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Biochemistry and Pharmacology of the Crotoxin Complex

I. Subfractionation and Recombination of the Crotoxin Complex

K. Rübsamen, H. Breithaupt, and E. Habermann

Pharmakologisches Institut der Universität Gießen

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Summary. 1. Crotoxin, the crystallizable, proteinaceous, approximately electroneutral fraction from the venom of *Crotalus durissus terrificus*, has been resolved further by chromatography on carboxymethyl cellulose.

We obtained: a) A basic phospholipase A with comparatively low toxicity in mice (LD_{50} i.v. 0.54 mg/kg; LD_{50} s.c. > 100 mg/kg), whereas the toxicity of crotoxin was 0.108 mg/kg i.v. and 0.50 mg/kg s.c., respectively. In contrast, the specific enzymatic activity of phospholipase A was about 4 times higher than in crotoxin, depending on the dosage used. Overall recovery of activity was 140% to 170%, which suggests the removal of an inhibitor.

b) An acidic substance ("Crotapotin")[1] devoid of toxicity (LD_{50} i.v. > 50 mg/kg, mice) and phospholipase activity.

c) A mixture (called fraction III) of phospholipase A, crotapotin and a third basic substance. The latter, being inactive in our tests, is present in small amounts only (about 1% of crotoxin).

2. Recombination of crotapotin with phospholipase A increases the i.v. toxicity of phospholipase A approximately 12 fold to 0.042 mg/kg; the s.c. toxicity of the enzyme is raised approximately 1000 fold to 0.13 mg/kg.

3. In contrast to potentiating the phospholipase toxicity, crotapotin strongly inhibits the enzymatic activity in vitro.

4. These findings help in explaining the former reports on the chemical heterogeneity of crotoxin and on the preparation of toxic compounds with low phospholipase activity from it. Crotoxin is to be considered as a complex consisting of phospholipase A, crotapotin, and small amounts of impurities.

Key-Words: Snake venom — Crotoxin — Phospholipase A — Toxicity — Potentiation.

For a long time, crotoxin has been regarded as the prototype of a homogeneous, even crystallizable protein with both neurotoxic and phospholipase properties (Slotta and Fraenkel-Conrat, 1938). Reinvestigation of this "toxic enzyme" became necessary when neuro-

1 Substance from CROTAlus venom, which POTentiates the toxicity and INhibits the enzymatic activity of phospholipase A.

toxicity and enzymatic activities of bee and snake venoms (for review see Habermann, 1968) were attributed to different constituents. Neumann and Habermann (1955) partially succeeded in preparing a toxic fraction with low phospholipase activity. They thought the toxicity to be connected with a true toxin which they called crotactin. However, this substance never could be obtained in sufficient purity and amounts. These initial experiments lost some of their relevance since they did not allow for the inhibition of crotalus terrificus phospholipase by the phosphate buffer used.

Slotta (1953, 1955) reported on findings of himself and other authors which contradicted the previously assumed homogeneity of crotoxin. He already discussed, but could not prove the concept that crotoxin represents an ionic complex between a strongly basic and a strongly acidic protein. Habermann (1957b) prepared a basic phospholipase A with low toxicity from the acidic, highly toxic crotoxin by chromatography on alumina. The recovery of enzyme activity exceeded 100%, which indicated the removal of an inhibitor simultaneously present in the venom. No phospholipase-free toxin appeared, so the question concerning the whereabouts of the toxicity remained unanswered. Inhomogeneity of crotoxin was also evident from some chemical findings. Fraenkel-Conrat and Singer (1956) treated crotoxin with fluorodinitrobenzene. They obtained a watersoluble dinitrophenyl derivative and another insoluble fraction. The soluble component contained fewer basic amino acids than the insoluble component. More recently, Hendon *et al.* (1970) obtained similar results when they blocked the amino groups of crotoxin with maleic or methyl-maleic anhydride. Although neurotoxicity seemed to be more sensitive to these substitutions than phospholipase was, both activities were recovered in the pH 4-insoluble fraction, from which the maleic groups could be removed by gentle treatment. They concluded that crotoxin consists of one or two closely related acidic and basic proteins, which can be separated only after their amino groups have been blocked. However, phospholipase A and toxicity were assumed to remain associated with the basic moiety. Habermann and Rübsamen (1971), and Breithaupt *et al.* (1971) reported the separation of unsubstituted crotoxin on carboxymethyl cellulose into various fractions. The two main components were identified as a strongly basic phospholipase A with relatively low toxicity, and an acidic protein devoid of activity in the tests used. Recombination of the two substances restored the toxicity to that of crotoxin, indicating a potentiation of the toxicity of phospholipase A. In vitro, however, the potentiator of toxicity inhibited the phospholipase A activity.

This paper describes the analysis and the recombination of the crotoxin complex.

Methods and Substances

Preparation of Crotoxin

Crotoxin was prepared from *Crotalus durissus terrificus* venom (Miami Serpentarium, Miami, Florida) according to Neumann and Habermann (1955), who used a slight modification of the procedure of Slotta and Fraenkel-Conrat (1938). In a typical run we obtained 600 mg crotoxin and 40 mg crude crotamine from 800 mg dry venom. The LD_{50} (mice, i.v.) of the starting venom was about 0.13 mg/kg and that of crotoxin 0.108 mg/kg ($0.082 < LD_{50} < 0.142$ mg/kg; $p < 0.05$). S.c. toxicity of crotoxin was 0.50 mg/kg ($0.34 < LD_{50} < 0.74$ µg/kg; $p < 0.05$). Phospholipase activity (egg yolk test) of crotoxin was in the same range as that of whole venom.—The earlier experiments (as reported by Habermann and Rübsamen, 1971, and Breithaupt *et al.*, 1971) were done with a Crotalus terrificus venom from Instituto Butantan (Sao Paulo, Brazil). Both samples were useful for the analytical and preparative work described below.

Fractionation of the Crotoxin Complex

500 mg crotoxin were dissolved in 80 ml 0.1 M ammonium formate buffer, pH 3.5, and applied to a 2.8×10.5 cm colum, packed with carboxymethyl cellulose (CM 32, Whatman), and equilibrated with the same buffer. The molarity of the eluting buffer was continuously raised, first to 1.0 M, then to 2.0 M ammonium formate buffer, pH 3.5, using a closed 500 ml mixing device. The flow rate was 0.5 ml per minute and the effluent was collected in 8.0 ml fractions. According to the elution pattern, the fractions were pooled and four times lyophilized. We obtained 81.30 mg of fraction I (crotapotin), 45.96 mg of fraction II (crotapotin), 61.55 mg of fraction III (subdivided in 28.60 mg III A, 17.43 mg III B and 15.52 mg III C), 5.47 mg of fraction IV (crotamine) and 196.71 mg of fraction VI (phospholipase A). The fraction previously labelled as "V" (Habermann and Rübsamen, 1971) was lacking in the crotoxin samples used. The total yield after chromatography was 390.99 mg, which is 78% of the material applied. Recovery of phospholipase A activity was calculated by comparing the enzymatic activity of all eluates with the activity of the crotoxin applied to the column. In two experiments, the recovery was 140% and 170% of the activity applied.—The recovery of toxicity was determined by comparing the total toxicities (LD_{50}/g mouse i.v.) in fractions III, IV and VI with that of the crotoxin applied. A recovery of 13% was calculated from the quantities of LD_{50}/g (mouse, i.v.): 4,854,368 in crotoxin, 314,277 in phospholipase A (fraction VI), 307,750 in fraction III, and 10,940 in fraction IV.

Further Purification of Crotoxin Fractions

a) Rechromatography of Fraction I and II (Crotapotin). 25.92 mg of fraction I were dissolved in 0.02 M phosphate buffer, pH 6.9, and absorbed on a DEAE cellulose column (0.7×10 cm; DE 32, Whatman), equilibrated with the same buffer. Using a closed 50 ml mixing device, a gradient was generated from 0.02 M phosphate buffer, pH 6.9, to 1.0 M sodium chloride dissolved in the same buffer. Fractions of 1.5 ml per 10 min were collected. The fractions containing protein were pooled, dialyzed against water for 36 h, and lyophilized. Recovery was 15.91 mg.—20.16 mg of fraction II, processed in the same way, yielded 14.67 mg.

b) Rechromatography of a Mixture of Fractions I and II (Crotapotin). 0.526 mg fraction I and 0.538 mg fraction II were dissolved in 0.20 ml 0.02 M phosphate buffer, pH 6.8, absorbed on a DEAE cellulose column (0.6×9.cm) and processed as described. The elution pattern showed a single peak only.

Fractions I and II (1.085 mg and 1.235 mg) were rechromatographed also on carboxymethyl cellulose (0.6×10 ,cm 1.0 M ammonium formate equilibration buffer, pH 3.5) by gradient elution (0.1 to 0.5 M ammonium formate buffer, pH 3.5). One peak only (1.223 mg; 53%) appeared in the conductivity range of 0.1 M ammonium formate buffer.

c) Rechromatography of Phospholipase A. 24.6 mg of fraction VI were rechromatographed on a carboxymethyl cellulose column (0.7×10 cm) by gradient elution with ammonium formate buffer, pH 3.5, from 0.1 to 2.0 M. The fractions containing protein were pooled and three times lyophilized with a yield of 17.69 mg phospholipase A.

d) Gel Filtration of Phospholipase A. 5.0 mg of fraction VI were dissolved in 0.5 ml 3.0 M ammonium formate buffer, pH 3.5, and filtrated through a Sephadex G 50 column (0.9×80 cm; medium, Pharmacia Uppsala) equilibrated with the same buffer. The yield was only 0.74 mg (15%) which indicates adsorption phenomena.

e) Rechromatography of the Subfractions from Fraction III. Fractions III A, III B and III C were rechromatographed using the same device as described under a). 16.01 mg of fraction III A yielded 7.59 mg of a substance which was, upon disc electrophoresis, identical with fraction I. In addition, 3.06 mg of a phospholipase A fraction appeared at an ionic strength lower than expected for fraction VI. Thus it resembled fraction V from former preparations (Habermann and Rübsamen, 1971). Furthermore, 1.14 mg of a substance inactive in our tests was eluted between fraction I and phospholipase.—11.87 mg of fraction III B yielded traces of fraction I, 0.20 mg of phospholipase A in the position of fraction V, 1.55 mg of phospholipase A in the position of fraction VI, and 3.20 mg of the intermediate fraction.—The elution pattern of 10.0 mg of fraction III C showed only traces of fraction I and the phospholipases. Fraction III C reappeared unchanged in its former position of conductivity and seems to be identical with the intermediate substance mentioned.—2.08 mg of the intermediate fraction from the chromatography of fraction III B were rechromatographed again on a carboxymethyl cellulose column (0.5×10 cm). Only traces of fraction I, but no phospholipase activity were found. The bulk of the substance reappeared in the same position as during the preceding chromatography of III B.

f) Chromatography of the Crude Crotamine Fraction. 25 mg of crude crotamine (for preparation see Neumann and Habermann, 1955) were chromatographed on a carboxymethyl cellulose column (1.0×10 cm) by gradient elution (0.1 to 2.0 M ammonium formate buffer, pH 3.5). The eluates (1.5 ml per 7 min) were collected and tested as described above. Four protein fractions were obtained. Only the last one showed spasmodic actions.

g) Chromatography of a Mixture of the Fractions I, II and VI. 2.00 mg of fraction I, 2.27 mg of fraction II and 5.37 mg of fraction VI were dissolved in 8.0 ml 0.05 M ammonium formate buffer, pH 3.5, and 2 h later adsorbed on a carboxymethyl cellulose column (0.9×10 cm) which was equilibrated with 0.1 M ammonium formate buffer, pH 3.5. A gradient elution from 0.1 to 1.0 M, then to 2.0 M ammonium formate buffer, pH 3.5, was performed by using a closed 50 ml mixing device. The flow rate was 1.05 ml per 8 min. The elution pattern showed single peaks in the positions of fractions I and VI. The yield was about 60% of fraction I and II and about 56% of fraction VI. Fraction VI showed phospholipase activity and toxicity, according to its protein content, whereas fractions I and II were inactive.

Disc Electrophoresis in Polyacrylamide Gel

The pooled crude fractions as well as the purified substances were, without exception, subjected to disc electrophoresis according to the prescription given by

Maurer (1968). Two 7.5% gels (pH 2.9 and 8.6) were used. About 0.1 mg of protein was applied to each gel and stained with Amidoschwarz 10 B in 7% aqueous acetic acid.

Toxicity

Toxicity tests were done in mice (NMRI, Hannover; 20—30 g) by i.v. or s.c. injection of 0.1 ml test solution per 10 g body weight. Up to 1.0 ml were injected intravenously or subcutaneously into rats (Wistar AF-Hannover; 150—250 g). Rabbits (2—3 kg) received up to 2.5 ml via the ear vein.

All solutions were made in saline. Mortality during the first 24 h was used for calculation.

At least 30 mice were needed for each determination of the LD_{50} by the method of Litchfield and Wilcoxon (1949).

Enzymatic Activity

Phospholipase A was assayed by the method of Habermann and Neumann (1954). 2.0 ml of 50% (v/v) egg yolk suspension in saline were incubated with 0.5 ml of the test solution for 2 h at 37° C. Then the coagulation time was measured in a boiling water bath. 1 μg of crotalus terrificus phospholipase A was just demonstrable.

Phospholipase A activity was also measured by titrating the fatty acids liberated from lecithin. We recorded the rate of delivery of 0.01 N NaOH necessary to keep the pH of the reaction mixture constant at 8.04. This was accomplished by a Combititrator 3-D, Metrohm Ltd., Herisau, Switzerland.—20.0 mg L-α-dipalmitoyl-lecithin (synth., purum; Roth, Karlsruhe, BRD) were dissolved in 25 ml 40% aqueous methanol, containing 3.4 mg $CaCl_2 \cdot 2H_2O$ and 0.5 ml Tween 20 (Serva, Heidelberg, BRD), adjusted to pH 8.04 with 0.01 N NaOH and made up to a total volume of 30 ml with 40% (v/v) aqueous methanol. 3.0 ml of this solution were stirred vigorously at 25° C, then the aqueous solutions of fraction I and phospholipase A, separately or in a preincubated mixture, were added.

Protein content of the eluates was determined by the modified Folin reaction.

Conductivity, was measured with a conductivity meter Type CDM 2d Radiometer, Kopenhagen, Denmark. Hundredfold dilutions of buffers and eluates were used.

Results

I. Chromatographic Analysis of the Crotoxin Complex

Some material appears with the void volume of the carboxymethyl cellulose column and has been called fraction I (Fig. 1). It is followed immediately by fraction II. Both fractions are devoid of toxicity and phospholipase activity, which characterize crotoxin. However, at the tail of fraction II considerable toxicity appears. The egg yolk test indicates some enzymatic activity. Therefore, this part of the protein pattern has been denominated tentatively as fraction III. This fraction again is followed by some minor components, which appear to be contaminants of the crotoxin complex. Area IV produces spasms in mice and thus resembles crotamine. Purified crotamine emerges from the column with the same conductivity. The bulk of phospholipase A appears with

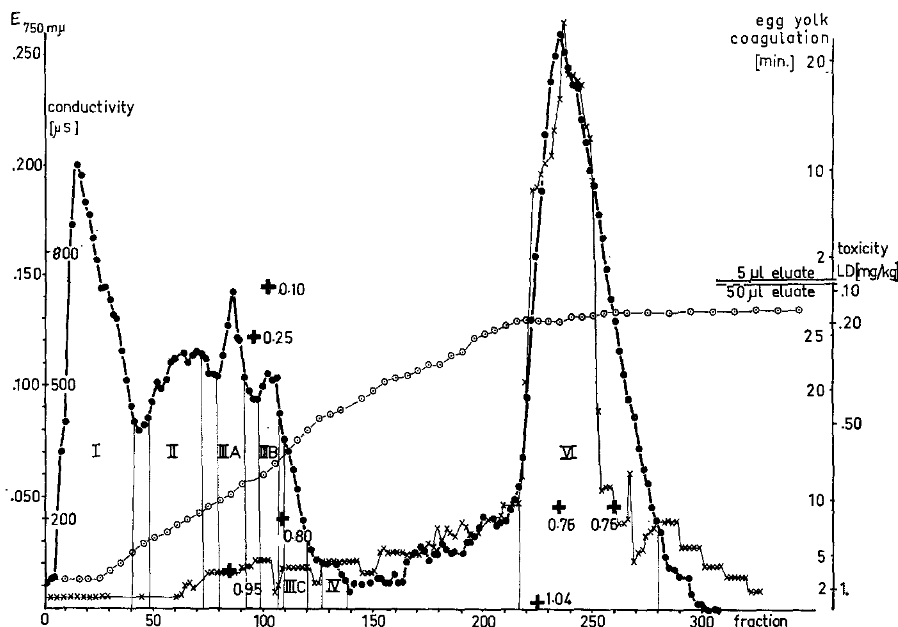

Fig. 1. *Chromatography of crotoxin on carboxymethyl cellulose.* •——• protein content (Folin reaction); ⊙——⊙ conductivity; ×——× egg yolk coagulation, + LD_{50} (white mice, i.v.). Exclusion limit of toxicity of the other fractions is above 1.1 mg/kg. For further details see "Methods". Fraction I and II consist of crotapotin mainly, fractions III A, B, C represent the intermediate fractions, fraction IV has crotaminelike actions. Fraction VI is phospholipase A. Phospholipase A activity was tested with the eluates diluted 1:10 (below =) and 1:100 (above =)

relatively high ionic strength. The recovery of the enzymatic activity of phospholipase A was calculated as 140% and 170% of that of the crotoxin applied to the column (two experiments). The crotoxin samples, used during this work, yielded only one peak of enzymatic activity. Earlier experiments had shown the occurrence of two subfractions of phospholipase in other venoms. The overall recovery of the toxicity applied to the column was low (about 13%). Some of it appeared with phospholipase A, another part with the intermediate fraction III (see page 277). The symptomatology of envenomation resembled that produced by the crotoxin complex. The disappearance of most of the toxicity was the crucial finding in the chromatographic experiments; it stimulated the recombination experiments described below.

The main fractions were checked by rechromatography. Phospholipase A appeared as a homogeneous peak when applied to a second carboxymethyl cellulose column or to Sephadex G 50 (see "Methods").—

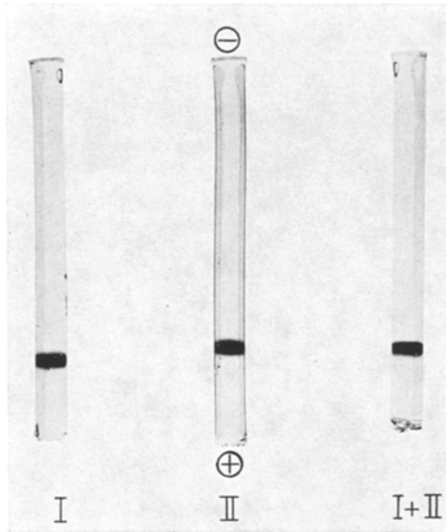

Fig. 2. *Disc gel electrophoresis.* 0.1 mg of fraction I, 0.1 mg of fraction II, and a mixture of 0.05 mg of fraction I and II were applied. The proteins migrated to the anode at pH 8.9. 2 h run; 3 mA/gel

Since fractions I and II were strongly acidic, their rechromatography was performed on DEAE cellulose. Both fractions appeared with the same ionic strength when applied alone or combined. Chromatographed on carboxymethyl cellulose, fractions I and II were recovered as one single peak at the ionic strength of the equilibration buffer. Fraction I and II also could not be differentiated by disc electrophoresis (Fig. 2), which can be taken as additional evidence for their identity. The strong acidity of fractions I and II is apparent from their lack of adsorption to carboxymethyl cellulose and also from their fast anodal migration on disc gel electrophoresis. Since both fractions proved to be identical in every test applied, they can be taken as a single substance which we call crotapotin.

Fraction III was divided into three subfractions (see Fig. 1) which were rechromatographed separately on CM cellulose (Fig. 3). Fraction III B contained the main part of the toxicity of fraction III. In the elution range of the former fraction III, each of the subfractions yielded a protein peak which was neither toxic nor active as phospholipase A. In addition, subfraction III A contained fraction I and phospholipase A (for quantitative details see "Methods"). This was true also for subfraction III B. Subfraction III C was devoid of fraction I and contained only traces of phospholipase activity.—It should be noted that the phospholipase peaks of the various subfractions appear at different ionic strengths. This might reflect the presence of isoenzymes. They had been observed previously

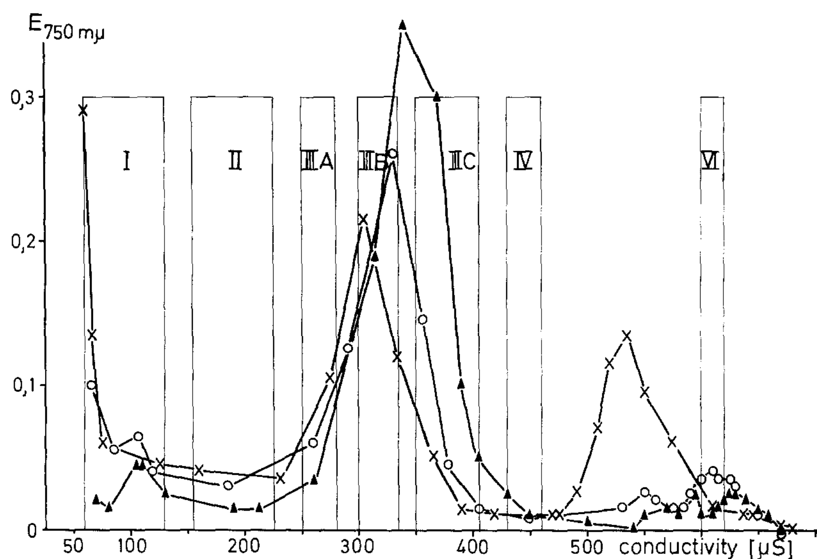

Fig. 3. *Rechromatography of the intermediate fractions III A, III B, III C from Fig. 1 on carboxymethyl cellulose.* The protein concentration of the eluates of equal conductivity are compared. Elution pattern of the fractions III A ×——×; III B o——o; III C ▲——▲. The columns indicate the positions of the crotoxin fractions as shown by Fig. 1

in other crotoxin samples to a larger degree and labelled as fractions V and VI respectively (Habermann and Rübsamen, 1971).—The protein peak of subfraction III B appearing with about 300 μS, was rechromatographed again on CM cellulose. No further resolution took place.

Since this part of fraction III was inactive in our tests and amounted, by weight, to only 1% of crotoxin, it has not been studied further.

By chromatographic analysis of the crotamine fraction, prepared according to Neumann and Habermann (1955) (see "Methods"), we found a protein with a disc electrophoresis pattern quite similar to that of the 300 μS peak of fraction III.

The main peak of fraction III cannot be reconstituted by combination of fraction I, II and VI (Fig. 4). On CM cellulose, neither a "fraction III" could be found nor was there any separation of fractions I and II.

II. Reconstitution of Toxicity by Recombining Phospholipase A with Crotapotin

Crotapotin was practically non-toxic. 50 mg/kg, given intravenously, did not affect the behaviour of mice. Phospholipase A (LD_{50} i.v., 0.54 mg/kg; see Fig. 5) was considerably less toxic in mice than our crotoxin preparation (0.108 mg/kg i.v.). The s.c. LD_{50} of the enzyme was higher than 100 mg/kg, whereas the s.c. LD_{50} of crotoxin was 0.50 mg/kg.

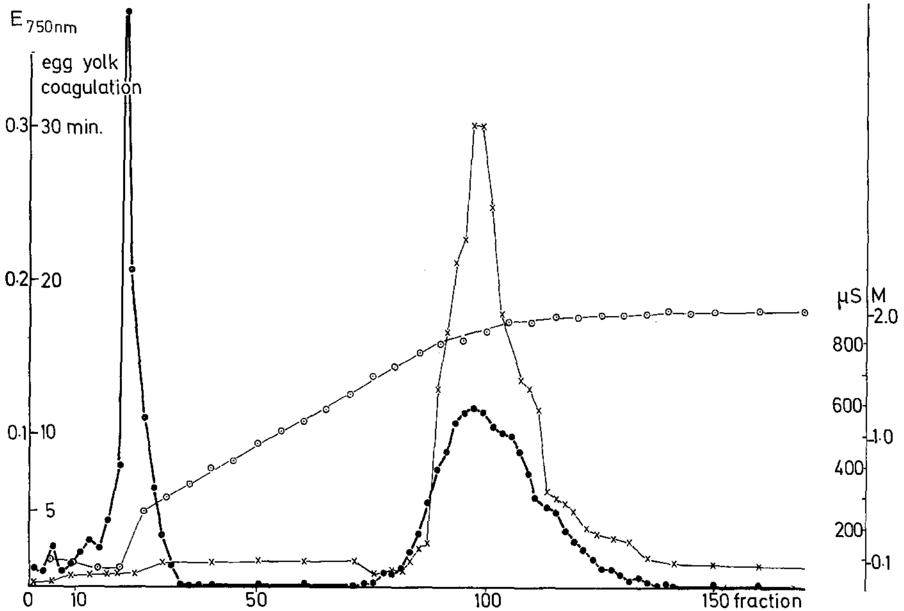

Fig. 4. *Chromatography of a mixture of fractions I, II and VI on CM cellulose.* Left ordinate: protein content (E_{750} nm, 1 cm) •——•; activity in the egg yolk test ×——×. Right ordinate: conductivity (µS) and molarity (M) of the eluting buffer ⊙——⊙

As described above, fraction III has been resolved into fraction I, phospholipase A and an inert protein. Therefore, this fraction also cannot be regarded as a toxic entity.

In order to find out if the severe loss of toxicity during chromatography was due to separation of phospholipase from accompanying substances, we recombined the various fractions. Mixtures of fraction I or II with phospholipase indeed were, by weight of the enzyme, more toxic than phospholipase A alone and even more toxic than crotoxin (Fig. 5). The addition of crotapotin yielded a twelve fold increase in the toxicity of phospholipase A on i.v. injection. The LD_{50} of the enzyme was diminished, in this manner, to 0.042 mg/kg. On subcutaneous injection the toxicity of phospholipase A could be potentiated approximately thousandfold up to an LD_{50} of 0.132 mg/kg.

The toxicity of phospholipase A combined with crotapotin approaches, by weight of both proteins, the toxicity of crotoxin. For instance, the LD_{50} of the enzyme decreased to 0.087 mg/kg, when combined with 0.050 mg/kg crotapotin (see Fig. 5). So 0.137 mg/kg of this mixture of both proteins show the same toxicity as 0.108 mg/kg crotoxin.

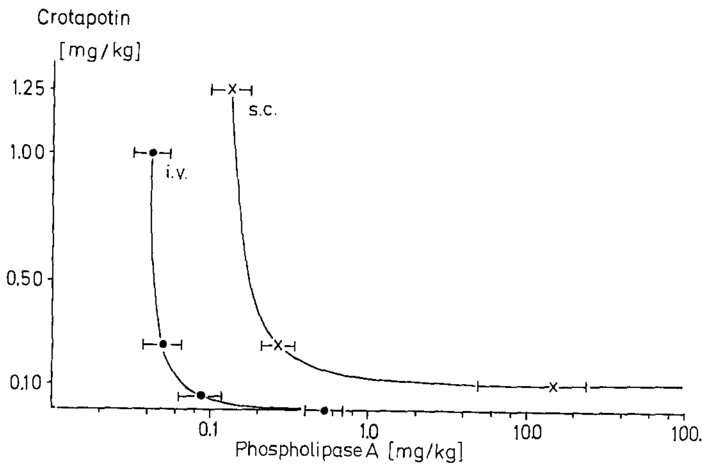

Fig. 5. *Potentiation of the toxicity of phospholipase A by crotapotin.* Each point represents the LD_{50} of phospholipase A or of a mixture with crotapotin ($p < 0.05$) on intravenous or subcutaneous injection into mice

In rats, the toxicity of phospholipase A is less than in mice. 40 mg/kg i.v. produced prostration only. In combination, however, with crotapotin (1.25 mg/kg) as little as 1.25 mg/kg phospholipase A was lethal. For instance, one animal given a combination of 2.5 mg/kg of phospholipase and 2.5 mg/kg of crotapotin and two animals given 1.25 mg/kg of each substance died, whereas four animals survived a combination of 0.625 mg/kg phospholipase A and 0.625 mg/kg crotapotin.

One rabbit died after i.v. injection of 3.0 mg/kg phospholipase A, while another survived 0.5 mg/kg. One rabbit was injected with a combination of 0.25 mg/kg crotapotin and 0.10 mg/kg phospholipase A; it dies after 7—13 h. The symptoms of intoxication were the same as decribed in mice.

Table. *Toxicity of phospholipase A and crotapotin, injected together or separately (mice; i.v.)*

Line	Doses (mg/kg)	Substances	Interval between the applications	Death time (h) 3 animals per dose		
1	0.1 0.2	crotapotin phospholipase	Injected as a mixture	1.1	1.2	3
2	a) 0.1 b) 0.2	crotapotin phospholipase	Injected immediately one after another	3	5—7	5—7
	a) 0.2 b) 0.1	phospholipase crotapotin	(lag period less than 10 sec)	2.2	3	3.1
3	a) 0.1 b) 0.2	crotapotin phospholipase	Second injection (b) 30 min after the	—	—	18—24
	a) 0.2 b) 0.1	phospholipase crotapotin	first (a)	—	—	—

a) first injection; b) second injection; — = survived.

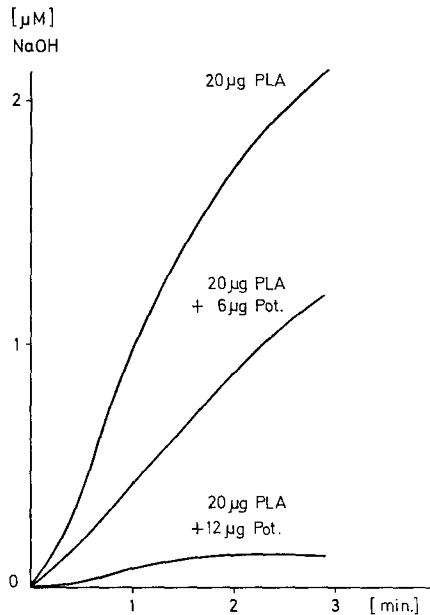

Fig. 6. *Inhibition of phospholipase A—activity by crotapotin in vitro.* Titration of the fatty acids liberated from dipalmitoyllecithin. *PLA* phospholipase A, *Pot* crotapotin. Preincubation time: 1 min, further details: see "Methods". Ordinate: NaOH consumption. Abscissa: incubation time (min)

In order to test whether phospholipase and crotapotin must be premixed in vitro or whether they interact in vivo too, the experiment given in the Table was performed. By comparison of line 2 and 3, it is evident that crotapotin, applied immediately (10 sec) before or after phospholipase A, does indeed increase the toxicity of the enzyme. A lag period of 30 min, however, weakens the interaction considerably.

By injecting crotoxin, phospholipase alone or phospholipase combined with crotapotin the resulting symptoms of envenomation were similar and relatively non-characteristic. The animals died after a dosage-dependent latent period, showing prostration, shortness of breath and prefinal spasms, probably due to hypoxia. The death time depended on the dosage and ranged from 5 min to 24 h.

III. Inhibition of Phospholipase A Activity in vitro by Recombination with Crotapotin

In earlier experiments with alumina chromatography (Habermann, 1957 b), and also in our present preparative work, the recovery of in vitro phospholipase A activity surpassed 100% which indicated the removal

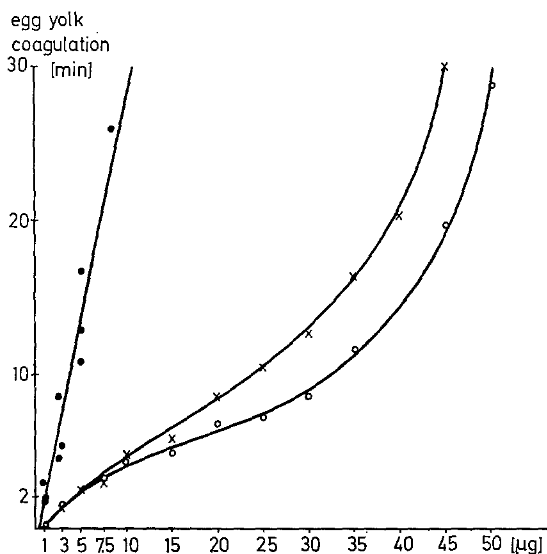

Fig. 7. *Lecithinase A—activity of crotalus phospholipase A, crotoxin and crude venom.* Details of the egg yolk test: see "Methods". Phospholipase A •——•; crotoxin ×——×; crude venom o——o. Ordinate: coagulation time (min). Abscissa: µg enzyme added

of an inhibitor. In order to study the possible effect of crotapotin on phospholipase in vitro, the release of fatty acids from lecithin by various combinations of both substances was determined. Crotapotin strongly inhibited the enzyme in vitro (Fig. 6). The activity of 20 µg of phospholipase was slightly depressed by 6 µg of crotapotin, whereas 12 µg nearly completely inhibited the enzyme. Therefore crotapotin cannot be called simply a "potentiator" of the enzyme, as we have done previously. It potentiates in vivo, but inhibits in vitro.

Whereas the dose-response relationship of purified phospholipase A is approximately linear, this is not true for crotoxin or whole rattlesnake venom in the egg yolk test (Fig. 7). With his manometric phospholipase assay, Habermann (1957c) had observed a lag period for the activity of crotoxin, but not for phospholipase A prepared from crotoxin (see Habermann, 1957b). Both findings may be taken as additional hints at a special bonding of phospholipase A in crotoxin which resembles an enzyme-inhibitor complex.

Discussion

Our experiments demonstrate for the first time that unsubstituted crotoxin can be separated into two opposite main components and some

minor fractions. One major component is not or only slightly adsorbed on carboxymethyl cellulose, which argues for its acidic character; it has neither toxic nor known enzymatic properties. Phospholipase A of crotalus terrificus venom is, in contrast to other phospholipases A, a strongly basic protein. Toxicity of crotoxin depends on the presence of the phospholipase A but the enzyme is, by weight, about five times less toxic than crotoxin when given intravenously. The toxicity of crotalus terrificus phospholipase A is potentiated by the acidic protein, while the in vitro activity of the enzyme is inhibited. Therefore we would like to call the acidic protein "crotapotin". The term "potentiator" which we used previously is misleading with respect to the enzymatic activity.

The observations mentioned explain not only some pharmacological and biochemical properties of crotoxin but also the partially contradictory reports in the literature. In future, crotoxin cannot be regarded as a homogeneous, crystallizable protein but as a complex consisting mainly of two differently charged proteins, held together by acid-base interactions. The unique role of crotapotin—enhancement of toxicity of phospholipase in vivo and inhibition of its enzymatic activity in vitro—represents a new principle of pharmacological interactions. Retrospectively, we did in 1957 nothing else than to remove the crotapotin by alumina. This necessarily resulted in a decreased toxicity and in a recovery of more than 100% of the enzymatic activity applied (Habermann, 1957b).

The former crotactin (Neumann and Habermann, 1955) was said to be more acidic than phospholipase A. Perhaps it was a complex between high amounts of crotapotin and relatively low amounts of phospholipase A. This would have resulted in considerable toxicity and low enzymatic activity in vitro. In the light of the present findings, the term "crotactin" should be deleted.

As far as we know, crotalus terrificus phospholipase A is the only neurotoxic enzyme, and it is the only enzyme known whose toxicity can be increased considerably by crotapotin. In mice, bee venom phospholipase A is at least ten times less toxic (LD_{50} i.v. about 7.5 mg/kg; Habermann, 1957a; Stockebrand, 1965) than the crotalus terrificus enzyme (0.54 mg/kg i.v.) without crotapotin. Furthermore, neurotoxic symptoms prevail with crotalus terrificus enzyme, whereas bee venom phospholipase A acts mainly in the periphery. One is tempted to correlate the neurotoxicity of crotalus phospholipase with its basic character, which it shares with the classical neurotoxins of elapid or scorpion venoms. This working hypothesis would explain the findings of Hendon *et al.* (1970), that the phospholipase A fraction from crotalus terrificus venom looses its residual toxicity but retains its phospholipase activities

when maleylated. We agree with the statement of Hendon *et al.* (1970), that the basic protein is indispensible for neurotoxity and phospholipase A activity, but stress the considerable modifications of both toxicity and enzymatic activity of rattlesnake phospholipase A by crotapotin.

Initially, our so-called fraction III seemed to be a suitable candidate for the former crotactin, since it had only negligible phospholipase activity despite its considerable toxicity. Its careful analysis, as described above, showed the presence of a hidden phospholipase A together with crotapotin and a third protein which was inactive in our tests. Since the latter contributes, by weight, to about 1% of crotoxin, it can be put aside as an impurity. Also crotamine (fraction IV) must be considered as a slight contamination of crotoxin.

In the experiments reported previously (Habermann and Rübsamen, 1971) a second phospholipase peak (fraction V) appeared just before the main enzyme peak (fraction VI). The venom used at present contains only traces of fraction V. Nevertheless, during rechromatography of fraction III a phospholipase peak emerged from the column at the position of fraction V. It should be recalled from the previous experiments that the slight toxicity of this phospholipase peak also was increased by adding crotapotin.

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E. Habermann
Pharmakologisches Institut
der Universität
BRD-6300 Gießen
Rudolf Buchheim-Straße 4
Germany