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ISOLATION OF SNAKE VENOM TOXINS AND STUDY OF THEIR MECHANISM OF ACTION

Final Technical Report

by

Prof. André de Vries

January 1970

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EUROPEAN RESEARCH OFFICE

United States Army

Contract Number DAJA 37-69-C-0252

Rogoff-Wellcome Medical Research Institute, Beilinson Hospital,

Petah Tikva, Israel

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Abstract:

In this study on snake venoms a threefold approach was made: isolation of venom toxins, elucidation of their mode of action, raising antivenin potency. Attention was directed to phospholipase, hemolytic polypeptide, kinin-releasing enzyme, hemorrhagin, and antihemorrhagin antibody. Methods included exchange chromatography, gel filtration, histochemical, electron microscopical, phospholipid hydrolysis, microsomal ATPase, enzymatic antibody fragmentation. The conclusions are: Naja naja venom contains several phospholipase A iso-enzymes; Echis coloratus venom contains two kinin-releasing enzymes; fibrin deposition in the kidney by Echis carinatus venom is promoted by epsilon aminocaproic acid; Vipera palestinae hemorrhagin impairs blood coagulation and platelet function by its proteolytic activity; Ringhals phospholipase A inhibits microsomal ATPase; lytic peptide of cobra venom activates hydrolysis of membrane phospholipids but not of soluble phospholipids; bivalence of antibody appears significant in hemorrhagin neutralization. It is recommended that further studies in the above areas are carried out.

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Report

Introduction

Snake bite produces varying symptomatology, grossly classified as neurotoxic, cardiotoxic, hemorrhagic, hemolytic, tissue necrosis, and causally related to different toxins termed, respectively, neurotoxins, cardiotoxins, hemorrhagins, hemolysins, cytotoxins. It has become evident that a specific venom may contain various toxins, thus producing a complicated clinical picture produced by the combined action of more than one toxin. Indeed, in recent years, modern methods of fractionation have made it possible to isolate different toxins from one single venom and to obtain them in purified form. Thus, it is now feasible not only to chemically characterize different venom toxins, but also to study their specific action both in in vitro systems and in vivo. In addition to the progress achieved, in the understanding of the mechanisms underlying the varying symptomatology, it has now become possible to evaluate the antigenicity of the isolated toxins, that is their aptitude to elicit therapeutically adequate antibody response. The availability of isolated toxins may render it possible to

approach the problem of increasing the neutralizing potency of specific antitoxins. Moreover the way is opened for a more rational pharmacological approach to the prevention and treatment of pathology induced by the specific toxins.

Still, whereas progress in these fields is considerable, the final goal of clarification of the mode of action of the various toxins and rational therapy, both immunologic and pharmacological, is still far off. It is therefore that our team has attacked the problems concerned with snake bite, specifically that in the Near East, by a threefold approach: isolation and purification of venom toxins, study of their mode of action and of their immunologic properties.

The specific problems, methods of study, results and conclusions in the various areas investigated are given in the chapters listed below. A final general comment surveys the results obtained and conclusions made.

Studies on various venom components have been carried out on venoms belonging to two main snake species: the Viperidae - Vipera palestinae, Echis carinatus and coloratus - and the Elapidae - Hemachatus haemachates (Ringhals) and Naja naja.

The specific areas investigated were as follows:

1. Isolation and characterization of phospholipase A from Cobra Naja naja and Vipera palestinae venoms.
2. Action of calcium and of Ringhals venom direct lytic factor on the activity of the venom phospholipase A.
3. Action of Ringhals venom phospholipase on microsomal ATPase.
4. Isolation and characterization of kinin-releasing enzyme of Echis coloratus venom.
5. Effects of purified Vipera palestinae hemorrhagin on blood coagulation and platelet function.
6. Intravascular coagulation induced by Echis carinatus venom.
7. Molecular basis of neutralization of Vipera palestinae hemorrhagin by specific antibodies.

1. Isolation and Characterization of Phospholipase A from Cobra Naja naja and Vipera palestinae Venoms

The generic term phospholipase designates a class of enzymes, which specifically hydrolyze phospholipids. Phospholipase A (E.C. 3.1.1.4) catalyzes the hydrolysis of one ester bond in a 1,2 diacyl-sn-glycero-3-phosphatide with formation of a lyso-derivative and release of one fatty acid.

Phospholipases A from various snake venoms (direct and indirect, belonging to the Elapidae and Viperidae families) exhibit almost no difference in activity when applied on soluble substrates, such as lipoprotein molecules in blood serum or egg yolk (1). On the contrary, a marked difference in the action of the Elapid cobra Naja naja and Vipera palestinae phospholipases was revealed when applied to phospholipids in cell membranes (2). Whereas cobra phospholipase A is able to split the phospholipids of human and animal osmotic red cell ghosts (2), human platelets (3) and animal brain mitochondria (4), Vipera palestinae phospholipase is ineffective on these systems.

In view of the yet unclear relationship between the phospholipases' molecular structure and their phospholipid splitting ability of structural lipoproteins, it appeared indicated to study this problem with purified phospholipase A obtained from the Elapid Naja naja and Vipera palestinae venoms.

Methods

The methods applied for purification were ion exchange chromatography and gel filtration.

The purified proteins were subjected to hydrolysis in vacuo in 6 M HCl at 110°C for 22, 48, 72 and 96 hrs. The amino acid composition was determined using Beckman Spinco automatic amino-acid analyser Model 120B.

The N-terminal amino acid was estimated using the dansyl-chloride procedure (5). Applying dansyl-chloride, a fluorescent, stable (towards acid hydrolysis) sulfonamide complex is formed with the N-terminal amino acid. The C-terminal amino acid was estimated either by hydrazinolysis (6) or by carboxypeptidase A (7). By hydrazinolysis transamidation of all peptide bonds occurs and only the C-terminal amino acid is liberated as free - amino, -carboxylic acid. The SH bonds were estimated by titration with parachloromercurybenzoate according to Boyer (8).

The molecular weights were estimated in a Spinco Model E centrifuge with a schlieren optical system or by diffusion measurements. Disc electrophoresis on acryl-amide gel was carried out by the method of Reisfeld et al.(9). Immuno-diffusion technique of Ouchterlony (10) was used and immuno-electrophoresis according to Scheidegger (11) was performed.

Results and Discussion

The phospholipase A from Cobra Naja naja venom was isolated and purified by exchange chromatography on CM-cellulose, using for elution ammonium acetate pH 6.0, in a linear gradient between 0.005M - 0.5M. The enzymatic activity could be separated into four defined fractions. The fraction which emerged at the initial ionic strength (0.005M) represented about 63% of the total activity of the isoenzyme complex, and the other three emerging at ionic strength of 0.065M, 0.05M and 0.15M represented about 11%, 7% and 19% respectively. Each of the above isoenzymes was rechromatographed on the CMC column at appropriate ionic strength and purified further by gel filtration on Sephadex G-50.

Each of the above fractions was homogeneous when examined on acrylamide gel electrophoresis. The first three isoenzymes had similar electrophoretic mobilities. All of the isoenzymes were antigenically identical when tested by immuno-diffusion. Furthermore, similarly to the whole phospholipase A complex, each of the isoenzymes was active in splitting phospholipids of red blood cell ghosts (2). The isoenzyme I, representing 63% of the complex, appears to consist of one polypeptide chain having no free SH groups. The N-terminal residue is aspartic acid or asparagine, the C-terminal glutamine. The amino acid composition of the isoenzyme I and IV is given in Table 1. The amino acid composition of the isoenzymes II and III is still under investigation.

Vipera palestinae phospholipase A was purified first on DEAE-cellulose. The active fraction was rechromatographed on Sephadex G-50. The purified phospholipase gave one homogeneous band on acrylamide gel electrophoresis. The enzyme consists of one single polypeptide chain having no free SH groups. The C-terminal residue is a half cystine. The nature of the N-terminal group is still under investigation. The molecular weight of the purified enzyme was about

20,000. The purified phospholipase behaves similarly to the crude preparation in its inability to split phospholipids of red blood cell ghosts.

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Table 1

Amino Acid Composition of Naja naja (Isozymes I and IV) and Vipera palestinae Phospholipases A *

Amino acid	Naja naja						Vipera palestinae		
	Isozyme I			Isozyme IV					
	Residues per 6 leucine residues	Nearest integer	Molar % of total amino acid	Residues per 6 leucine residues	Nearest integer	Molar % of total amino acid	Residues per 12 aspartic acid residues	Nearest integer	Molar % of total amino acid
Lysine	7.9	8	5.84	12.3	12	7.10	7.2	7	7.53
Histidine	1.7	2	1.46	2.3	3	1.78	1.6	2	2.15
Arginine	5.1	5	3.65	5.3	5	2.96	2.2	2	2.15
Aspartic acid	22.7	23	16.79	25.2	25	14.79	12	12	12.90
Threonine	5.9	6	4.38	7.9	8	4.73	4.9	5	5.38
Serine	11.3	11	8.03	20.7	21	12.43	7.3	7	7.53
Glutamic acid	10.3	10	7.30	15.8	16	9.47	8.3	8	8.60
Proline	4.2	4	2.92	3.7	4	2.37	2.4	2	2.15
Glycine	13.7	14	10.22	21.3	21	12.43	11.2	11	11.83
Alanine	11.8	12	8.76	14.8	15	8.88	5.9	6	6.45
Half cystine **	11.8	12	8.76	8.1	8	4.73	6.0	6	6.45
Valine	4.7	5	3.65	6.2	6	3.55	5.1	5	5.38
Methionine	1.0	1	0.73	1.0	1	0.59	1.9	2	2.15
Isoleucine	4.2	4	2.92	4.9	5	2.96	2.0	2	2.15
Leucine	6.0	6	4.38	6.0	6	3.55	5.0	5	5.38
Tyrosine	8.3	8	5.84	6.9	7	4.14	4.8	5	5.38
Phenylalanine	3.9	4	2.92	4.3	4	2.37	4.2	4	4.30
Tryptophan ***		2	1.46		2	1.18		2	2.15

* The above results are the average of four analyses after hydrolysis for 22, 48, 76 and 96 hrs.

** Cystine determined as cysteic acid.

*** Tryptophan determined colorimetrically.

2. Effects of calcium ions and of cobra venom direct lytic factor on phospholipases A

It has been found in a previous study (1) that a basic protein isolated from cobra venoms termed "direct lytic factor" (DLF) induces a moderate degree of hemolysis in human erythrocytes and acts synergistically with purified snake venom phospholipases A in promoting the splitting of phospholipids in a number of cellular and subcellular membranes (1,2,3,4).

The mode of action of DLF as an activator of the phospholipase is largely unknown. We thought it of interest to investigate whether the synergistic action of DLF with phospholipase A applies also to phospholipid substrates other than those in membranes, and, in this respect, to compare the action of DLF with that of the calcium ion, a specific phospholipase A activator.

The direct lytic factor is a small molecular weight, strongly basic protein (5) which becomes attached to intact red cells and to red cell osmotic ghosts (6). For the synergistic effect of DLF and phospholipase A to occur, simultaneous presence of both factors is required (1).

It had been found that in the splitting of red cell ghost phospholipids by phospholipase A, DLF can be substituted by a number of natural and synthetic basic polypeptides and a common mode of action through disturbance of lipid-protein hydrophobic bonding has been proposed (7).

Venom phospholipase A was supplemented with either calcium or DLF, and their effects on hemolysis and phospholipid splitting in human erythrocytes were compared. Phospholipid splitting mediated by either DLF or calcium was also investigated in red cell osmotic ghosts, as well as in non-membranal substrates such as egg yolk lipoproteins and micellae of purified egg yolk lecithin.

Methods

The DLF containing fraction was isolated from Ringhals venom by paper electrophoresis (1), dissolved in 5 mM EDTA solution and further purified by ammonium sulfate fractionation and filtration through a Sephadex G-50 column as described by Aloof-Hirsch et al (5). The phospholipase A of Ringhals venom was obtained by paper electrophoresis (1) and that of Vipera palestinae venom

by fractionation on DEAE-cellulose (8). All phospholipase fractions were supplemented with EDTA, subsequently removed by dialysis. Lecithin was prepared by fractionation on an alumina column (9) of a total egg yolk phospholipid extract.

The splitting of phospholipid was followed both by titration of the free fatty acids released, according to the procedure of Dole (10) and by chromatographic analysis (11).

Results and Discussion

The splitting of the red cell phospholipids by snake venom phospholipase A was found to be promoted by either Ca^{2+} or DLF, the latter being more efficient. The hemolysis promoted by DLF was of a very low magnitude. Hydrolysis of phospholipids in red cell ghosts by Vipera palestinae phospholipase A was equally promoted by DLF and by Ca^{2+}. The effects were similar both in the extent of phospholipid splitting and in the degree of hydrolysis of each individual phospholipid species. Ringhals venom phospholipase A catalyzed splitting of red cell ghost phospholipids in the absence of Ca^{2+} or DLF (Table 2).

With egg yolk lipoproteins and lecithin preparations as substrate, both Vipera palestinae and Ringhals phospholipases exhibited a strict requirement for Ca^{2+} added. On these non-membranal substrates, Ca^{2+} could not be replaced by DLF.

The effect of a specific phospholipase inhibitor EDTA, was tested in all the above systems. Although EDTA inhibition is presumably due to Ca^{2+} chelation, it was found that EDTA inhibited the action of the phospholipase also in Ca-free systems. These data suggest that the inhibitory effect of EDTA bears directly on the catalytic function of the enzyme itself.

It is concluded that the observed disparities in the requirement and specificity for Ca^{2+} or DLF of the diverse systems studied are determined both by the dissimilar molecular properties of the various phospholipases A and the differences in the physical state of the phospholipid substrate.

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Table 2

Patterns of Phospholipid Splitting in Red Cells Osmotic Ghosts by Venom Phospholipase A: Effects of Ca^{2+} and DLF

Phospholipid composition %		Phospholipid splitting %			
		V. palestinae phospholipase			Ringhals phospholipase
		No addition	Plus DLF	Plus Ca^{2+}	No addition
PE	26.3±1.46	34	79	85	100
PC	29.7±0.85	31	84	91	100
PA	1.4±0.63	43	100	100	100
PS	13.8±1.07	76	100	100	100
PI	1.2±0.60	0	0	0	0
SM	27.7±1.21	0	0	0	0

The values for the individual phospholipids represent means ± S.D. of 5 determinations done in separate experiments. Abbreviations: PE, phosphatidyl ethanolamine; PC, phosphatidyl choline; PA, phosphatidic acid; PS, phosphatidyl serine; PI, phosphatidyl inositol; SM, sphingomyelin.

3. Action of Ringhals phospholipase on microsomal ATPase.

This study was undertaken to detect a possible effect of venom phospholipase A on membrane enzymatic activity, specifically microsomal ATPase.

It is well established that cobra phospholipase A is able to split phospholipids in biological membranes: 500 µg/ml upon 2 hours incubation completely hydrolyzes phosphatidyl ethanolamine, phosphatidyl serine and lecithin of red blood cells or platelets (1,2). It is also known that ATPase is an integral component of membranes such as the red cell ghost or the reticuloendothelial membrane of microsomes from mammalian cells. It has been shown that phospholipids are essential for ATPase activity of a membrane fraction from beef brain (3) and also for that of red cell ghosts (4).

ATPase activity of rat brain microsomes following incubation with venom phospholipase A was assayed and compared with that of untreated microsomes. Since a pure phospholipase A devoid of other activities was used, any change in ATPase activity should correlate with membrane phospholipid hydrolysis.

Methods

1. Microsomes. Microsomes were prepared by a procedure modified from that used by Skou (5). Male albino rats (weight 120-130 g.) were killed by decapitation. The brains were removed, rinsed and homogenized in a 0.25 M sucrose solution at pH 7.5, containing 1 mM EDTA. The brain homogenate was centrifuged in the cold for 10 minutes at 10,000 rpm to sediment unbroken cells and heavy debris. Mitochondria were removed from the supernatant by further centrifugation at 12,500 rpm for 15 minutes. The postmitochondrial supernatant was then centrifuged at 40,000 rpm for 60 minutes. The microsomal pellet obtained was suspended in sucrose solution and stored at $-20^{\circ}C$. An aliquot was removed for protein determination according to Lowry (6).

2. Assay of microsomal ATPase. ATPase was determined according to Swanson et al. (7). Incubation mixtures at a final volume of 1 ml contained 100 µM Tris-HCl buffer pH 7.5; 3 µM magnesium chloride; 3 µM ATP and in addition - when indicated - 30 µM potassium chloride and 100 µM sodium chloride. Microsomes were introduced at a concentration of 75-150 µg protein and the mixture was incubated at $37^{\circ}C$

for 30 minutes. The reaction was stopped by adding 1 ml of 10% TCA. Following incuabtion in an ice bath for 30 minutes the mixture was centrifuged at 10 000 rpm for 15 minutes. Inorganic phosphorus in the supernatant was determined according to Gomori (8).

3. Phospholipase A was isolated from the venom of Hemachatus haemachatus by a procedure described by Aloof (9). The material migrated as a single band on polyacrylamide gel electrophoresis.

Results and Discussion

The results of a typical experiment are given in Table 3. Microsomes suspended in buffer were preincubated with phospholipase A at 35°C for various periods. Metals and substrate were then added, the volume made up to 1 ml with water and the tubes transferred to 37°C, where they were incubated for another 30 minutes. The reaction was stopped with trichloroacetic acid and inorganic phosphorus in the supernatant determined. When phospholipase is added directly into complete incubation mixture at a concentration of 0.5 µg/ml both ATPase activities are reduced by about 14%. Preincubation of the microsomes with the enzyme at

that same concentration for only 15 minutes results in a 50% inhibition of the magnesium dependent activity and a 70% inhibition of the sodium and potassium dependent activity. Further preincubation or a higher concentration of phospholipase A cause even more drastic inhibitions. The basis for the remarkable effect of preincubation was further studied. The results of an experiment given in Table 4 demonstrate that the substrate (ATP) as well as a mixture of the monovalent cations (sodium and potassium) efficiently protect microsomal ATPase from loss of activity by phospholipase A. The other essential metal, magnesium, is uneffective.

The experiments described confirm former findings concerning the ability of cobra phospholipase A to split membrane phospholipids and also indicate that phospholipids, located at sites for binding substrate and the monovalent cations sodium and potassium, are essential for functional membrane ATPase.

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Table 3

Action of Phospholipase on Microsomal ATPase. Effect of Preincubation

Preincubation (min.) \ Treatment	Untreated		Phospholipase 0.5 ug/ml		Phospholipase 5 ug/ml	
	Mg	Mg, Na, K	Mg	Mg, Na, K	Mg	Mg, Na, K
0	22*	30.4	19	26	13	17.1
15	21.7	27	10.6	8.2	4.6	5.6
30	19.6	26	6.8	5	4.6	4.6
45	21.2	26	5	5	4.6	4.2
60	19.6	26	5.2	4.2	4.6	3.7

* ug inorganic phosphorus liberated / 75 µg microsomal protein / 30 min.

Table 4

Action of Phospholipase on Microsomal ATPase. Effect of Substrate and Metals

Contents of preincubation*	Pi liberated in presence of	
	Mg	Mg, Na, K
—	19.4	30.4
Phospholipase	9.2	8.2
ATP, phospholipase	19.4	27.4
Na, K, phospholipase		27.0
Mg, phospholipase	11.8	9.2

* Preincubation at 35°C was carried out for 30 minutes. Concentrations: phospholipase, 0.5 µg/ml; ATP, 3 µM/ml; Na, 100 µM/ml; K, 30 uM/ml; Mg, 3 uM/ml; microsomes, 75 µg protein/ml.

4. Isolation and characterization of kinin-releasing enzyme of Echis coloratus venom

Snake venoms contain enzymes capable of releasing pharmacologically active peptides, termed kinins, from plasma globulin. These peptides cause pain, edema, leukocyte migration, hypotension and contraction of various smooth muscles. The kinin-releasing enzymes (KRE) from various sources differ in physical characteristics, such as molecular weight and electrophoretic mobility, as well as in chemical properties, determining the type of kinin released, inhibitors and reaction with specific antibody. The KRE are both species specific and organ specific.(1). Rocha e Silva et al. (2), demonstrated bradykinin-releasing activity in the venom of Bothrops jararaca. Recently, Suzuki et al. (3) partially purified a KRE from the venom of Agkistrodon halys blomhoffii. Final elucidation of the postulated relationship between kinin release and some aspects of the clinical symptomatology of envenomation f.i. hypotension and shock, would require as the first step the procurement of a purified kinin-releasing enzyme, as a second step the characterization of the kinin released,

and finally a study of the toxicological effects of the purified kinin-releasing enzyme in vivo. In the present communication we report the purification and some biochemical properties of the KRE from Echis coloratus venom.

Kinin-releasing enzyme was isolated from Echis coloratus venom by exchange chromatography and gel filtration and its molecular weight and mode of action on amino acid esters and synthetic peptides determined. The kinin released by KRE from globulin was characterized and the properties of the enzyme, as regarding sensitivity to inhibitors, were compared to those of other kallikreins.

Methods

Kinin-releasing enzyme was purified by chromatography on DEAE-cellulose following the method of Sato et al. (4) applying gradient elution. Disc electrophoresis was done on acrylamide gel according to Davis (5). Sedimentation velocity measurements were performed in a Spinco model E centrifuge. Molecular weight was calculated from the sedimentation coefficient and the diffusion coefficient. For amino acid analysis of the peptide released from equine kininogen substrate, peptide hydrolysates were chromatographed in a Spinco amino acid analyzer and high

voltage electrophoresis carried out. Capillary permeability increasing activity was estimated according to Miles and Wilhelm (6), kinin-releasing enzyme activity was tested on guinea pig ileum according to Henriques et al. (7), esterase activity was assayed by modified Hestrin's colorimetric method (8), peptidase activity was detected by high voltage electrophoresis (9), kininase activity was estimated by incubating the chromatographic fraction with synthetic bradykinin. Kinin released from equine kininogen substrate was determined using the extraction procedure of Habermann (10).

Results and Discussion

Two KRE fractions were obtained by chromatography on DEAE cellulose followed by gel filtration on G-75 Sephadex. On disc electrophoresis KRE fraction I showed at least 3 bands while fraction II was more homogeneous. The molecular weight of the latter was estimated to be about 22,000. Their specific KRE activities are summarized in Table 5. The relatively low specific activity of fraction II may possibly be ascribed to instability of the highly purified material. Both

KRE fractions were capable of hydrolyzing arginine ester bonds (T [illegible] 6) while neither of the two hydrolyzed clupein, poly-arginine or polylysine (Table 7). Lysine peptide bonds in short synthetic peptides were split. The two KRE fractions possessing a considerable capillary permeability increasing activity were also similar in respect to the action of various inhibitors, heat stability and absence of hemorrhagic activity. The sensitivity of the ECV-KRE proteins to inhibitors was found to differ from that of various other kallikreins, KRE and arginine ester hydrolase of the purified ECV fractions were not inhibited by trasylol, soybean trypsin inhibitor or pancreatic trypsin inhibitor. In contradistinction, the KRE of Agkistrodon halys blomhoffi is sensitive to trasylol, and pancreatic, plasma and urinary kallikrein are inhibited by trasylol, pancreatic trypsin inhibitor and STI (11). The inhibition of the KRE and arginine ester hydrolase activity of the purified ECV fractions by DFP seems to indicate involvement of a serine residue in the active center of the enzyme.

The biologically active principle released by ECV from equine kininogen was identified as kinin. The purified kinin fraction was chemically characterized and found to be identical in its amino acid composition with the synthetic bradykinin (Table 8).

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Table 5

Data on KRE Fractionation

Enzyme preparation	KRE specific activity, μg bradykinin-eq.min^{-1}/mg	Total protein, mg
Echis coloratus venom	1.1	650
DEAE-cellulose fraction	6.6	47.5
Sephadex - G 75		
Fraction I	8.0	1.25
Fraction II	2.5	11.25

Table 6

Hydrolysis of Low Molecular Weight Synthetic Substrates by Purified KRE Fraction II, and Competitive Inhibition of Kinin Release

Substrate	Hydrolysis (u moles min^{-1}/mg)	Ester concentration in inhibition assay, molar	Inhibition of KRE, %
BAEE	4.2	2.10^{-3}	100
BAEE		2.10^{-4}	20
TAME	2.1	4.10^{-3}	100
TLME	0	4.10^{-3}	0
LME	0	4.10^{-3}	0
LEE	0	4.10^{-3}	0
BAA	0	4.10^{-3}	0

Table 7

Peptidase Activity of Echis coloratus Venom-KRE Fractions I and II

Substrate	Cleavage
Phe-Lys-Ala NH_2	+
Ala-Phe-Lys-Ala NH_2	+
Phe-Ala-Lys-Ala NH_2	+
Ala-Ala-Lys-Ala NH_2	+
Polylysine	-
Polyarginine	-
Clupein	-

Table 8

Amino Acid Analysis of the Purified Kinin Released by Echis coloratus Venom from Equine Werle's Substrate

Amino acid	umoles	Residues per mole glycine	
		Experimental	Theoretical
Lysine	0.003		
Histidine	0.003		
Arginine	0.506	1.95	2
Aspartic acid	0.003		
Threonine	0.003		
Serine	0.234	0.90	1
Glutamic acid	0.003		
Proline	0.770	2.97	3
Glycine	0.260	1	1
Alanine	0.003		
Half cystine	-		
Valine	0.003		
Methionine	0.003		
Isoleucine	0.003		
Leucine	0.003		
Tyrosine	0.003		
Phenylalanine	0.533	2.05	2

5. Effect of Purified Vipera palestinae Hemorrhagin on Blood Coagulation and Platelet Function

Snake bite hemorrhage is caused primarily by the venom hemorrhagins (1,2), whereas coagulation disturbances may aggravate the bleedings (3). The present study was undertaken to elucidate whether in addition to its vessel wall damaging principle Vipera palestinae hemorrhagin (VPH), possibly by virtue of its proteolytic activity, also affects blood coagulation or platelets.

Hemorrhage is a prominent clinical sign of Vipera palestinae bite (4). Purified VPH was found to posses a strong proteolytic activity (5). However, inhibition of the proteolytic activity of VPH by diisopropyl fluorophosphate (DFP) or soybean trypsin inhibitor did not impair its ability to cause hemorrhage (5).

The following effects of VPH were studied:

1) Effect of VPH on human plasma
2) Effect of VPH on bovine fibrinogen
3) Effect of VPH on platelet clot retracting activity, platelet adhesiveness, ADP and connective tissue-induced platelet aggregation, and release of ADP from platelets by connective tissue.
4) Effect of VPH in guinea pigs.

Methods

Treatment of VPH with DFP was performed according to Grotto et al. (5). Platelet rich plasma (PRP): Human venous blood was collected over 1/10 vol of 3.8% sodium citrate and centrifuged at 170g for 10 minutes at room temperature. Platelet counts were according to Brecher and Cronkite (6). A final concentration of 10^5 - 2 x 10^5 platelets/mm^3 was used. Clot retraction was tested according to Wintrobe (7). Thrombin clotting time: One volume of thrombin (100 NIH U/ml) was added to 9 volumes of PRP or fibrinogen solution (10 mg/ml) at 37°C. Recalcification time was measured according to Biggs and Macfarlane (8). Thromboplastin generation test was performed according to Biggs and Douglas (9). Plasma fibrinogen was assayed according to Ratnoff and Menzie (10). Enzymatic action of thrombin on fibrinogen was tested according to Latallo et al. (11). Released peptides were determined according to Lowry (12). FSF activity was measured according to Loewy et al. (13). FSF activation performed according to Lorand et al. (14). Released ADP from platelets was determined quantitatively according to Haslam (15) using the enzymatic method of Adam (16). Degradation of fibrinogen by VPH: 0.9 ml of bovine fibrinogen (10 mg/ml) was

incubated with 0.1 ml VPH (1mg/ml) at 37°C. 0.01 ml of STI (4mg/ml) was added to 0.09 ml aliquots of the mixtures removed at varying intervals of time. Degradation products were examined by immunoelectrophoresis (17) and by gel diffusion (18), using antifibrinogen sera and by acrylamide gel disc electrophoresis (19). In vivo studies: 0.5 ml of VPH, VPH treated with DFP (150 µg/250g guinea pigs) or buffer were injected intracardially. 1 hr later blood was obtained by heart puncture.

Results and Discussion

Effect of VPH on human plasma. Incubation of platelet rich plasma with VPH (150 µg/ml) caused a prolongation of recalcification and thrombin clotting times, a reduced opacity and tensile strength of the clot and impairment of thromboplastin generation and yield. DFP treatment of VPH, which had been previously shown to inhibit the proteolytic activity (5), abolished all the above effects. This indicated that the coagulation disturbances induced by VPH are probably due to its protease.

Effect of VPH on fibrinogen and its conversion to fibrin. Incubation of fibrinogen with VPH (100-240 µg/ml) for various periods of time caused a progressive prolongation of thrombin-clotting time; after 18 hours incubation the fibrinogen became unclottable. The activity of VPH on fibrinogen was accompanied by release of TCA-soluble peptides, which did nor prevent thrombin to release further fibrinopeptides. The bonds cleaved by VPH in the fibrinogen molecule were therefore different from those susceptible to thrombin. The fibrinogen degradation product obtained after incubation with VPH had electrophoretic mobility and antigenic specificity similar to those of the intact molecule as demonstrated by acrylamide disc electrophoresis, gel diffusion and immunoelectrophoresis. This proves a limited proteolysis.

VPH interfered with clot stabilization as manifested in urea-solubility of the clot. This was due to both the action of VPH on fibrinogen and its capability to inactivate fibrin stabilizing factor.

Effect of VPH on platelets. The interference of VPH with platelet functions was manifest in impaired clot retraction, diminished adhesiveness, delay of ADP and

connective tissue-induced aggregation as well as decrease of aggregate size and diminished release of ADP by connective tissue. These effects might be due to either an action on the platelet surface or to an action on plasma fibrinogen. Treatment of VPH with DFP only partially inhibited its effect on the platelets, therefore the VPH interference with platelet functions cannot be solely explained by its proteolytic activity. The action of VPH on the platelet surface seems to be selective since it did not interfere with platelet factor 3 availability nor with the release of platelet factor 4.

Effect of VPH in vivo. Intracardial injection of VPH (150 µg/250 g body weight) into guinea pigs caused widespread hemorrhage and hypofibrinogenemia without affecting clotting time, clot retraction and platelet count. The effective concentrations of VPH in vitro exceed by far that of VPH estimated to exist in the circulating blood following administration of one LD_{50} (150 µg).

DFP-treated VPH, while producing hemorrhage, had no significant hypofibrinogenic effect. These facts support the assumption that the hemorrhagic effect of VPH is primarily due to its action on the vessel wall.

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6. Intravascular coagulation induced by Echis carinatus venom (ECV).

The bite of the snake Echis carinatus may result in severe hemorrhage which is accompanied by disseminated intravascular coagulation (DIC) (1). The aim of the present study was to investigate the effect of ECV inoculation to rabbits and the conditions which might favor fibrin deposition in the glomerular capillaries of the kidneys. Rabbits were treated with either the fibrinolytic inhibitor epsilon aminocaproic acid (2) or by cortisone acetate which blocks the phagocytic ability of the reticuloendothelial system (3).

Certain snake venoms possess a coagulant activity, others anticoagulant activity or both (4). The bite of certain snakes or the administration of their venoms or isolated coagulants may cause disseminated intravascular coagulation. Such a phenomen may lead to fibrin deposition in the glomerular capillaries of the kidneys which characterizes the generalized Shwartzman reaction (5). Echis carinatus venom possesses a coagulant factor and an activator of plasminogen (1).

The effects of ECV were studied by its inoculation into rabbits, specifically: a) Hematological values of ECV inoculated rabbits; b) Fibrin deposition in the glomerular capillaries of the kidneys; c) Effect of ECV inoculation on cortisone treated rabbits.

Methods

Blood was drawn by heart puncture. Whole blood clotting time was determined according to Lee and White (6).

Plasma was prepared from blood taken either into 1/10 volume of 3.8% sodium citrate or into 1/100V of 5000 UI/ml heparin, and centrifuged according to Corrigan et al.(7).

Recalcification time was determined according to Biggs and Macfarlane (8).

Thrombin clotting time was determined according to Jerushalmy and Zucker (9). One-stage prothrombin time was determined according to Quick (10).

Fibrinogen was assayed according to Ratnoff and Menzie (11). Hemoglobin in the plasma was determined according to Dacie and Lewis (12). Platelet and leukocyte counts were performed according to Joshua (13).

Rabbits were sacrificed by pentobarbital (162 mg). Histological studies: The kidneys were fixed in formalin, embedded in paraffin and sections stained with hematoxylineosin or phosphotungstic acid hematoxylin and Gram stain for determination of fibrin. Hundred glomeruli of each kidney were examined.

Electronmicroscopical studies: Small pieces of renal cortex were fixed in OsO_4, dehydrated and fixed in Epon resin. Ultrathin sections were cut with LKB ultratome and examined in a RCA G-2 EM.

Results and Discussion

A. In preliminary studies it was observed that ECV has a quite potent coagulant activity towards citrated rabbit plasma. Its coagulant activity was also manifest in the presence of heparin but only in high concentrations of the venom and to a lesser degree.

Saline or 32 mg epsilon amino caproic acid (EACA) per kg body weight were injected into the marginal ear veins of young rabbits. Fifteen minutes later a solution of ECV (5 ug/ml) was administered by the same route. Doses of 20 µg of ECV were injected at 15 minute

intervals. One hour after the last injection blood was drawn by heart puncture. The hematological findings are summarized in Table 9. The blood of rabbits which received 25 µg ECV per kg body weight was usually incoagulable. The blood of some rabbits clotted after 10 minutes but lysed thereafter. A significant drop in the amount of clottable fibrinogen occured. Plasma hemoglobin, platelet and leukocyte count remained within the normal range. Administration of EACA prior to venom inoculation brought about a drop in platelet count.

Inoculation of 45 µg ECV/kg resulted in a marked hypofibrinogenemia which was usually accompanied by thrombocytopenia and a rise in the amount of plasma hemoglobin. These phenomena may be attributed to the fact that ECV causes disseminated intravascular coagulation (1). Under such conditions hemolysis of red blood cells occurs (14).

B. Rabbits were treated as above. Those animals which did not die after the drawing of the blood, were sacrified. Sections of kidneys and lungs were stained for the detection of fibrin. Samples of renal cortex were examined with the electronmicroscope.

No fibrin deposition was detected in the glomerular capillaries of rabbits which were treated with saline and 25 ug ECV per kg. However fibrin deposition occurred in rabbits treated with EACA prior to the administration of the venom. The incidence of positive glomeruli varied between 20 and 50 percent, in one rabbit fibrin deposition occured in 80% of the glomeruli.

Fibrin deposition occured in one out of seven rabbits, treated with saline and 45 ug ECV per kg. Administration of EACA prior to venom inoculation resulted in fibrin deposition in the glomerular capillaries of the kidneys in 7 out of 9 rabbits. The incidence of positive glomeruli was between 25 and 95 percent.

It appears that the disseminated intravascular coagulation induced by ECV does not usually result in fibrin deposition. It is suggested that the fibrin which was formed in the blood was thereafter lysed by the fibrinolytic system, which is commonly activated following disseminated intravascular coagulation (15). ECV itself is capable of activating plasminogen (1) and might have therefore potentiated the fibrinolysis.

The administration of EACA brought about the blocking of the fibrinolytic system thus enabling the fibrin to sediment in the kidneys.

C. Following three successive days of cortisone treatment, the rabbits were inoculated with either 35 or 45 ug ECV per kg in the manner described above.

Fibrin deposition in the glomerular capillaries of the kidneys was rare and did not exceed the rate of non-treated rabbits.

It seems therefore that deposition of fibrin in the kidneys is not affected by agents acting on the reticuloendothelial system. Under our conditions fibrin deposition occurred after blocking the fibrinolytic system.

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Table 9

Hematological Values of Rabbit Blood Following Inoculation of ECV

1st injection	ECV µg/kg body weight	Whole blood clotting time	Thrombin time, sec.	Recalcification time, sec.	One stage prothrombin time, sec.	WBC per cmm	Platelets per cmm	Fibrinogen mg%	Hemoglobin mg%
Saline*	25	0-10'	0	0	0	4000-5000	$1.2 \times 10^5 - 3.2 \times 10^5$	12-80	8-14
EAC**	25	0-10'	0	0	0	4000-9000	$6.5 \times 10^4 - 9.5 \times 10^4$	6-83	10-14
Saline*	45	0	0	0	0	3000-8000	$4.5 \times 10^4 - 2.2 \times 10^5$	14-23	32-62
EACA**	45	0	0	0	0	3000-7000	$3.8 \times 10^4 - 1.0 \times 10^5$	11-28	43-72
EACA**	Saline***	2'30"-4'45"	11-15	85-125	8-11	4000-8000	$1.2 \times 10^5 - 4.2 \times 10^5$	134-178	6-9
Saline*	Saline***	2'30"-4'30"	11-15	80-100	8-10	3000-8500	$1.6 \times 10^5 - 4.2 \times 10^5$	200-380	6-13

* [redacted] ml

** 32 mg per kg body weight, the whole amount was included in 3 ml

*** 18 ml of saline

7. Molecular basis of neutralization of snake venom hemorrhagin by specific antibodies

The lethal action of Vipera palestinae venom is largely due to its hemorrhagin i.e. a toxic component causing bleeding by action on the blood vessel wall. The hemorrhagin isolated from Vipera palestinae venom had toxic activity in mice and exhibited hydrolytic activity on gelatin. Antisera produced in rabbits against the hemorrhagin had both antitoxic and anti-gelatinase activities. The present study was undertaken with the aim to gain insight in the molecular basis of toxin neutralization and to increase the specific neutralizing activity of the antibody.

In recent years it has become possible to split the 7 S antibodies especially from rabbit (1.2) by means of papain or pepsin into 3.5 S and 5 S fragments, respectively. The 3.5 S fragments, in contrast to the 5 S and 7 S antibodies, are monovalent, do not precipitate with the homologous antigen although they are capable of binding to it, as shown for 3.5 S fragments of anti hapten antibodies (3). Studies on the neutralizing activity of enzyme digested antibody have been

carried out for other antitoxins such as antitetanus, antidiphteria and anticlostridium Welchii alpha toxin (4).

Methods

Vipera palestinae hemorrhagin was obtained by chromatography of whole venom on DEAE cellulose column followed by ammonium sulphate precipitation and chromatography on Sephadex G-200 (5). One LD_{50} of the isolated hemorrhagin by intravenous route in 12 g white mice was 4.5 - 5 µg protein.

The proteolytic activity of the hemorrhagin was estimated on a gelatin plate with subsequent amido-black staining (6). One unit of protease activity was 0.5-1 µg. Esterase activity was tested on p-toluene-sulfonyl-L-arginine methyl esters (TAME) according to the method of Roberts (7). Antihemorrhagin antibodies were obtained in rabbits. 2 mg/ml of hemorrhagic protein were premixed with Freund's adjuvant v/v and injected into the four foot pads and the back skin. Booster injections were given intramuscularly after 14 and 24 days. Ten days after the last booster injection the rabbits were bled by cardiac puncture and antisera collected. IgG was isolated from hyperimmune sera by precipitation with ammonium sulphate followed by DEAE cellulose chromatography (1).

Antihemorrhagin IgG was digested with papain according to the method of Porter et al. (1).

Antihemorrhagin IgG was digested with pepsin according to the method of Utsumi and Karush (8).

Antitoxic activity of the different antibody preparations was determined after incubating mixtures of hemorrhagin and antibodies in 0.9% NaCl pH 7.0 at 37°C for 1 h. The mixture was spun at 3000 rpm for 10 min and the supernatants injected intravenous into groups of 5 mice weighing 12 gr each.

Inhibition of the proteolytic activity of the hemorrhagin by the antibody preparations was measured following incubation of the hemorrhagin with different amounts of antibodies for 1 h at 37°C. The mixtures were spun at 3000 rpm for 10 min and the supernatants tested for proteolytic activity as described above.

Results and Discussion

The neutralizing capacity of 3.5 S monovalent antibody fragment obtained by papain digestion, and the bivalent 5 S antibody fragment obtained by pepsin digestion, were compared to that of the intact antibody and the mildly

reduced and alkylated antibodies. It was found (Table 10), that the antihemorrhagic activity of the monovalent 3.5 S antibody (F'ab fragment) decreased to 55% of that of the complete antibody. The bivalent 5 S fragment showed an increase in antihemorrhagic activity of about 40%. However when the antihemorrhagic activity of the 3.5 S and 5 S fragments were compared on a molar basis, it appeared that the neutralizing activity of the monovalent antibody was 37% compared to that of the complete IgG, and that the 5 S antibody had similar activity to the bivalent complete antibody. The mildly reduced antibody exhibited only a slight loss of antitoxic activity.

Since the isolated hemorrhagin exhibited hydrolytic activity on gelatin and on theester substrate TAME, all antibody preparations were tested for antiproteolytic activity on these substrates.

The antiproteolytic activity of the monovalent and bivalent antibody, when tested on gelatin, showed results similar to those of the antihemorrhagic activity (Table 10). Whereas, 1 mg of the complete IgG antibody neutralized 266 protease (gelatinase) units, the monovalent

antibody fragment (F'ab) neutralized 110 protease units per mg and the bivalent 5 S fragment $(Fab)_2$ exhibited an increased activity up to 440 units per mg. However, when calculated on a molar basis the monovalent antibody fragment activity was 73 units per mg and the bivalent fragment activity 293 units per mg., i.e. the neutralizing activity of the bivalent fragment was only slightly higher in comparison to the complete antibody. The antiproteolytic activity of the reduced and alkylated complete antibody was unchanged.

When tested on the low molecular weight synthetic substrate TAME, neither the complete antibody nor the various antibody fragments neutralized the proteolytic activity of the hemorrhagic preparation. This suggests that the antibodies obtained by immunization with hemorrhagin combine to the protease near to the active site, and that neutralization when tested on gelatin is caused by steric hindrance.

The results obtained point to the significance of the bivalence of the antibody in the neutralization of the toxic and proteolytic activities of the venom hemorrhagin.

Since the F'c fragment (non-active part of the antibody) does not take part in neutralization whereas the antigenicity of the antibody molecule lies within this part, the possibility is considered that a peptic digest of antivenom antibodies would enhance its potency per protein weight and thus decrease the occurrence of anaphylactic reactions in the treated patient.

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Table 10

Neutralization of Toxic and Gelatinase Activities of Venom Hemorrhagin by Specific Antibodies and Antibody Fragments

Bleeding No.	Immunoglobulin preparation	Neutralizing activity							
		No LD_{50}/mg		Per cent		No. gelatinase units/ mg		Per cent	
1	IgG	14,5		100		266		100	
	3.5 S fragment	8	5.3*	55	37*	110	73*	41	27*
	5 S fragment					440	293*	165	110*
2	IgG	17		100		428		100	
	Reduced IgG	15		96		400		93.4	
	5 S fragment	23	15*	135	90*	888	558*	200	133*
	3.5 S fragment					324	216*	75	50*

* Corrected for molar ratios: 7 S antibody M.W. 150,000, 5 S fragment 100,000, two 3.5 S fragments 100,000 (2 x 50,000).

General comment and conclusions

The studies on snake venom presented in this report were concerned with the following: isolation of venom phospholipases, their mode of action on membrane phospholipids - alone and jointly with direct lytic basic polypeptide, their action on a microsomal enzyme; isolation of kinin-releasing enzyme, its mode of action on kininogen and characterization of the kinin produced; effects of hemorrhagin on blood coagulation and platelet function; venom induced intravascular coagulation; molecular basis of neutralization of venom hemorrhagin by specific antibodies.

The main conclusions reached may be summarized as follows:

Area 1: Isolation and characterization of phospholipase A from Naja naja and Vipera palestinae venoms.

Naja naja venom contains several phospholipase A enzymes, whereas Vipera palestinae venom contains only one. The main iso-enzyme of Naja naja venom was found to consist of one polypeptide chain having no free SH

groups. The amino acid composition was determined. Vipera palestinae phospholipase A consists also of one single polypeptide chain devoid of free SH groups. Its estimated molecular weight is about 20,000.

Area 2: Effects of calcium ions and of Cobra direct lytic factor on phospholipase A

Direct lytic factor as well as calcium promote the phospholipid hydrolysis by venom phospholipase A in human erythrocytes and their osmotic ghosts. In contrast the hydrolysis of phospholipids in lipoprotein form as well as purified, by venom phospholipase A is enhanced by calcium ions but not by the direct lytic factor. EDTA inhibits phospholipase A activity also in systems devoid of calcium, suggesting a direct action on the catalytic function of the enzyme.

Area 3: Action of Ringhals phospholipase on microsomal ATPase

Ringhals venom phospholipase A inhibits rat brain microsomal ATPase activity, the Na^+, K^+ dependent ATPase activity being the more sensitive. ATP as well as a

sodium potassium ion mixture protect microsomal ATPase from loss of activity induced by phospholipase A. These observations indicate that phospholipids located at substrate and sodium, potassium binding sites in the microsomal membrane, are essential for functional membrane ATPase.

Area 4: Isolation and characterization of kinin-releasing enzyme (KRE) of Echis coloratus venom

Echis coloratus venom releases bradykinin upon incubation with a equine plasma kininogen. Two KRE preparations were obtained from the venom, one of them, having a molecular weight of 22,000, hydrolyses arginine and tyrosine esters, and synthetic lysine peptides, but not arginine amide or lysine esters. The properties of the enzyme are compared to those of other kallikreins.

Area 5: Effect of purified Vipera palestinae hemorrhagin in blood coagulation and platelet function

Vipera palestinae venom hemorrhagin interferes with blood coagulation and platelet function in vitro - specifically it impairs thrombin formation, degrades fibrinogen, inactivates fibrin stabilizing factor, impairs platelet clot retracting activity, adhesiveness and aggregation. These effects are related mainly to the proteolytic activity of the hemorrhagin. In vivo the purified hemorrhagin produces moderate hypofibrinogenemia, whereas DFP-treated hemorrhagin had no such effect.

Area 6: Intravascular coagulation induced by Echis carinatus venom

Intravenous administration of ECV into rabbits produces hypofibrinogenemia, thrombocytopenia and hemoglobinemia. Prior treatment with epsilon amino caproic acid leads to wide spread fibrin deposition in the glomerular capillaries of the kidney. Prior treatment with cortisone had no such effect, but appeared to increase hemorrhage.

Area 7: Molecular basis of neutralization of snake venom hemorrhagin by specific antibodies

Immunoglobulin obtained from rabbit antiserum prepared against purified Vipera palestinae hemorrhagin had both antitoxic and antigelatinase activities. 5 S bivalent antibody fragments obtained by pepsin-digestion of the 7 S immunoglobulin exhibited an increase in specific antitoxic and antigelatinase activity, whereas 3.5 S monovalent antibody fragments obtained by papain digestion showed a marked decrease in both. Neither the 7 S immunoglobulin nor the 5 S and 3.5 S antibody fragments were capable of inhibiting the esterase activity of the hemorrhagin on TAME.

A general statement may be made that due to the availability of isolated toxins, some progress has been achieved in the disentanglement and understanding of their modes of action mainly on in vitro systems as well as in vivo for one toxin, a hemorrhagin. Yet only a beginning appears to have been made in the clarification of the mechanisms involved in the action of the various toxins on cell membranes and biochemical systems. Enzymatic

digestion of specific antitoxin has opened the way for an understanding of the molecular basis of venom toxin neutralization and possibly for the enhancement of antivenin potency.

It is recommended that further studies in the above areas be carried cut, specifically as to the in vivo and vitro mode of action of the isolated toxins and their immunologic properties.

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13. ABSTRACT

In this study on snake venoms a threefold approach was made: isolation of venom toxins, elucidation of their mode of action, raising anti-venin potency. Attention was directed to phospholipase, hemolytic polypeptide, kinin-releasing enzyme, hemorrhagin, and antihemorrhagin antibody. Methods included exchange chromatography, gel filtration, histochemical, electron microscopical, phospholipid hydrolysis, microsomal ATPase, enzymatic antibody fragmentation. The conclusions are: Naja naja venom contains several phospholipase A iso-enzymes; Echis coloratus venom contains two kinin-releasing enzymes; fibrin deposition in the kidney by Echis carinatus venom is promoted by epsilon aminocaproic acid; Vipera palestinae hemorrhagin impairs blood coagulation and platelet function by its proteolytic activity; Ringhals phospholipase A inhibits microsomal ATPase; lytic peptide of cobra venom activates hydrolysis of membrane phospholipids but not of soluble phospholipids; bivalence of antibody appears significant in hemorrhagin neutralization. It is recommended that further studies in the above areas are carried out.

DD FORM 1 NOV 1473 REPLACES DD FORM 1473, 1 JAN 64, WHICH IS OBSOLETE FOR ARMY USE.

UNCLASSIFIED
Security Classification

14. KEY WORDS	LINK A		LINK B		LINK C	
	ROLE	WT	ROLE	WT	ROLE	WT
Snake venom						
Venom						
Snake						
Hemorrhagin						
Phospholipase						
Lytic factor						
Coagulation						
ATPase						
Kinin						
Vipera palestina						
Echis carinatus						
Naja naja						
Cobra						
Echis coloratus						
Antiserum						
Antibody fragmentation						