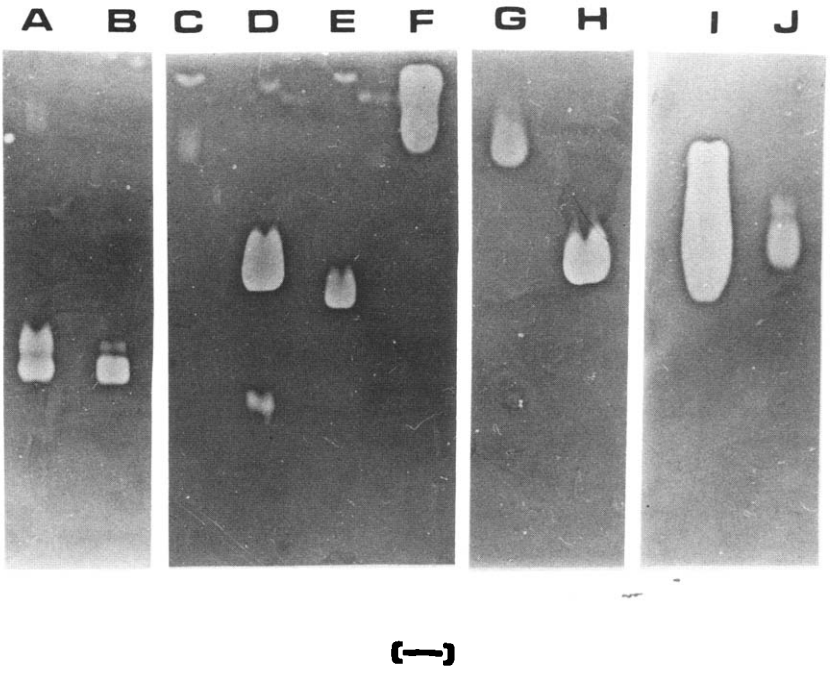

FIG. 4. PHOSPHOLIPASE A_2 ISOENZYMES IN CENTRAL AMERICAN CROTALINE SNAKE VENOMS. Separation of venom proteins was carried out by electrophoresis on polyacrylamide gels, pH 4.3. Then, proteins were transferred onto nitrocellulose paper and tested for indirect hemolytic activity. A = *Bothrops asper* (Pacific population); B = *B. asper* (Atlantic population); C = *B. nummifer*; D = *B. schlegelii*; E = *B. godmani*; F = *B. lateralis*; G = *B. picadoi*; H = *Agkistrodon bilineatus*; I = *Lachesis muta stenophrys*; J = *Crotalus durissus durissus*.

TABLE 2. NEUTRALIZATION OF INDIRECT HEMOLYTIC ACTIVITY OF CENTRAL AMERICAN CROTALINE SNAKE VENOMS BY POLYVALENT ANTIVENOM

Venom	Effective dose 50% (μl antivenom/mg venom)*
Bothrops asper (Pacific)	619 ± 43
Bothrops asper (Atlantic)	543 ± 14
Bothrops godmani	2029 ± 160
Bothrops schlegelii	4411 ± 276
Bothrops lateralis	2215 ± 196
Bothrops nasutus	812 ± 42
Bothrops nigroviridis	1608 ± 472
Bothrops nummifer	843 ± 66
Bothrops picadoi	1662 ± 394
Bothrops ophryomegas	906 ± 425
Lachesis muta stenophrys	666 ± 27
Crotalus durissus durissus	508 ± 47
Agkistrodon bilineatus	2434 ± 30

*Effective dose 50% (ED_{50}): ratio μl antivenom/mg venom at which the diameter of hemolytic halo is reduced 50% when compared to the hemolytic halo resulting from the action of venom alone (see Materials and Methods for details). Results are presented as mean ± S.E. ($n = 4$).

In the case of *B. asper* venom, there is evidence of individual variability in the electrophoretic pattern of phospholipase A_2 isoenzymes. This finding agrees with other studies showing individual variations in the electrophoretic profile of venoms from single species (JIMÉNEZ-PORRAS, 1964*a,b*; LOMONTE and CARMONA, 1992). Therefore, when establishing the phospholipase A_2 isoenzyme pattern of the venom of a particular species, it is necessary to work with pools obtained from a large number of specimens.

Crotaline venoms used in this study showed prominent differences not only in the total phospholipase A_2 activity but also in the number and isoelectric points of isoenzymes. The venoms of *B. nigroviridis, B. nasutus, B. nummifer, B. ophryomegas* and *B. picadoi* had the lowest phospholipase A_2 activity when tested in the gel hemolytic assay. Interestingly, these venoms also had the least number of phospholipase A_2 isoenzymes. In contrast, the venoms of *B. asper, B. godmani, B. schlegelii, L. muta* and *A. bilineatus* had the highest indirect hemolytic activity, also presenting the highest number of phospholipase A_2 isoenzymes, with the exception of the venom of *L. muta* which had only two isoenzymes.

Neutralization assays carried out by incubating venom and antivenom in the fluid phase before testing activity by indirect hemolysis in gels demonstrated the effectiveness of polyvalent antivenom against all venoms tested. The highest neutralization was achieved with the venoms of *B. asper, L. muta* and *C. durissus*, the three venoms used in the immunization mixture to produce this antivenom (BOLAÑOS and CERDAS, 1980). However, the fact that it also neutralizes other venoms indicates that there are immunological similarities between phospholipases A_2 of different crotaline venoms. Previous studies have shown the ability of antivenoms to neutralize phospholipase A_2 activity of heterologous venoms (NAIR *et al.*, 1975; GENÉ *et al.*, 1985). In conclusion, this work demonstrates a conspicuous interspecies variability in the number and isoelectric points of phospholipases A_2 present in Central American crotaline snake venoms.

Acknowledgements—The authors thank REYNALDO PEREIRA, EDGAR BOLAÑOS and CARLOS COTO for their valuable collaboration in the photographic and laboratory work. This study was supported by Vicerrectoría de Investigación, Universidad de Costa Rica, project 741-89-057 and by the International Foundation for Science, grant F/0883-3. C. VALIENTE, E. MORENO, J. M. GUTIÉRREZ and B. LOMONTE are recipients of research career awards from the Costa Rican National Scientific and Technological Research Council (CONICIT).

REFERENCES

BOLAÑOS, R. and CERDAS, L. (1980) Producción y control de sueros antiofídicos en Costa Rica. *Bol. Of. Sanit. Panam.* **88**, 189–196.

DUBOURDIEU, D. J., KAWAGUCHI, H. and SHIER, W. T. (1987) Molecular weight variations in the diversity of phospholipase A_2 forms in reptile venoms. *Toxicon* **25**, 333–343.

DURKIN, J. P., PICKWELL, G. V., TROTTER, J. T. and SHIER, W. T. (1981) Phospholipase A_2 electrophoretic variants in reptile venoms. *Toxicon* **19**, 535–546.

GENÉ, J. A., GÓMEZ, M., GUTIÉRREZ, J. M. and CERDAS, L. (1985) Neutralization of hyaluronidase and indirect hemolytic activities of Costa Rican snake venoms by a polyvalent antivenom. *Toxicon* **23**, 1015–1018.

GUTIÉRREZ, J. M., AVILA, C., ROJAS, E. and CERDAS, L. (1988) An alternative *in vitro* method for testing the potency of the polyvalent antivenom produced in Costa Rica. *Toxicon* **26**, 411–413.

JIMÉNEZ-PORRAS, J. M. (1964*a*) Intraspecific variations in composition of venom of the jumping viper, *Bothrops nummifera*. *Toxicon* **2**, 187–195.

JIMÉNEZ-PORRAS, J. M. (1964*b*) Venom proteins of the fer-de-lance, *Bothrops atrox*, from Costa Rica. *Toxicon* **2**, 155–166.

KOCHVA, E. (1987) The origin of snakes and evolution of the venom apparatus. *Toxicon* **25**, 65–106.

LOMONTE, B. and CARMONA, E. (1992) Individual expression patterns of myotoxin isoforms in the venom of the snake *Bothrops asper*. *Comp. Biochem. Physiol.* (in press).

LOMONTE, B. and KAHAN, L. (1988) Production and partial characterization of monoclonal antibodies to *Bothrops asper* (terciopelo) myotoxin. *Toxicon* **26**, 675–689.

MEBS, D. (1978) Pharmacology of reptilian venoms. In: *Biology of the Reptilia*, Vol. 8, pp. 437–560 (GANS, C., Ed.). London: Academic Press.

MORENO, E., ALAPE, A., SÁNCHEZ, M. and GUTIÉRREZ, J. M. (1988) A new method for the detection of phospholipase A_2 variants: Identification of isozymes of the venoms of newborn and adult *Bothrops asper* (terciopelo) snakes. *Toxicon* **26**, 363–371.

NAIR, B. C., NAIR, C. and ELLIOT, W. B. (1975) Action of antisera against homologous and heterologous snake venom phospholipases A_2. *Toxicon* **13**, 453–456.

REISFELD, R. A., LEWIS, U. J. and WILLIAMS, D. E. (1962) Disk electrophoresis of basic proteins and peptides on polyacrylamide gels. *Nature* **195**, 281–283.

ROSENBERG, P. (1990) Phospholipases. In: *Handbook of Toxinology*, pp. 67–277 (SHIER, W. T. and MEBS, D., Eds). New York: Marcel Dekker.

SALACH, J. I., TURINI, P., SENG, R., HAUBER, J. and SINGER, T. P. (1971) Phospholipase A_2 of snake venoms. I. Isolation and molecular properties of isoenzymes from *Naja naja* and *Vipera russelli* venoms. *J. biol. Chem.* **246**, 331–336.

SHIER, W. T. and TROTTER, J. T. (1978) Phospholipase A_2 electrophoretic variants and their detection after polyacrylamide gel electrophoresis. *Analyt. Biochem.* **87**, 604–611.

SHILOA, J., KLIBANSKY, C. and DE VRIES, A. (1973) Phospholipase isoenzymes from *Naja naja* venom. I. Purification and partial characterization. *Toxicon* **11**, 481–490.

SITTENFELD, A. and MORENO, E. (1988) A sensitive blotting system for detection of alpha fetoprotein variants with monoclonal and polyclonal antibodies. *J. immunol. Meth.* **106**, 19–26.

TOWBIN, H., STAEHELIN, T. and GORDON, J. (1979) Electrophoretic transfer of proteins from polyacrylamide gels to nitrocellulose sheets. *Proc. natn. Acad. Sci. U.S.A.* **76**, 4350–4354.

TU, A. T. (1977) *Venoms: Chemistry and Molecular Biology*. New York: John Wiley.