

THE ANTIPHOSPHATIDASE A ACTIVITY IN  
RELATION TO THE ANTITOXIC PROPERTIES  
OF VIPERA AMMODYTES ANTIVENIN

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*(Received for publication June 5, 1957)*

Phosphatidase A of *Ammodytes viper* venom shows the properties of a good antigen and antihaemolytic antibodies can be detected in corresponding antivenin. When *Ammodytes viper* venom was neutralized with antivenin the same value for antivenin was obtained irrespective of which property of venom haemolysis or killing power in animals was used for indicating the endpoint. Haemolysis and toxicity could be considered from the immunological standpoint as two actions of a single substance. A method for the determination of antihaemolytic power of antivenin is described.

The snake venoms may be considered as proteic mixtures of toxins and enzymes, varying to some extent in chemical composition with the snake species. The most interesting unsolved problem is whether enzymes take any significant part in the complex intoxication symptoms produced by the venom in the bitten animal. In spite of the great importance of enzymes in snake venoms, results of various investigations suggest the conclusion that enzymes could scarcely be considered as real toxic principles of venoms.

*Slotta* and *Fraenkel-Conrat* isolated from the *Crotalus terrificus terrificus* venom (1) crystalline crotoxin and considered it identical with phosphatidase A. Crotoxin accounted for all the haemolytic and neurotoxic activity of crude venom, and both these activities ought, therefore, to be due to its phosphatidase A activity. This work suggests that the neurotoxic and the haemolysis producing enzyme phosphatidase A of *Crotalus terrificus terrificus* venom are identical. Meanwhile, at the same time (1938) *Gosh* and *De* (2) reported their observations on the same subject that appeared to be contradictory to the results obtained by *Slotta* and his co-workers. They suggested that crotoxin was a mixture of a haemolytic and a neurotoxic component.



*Goncalves* (1952) isolated crotamin from crotoxin by means of electrophoresis (3). Recently (1955) *Neumann* and *Habermann* (4, 5) succeeded in separating crotactin as the most important proteinic constituent of crotoxin using Amberlite resin chromatography. Crotactin showed no other haemolytic activity.

In order to solve similar problems *Zeller* (6) suggested that quantitative data on the enzyme activity of various snake venoms should be accumulated, and the results compared with the corresponding biological activities of the venoms.

In this communication we report the results of our investigation of the inhibitory effect of immune horse serum on the haemolytic activity of *Ammodytes viper* venom. The results so obtained have been compared with the antitoxic potency of the same immune serum when tested on white mice against same venom.

Though the haemolytic activity of the *Vipera ammodytes* venom is neutralized by antivenin, no evidence is yet available to determine whether this activity is due to neurotoxin of *Vip. ammodytes* so far described or whether more than one substance is responsible for it. We tried to tackle these problems from the immunochemical standpoint.

#### *Materials and methods*

The utilized toxin of *Vipera ammodytes* was a pooled sample of venom collected from living snakes, lyophilized or vacuum dried and ground in a mortar. Nitrogen content 13.5 per cent, sulphur 4.2 per cent; LD<sub>50</sub> is considered to be the amount of venom killing 50 per cent of mice within 48 hours. For each serial dilution twelve mice, and for the experiment 54 animals in all were used. A fresh solution was made up on each experimental occasion. The injected volumes were 0.25 ml. in the toxicity test and 0.50 ml. in the serum titration test. This was necessary to avoid large variations in the concentration of the snake venom solution.

*Sheep erythrocytes* have been used in all experiments. The blood was defibrinated by shaking with glass beads under sterile conditions. The erythrocytes were washed three times with 0.9 per cent NaCl solution and centrifuged (10 min./2500 r. p. m.). They were used only two or three days following the withdrawal.

*Titration of immune serum.* A series of toxin-immune serum combinations has been prepared. To 2.5 mg. of toxin as 0.1 per cent venom dilution (in an isotonic buffer solution pH 7.2) was added diluted serum (1:1 in a phosphate buffer) in progressive doses (1.1; 1.2; 1.3; 1.4; 1.6; 1.8 ml.). All venom-serum combinations were diluted with an isotonic buffer solution to 5 ml. After standing one hour in an incubator (37° C), the mixtures were used for intravenous injection (inject-



ed vol. 0.5 ml.). Five sets, each containing six animals, have been employed at least for each serial dilution of venom. The animals were weighed by the experimenter and only healthy male mice between 16 and 18 g. in weight were used. The deaths were counted after 48 hours.

*Lecithin* has been prepared by the method of Pangborn (7). 0.05 per cent of lecithin emulsion or 1 per cent of a fresh egg yolk solution in phosphate buffer pH 7.1 have been used (8).

*Immune antitoxic serum* was pepsin digested purified immune horse serum concentrated by ammonium sulphate precipitation and ultrafiltration (9). In dilution 1:450 with saline this serum protected the mice against 20 microgramms of Ammodytes viper venom.

All our experiments have been carried out with one preparation of immune serum (Institute for Production of serums, Zagreb).

*Determination of phosphatidase A activity.* In all experiments two series of tubes containing 0.2 ml of venom-buffer dilution of various concentrations (4–20 microgramms of snake venom) were applied. To the first set ( $S_1$ ) of tubes 0.5 ml. of horse immune serum diluted with saline 1:500 and in another set ( $S_2$ ) 0.5 ml. of serum dilution 1:250 was added. After standing 24 hours at 20°C 3 ml. of erythrocytes and lecithin emulsion in isotonic phosphate buffer was added (2 ml. of erythrocytes centrifugate, 10 ml. of 0.05 per cent lecithin or 10 ml. of 1 per cent egg yolk emulsion were diluted with 88 ml. of isotonic phosphate buffer, pH 7.1).

After standing for 2 or 3 hours in an incubator at 37°C all tubes were simultaneously centrifuged (10 min. at 2500 r. p. m.) and 0.5 ml. of clear haemoglobine supernatant from each tube was withdrawn and diluted with distilled water 1:7. The optical density of the samples was measured with Fisher's electrophotometer (filter 425 B), and the degree of haemolysis in each tube was expressed in percentages of total haemolysis.

### Results

In order to compare the potency of the antiphosphatidase A in immune serum with the potency of all antibodies neutralizing the toxic activity of snake venom in white mice test, we determined the amount of snake venom neutralized by 1 ml. of immune serum, using partial haemolysis of sheep red blood cells as an indicator for controlling the residual venom after the formation of the toxin-antitoxin complex in the reaction mixture.

The haemolysis induced by the presence of lysolecithin is a process governed not by laws of stoichiometry but of probability (10, 11). If the amount of erythrocytes lysed out of a certain total dose of red blood cells is plotted against the dose of the venom a sigmoid curve describing the course of the haemolytic process is obtained.



Because of variations in resistance of the various samples of erythrocytes, different degrees of haemolysis were produced by the same snake venom in successive experiments carried out under, otherwise, same experimental conditions (12). The measurements obtained simultaneously in the same experiment showed a satisfactory grade of reproducibility.

Figure 1 A and 1 B show the curves representing the course of haemolysis of sheep erythrocytes when the phosphatidase A activity of the snake venom was partially inhibited by serum antibodies.

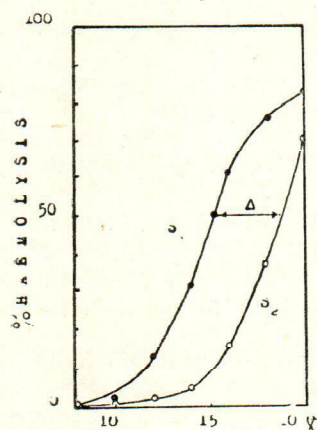


Fig. 1a The course of haemolysis of sheep erythrocytes caused by *Vip. ammodytes* venom and partially inhibited by antivenin.  
abscissa: venom in microgramms  
ordinate: per cent of haemolysis.

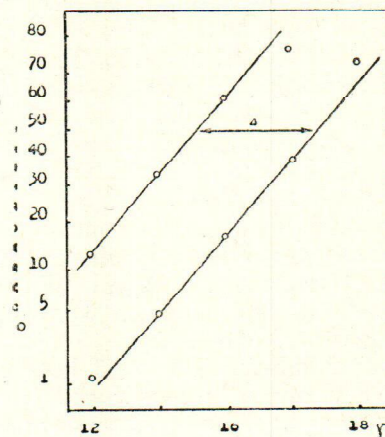


Fig. 1b. The curves of haemolysis presented in fig. 1a transformed in system parallel straight lines.  
abscissa: venom microgramms.  
ordinate: per cent of haemolysis (in probability units).

In each haemolysis experiment we used two sets ( $S_1$  and  $S_2$ ) of tubes containing venom-buffer solution in identical progressive doses. Each tube of the first set contained a certain amount of immune serum while to the second set twice this amount was added. After the formation of toxin-antitoxin complex, a lecithin and erythrocytes suspension in isotonic phosphate buffer to each tube has been admixed. Both sets of tubes were then simultaneously incubated until a satisfactory partial haemolysis developed.

The degree of hemolysis produced by the phosphatidase A of residual toxin in each tube was determined photometrically under the same experimental conditions.



From the sigmoid shape of the haemolysis curve it is evident that a certain amount of toxin is without haemolytic effect, and consequently the curve does not begin at the origin. These sigmoid curves can be transformed to a system of parallel (13) straight lines with the dose as abscissa and some univariant function, the probit or the logit, as ordinate (14). The shift (Fig 1 A and 1 B) of the second curve in the direction parallel to the abscissa is caused by the excess of antitoxin in the second set of tubes. This shift corresponds to the difference of the venom doses ( $\Delta$ ) in two simultaneous experiments ( $S_1$  and  $S_2$ ), necessary to produce 50 per cent of haemolysis in shccp erythrocytes. This difference can be computed (15) or graphically estimated as a horizontal distance between parallel lines obtained by transforming haemolysis curves, and represents the amount of snake venom neutralized by an excess of immune serum in the second set of tubes.

Table 1.

*Inhibitory effect of immune serum on phosphatidase A of Vipera ammodytes venom*

Experiment No.	1	2	3	4	5	6	7	8	9	10
Set 1 $d_1$	14.0	11.6	16.0	15.2	8.0	14.2	13.8	15.2	15.1	12.6
Set 2 $d_2$	17.2	15.2	19.4	18.6	11.4	17.6	17.2	18.6	18.5	16.2
$d_2 - d_1 = \Delta$	3.2	3.6	3.4	3.4	3.4	3.4	3.4	3.4	3.4	3.6
	$\bar{\Delta} = 3.42$									

Note:  $d_1$  and  $d_2$  are doses (in microgramms) producing 50 per cent haemolysis in sets  $S_1$  and  $S_2$ ;  $S_1$  contains 0.002 ml. and  $S_2$  0.004 ml. of immune serum.

Table 2.

*Neutralization of Vipera ammodytes venom by antivenin tested on white mice*

Immune serum (dilution 1:1) ml.	1.8	1.6	1.4	1.3	1.2
0.1 per cent sol. Vip. Amm. ven. ml.	2.5	2.5	2.5	2.5	2.5
Isotonic buffer ml.	0.7	0.9	1.1	1.2	1.3
Number of mice (injected)	30	36	42	30	36
Proportion killed per cent	0	8.3	50	76.6	94.3

The results of different experiments presented in the table 1 indicate that 0.5 ml of an immune serum dilution 1:500 neutralizes 3.42 microgramms of toxin, that is 1 ml of the immune serum neutralizes 3.42 mg of snake venom, when a 50 per cent haemolysis of the red blood cells was used as endpoint indicator for the formation of toxin-antitoxin complex in the reaction mixture.



In order to compare the results obtained from the haemolysis test we investigated the inhibition of the venom toxic activity with the same immune serum measuring the combinations of toxin and antitoxin in terms of the neutralization of killing power in white mice. The animal test (Fig. 2) has been performed under experimental conditions explained in table 2. The final results of five independent experiments are recorded. For the estimation  $LD_{50}$  in the toxicity test and for the titration of the immune serum a fairly large sample of white mice was used.

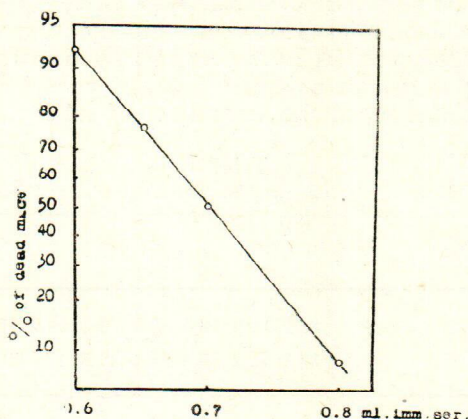


Fig. 2. Neutralization of *Vip. ammodytes* venom by antivenin tested on white mice. abscissa: dose of immune serum in ml. ordinate: proportion of dead mice (in probability units).

At 50 per cent fatality rate, 2.5 mg. of snake venom was neutralized by 0.7 ml. of immune serum, or, for 3.57 mg. of snake venom 1 ml. of serum was required. This animal test is only efficient in measuring within a certain range of toxin-antitoxin proportions since a constant indicating dose of free toxin is required for the measurement of  $LD_{50}$  in a white mice population.

This constant dose is likely to be approximate of the same a magnitude as the dose necessary to produce 50 per cent fatality rate in toxicity tests on white mice, and amounts to 16 microgramms for our sample of the snake venom.

Taking this into consideration the real amount of *Vip. ammodytes* venom neutralized by 1 ml. of immune serum in animal test was 3.31 mg. It is very interesting that this result tallies with that obtained from haemolysis test.



*Discussion*

The principle we employed to throw light on the chemical nature of toxic and haemolytic activities in Vip. ammodytes venom can be explained as follows: when two different actions of an antigen are due to a single substance and an antiserum is titrated against this antigen, the same value should be obtained for serum irrespective of which action of the antigen is used indicating the endpoint.

The results of our investigations have shown that nearly the same value of serum has been obtained for a neutralization of Vip. ammodytes venom irrespective of which activities of venom, haemolysis or toxicity in mice were used for indicating the endpoint. Consequently the active principles of snake venom producing haemolytic and toxic effects could be considered as a single substance.

We made use of immunochemical experimental methods for we experienced that the venom proteins in a salt-free state showed a great tendency to spontaneous denaturation. The most delicate methods as partition chromatography on Hyphlo-Supercel kieselguhr column (16) or Amberlite synthetic resins (4) or endgroup determination with fluorodinitrobenzene, when applied on crystalline crotoxin did not yield reliable results (17).

We succeeded (12) in separating the Vip. ammodytes venom proteins by means of electrophoresis into seven components, but Oudin's antigen-antibody precipitation test in agar gel (18) has given evidence only for the presence of three antigenic components. One proteinic component isolated from the venom showed neither haemolytic nor neurotoxic properties (19).

Haemolytic action of snake venom in relation to its neurotoxic activity has been investigated and the results of our experiments are presented in this communication.

*Acknowledgments.* We wish to express our thanks to Prof. Dr. V. Vouk for aid in statistical interpretation of experimental results and Dr. D. Ikić for aid in immunological investigations.

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### Sadržaj

## ANTIHEMOLITIČKA I ANTITOKSIČKA SVOJSTVA IMUNOG SERUMA PROTIV OTROVA VIPERAE AMMODYTES

Fosfatidaza A u otrovu *Viperæ ammodytes* posjeduje svojstva antigena, pa imuni serum, dobiven imunizacijom konja tim otrovom, inhibira hemolitički encim otrova *Vip. ammodytes*. Količina imunog seruma, potrebna da se neutralizira neka određena količina otrova ista je, bez obzira na to, da li se kod titracije seruma kao indikator neutralizacije upotrebljava 50 posto'ni letalitet miševa ili parcijalna hemoliza ovčjih eritrocita u smjesama toksina i antitoksina. Prema tim rezultatima moglo bi se zaključiti, da su hemolitička aktivnost i toksična svojstva otrova osobina jedne te iste supstancije.

Opisana je metoda za određivanje antihemolitičkog aktiviteta imunog seruma.

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Zagreb

Primljeno 5. VI. 1957.