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CROTOXIN. I. IMMUNOLOGY AND INTERACTION OF THE SUBUNITS

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1. Phospholipase A and crotapotin were purified by gel filtration and ion-exchange chromatography from *Crotalus durissus terrificus* venom. The minimal molecular weights obtained by amino acid analysis were 13,400 daltons for phospholipase A and 8,300 daltons for crotapotin.

2. Phospholipase A and crotapotin form a 1:1 molar complex that was stable during gel filtration. The LD_{50} of phospholipase A was increased about 10-fold when in a 1:1 complex with crotapotin.

3. Crotapotin, phospholipase A and crotoxin (crotapotin + phospholipase A) gave precipitin reactions when tested with specific rabbit antisera. Immunological studies of these proteins using crotalus antiserum confirmed 1:1 molar ratio of the complex.

4. Crotoxin antiserum also reacts with either crotapotin or phospholipase A separately. Similar antigenic determinants were observed in the subunits and it seems that some antigenic determinants are masked in the phospholipase A-crotapotin complex.

Key words: crotoxin, phospholipase A, crotapotin, *Crotalus durissus terrificus* venom, crotapotin-phospholipase A complex.

Introduction

Crotoxin, the neurotoxic protein of *Crotalus durissus terrificus* venom, was isolated and crystallized by Slotta and Fraenkel-Conrat (1938). The results of amino-terminal analysis by the fluoridinitrobenzene (FDNB) reaction indicated the presence of two different proteins (Fraenkel-Conrat and Singer, 1956). Using ion-exchange chromatography, Rubsamen *et al.* (1971) and Hendon and Fraenkel-Conrat (1971) separated crotoxin into two proteins: the basic protein phospholipase A (EC 3.1.1.4.) and the acidic protein crotapotin. The characteristics of these proteins and their interactions have been studied in order to determine how two proteins with little or no toxicity alone can be extremely toxic when combined (Habermann *et al.*, 1972; Horst *et al.*, 1972; Breithaupt *et al.*, 1974; Paradies and Breithaupt, 1975; Hendon and Fraenkel-Conrat, 1976).

The objective of the present research was to study the interaction of crotapotin and phospholipase A and their biological activity separately and in complex. The use of gel filtration associated with immunological methods indicated that the 1:1 molar mixture of the subunits of crotoxin increased the ID_{50} of phospholipase A about 10-fold. Crotapotin labelled with ^{125}I was used to detect the presence of the complex in the gel filtration effluent. The immunological reactions of the anticrotalic serum with [^{125}I]-crotapotin, and the cross reactions among the specific antisera against crotoxin, phospholipase A and crotapotin also demonstrated that interaction with crotapotin was important for the increase in the toxicity of phospholipase A.

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Some of these data were presented at the II Reunión Regional de PAABS, Córdoba, Argentina, 1977 (Rogero *et al.*, 1977 and Nakazone *et al.*, 1977).

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Material and Methods

Protein purification

The first step in the purification of crotoxin was gel filtration using a procedure similar to that we employed for crotamine (Cheymol *et al.*, 1971). About 200 mg of dry *Crotalus durissus terrificus* venom was dissolved in 10 ml 0.1 M acetic acid and centrifuged at 12,100 *g* for 10 min. The supernatant solution was applied to a column of Sephadex G-75 fine (3 x 80 cm), equilibrated with 0.1 M acetic acid at 4°C. The flow rate was 0.5 ml/min and the effluent was collected in 10 ml fractions. The effluent, containing crude crotoxin detected by UV absorbance and biological activity measurements, was pooled and lyophilized. The material was dissolved in 5 ml of 50 mM ammonium formate buffer, pH 3.3, and applied to a 0.9 cm x 40 cm column of SP Sephadex C-25 equilibrated with the same buffer at 4°C. The column was eluted with a linear gradient from 0 to 3 M NaCl in the same buffer at 0.2 ml/min and fractions of 4 ml were collected. The elution profile was obtained with a Gilson UV monitor at 280 nm. The effluent containing crotapotin and phospholipase was pooled separately and dialyzed against deionized water in cellophane dialysis tubing (treated overnight with pyridine:acetic anhydride (1:1) to decrease pore size) until salt-free, and the proteins were lyophilized. Phospholipase A was rechromatographed several times under the same conditions until a single symmetrical peak was obtained. Similarly, crotapotin was rechromatographed several times on a 0.9 cm x 30 cm column of DEAE Sephadex A-25 equilibrated with 0.05 M ammonium formate buffer, pH 3.3, and eluted with a linear 0-3 M NaCl gradient in the same buffer until a single symmetrical peak was obtained. After each purification step the protein was extensively dialyzed against water, lyophilized and stored in a desiccator under vacuum at 0°C.

Polyacrylamide gel electrophoresis

All purification steps were monitored by disc electrophoresis analysis in polyacrylamide gel by the method of Davis (1964) using 7 and 15% gels at pH 4.3 and 9.5. About 0.1 mg of protein was applied to each gel and, after electrophoresis, the gels were treated with 12% aqueous trichloroacetic acid and stained with Brilliant Blue R 250.

Amino acid analyses

Amino acid analyses were carried out with a Beckman model 120 C amino acid analyzer. Five hundred micrograms crotapotin or phospholipase A were hydrolyzed in 6 N hydrochloric acid in a sealed evacuated tube for 20 h at 110°C. The hydrolysates were evaporated to dryness and twice-distilled water was added. The process was repeated three times. The dry hydrolysate was dissolved in the sodium citrate buffer and applied to the amino acid analyzer.

Phospholipase A activity

The enzymatic activity of phospholipase A was measured by the method of Haberman and Neuman (1954). An egg yolk suspension in saline solution (50 μl) was incubated with 50 μl of the protein for 2 h at 37°C. Then 4.9 ml of a 6% (v/v) red blood cell suspension was added and after 40 min at 37°C hemolysis was measured at 560 nm with a Zeiss model PMQ II spectrophotometer.

Coagulase activity

The possible contamination of the proteins with the enzymatic complex "coagulase" was tested using the fibrinogen coagulation method described by Gonçalves (1956). None of the purified proteins had detectable coagulase activity.

Protein determination

The method of Lowry *et al.* (1951) was employed using bovine serum albumin (Fraction V) as a standard.

Iodination of crotapotin

Crotapotin was labelled with ^{125}I by the method of Greenwood *et al.* (1973). About 5 μg of crotapotin was dissolved in 0.5 ml of 0.25 M phosphate buffer, pH 7.4 and mixed with 1.0 mCi carrier-free Na ^{125}I (Union Carbide). The reaction was started by adding 20 μl of chloramine T (2.65 mg/ml in the same buffer) and stopped after one min by adding 20 μl of sodium metabisulfite (4.8 mg/ml in the same buffer). Only protein having 5% or less free ^{125}I was used in the assays.

Determination of LD_{50}

The toxicity of phospholipase A, crotapotin and crotoxin was determined by intraperitoneal injection into 20 g male Swiss mice. Appropriate dilutions of a 50 μg/ml solution of each protein were prepared and injections of 0.2 ml/mouse were used. The LD_{50} was calculated by the method of Reed and Muench (1937) after a 24 h period of observation.

Interaction studies

Sephadex G-75 fine, 0.9 cm x 100 cm, was used to study the formation of the crotapotin and phospholipase A complex at 4°C. The proteins were mixed in different proportions and applied to the column equilibrated and developed with 0.1 M ammonium formate buffer, pH 3.5, at 0.2 ml/min. In some experiments, a small amount of radiolabelled crotapotin was added to the cold protein and used as a marker to detect the complex in the effluent. Chromatographic profiles were determined by absorption at 280 nm with an LKB UV monitor Uvicord II 8300. A planimeter (Keuffel & Esser Co., New York) was used to measure the peak areas for the calculation of protein concentration.

Immunological studies using anticrotalus serum

An anticrotalus serum produced by the Butantan Institute (São Paulo, Brazil) was used to study the immunological aspects of the phospholipase A and crotapotin interaction. The precipitation method was the same as that used by Kabat and Mayer (1961). Tubes containing 0, 50, 75, 100, 150, 200, 300, 400 and 600 μg of crotapotin, phospholipase A, crotoxin (1:1 molar ratio of crotapotin and phospholipase A) or crystallized crotoxin in 0.8 ml of 0.1 M phosphate buffer, were incubated with 0.2 ml of anticrotalus serum for one h at 37°C and 24 h at 4°C. The precipitates were centrifuged, washed several times with the same buffer and dissolved in 1.0 ml of 0.1 N NaOH. The absorption at 280 nm was used to measure the amount of protein in the precipitate. Mixtures with different molar ratios of crotapotin and phospholipase A were tested to determine the maximum level of precipitin.

Immunoelectrophoresis

[^{125}I]-crotapotin was incubated with anticrotalus serum and spotted on strips of Whatman No. 1 filter paper for electrophoresis in 0.1 M phosphate buffer, pH 7.0 at 350 V and 3 mA per strip, for 4 h. Half of these strips were dried and divided into small pieces and counted in a Nuclear Chicago gamma counter model 120 C. The other half of the strips were stained with amido black stain.

Antisera against crotapotin, phospholipase A and crotoxin (1 crotapotin + 1 phospholipase A)

The antisera were produced in rabbits by subcutaneous injection of 4 mg of the antigen dissolved in 0.5 ml of 0.15 M phosphate buffer, pH 7.0, emulsified with the same volume of complete Freund adjuvant. Two boosters of 2 mg antigen were injected subcutaneously at 15 day intervals. The blood was drawn from rabbits one month after the last injection and the sera were stored at -20°C.

Immunological studies with specific antisera against crotapotin, phospholipase A and crotoxin

Precipitation reactions were carried out by adding 0.2 ml of specific antiserum to 0, 50

75, 200, 300, 400 and 600 μg of crotapotin, phospholipase A and crotoxin per 0.8 ml 0.1 M phosphate buffer, pH 7.0. After incubation for one h at 37°C, the tubes were stored overnight at 4°C. The precipitates were dissolved in 1 ml 0.1 M NaOH, and protein was measured spectrophotometrically at 280 nm.

Cross reactivity

Tests for cross reactivity between crotapotin, phospholipase A, and crotoxin with the three specific antisera were carried out under the same conditions.

Immunodiffusion

The double diffusion method of Ouchterlony (1958) was performed on 2% agarose plates in 0.1 M phosphate buffer, pH 7.0, to characterize purified crotapotin, phospholipase A and crotoxin.

Results and Discussion

Purification and characterization of the proteins

The basic step for the purification of crotoxin is shown in Figure 1A. Five peaks were

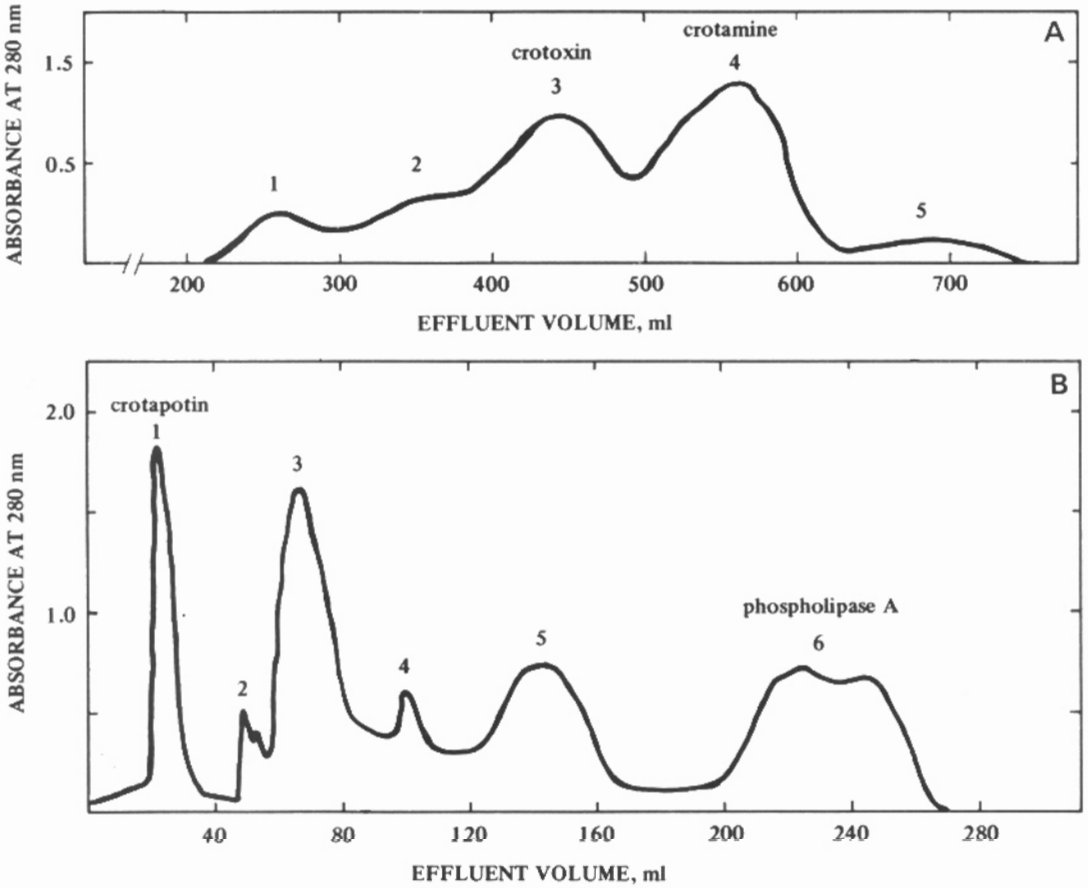

Figure 1 - Preparation of crotoxin, crotapotin and phospholipase A. *A*, Sephadex G-75 filtration of 200 mg dry crude venom dissolved in 10 ml 0.1 M acetic acid. The column, 3.0 cm x 80 cm, was equilibrated and eluted with the same solution at 0.5 ml/min. The effluent was collected in 10 ml fractions. *B*, chromatographic dissociation of crotoxin into crotapotin and phospholipase A. One hundred milligrams of crude crotoxin (peak 3, Figure 1A), was dissolved in 0.1 M ammonium formate buffer, pH 3.3, and chromatographed on SP-Sephadex C-25, 0.9 cm x 40 cm, eluted with a linear gradient of 0-3 M NaCl in the same buffer. Flow rate was 0.2 ml/min, 4 ml/tube.

obtained after Sephadex G-75 gel filtration of the crude *Crotalus d. terrificus* venom. Peak 3 corresponds to crotoxin. Other components such as crotamine (Gonçalves and Vieira, 1950) will be described in detail in future publications. Recently, several effective methods for isolating crotapotin and phospholipase A have been described but different elution patterns have been obtained containing peaks of unknown proteins when different crude venoms are analyzed. Ion exchange chromatography following gel filtration of the crude venom to isolate crotoxin proved to be a good method with a good recovery ratio and is an easy way to identify crotapotin and phospholipase A.

The results of SP-Sephadex ion-exchange chromatography of crotoxin (Figure 1A, peak 3) are shown in Figure 1B. Crotapotin was identified as peak 1 and phospholipase A as peak 6. Peaks 2, 3 and 4 did not contain phospholipase A activity and were not studied further. Cross reactivity between crotamine and crotoxin was observed in spite of the fact that the crotoxin "pool" was rechromatographed several times before the ion-exchange chromatography step. Peak 5 in Figure 1B was shown to be crotamine. Phospholipase A (6, in Figure 1B) appeared as a double peak, but even after several attempts it was not possible to demonstrate two different enzymes as reported by Breithaupt *et al.* (1975). The proteins were pooled and rechromatographed under the same conditions until only a single symmetrical peak was obtained.

Polyacrylamide gel electrophoresis showed only one band for each protein as illustrated in Figure 2.

The amino acid analysis of crotapotin and phospholipase A, reported in Table 1, is essentially the same as that reported by Hendon and Fraenkel-Conrat (1971).

Table 2 shows the LD_{50} of intraperitoneal injections of the purified proteins for mice (0.5 mg/kg for phospholipase A and 0.1 mg/kg for crotoxin). The toxicity of phospholipase A dramatically increased about 10 times (0.06 mg/kg) when crotapotin was present in a 1:1 molar ratio. However, no further increase in toxicity was detected with molar ratios in excess of 1:1.

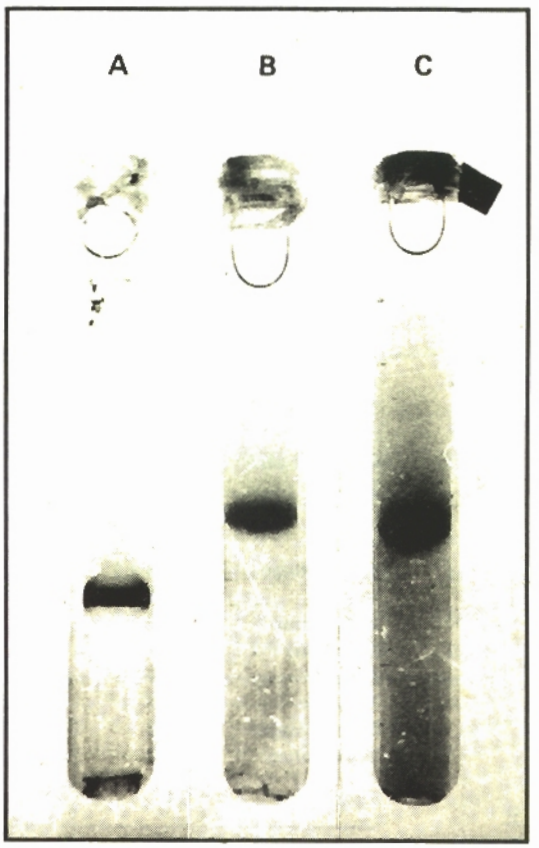

Figure 2 - Polyacrylamide gel electrophoresis. The samples were 0.1 mg crotapotin (A) and crotoxin (C) in Tris-glycine buffer, pH 9.5 (cathode at top), and 0.1 mg phospholipase A (B) in alanine-acetic acid buffer, pH 4.3 (cathode at bottom). After electrophoresis, the gels were treated with 12% trichloroacetic acid and stained with Brilliant Blue R 250.

Phospholipase A-crotapotin interactions

Sephadex G-75 gel filtration was used to demonstrate complex formation and determine the stoichiometry of the interaction. A typical experiment is illustrated in Figure 3. It shows that the elution volume of the crotoxin complex is the same when prepared from an excess of phospholipase A or an excess of crotapotin. Even though there is only a small difference between the molecular weights of crotoxin and phospholipase A, Sephadex G-75 gel filtra-

Table 1 - *Amino acid composition of crotapotin and phospholipase A.*

The data reported are the average of three determinations. Tryptophan was determined spectrophotometrically by the method of Beavan and Holiday (1952).

Amino acid	Crotapotin		Phospholipase A	
	Present study	Hendon and Fraenkel-Conrat (1971)	Present study	Hendon and Fraenkel-Conrat (1971)
Lysine	1.7	1.8	9.3	9.0
Histidine	0.8	0.9	1.5	1.8
Arginine	1.6	1.8	9.2	8.0
Aspartic acid	10.0	10.0	9.0	9.0
Threonine	3.6	3.5	6.4	6.0
Serine	4.3	4.8	5.9	6.0
Glutamic acid	12.5	12.5	9.2	8.2
Proline	4.2	4.5	4.6	4.6
Glycine	8.6	8.6	10.9	10.4
Alanine	5.1	5.2	5.8	5.8
1/2 Cysteine	11.8	10.6	12.3	10.6
Valine	0.5	1.0	1.9	2.0
Methionine	0.7	0.8	2.1	1.6
Isoleucine	1.8	2.5	3.9	4.4
Leucine	0.9	1.0	5.8	5.6
Tyrosine	2.2	2.5	9.6	9.2
Phenylalanine	2.2	2.5	5.9	5.6
Tryptophan	1.0	1.0	2.0	2.0
Total	74	76	113	110

Table 2 - *LD_{50} of phospholipase A and reconstituted crotoxin.*

The crotoxin used was a mixture of purified crotapotin and phospholipase A in 1:1 molar proportions. Six mice were used for each determination. Mortality is based on a 24 h observation period.

Phospholipase A		Crotoxin	
mg/kg	% mortality	mg/kg	% mortality
3.0	100	1.0	100
1.5	83.3	0.5	83.3
0.75	66.6	0.25	83.3
0.375	33.3	0.125	66.6
0.187	0	0.0625	0

tion separated the complex from phospholipase A.

The stability of the resulting phospholipase A-crotapotin complex and the 1:1 stoichiometry of both proteins are shown in Table 3. In order to eliminate the possibility of a chromatographic artefact in these experiments, a small amount of radiolabelled [^{125}I]-crotapotin was sometimes used to monitor complex formation, as shown in Figure 4. The iodination of crotapotin did not interfere with the interaction and this was also later confirmed for the reaction of crotapotin with specific antiserum.

Immunological studies were used to confirm the results obtained by the gel filtration technique. Antiserum against the whole venom produced by the Butantan Institute of São Paulo and specific antiphospholipase A, anticrotapotin and anticrotoxin antisera obtained in our laboratory were used to carry out the studies. Figure 5 shows the qualitative measurement of purified crotapotin, phospholipase A, and a 1:1 molar mixture of crotapotin and phospholipase A by double diffusion immuno-

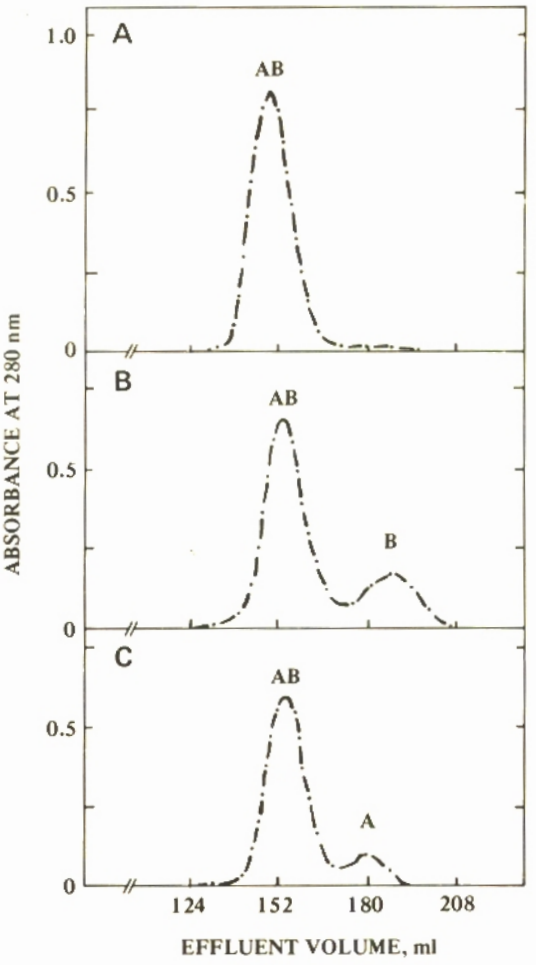

Figure 3 - Interaction of crotapotin and phospholipase A demonstrated by gel filtration. The column of Sephadex G-75, 0.9 cm x 100 cm, was equilibrated and eluted with 50 mM ammonium formate buffer, pH 3.5, at 0.2 ml/min and fractions of 4 ml were collected. *A*, 2.8 mg crotoxin; *B*, elution diagram of 2.6 mg phospholipase A + 0.8 mg crotapotin: *C*, elution diagram of 1.3 mg phospholipase A + 1.6 mg crotapotin. AB = crotoxin, B = phospholipase A, A = crotapotin.

Table 3 - *Determination of the stoichiometry of the phospholipase A-crotapotin complex by gel filtration on Sephadex G-75.*

Protein eluted in the position of the complex was determined by the Lowry method and/or peak area (absorbance at 280 nm).

Experiment No.	Amount applied to column (mg)	Phospholipase A/ crotapotim molar ratio of sample	Complex formed (mg)	Molar ratio of complex
1	2.9	2/1	2.1	1/1
2	3.4	1/2	2.1	1/1
3	4.8	1/12	3.46	1/1.03
4	2.5	1.1/1	2.34	1/1.04
5	2.5	1.1/1	2.24	1/0.81
6	2.5	1.1/1	2.05	1/1.05
7	2.5	1.1/1	2.32	1/0.89
8	2.5	1.1/1	2.13	1/1.6

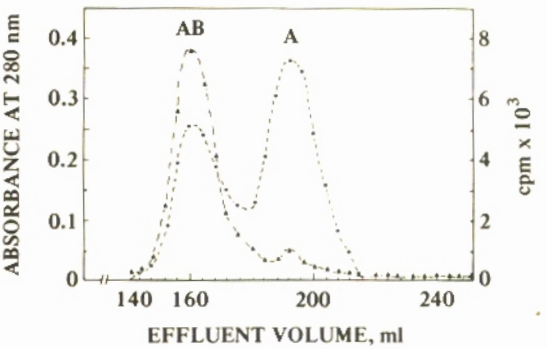

Figure 4 - Interaction of [^{125}I]-crotapotin and phospholipase A. Sephadex G-75 gel filtration of 1.5 mg phospholipase A in 0.5 ml buffer + 1.0 ng crotapotin + 60 μl [^{125}I]-crotapotin in 0.5 ml of the same buffer. The conditions for gel filtration were the same as in Figure 3. AB = crotoxin, A = crotapotin, –•–, cpm, –▲–, absorbance at 280 nm.

assay (Ouchterlony, 1958). These data not only provide criteria of homogeneity for the individual proteins, but also support the 1:1 stoichiometry determined by gel filtration.

Figure 6 shows the precipitation reactions of phospholipase A and crystallized crotoxin with anticrotalic serum of Butantan Institute. Crotapotin does not precipitate. Different patterns were obtained for crotoxin and phospholipase A, showing the importance of crotapotin in the immunological reaction of crotoxin, but it is clear that phospholipase A is generally responsible for the precipitation of the complex. The results of the reaction for three different molar ratios with an excess of phospholipase A or an excess of crotapotin

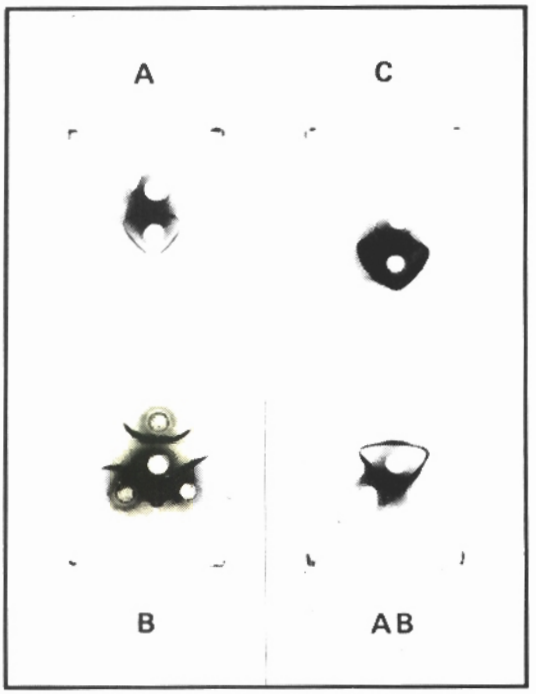

Figure 5 - Double diffusion method. The inner well contained anticrotalus serum. The plates were 2% agarose in 0.1 M sodium phosphate buffer, pH 7.0. Anticrotalus serum was added to the center well of each plate. Each of the 3 wells contained different quantities of the same protein. *A*, crotapotin; *B*, phospholipase A; *C*, crotoxin; *AB*, crotapotin and phospholipase A (1:1 molar ratio).

indicate that only the interaction of the 1:1 molar ratio gives a crotapotin pattern which agrees with the data obtained by gel filtration.

The contribution of crotapotin to the immunological properties of crotoxin was also demonstrated using [^{125}I]-crotapotin and paper electrophoresis. Figure 7 shows that crotapotin reacts with anticrotalic serum, forming a soluble complex that cannot be detected by the classical precipitation method. [^{125}I]-Crotapotin incubated with normal horse serum migrates with serum albumin. However, when [^{125}I]-crotapotin is incubated with anticrotalus horse serum it has the mobility of gammaglobulin, as expected.

Figure 8 illustrates the quantitative precipitation of crotapotin, phospholipase A and crotoxin with specific rabbit antisera. The same specific antisera also give cross immunoreac-

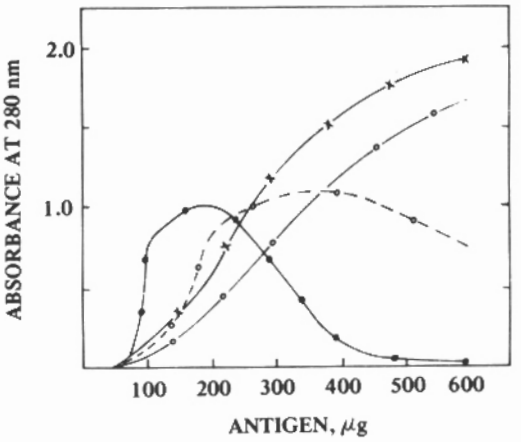

Figure 6 - Immunoprecipitation curves. Crotapotin, phospholipase A, crotoxin and a crotapotin-phospholipase A mixture were reacted with 0.2 ml anticrotalus serum in 0.1 M phosphate buffer, pH 7.0, for 1 h at 37°C. The precipitates, dissolved in 1 ml 0.1 M NaOH, were measured spectrophotometrically. ●—●, Crotoxin, ○---○, phospholipase A; ○—○, 2 crotapotin + 1 phospholipase A; x—x, 1 crotapotin + 2 phospholipase A. Crotapotin alone did not form a precipitate.

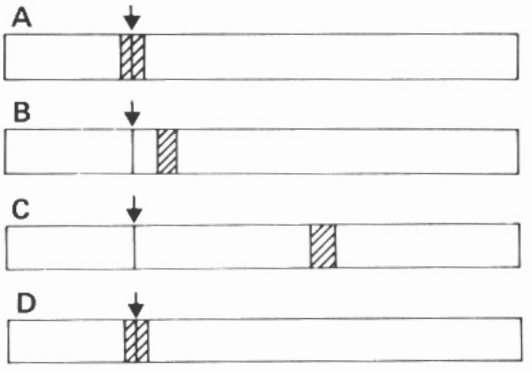

Figure 7 - Interaction of [^{125}I]-crotapotin with anticrotalus serum. Paper electrophoresis was carried out in 0.25 M phosphate buffer, pH 7.0, using 2.5 x 33 cm strips of Whatmann no. 1 paper. The strips were counted in a Nuclear Chicago gamma counter and stained with amido black. The hatched areas show the radioactivity. *The arrow indicates the site of sample* application. The cathode is on the left side of the strip. *A*, [^{125}I]-crotapotin, electrophoresis for 1 h; *B*, [^{125}I]-crotapotin, electrophoresis for 4h; *C*, [^{125}I]-crotapotin + normal horse serum, electrophoresis for 4 h. Radioactivity was present in the same position as serum albumin; *D*, [^{125}I]-crotapotin and anticrotalus serum, electrophoresis for 4 h. Radioactivity was present in the same position as gammaglobulin.

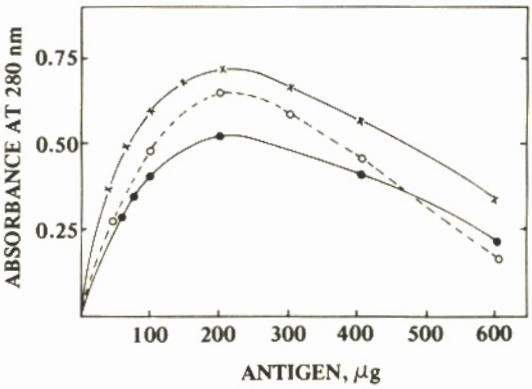

Figure 8 - Immunoprecipitation curves. Crotapotin, phospholipase A and crotoxin were incubated with specific antisera for 1 h at 37°C and overnight at 4°C in 0.1 M phosphate buffer, pH 7.0. The protein in the precipitate was determined by absorbance at 280 nm. ●—●, Crotapotin-anticrotapotin serum: ○---○, crotoxin-anticrotoxin serum; x—x, phospholipase A-antiphospholipase serum.

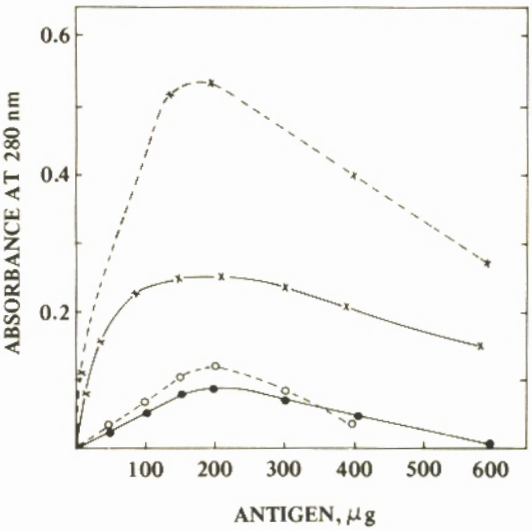

Figure 9 - Cross immunoreaction. Crotapotin was reacted with antiphospholipase A serum and anticrotoxin serum. Also, phospholipase A was reacted with 0.2 ml of anticrotapotin and anticrotoxin serum in 0.1 M phosphate buffer, pH 7.0, for 1 h at 37°C and 24 h at 4°C. The precipitates dissolved in 0.1 M NaOH were measured spectrophotometrically. x---x, Phospholipase A-anticrotoxin serum; x—x, crotapotin-anticrotoxin serum; ○---○, crotapotin-antiphospholipase A serum; ●—●, phospholipase A-anticrotapotin serum.

tions which are shown in Figure 9. The comparison of the quantitative precipitations of anticrotoxin - phospholipase A, anticrotoxin - crotapotin and anticrotoxin - crotoxin systems indicates that some antigenic determinants are masked in the crotapotin - phospholipase A interaction. However, it seems that crotapotin and phospholipase A show at least one similar antigenic determinant.

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