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THE EFFECT OF pH, TEMPERATURE, ANTIVENIN AND FUNCTIONAL GROUP INHIBITORS ON THE TOXICITY AND ENZYMATIC ACTIVITIES OF CROTALUS ATROX VENOM

by

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In conducting the research described in this report, the investigators adhered to the "Principles of Laboratory Animal Care as established by the National Society for Medical Research."

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ABSTRACT

THE EFFECT OF pH, TEMPERATURE, ANTIVENIN AND FUNCTIONAL GROUP INHIBITORS ON THE TOXICITY AND ENZYMATIC ACTIVITIES OF *CROTALUS ATROX* VENOM

OBJECT

To determine the effects of certain group-specific reagents on the toxicity of *Crotalus atrox* (Western diamondback rattlesnake) venom and the effect of pH, temperature, and antivenin on the toxicity and enzymatic activities of this venom.

RESULTS

Crotalus atrox venom has been completely detoxified by 50 mM N-bromosuccinimide. This venom cannot be detoxified with the sulfhydryl group reagents, N-ethylmaleimide or iodoacetamide, or the metal-binding agent EDTA. It is very slightly detoxified by reaction with O-methylisourea, diisopropylfluorophosphate, or British antilewisite. The toxic principle in *C. atrox* venom is resistant to low pH treatment, but is destroyed by heating at 75° for one hour. Under the same conditions the following enzymes are destroyed: phosphodiesterase, esterase (TAMEase), and proteolytic enzyme activity on azocoll, congocoll, casein, and N-benzoyl-DL-arginine p-nitroanilide. Phospholipase A activity is only partially destroyed under these conditions.

CONCLUSIONS

The effects observed on various enzymes with functional group inhibitors and the results of the heat inactivation and acid treatment of *C. atrox* venom indicate that the main toxicity of this venom is not associated with the enzymatic components but may reside with a peptide of relatively low molecular weight.

RECOMMENDATIONS

Attempts should be made to separate the proteolytic, phospholipase A, anticoagulant and toxic activities of *C. atrox* venom and

their physiological effects determined in order to learn whether the toxicity of this venom is due to separated toxin(s) or a synergistic action of one or more enzymes. Detoxification with N-bromosuccinimide and antibody production should be studied with the detoxified venom produced.

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THE EFFECT OF pH, TEMPERATURE, ANTIVENIN AND FUNCTIONAL GROUP INHIBITORS ON THE TOXICITY AND ENZYMATIC ACTIVITIES OF CROTALUS ATROX VENOM

INTRODUCTION

Various chemical compounds have been used to detoxify snake venoms. Trethewie (1) found that tetra-hydro-aminoacridine and poly-vinyl-pyrollidone increases the survival time of mice following injection of cobra venom *in vitro*. Heparin and other sulphated mucopoly-saccharides can inhibit the lethal effects of Russell's viper venom (*Vipera russellii*) (2). Also unsaturated fatty acids can protect mice against *Crotalus terrificus* venom (3). Hydro-cortisone, in large doses will ameliorate the lethality of cobra venom in dogs (4). Deichmann *et al*. (5) found similar results with hydro-cortisone on *Crotalus adamanteus* venom. Sawai *et al*. (6) have shown that treatment of snakebite with ethylenediaminetetraacetic acid (EDTA) enhances the effect of antivenin in Habu (*Trimeresurus flavoviridis*) bite. Both the necrotic and lethal effects of Habu venom have been partially neutralized by dihydrolipoic acid and tetracycline *in vivo* (7). The mechanisms by which these compounds alter the toxicity of snake venoms is unknown.

Kochwa *et al*. (8) showed that horse or rabbit antivenin prepared from whole *Vipera xanthina palestinae* venom will not neutralize the neurotoxic components of that venom but will neutralize the hemorrhagic activity. Pooled sera of rabbits immunized with either *Walterinnesia aegyptia* or *Pseudocerates fieldii* venom inactivated the venom phospholipase activities (9). Rabbit antivenin for *Echis colorata* venom was capable of neutralizing proteolytic activity, but not the amino acid oxidase, phospholipase, erythrocyte sphering factor, the anti-coagulant, the procoagulant or the fibrinogenolysin-fibrinolysin activity (10). Hβ-Proteinase of *Trimeresurus flavoviridis* (Habu) venom is completely inhibited by anti-Habu serum (11). It is possible that some of the antigens which combine with snake venom antivenin play no significant role in the toxicity of the venom.

This study describes the effects of certain group-specific reagents on the toxicity of *Crotalus atrox* venom, the effect of pH, temperature, and Antivenin Crotalidae Polyvalent (Wyeth Laboratories) on the toxicity and enzymatic activities of this venom. Evidence is also presented which indicates that one enzyme of *C. atrox* venom has both esterolytic and proteolytic activities.

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MATERIALS AND METHODS

Venom of *C. atrox* was collected from several hundred rattlesnakes, immediately frozen, and *lyophilized* within two weeks after collection. It was sieved and stored in the cold in a desiccator. Immediately before use the venom was reconstituted by dissolving in distilled water or appropriate solvent and centrifuged to remove undissolved matter, if any.

Studies on toxicity of lyophilized *C. atrox* venom were carried out in 20 g albino mice by intraperitoneal injection of 0.5 ml of venom solutions. Four mice were used for each of ten doses: 50, 62, 73, 80, 89, 100, 114, 160, 200, and 400 μg per mouse. The LD_{50} was estimated from a log dose-probit curve to be 112 γ of lyophilized venom per mouse.

Enzyme activities were measured as previously described (12-14). Venom and antivenin were incubated at 37°C for 60 minutes in 0.025 M Tris-HCl (tris-(hydroxymethyl) aminomethane), pH 8.0 in various proportions. Aliquots were withdrawn, diluted appropriately, and the enzymic activities measured. Controls without antivenin and without venom were determined with the venom-antivenin mixtures at the same time to standardize venom-enzyme activities and to determine whether antivenin had any enzyme activity.

All chemicals used were reagent grade.

RESULTS

Table 1 lists the effects of several functional group reagents on the toxicity of *C. atrox* venom. O-Methylisourea (OMIU), diisopropylfluorophosphate (DFP), and British anti-lewisite (BAL) (2,3-dimercaptopropanol) showed a slight inhibition of toxicity. With BAL, however, a precipitate was formed which could indicate a denaturation of the toxin(s). Only with 50 mM N-bromosuccinimide (NBS) was there complete detoxification of the venom. This compound, NBS, has been shown to react fairly specifically with tryptophan residues of proteins (15,16). N-Ethylmaleimide (NEM), EDTA, and iodoacetamide (IAA) had little effect on toxicity.

The toxic principle in *C. atrox* venom is quite resistant to treatment at low pH values, as shown in Table 2. Venom treated with 0.10 N HCl for one hour at 37°C was still toxic.

TABLE 1

EFFECT OF FUNCTIONAL GROUP INHIBITORS ON THE TOXICITY OF *CROTALUS ATROX* VENOM

Venom (11.25 mg/ml) was incubated at 37°C for one hour with the concentrations of inhibitors listed then dialyzed overnight in the cold with one or two changes of distilled water. One-half ml of each of these solutions, after dilution containing the number of LD_{50} listed was injected into 20 g albino mice intraperitoneally. With the exception of DFP and NBS, which were incubated in 0.1 M sodium phosphate, pH 8.0, the compounds were incubated with venom in 0.05 M Tris-HCl, pH 8.0. The fractions in parentheses indicate the number of deaths over the number of mice injected.

Inhibitor	Conc. mM	LD_{50} Injected No.	Survival %
OMIU	*	5	20 (4/5)
"	*	10	0 (5/5)
EDTA	1	5	0 (4/4)
NEM	100	5	0 (5/5)
IAA	100	5	0 (5/5)
DFP	50	5	20 (4/5)
"	50	10	0 (5/5)
NBS	10	10	0 (5/5)
"	50	150	100 (0/5)
BAL	20	5**	100 (0/5)
"	20	10**	0 (5/5)

*Venom (11.25 mg/ml) was incubated for 72 hours at 2°C with a 50:1 excess of O-methylisourea (OMIU). The unreacted OMIU was removed by dialysis at 2°C against distilled water. The OMIU-treated venom was then incubated at 37°C and samples were removed for assay after 60 minutes.

**Slight precipitate noted.

TABLE 2

EFFECT OF pH ON THE TOXICITY OF CROTALUS ATROX VENOM

Venom (4 LD_{50}/ml) was treated for 15, 30, and 60 minutes at 37°C in the following solvents: pH 1.0, 0.10 N HCl; pH 3.0, 0.05 M glycine; pH 4.0, 5.0, 6.0, 0.05 M sodium acetate; pH 8.0, 0.05 M Tris-HCl. Following incubation, 0.5 ml of venom (in the respective solvent) was injected. Controls contained no venom. Numbers in parentheses are deaths over the total number of mice injected.

LD_{50} Injected No.	Incubation Time Min.	pH 1	3	4	5	6	8*
2	15	(3/3)	(3/3)	(2/3)	(3/3)	(3/3)	-
2	30	(3/3)	(2/3)	(3/3)	(3/3)	(3/3)	-
2	60	(3/3)	(3/3)	(3/3)	(2/3)	(3/3)	(3/3)
0							
0	60	(0/3)**	(0/3)	(0/3)	(0/3)	(0/3)	(0/3)

*Treated with 0.05 M Tris-HCl, pH 8.0, at 25°C.

**These controls died about four days after injection.

The toxic principle in C. atrox venom is not very resistant to heat, as shown in Table 3. Treating the venom at 75°C for one hour destroys most of the toxicity and treatment at 100°C for the same time completely inactivates the toxic component(s).

Table 4 lists the effect of heat on the enzymes in C. atrox venom. Most of the enzymes are inactivated at temperatures between 50° and 75°C with the exception of phospholipase A which retains more than half of its activity after heating it for one hour at 100°C. Also, 17% of the proteolytic activity on benzoyl-DL-arginine p-nitroanilide (BAPNA) is retained after treatment at 75°C for one hour.

Table 5 lists the effect of antivenin on the hydrolysis of azocoll, BAPNA, casein and congocoll by C. atrox venom. With a 10:1 ratio (w/w) of antivenin:venom only slight inhibition was observed. Higher ratios (50:1, 100:1), however, produced significant inhibition. There is an obvious difference in the effect of antivenin on the hydrolysis of

TABLE 3

EFFECT OF TEMPERATURE ON THE TOXICITY OF CROTALUS ATROX VENOM

Venom (11.25 mg/ml) was treated in 0.05 M Tris-HCl, pH 8.0, at 0°, 25°, 50°, 75°, and 100°C for 60 minutes then, after dilution, 0.5 ml was injected into mice. Numerators are deaths; denominators are total of mice injected.

LD_{50} Injected No.	0°	25°	50°	75°	100°
2	3/3	3/3	3/3	0/3*	0/3*
5	-	-	-	0/3	0/3
10	-	-	-	0/3	0/3
15	-	-	-	1/8	0/3
20	-	-	-	5/8	0/5
30	-	-	-	7/8	0/5
50	-	-	-	7/8	0/8

*Precipitates were formed at these temperatures. The supernatant liquid obtained after centrifugation, contained 65% of the biuret positive material. This was used for injection.

TABLE 4

EFFECT OF TEMPERATURE ON THE ENZYMATIC ACTIVITIES OF CROTALUS ATROX VENOM

Venom (11.25 mg/ml) was incubated in 0.05 M Tris-HCl, pH 8.0, at 0°, 25°, 50°, 75°, and 100°C for 60 minutes and then diluted appropriately for assay. Enzymic assays were carried out as described previously (12-14).

Temperature	0	25	50	75*	100*
	Per Cent Activity				
Proteolytic Substrates					
Asocoll	100	100	95	0	0
Congocoll	100	119	81	0	0
BAPNA	100	85	79	17	0
Casein	100	80	31	0	0
Esterases					
Phosphodiesterase	100	100	49	0	0
Esterase (TAMEase)	100	107	77	0	0
Phospholipase A	100	-	-	67	58

*Precipitates were formed at these temperatures. The supernate, obtained after centrifugation, contained 65% of the biuret reading material. This was used for assaying the enzymes.

TABLE 5

EFFECT OF ANTIVENIN ON THE PROTEOLYTIC ENZYME ACTIVITIES OF *CROTALUS ATROX* VENOM

Substrate	mg Antivenin / mg Venom	Per Cent Inhibition
Azocoll	50/5.0	12
"	50/0.5	92
BAPNA	50/5.0	9
"	50/1.0	27
Casein	50/5.0	26
"	50/1.0	57
Congocoll	50/5.0	16
"	50/0.5	100

BAPNA and casein by *C. atrox* venom. Hydrolysis of BAPNA is not inhibited appreciably, while caseinolytic activity is inhibited almost 60 per cent at the 50:1 ratio of antivenin:venom.

Table 6 lists the effect of antivenin on the esterase activities of *C. atrox* venom. At a low ratio of antivenin to venom (10:1), phosphodiesterase is markedly inhibited while hydrolysis of TAME (N-p-toluenesulphonyl-L-arginine methyl ester) is only slightly inhibited by a 17:1 ratio of antivenin to venom. Phospholipase A activity was not inhibited by a 10:1 ratio of antivenin to venom.

Figure 1 shows the effect of diisopropylfluorophosphate (DFP) on the hydrolysis of TAME and BAPNA by *C. atrox* venom. The hydrolytic activity on TAME or BAPNA was essentially equally inhibited at any concentration of DFP used. It thus appears that *C. atrox* venom contains an enzyme which can hydrolyze both — CONH — and — COOR — bonds, *viz.* amide and ester linkages.

DISCUSSION

The toxic principle in *C. atrox* venom has been shown to be quite resistant to treatment at low pH values. If the toxic principle

TABLE 6

EFFECT OF ANTIVENIN ON THE ESTERASE ACTIVITIES OF CROTALUS ATROX VENOM

For experimental details see Materials and Methods.

Esterase	mg Antivenin / mg Venom	Per Cent Inhibition
Phosphodiesterase	50/5.0	86
Esterase	50/5.0	18
"	50/3.0	24
Phospholipase A	50/5.0	0

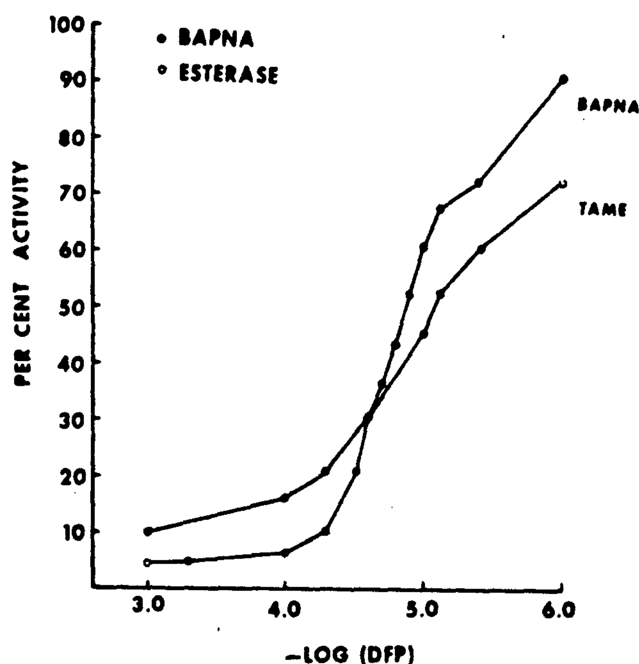

Fig. 1. Effect of diisopropylfluorophosphate on the hydrolysis of BAPNA and TAME by Crotalus atrox venom. Venom (6.0 mg/ml) and DFP were incubated for 60 minutes in 0.05 M Tris-HCl buffer, pH 8.0, at 37°C. Aliquots were removed, diluted with water to 1.0 mg/ml and assayed for enzymic activity.

were a low-molecular weight peptide, it could be non-denaturable at low pH values simply because it has little secondary and tertiary structure to be altered. Yang (17) has recently shown that more than four-fifths of Russell's viper and Formosan Habu venoms are composed of components with molecular weights less than 10,000. He also demonstrated that about one-third of Green Habu (Trimeresurus gramineus formensis), Hundred-pace snake (Agkistrodon acutus), and Eastern diamondback (C. adamanteus) venoms are composed from protein components larger than 50,000; more than 50% of them were low-molecular weight proteins ranging 4,500 - 10,000. Preliminary separation studies* on C. atrox venom with Bio-Gel (P-60) indicate that separated and lyophilized components with molecular weights 5,000 - 14,000 have about twice the toxicity as whole lyophilized venom in terms of either protein or dry weight. These low-molecular weight components constitute approximately 43% of the dry venom. This increase in toxicity is paralleled by increases in proteolytic enzyme activities and phosphodiesterase activity.

Previous work in this laboratory (12-14) demonstrated that most of the enzymes in C. atrox venom could be almost completely inactivated by one or more of the functional-group inhibitors listed in Table 1, which have little or no effect on the toxicity of this venom. One millimolar EDTA, although it has little effect on the toxicity, completely inhibits the proteolytic activity of C. atrox venom on casein, azocoll, and congocoll, as well as phosphodiesterase activity. Ten millimolar NBS has been shown to inhibit 94% of the proteolytic activity on BAPNA, yet this does not affect toxicity. Esterase (TAMEase) activity is completely inhibited by DFP; this compound has little effect on toxicity. From these observations and from the data in this paper, one can presume that the toxic principle in C. atrox venom is not associated with any of the enzymes listed in Table 4.

Of the functional group reagents used in an attempt to detoxify C. atrox venom only NBS was completely effective in reducing the toxicity to nil. This compound has been used by Boroff et al. (18) to detoxify Clostridium botulinum toxin. It has also been used by Okada et al. (19) to inactivate Bacillus subtilis α-amylase with no loss of antigenicity and loss of one mole per mole of tryptophan. In preliminary experiments with the NBS-detoxified C. atrox venom there was some indication that the treated venom could elicit antibody formation.

L-Amino acid oxidase and adenosine triphosphatase, which are present in C. atrox venom (20, 21), have not, however, been characterized with respect to these functional-group inhibitors. Other enzymes

*Unpublished results.

which have not been investigated and could be present in this venom, based on their presence in other crotalid venoms, include deoxyribonuclease (DNase), ribonuclease (RNase), diphosphopyridine nucleotide-pyrophosphatase, hyaluronidase and 5'-nucleotidase. We have observed that C. atrox venom has little, if any, hyaluronidase activity,* using the colorimetric method of Slotta and Ballester (22).

Furthermore, phospholipase A activity is only partially inactivated by heating venom at 100°C, whereas the toxicity is completely destroyed under these conditions.

The effect that antivenin has on the enzymes of C. atrox venom, under the conditions listed, indicates that phospholipase A has little antigenicity. The enzymes responsible for the hydrolysis of TAME, BAPNA, and casein are weakly antigenic and phosphodiesterase, azocollase, and congocollase are strongly antigenic.

Some of these enzymic activities in this and other venoms have been shown to be toxic per se (23-27). Whether the toxicity of this venom is due to separate toxin(s) or the synergistic action of several enzymes has yet to be determined.

Mandelbaum and Henriques (28) have shown the Bothrops protease A of Bothrops jararaca venom is the enzyme responsible for the hydrolysis of N-benzoyl-L-arginine amide (BAA) and that this crude enzyme also contains the bulk of TAMEase activity. It may be that one enzyme is hydrolyzing both TAME and BAA. Evidence in this paper (Fig. 1) indicates that one enzyme might have both proteolytic and esterolytic activity similar to chymotrypsin and trypsin (29).

SUMMARY

1. C. atrox venom has been completely detoxified by 50 mM N-bromosuccinimide.

2. This venom cannot be detoxified by reaction with the sulfhydryl group reagents, N-ethylmaleimide or iodoacetamide, or the metal-binding agent EDTA.

3. C. atrox venom is very slightly detoxified by reaction with O-methylisourea, diisopropylfluorophosphate, or British anti-lewisite.

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*Unpublished results.

4. The toxic principle in C. atrox is resistant to low pH treatment, but is destroyed by heating at 75° or 100°C for one hour. Under the same conditions the following enzymes are destroyed: phosphodiesterase, esterase (TAMEase), and proteolytic enzyme activity on azocoll, congocoll, casein, and N-benzoyl-DL-arginine p-nitroanilide. Phospholipase A activity is only partially destroyed under these conditions.

5. Polyvalent crotalid antivenin has been shown to inhibit those enzymic activities of C. atrox venom responsible for the hydrolysis of TAME, BAPNA, casein, azocoll, and congocoll. Phosphodiesterase was significantly inhibited by antivenin but phospholipase A, under the conditions studied, was not inhibited.

6. Diisopropylfluorophosphate was found to inhibit the hydrolysis of TAME and BAPNA by C. atrox venom almost identically over a wide range of DFP concentrations.

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13. ABSTRACT

Venom of Crotalus atrox can be detoxified with N-bromosuccinimide. The toxin(s) are not affected by treatment at low pH but are inactivated by heating at 75° or 100°C. At these temperatures all of the enzymes tested are inactivated except phospholipase A.

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Venoms, Biochemistry						
Enzymes						
Toxins and Antitoxins						
Temperature and Enzyme Activity						
Temperature and Toxicity						
pH and Toxicity						
Antivenin and Enzyme Activity						

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