THE COAGULATION OF BLOOD BY SNAKE VENOMS AND ITS PHYSIOLOGIC SIGNIFICANCE

By HARRY EAGLE, M.D.

(From the Department of Bacteriology, University of Pennsylvania Medical School, Philadelphia, the Department of Medicine, Johns Hopkins Medical School, Baltimore, and the United States Public Health Service, Washington, D. C.)

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It has recently been demonstrated (5 *a*) that certain proteolytic enzymes can cause the coagulation of blood or plasma by either of two mechanisms. Trypsin acts on prothrombin to form thrombin, and is thus the counterpart of the physiologic calcium-platelet system. Papain, however, acts directly on fibrinogen to form a fibrillar gel resembling fibrin, and is thus the counterpart of thrombin. In view of these findings, it was suggested that a calcium-platelet mixture (or a calcium-tissue extract mixture) contains a proteolytic enzyme which, like trypsin, reacts with prothrombin to form thrombin. It was further suggested that thrombin itself is a proteolytic enzyme analogous to papain which hydrolyzes fibrinogen to form an insoluble split product, fibrin. The kinetics of thrombin formation and of the resultant coagulation were found to be in accord with the thesis that physiological coagulation involves these two consecutive enzyme reactions (3, 4). Moreover, an analogy for this hypothesis was seen in the activation of chymotrypsinogen by trypsin to form a new enzyme, chymotrypsin (11).

Snake venoms are known to affect the phenomenon of blood coagulation profoundly. Some inhibit the clotting process when added to blood *in vivo* or *in vitro*, a few have no significant effect, and a large number have a marked coagulative action on whole blood, plasma, or fibrinogen. Most of those who have concerned themselves with the mechanism of this coagulation (1, 6, 9, 10, 13, 14, 21) have found the venoms to act directly on fibrinogen. Only in an early paper by Mellanby (15) does one find the suggestion that some other process may be involved. On adding the venom of either *Echis carinata* (Indian

viper) or *Notechis scutatus* (Australian tiger snake) to a solution containing prothrombin and fibrinogen, Mellanby noted that the coagulating activity of the mixture steadily increased, even after coagulation had occurred. He concluded that the venom constituted a kinase which functioned like a platelet suspension; and since platelets are effective only in the presence of calcium, he was constrained to assume that the prothrombin-fibrinogen mixture contained calcium as part of a non-ionizing complex with the protein, and that this calcium acted in conjunction with the venom kinase to convert prothrombin to thrombin. Later workers have been almost unanimous in considering these coagulant venoms to act directly on fibrinogen.

In view of the coagulative action of trypsin and papain already cited, and since most snake venoms are known to contain proteolytic enzymes (6, 14), it becomes of obvious interest and significance to ascertain whether the coagulative action of snake venoms depends on their enzyme content, and whether these venoms are of two types: one type which, like trypsin and like the calcium-platelet system, acts on prothrombin to form thrombin; and one which, like papain and thrombin, acts directly on fibrinogen to form fibrin. The experiments here reported indicate that such is, indeed, the case.

Methods and Materials[1]

Fibrinogen.—Citrated plasma was centrifuged at high speed and passed through a Berkefeld filter in order to remove platelets. The filtrate was precipitated with 1.5 volumes of saturated NaCl at room temperature. The precipitate was collected by centrifugation, the fluid discarded, and the glutinous protein precipitate allowed to express free fluid for half an hour at room temperature. It was then redissolved in a minimum volume of water, and reprecipitated by the addition of saturated NaCl (1.5 times the volume of water used for resolution). A trace of citrate was added to the water in order to prevent coagulation. Three such precipitations usually sufficed to yield a fibrinogen which did not coagulate on the addition of calcium and lung extract, but was promptly coagulated by thrombin. The final solution was rendered isotonic with blood by appropriate dilution before use.

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[1] The venoms used in these experiments were obtained through the courtesy of Dr. John Reichel and Dr. Thomas S. Githens of the Mulford Biological Laboratories, Glenolden, Pennsylvania. The fresh citrated horse plasma used in these experiments was furnished by the same laboratories.

Crude Prothrombin.—This was prepared by precipitation with acetic acid as described by Mellanby (16). One volume of plasma was diluted with 15 volumes of cold water and precipitated by the addition of approximately $\frac{1}{20}$ the original plasma volume N/1 acetic acid. The precipitate was collected by centrifugation, and contained fibrinogen, prothrombin, euglobulin, and some substance with a platelet-like action. It was then redissolved in 0.85 per cent NaCl and freed of fibrinogen by heating at 56°C. for 3 minutes (3). Because the resultant solution contained either traces of platelet derivative or a cephalin-like plasma factor, it was activated to thrombin by calcium alone.

Pure Prothrombin.—In order to prepare a "pure" prothrombin solution, which would not be activated to thrombin by either calcium alone or tissue extracts alone, plasma was passed through a Berkefeld filter prior to its dilution and precipitation with acetic acid, and the resultant precipitate was then extracted with $Ca(HCO_3)_2$ by the Mellanby (16) technic. The final solution, rendered isotonic by the addition of $\frac{1}{20}$ volume of 17 per cent sodium chloride, was a highly active prothrombin preparation which contained no fibrinogen, and which rapidly evolved large amounts of thrombin on the addition of $\frac{1}{20}$ volume of 1 per cent $CaCl_2$ and cephalin (or lung extract). The addition of calcium alone had either no demonstrable effect, or caused a very slow elaboration of minute quantities of thrombin, less than 2 per cent of the amount elaborated from the same prothrombin in the presence of an adequate amount of cephalin or tissue derivative.

Lung Extract.—Fresh rabbit lung tissue was washed as free as possible of blood and expressed in a large volume of 0.85 per cent NaCl. The minced tissue remained reactive for months if it was kept frozen in a sealed container over solid CO_2.

Quantitative Evaluation of Thrombin Activity.—This was accomplished by graphic interpolation on a curve correlating thrombin concentration and coagulation time (3).

Determination of Proteolytic Activity.—The proteolytic activity of the several venoms was determined with gelatin, using a modification of the Gates (7) technic. In this simple and accurate method a piece of exposed and fully developed photographic film is placed in contact with the solution to be tested. The enzyme digests away the gelatin from the surface of the film, and thereby removes some of the silver and increases the translucence of the film. The rate or degree of clearing thus effected is an accurate criterion of the gelatinase activity of the solution (2, 8).

The technic used in these experiments[2] differs only in several minor details from that used by Gates. The film was exposed as recommended by Gilman and Cowgill (8), developed with a hardener, dried, and again washed and dried in order to set the gelatin. The digestion cell was constructed with a flat brass ring instead

[2] Dr. William Mendelsohn rendered valuable assistance in the determination of proteolytic activity.

TABLE I

The Coagulant Action of Seventeen Snake Venoms

Coagulate citrated horse plasma		Do not coagulate citrated horse plasma	
Snake species	Common name	Snake species	Common name
Bothrops atrox	Fer de lance	*Agkistrodon piscivorus*	Cotton-mouth moccasin
Bothrops jararaca	Jararaca	*Bitis arietans*	Puff adder
Bothrops nummifera	Mano de Piedra	*Crotalus atrox**	Texas diamond back
Crotalus adamanteus†	Florida diamond back	*Crotalus horridus**‡	Timber rattler (1528) (1447)
Crotalus horridus‡	Timber rattler (A29091A)	*Naia flava*	Cape cobra
Crotalus terrificus basiliscus	Cascabel, Mexican	*Naia naia*	Spectacled cobra
Crotalus terrificus terrificus	Cascabel, Brazilian	*Sepedon haemachates*	Spitting cobra
Micrurus, mixed venoms	Coral snake	*Vipera ammodytes*	Sand viper
Notechis scutatus	Australian tiger snake	*Vipera russellii*	Daboia

* 2 specimens.

† 3 specimens.

‡ It is to be particularly noted that in the case of *Crotalus horridus*, individual lots of venom presumably obtained from different members of the same species, nevertheless differed in their coagulating activity. This variation in different lots of venom may explain the fact that although Link (13) found the venom of *Crotalus adamanteus* actively coagulant, a finding which was confirmed in the present experiments, Houssay and Sordelli (9) report it inactive *in vitro*. Similarly, the venom of Russell's viper was here found inactive; Arthus (1) also reported it inactive; Link (13) obtained variable results; and Houssay and Sordelli (9), as well as Taylor, Mallick, and Ahuja (21) found it to be actively coagulant. *Crotalus terrificus* venom was here found to be regularly active in high dilution, confirming Houssay and Sordelli (9) and Arthus (1); but Link (13) found it to be wholly inactive. Whether these variations are artefacts, caused by the varying methods of collecting and storing the venom or by the varying age of the preparations, or whether they reflect intrinsic differences in venoms obtained from different individuals of the same species, is an open question which need not concern us here.

of copper wire. The ring, 2 mm. thick, 25 mm. outside and 20 mm. inside diameter, was sealed with paraffin to a glass slide measuring 3.5 x 3.5 cm. The compartment so formed was filled with the solution to be tested, and a section of film 30 mm. square was placed on the solution, gelatin side down. Over the film was placed another glass slide, and the entire cell was then clamped together with two ordinary wooden spring-clamps, one on each side. The cell was then placed in a water bath at 28°C., the film exactly upright.

Instead of measuring the translucence of the washed film after a fixed interval with a colorimeter (8), complete clarification was used as a rough end-point of digestion (2). This end-point was made somewhat more accurate by observing the sediment of silver which gradually settled to the bottom of the cell as it was freed from the dissolved gelatin. The time at which this sediment formed a sharply demarcated layer which filled the lower $\frac{1}{4}$ of the cell, and left clear transparent film above, was taken as the end-point. The proteolytic activity of the several preparations was estimated by determining the maximum dilution of enzyme which produced this degree of clarification in an arbitrary time interval.

The Coagulation of Fibrinogen by Snake Venoms

The list of venoms tested, and their coagulative activity on plasma or on solutions containing both prothrombin and fibrinogen, are given in Table I. As is there shown, 8 of the 17 venoms tested proved active, and one (*Crotalus horridus*) yielded variable results. The discrepancies between the results there listed and the data in the literature are discussed in the footnote to Table I, and are not germane to the present paper.

In confirmation of previous reports, 7 of the 9 venoms which coagulated plasma were found capable of coagulating purified fibrinogen (Table II). These 7 venoms (*Bothrops atrox*, *Bothrops jararaca*, *Bothrops nummifera*, *Crotalus adamanteus*, *Crotalus terrificus terrificus*, *Crotalus terrificus basiliscus* and one of three specimens of *Crotalus horridus*) therefore contained a substance which, like thrombin or papain, reacted with fibrinogen to form a fibrillar gel indistinguishable from fibrin.

It is to be noted that this coagulative action is independent of the presence of calcium ions, as it occurs just as promptly in fibrinogen solutions containing 1 per cent sodium citrate. The coagulation is also independent of the presence of tissue or platelet derivatives, as it occurs in fibrinogen solutions which contain these factors only in minimal concentration. Moreover, the velocity of coagulation is

wholly unaffected by the addition of cephalin or tissue extracts to the fibrinogen. Finally, the coagulative action is independent of the presence of prothrombin, for fibrinogen which is prothrombin-free, and which is unaffected by Ca + cephalin, is nevertheless coagulated by the venoms. Clearly, the fibrinogen as such is attacked by the venoms directly.

An experiment to determine the optimum pH for this reaction is summarized in Fig. 1. As is there shown, each of the 3 venoms tested

TABLE II

*The Coagulation of Purified Fibrinogen by Snake Venoms**

To 0.4 cc. of a solution of purified horse fibrinogen were added 0.4 cc. of varying concentrations of snake venom. The figures in the body of the table indicate the coagulation time in minutes. Each horizontal row is an individual experiment.

Venom used	Concentration of venom added to an equal volume of purified fibrinogen							
	1:1000	1:2000	1:4000	1:8000	1:16,000	1:32,000	1:64,000	1:128,000
	min.	*min.*	*min.*	*min.*	*min.*	*min.*	*min.*	*min.*
Bothrops atrox	1½	1¾	2¼	2¾	4	5	8	12
Bothrops jararaca	1½	1½	2¼	2½	4	6½	3	—
Bothrops nummifera	¾	¾	1¼	1¾	3¾	7	14	—
Crotalus adamanteus (1483)	—	—	—	2	4½	10	15	—
(1336)	½	¾	1	2	3½	5¾	11	18
(1458)	½	¾	¾	1½	2	4	6¾	16
Crotalus horridus (A2091A)	½	¾	1¼	1¾	2¾	4¼	7½	19
Crotalus terrificus terrificus	¾	¾	1¾	2½	3¾	6	8½	
Crotalus terrificus basiliscus	2¼	2	3	5	10	21	37	—

* The following venoms caused no coagulation in 60 minutes: *Agkistrodon piscivorus, Crotalus atrox, Crotalus horridus* (lots 1528, 1447), *Micrurus* mixed, *Naia flava, Naia naia, Notechis scutatus, Sepedon haemachates, Vipera russellii, Bitis arietans, Vipera ammodytes.*

had a definite optimum at approximately pH 6.5; and it is to be noted that this optimum coincides with that for the action of thrombin on fibrinogen (Fig. 5, bottom curve). Unexpectedly, the coagulant action of snake venom on fibrinogen is unaffected by antithrombin. The intensely antithrombic plasma produced by anaphylactic shock in dogs (5 *b*) had no demonstrable effect on the coagulation of fibrinogen by *Crotalus adamanteus* or *Crotalus terrificus terrificus* (Table VI, page 629).

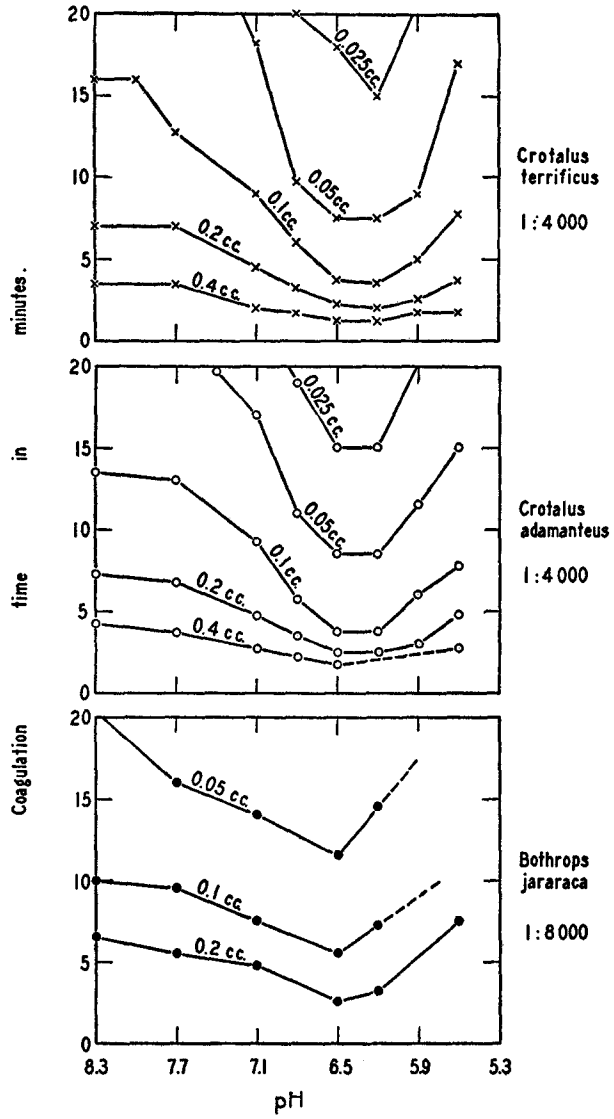

FIG. 1. The effect of pH on the fibrinogen-coagulating activity of several snake venoms.

Varying amounts of venom solution were made up to 0.4 cc. with 0.85 per cent NaCl solution, and 0.4 cc. of 0.1 M phosphate buffer was added to each tube. Finally, 0.4 cc. of unbuffered fibrinogen solution was added, and the coagulation time noted. The final experimental mixture therefore contained 0.033 M phosphate buffer and 0.105 M NaCl. In plotting the results, the pK′ of this buffer mixture has been taken as 6.8. A stated pH of 7.1 represents a 2:1 Na_2HPO_4 ratio, a pH of 7.4 is a 4:1 mixture, etc. These pH values are in error to the extent that the pK′ of these buffer solutions of varying ionic strength differs from 6.8, and the pH values are accordingly accurate only to ± 0.1 pH units.

TABLE III

The Destruction of Fibrinogen by Snake Venoms Which Do Not Cause Its Coagulation

Varying amounts of an 0.1 per cent solution of each venom were brought up to 0.4 cc., and 0.4 cc. of fibrinogen was added to each tube. After 1 hour at room temperature, 0.2 cc. of a 1:4 dilution of horse thrombin was added, and the coagulation time noted.

Venom used	Amount of 0.1 per cent venom solution added to 0.4 cc. fibrinogen (Total volume 0.8 cc.)							Conclusion
	0.4 cc.	0.2 cc.	0.1 cc.	0.05 cc.	0.025 cc.	0.0125 cc.	0	
	Coagulation time (in min.) on addition of thrombin 30 min. later							
Agkistrodron piscivorus	No coagulation				9	4	1¼	Fibrinogen destroyed by venom
Crotalus atrox (1476)	No coagulation				7 (shreds)	3	1¼	
(67248)	No coagulation			5 (shreds)	2½	1½	1¼	
Crotalus horridus (1528)	No coagulation			3 (shreds)	1¾	1½	1¼	
(1447)	No coagulation				5 (shreds)	2½	1¼	
Bitis arietans	No coagulation		9	—	4	3	1¼	
Vipera ammodytes	No coagulation				20	3	1½	
Naia flava	1½	1¼	1¼	1¼	1¼	1¼	1¼	Fibrinogen not destroyed by venom
Naia naia	3	2	1½	1½	1½	1½	1¼	
Micrurus mixed	4	—	1¾	—	1¾	1½	1¼	
Sepedon haemachates	2½	—	2¼	—	2¼	2	1¼	
Vipera russellii	3½	—	1¾	—	1½	1½	1¼	
Notechis scutatus	1½	1¼	1¼	1¼	1¼	1¼	1¼	

Of the 11 venoms which did not coagulate fibrinogen, 5 (*Agkistrodon piscivorus; Bitis arietans; Crotalus atrox; Vipera ammodytes;* 2 of the

TABLE IV

The Correlation between the Proteolytic Activity of the Several Venoms as Measured with Gelatin, and Their Ability Either to Coagulate or Destroy Fibrinogen

Venom used	Proteolytic activity* of the dry venom as compared with crystalline trypsin 1:10,000		Coagulate fibrinogen	Destroy fibrinogen
	Experiment 1	Experiment 2		
Agkistrodon piscivorus		96		+ (16)†
Bothrops nummifera	50	64	+	
Bothrops atrox	36	70	+	
Crotalus terrificus basiliscus	16	40	+	
Bothrops jararaca	12	25	+	
Crotalus atrox	8	12		+ (8)
Crotalus horridus (2091A)	5	8	+	
Crotalus horridus (1528)	4	4		+ (4–8)
Crotalus adamanteus	4	5	+	
Vipera ammodytes	3	4		+ (4)
Vipera russellii	2	2	‡	(3)
Bitis arietans	1½	1½		+ (2)
Crotalus terrificus terrificus	½	1¼	+	
Sepedon haemachates	¾	1		
Naia flava	½	½		
Micrurus mixed	½	⅛		
Naia naia	¼	1		
Notechis scutatus	¼	⅛		

* *Cf.* page 615 for the technic used in approximating the proteolytic activity of the various venoms.

† The numbers in this column indicate, very roughly, the relative fibrinogen-destroying activity of the several venoms as given in Table III. The differences in the coagulating activity (Table II) are too slight to justify quantitative analysis.

‡ In this list of 18 venoms, this constitutes the only exception to the observation that the capacity of the venom to coagulate or to destroy fibrinogen goes hand in hand with its proteolytic activity as measured with gelatin. It is to be noted that other observers have found this particular venom to be actively coagulant.

3 specimens of *Crotalus horridus*) were found to attack the protein, and render it non-coagulable by thrombin (Table III). The slight re-

tardation of coagulation caused by several of the remaining 6 venoms (Table III, lower half) is of questionable significance.

The question now arises as to whether the coagulative action of these snake venoms on fibrinogen is due to their proteolytic enzyme content. These venoms are heterogeneous mixtures of many different substances, and the demonstration of such enzymes does not prove them to be the factor responsible. Moreover, the actual demonstration of proteolysis in a fibrinogen-venom mixture is *a priori* difficult in view of: (*a*) the minute molecular concentration of protein in solution; (*b*) the possibility that only a few groups in the protein need be modified in order to produce a profound change in its biologic properties; and (*c*) the fact that one ends with a gel-like fibrinous mass which precludes precise formol titrations.

There is, nevertheless, a certain amount of collateral evidence that the coagulation of fibrinogen just described is due to proteolytic enzymes present in the venom. In the first place, the extraordinarily minute amounts of venom which may suffice is strongly suggestive. A 1:100,000 dilution of some of the crude venoms, in which the actual coagulant is only a small fraction of the total solid, regularly coagulated solutions containing 1:200 parts of fibrinogen, a minimum ratio of 500:1 between the two reagents.

Further evidence for the enzyme thesis is given by the data of Table IV. As is there shown, all 17 venoms used in these experiments contained proteolytic enzymes, capable of hydrolyzing gelatin. Their activity in this respect varied widely. The significant feature of Table IV is that only those venoms which contained comparatively large amounts of enzyme were able to coagulate or to destroy fibrinogen. If the particular samples of venom here used were arranged in the order of their gelatin-splitting activity, it was found that those venoms below a certain level of proteolytic activity had little or no effect on fibrinogen,[3] while those venoms above this level either actively coagulated the protein, or rendered it non-coagulable even by thrombin. This almost exact correlation between the hydrolytic activity of the venoms

[3] Within the time limits of the present experiments. It is not improbable that with a longer period of incubation, or at higher temperatures, even these weakly proteolytic venoms would have destroyed fibrinogen; but this possibility does not affect the validity of the argument.

as tested with gelatin on the one hand, and their ability either to destroy or to coagulate fibrinogen on the other, justifies the working hypothesis that the coagulation, like the destruction, is caused by one of the proteolytic enzymes present in the venom. One can only speculate as to why, given venoms of equal proteolytic activity, some hydrolyze fibrinogen to soluble products, while others hydrolyze it to an insoluble fibrillar gel indistinguishable from fibrin.

The Activation of Prothrombin to Thrombin by Snake Venoms

The venoms were now tested with respect to their ability to activate prothrombin to thrombin. Varying amounts of each venom were added to a fixed volume of purified prothrombin solution, and the coagulating activity of the mixture was tested after half an hour at room temperature (Table V). Of the 17 venoms tested, 3 were remarkably active (*Notechis scutatus*, *Bothrops atrox*, *Bothrops jararaca*), and regularly caused a definite transformation of prothrombin to thrombin when used in dilutions as high as 1:1,000,000 (the first 2 named were definitely active in 1:10,000,000 dilutions). Two other venoms (*Micrurus* mixed and *Crotalus terrificus basiliscus*) were weakly active.

Of the 5 venoms found capable of transforming prothrombin to thrombin, 2 (*Notechis scutatus* and a mixed *Micrurus* venom) had no effect on fibrinogen (Table II), and their coagulant action on whole plasma previously noted is apparently to be ascribed solely to the fact that they transform prothrombin to thrombin. The other 3 venoms which activated prothrombin acted also on fibrinogen. The activation of prothrombin in the case of these 3 venoms was demonstrated by the fact that a mixture of venom and prothrombin was 5, 50, or even 1000 times as active as the venom alone. For purposes of contrast, several venoms are included in Table V which actively coagulated fibrinogen but which did not activate prothrombin to thrombin.

That the coagulant produced in the venom-prothrombin mixtures was truly thrombin, and not the result of some wholly unrelated reaction, was indicated by the following observations.

1. The amount of coagulant formed with an optimum concentration of venom was of the same order of magnitude as that elab-

TABLE V

The Activation of Prothrombin to Thrombin by Various Venoms

To 1.2 cc. of purified prothrombin were added varying amounts of venom and the mixture brought to 1.6 cc. with salt solution. After 1 hour at room temperature, varying amounts of each mixture were added to 0.4 cc. of fibrinogen, and the coagulation time noted.

Venom used	0.1 per cent venom	Prothrombin	Final dilution of venom (total volume 1.6 cc.)	Coagulating activity of venom-prothrombin mixture. Amount of mixture added to 0.4 cc. fibrinogen in total volume of 0.8 cc.					Coagulating activity of venom alone, similarly diluted (Table II)				Approximate coagulative action of venom-prothrombin mixture as compared with venom alone as 1*
				0.4 cc.	0.2 cc.	0.1 cc.	0.05 cc.	0.025 cc.	0.4 cc.	0.2 cc.	0.1 cc.	0.05 cc.	
				Coagulation time					Coagulation time				
	cc.	*cc.*		*min.*	*min.*	*min.*	*min.*	*min.*	*min.*	*min.*	*min.*	*min.*	
Bothrops atrox	0.25	1.2	1:6400	½	½	¾	1½	3	2½	3½	5½	9	7
	0.062	1.2	1:25,000	½	½	¾	1½	3	5½	9	13	19	30
	0.016	1.2	1:100,000	½	½	1	2	3½	13	19	26	37	120
	0.0004	1.2	1:400,000	½	1	1½	2½	4	26	39			400
	0.0001	1.2	1:1,600,000			3	5	8	80				500
	0.000025	1.2	1:6,400,000	2	3½	6	12	—					900
	0.000006	1.2	1:25,000,000	6	9	17	—	—					1000
	0.0000015	1.2	1:100,000,000	18	—	—	—	—					1000
Bothrops jararaca	0.4	1.2	1:4000	1¾	2¼	3	5	—	2¼				2
	0.05	1.2	1:32,000	1¾	2½	4	7	—	6½				8
	0.025	1.2	1:64,000	2	3	4½	8	14	13				16
	0.015	1.2	1:128,000	2	3¼	5½	8	14	25				30
	0.0062	1.2	1:256,000	2¼	3½	6	10	—	70				50
	0.0031	1.2	1:500,000	4	7	12	20	—	120				30
Notechis scutatus	0.4	1.2	1:4000	½	¾	1¼	1¾	3¼					
	0.1	1.2	1:16,000	½	¾	1¼	1¾	3½	Venom did not act on purified fibrinogen				Venom-prothrombin mixture uniformly active; venom alone inactive
	0.025	1.2	1:64,000	—	—	—	2	4					
	0.0062	1.2	1:256,000	—	—	—	3½	7					
	0.0016	1.2	1:1,000,000				6½	9¼					
	0.0004	1.2	1:4,000,000	—	—	—	15						

Mixed *Micrurus*	0.4	1.2	1:4000	3	5	9	12						
	0.1	1.2	1:16,000	7	12	20	—			"		"	" "
	0.025	1.2	1:64,000	20	32	60	—						
Crotalus terrificus basiliscus	0.4	1.2	1:4000	1¼	2	3½	7		2¼				2
	0.1	1.2	1:16,000	2¾	5	9	16		7¾				5
	0.025	1.2	1:64,000	9½	18	35	60		36				4
Crotalus adamanteus	0.4	1.2	1:4000	1½					¾				Venom-prothrombin mixture uniformly less active than the venom alone. No formation of thrombin
	0.2	1.2	1:8000	2					1¼				
	0.1	1.2	1:16,000	3¼					2				
	0.05	1.2	1:32,000	4½					4				
	0.025	1.2	1:64,000	9½					7¾				
Crotalus horridus	0.4	1.2	1:4000	1¼					1¼				
	0.2	1.2	1:8000	1¾					1¾				
	0.1	1.2	1:16,000	3					2¾				" "
	0.05	1.2	1:32,000	7					4¼				
	0.025	1.2	1:64,000	10					7½				
Crotalus terrificus terrificus	0.4	1.2	1:4000	2					1¾				
	0.2	1.2	1:8000	2½					2½				
	0.1	1.2	1:16,000	5					3¾				" "
	0.05	1.2	1:32,000	8					6				
	0.025	1.2	1:64,000	16½					8½				

* The coagulation time was used as the index of thrombin concentration (page 615). Thus, in the first horizontal row 0.2 cc. of venom was as active as 0.028 cc. of the venom-prothrombin mixture, a ratio of 0.2:0.028 = 7:1.

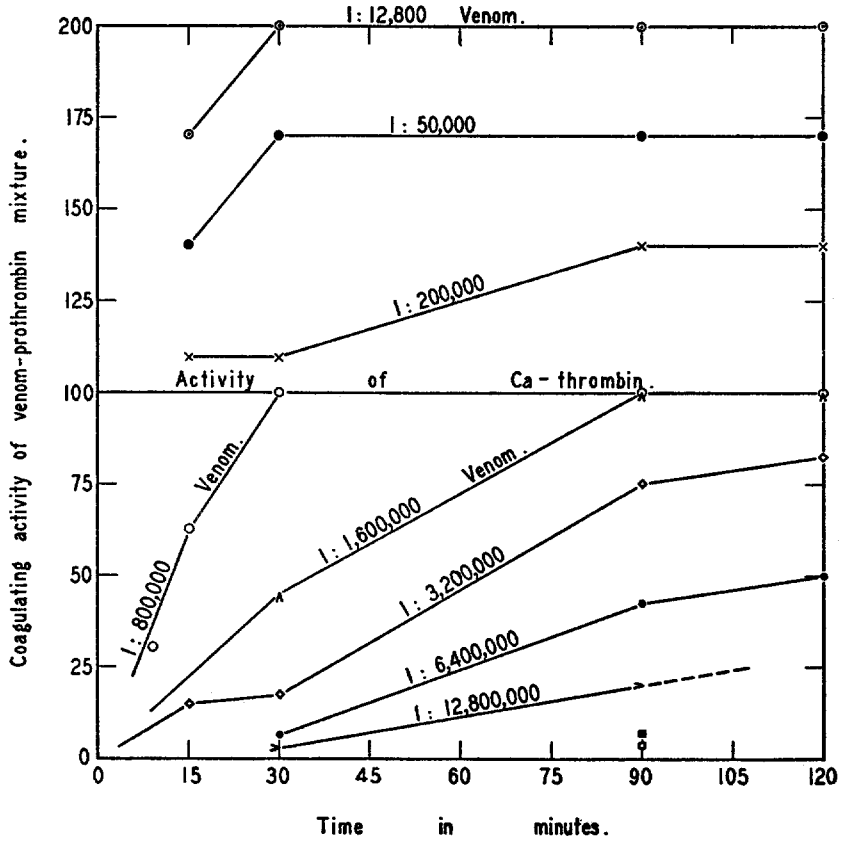

FIG. 2. The activation of prothrombin to thrombin by *Bothrops atrox* venom. Varying amounts of *Bothrops atrox* venom were brought up to 0.4 cc. with M/7 NaCl, and 2.8 cc. of prothrombin solution were added. At intervals thereafter, aliquot samples were withdrawn and tested for thrombin content by adding varying amounts to 0.4 cc. of fibrinogen solution in a total volume of 0.8 cc. The coagulation time served as an index of thrombin content. The dilutions indicated on the curves are the final concentration of venom in the venom-prothrombin mixture.

The apparent production of thrombin in excess of the amount formed from the same prothrombin by calcium and tissue extract is due to the fact that the venom in concentrations exceeding 1:500,000 has a definite coagulant action on fibrinogen directly, independent of its action on prothrombin, and additive thereto.

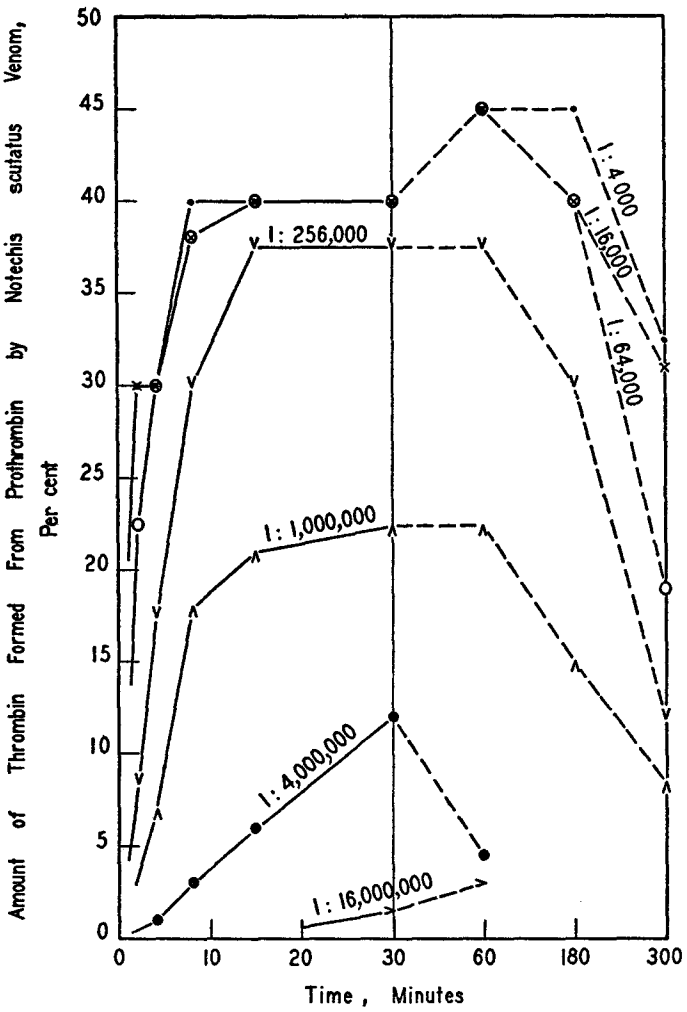

FIG. 3. The activation of prothrombin to thrombin by varying concentrations of *Notechis scutatus* venom.

Varying amounts of *Notechis scutatus* venom were brought up to 0.4 cc. with M/7 NaCl, and 1.2 cc. of horse prothrombin solution (Mellanby purified) were added. At intervals thereafter, aliquot samples were withdrawn and tested for thrombin content by adding varying amounts to 0.4 cc. of fibrinogen solution in a total volume of 0.8 cc. The coagulation time served as an index of thrombin content. The dilutions indicated on the curves are the final concentration of venom in the venom-prothrombin mixture.

In plotting the thrombin activity, the maximum amount formed from the same prothrombin by calcium and cephalin was taken as 100.

TABLE VI

The Neutralizing Effect of Antithrombin on the Thrombin Produced by the Action of Bothrops jararaca or Bothrops atrox Venom on Prothrombin; and the Absence of Such Neutralization on Adding Antithrombin to Venoms Which, Like Thrombin, Act Directly on Fibrinogen (Protocol 1)

	Fibrinogen	Normal plasma	Post-anaphylaxis dog plasma (antithrombin)	Type of thrombin added	Coagulation time, on adding varying amounts of thrombin to the fibrinogen-plasma mixtures								Conclusion
					0.4 cc.	0.2 cc.	0.1 cc.	0.05 cc.	0.025 cc.	0.0125 cc.	0.0062 cc.	0.0031 cc.	
	cc.	*cc.*	*cc.*		*min.*	*min.*	*min.*	*min.*	*min.*	*min.*	*min.*	*min.*	
A	0.4	—	—	*Bothrops atrox* + horse prothrombin	½	½	½	¾	1¼	2	3½	6	Antithrombin markedly inhibits the thrombin formed from prothrombin by snake venoms, to the same degree that it inhibits thrombin formed from prothrombin by calcium + tissue extract
	0.2	0.2	—		½	½	½	1	2	4	18	60	
	0.2	—	0.2		3¼	7	19	60	No coagulation				
	0.4	—	—	*Bothrops jararaca* + dog prothrombin	¾	1¼	2	3½	6	12	20	35	
	0.2	0.2	—		¾	1¾	4	19	60				
	0.2	—	0.2		4½	29	No coagulation after 4 hrs.						
	0.4	—	—	Calcium + tissue extract + horse prothrombin	½	½	½	¾	1	2	3¾	7	
	0.2	0.2	—		½	½	½	1¼	2½	4½	25	No coag.	
	0.2	—	0.2		4	9	25	90	No coagulation				
B	0.4	—	—	*Bothrops atrox* venom 1:4000	½	½	½	1	1¾	2¾	4	6½	Antithrombin has no effect on snake venoms which act directly on fibrinogen to form fibrin
	0.2	0.2	—		½	½	½	¾	1½	2¼	4	7	
	0.2	—	0.2		½	½	¾	1¼	2¼	6½	7	10	
	0.4	—	—	*Bothrops jararaca* venom 1:4000	½	1	2	4	7	15	25	60	
	0.2	0.2	—		½	1¼	2	3½	6¾	15	29	60	
	0.2	—	0.2		¾	1½	2¼	4½	9	17	35	80	
	0.4	—	—	*Crotalus adamanteus* venom 1:4000	—	1	2	3	7	14	—	—	
	0.2	0.2	—		—	1	2	3½	8	12	—	—	
	0.2	—	0.2		½	1¼	2½	4½	7	15	30	—	
	0.4	—	—	*Crotalus terrificus terrificus* venom 1:4000	½	¾	½	2½	5	9	—	—	
	0.2	0.2	—		½	¾	1¾	2½	6	12	19	—	
	0.2	—	0.2		½	¾	1½	2¾	5½	9	15	—	

orated from the same prothrombin by the addition of calcium and platelets (or tissue extract) (Figs. 2 and 3).

2. The addition of calcium and tissue extract to the optimum mixture of prothrombin and *Bothrops atrox* venom caused no further increase in its coagulating activity.[4] Conversely, if venom was added to the thrombin produced by the action of calcium and platelets on prothrombin, there was no increase in coagulating activity unless the venom was added in sufficient concentration to supplement the thrombin by virtue of a direct action on fibrinogen (Fig. 2).

3. The antithrombin which is elaborated in dogs after anaphylactic shock (5 *b*) is strictly specific for thrombin, and does not affect the coagulating activity either of papain or of those venoms which, like thrombin, act directly on fibrinogen. On the other hand, this antithrombin neutralized the coagulating activity of a prothrombin-venom mixture to exactly the same degree as it did a prothrombin-calcium-platelet mixture (Protocol 1 and Table VI).

Protocol 1

The neutralizing effect of antithrombin on the thrombin produced by *Bothrops jararaca* or *Bothrops atrox* venom acting on prothrombin, and the absence of such neutralization on adding antithrombin to venoms which, like thrombin, act directly on fibrinogen (Table VI, A and B).

The antithrombin used was citrated plasma obtained from dogs after anaphylactic shock, which contained approximately 25 times the normal amount of antithrombin (5 *b*). *Jararaca* thrombin was prepared by adding 0.4 cc. of a 1:10,000 solution of the venom to 4 cc. of horse or dog purified prothrombin. *Atrox* thrombin was prepared similarly, using a 1:50,000 solution of the venom. The final concentrations of venom (1:100,000 and 1:500,000, respectively) had no demonstrable effect on fibrinogen within the time limits of the present experiment. Physiologic thrombin was formed by adding 0.4 cc. of a 1 per cent $CaCl_2$ and a trace of lung extract to 4 cc. of prothrombin solution. The calcium in the physiologic thrombin was rendered inactive by the addition of $\frac{1}{10}$ volume of 3 per cent citrate just before its use in the experiment.

After 1 hour at room temperature, varying quantities of each thrombin were brought up to 0.4 cc. and added to: (*a*) 0.4 cc. fibrinogen; (*b*) 0.2 cc. fibrinogen +

[4] In the case of *Notechis scutatus*, even at the optimum prothrombin:venom ratio, only half of the prothrombin was converted to thrombin (Fig. 3); and on the subsequent addition of calcium and cephalin, the remainder of the prothrombin was converted.

0.2 cc. normal dog plasma; (*c*) 0.2 cc. fibrinogen + 0.2 cc. post-anaphylaxis dog plasma, and the coagulation time noted (Table VI, section A).

To test the effect of antithrombin on those venoms which act directly on fibrinogen, varying amounts of venom were brought up to 0.4 cc., and added to: (*a*) 0.4 cc. fibrinogen; (*b*) 0.2 cc. fibrinogen + 0.2 cc. normal plasma; (*c*) 0.2 cc. fibrinogen + 0.2 cc. post-anaphylaxis dog plasma. The results are given in Table VI, section B.

As is there shown, antithrombin did not affect the direct action of venoms on fibrinogen, but it did markedly inhibit the thrombins formed by the interaction of venom and prothrombin.

These several observations indicate that the venoms of *Bothrops atrox*, *Bothrops jararaca*, *Notechis scutatus*, and to a slight degree, *Crotalus terrificus basiliscus* and a mixed *Micrurus* venom, can transform prothrombin to thrombin. Indeed, despite the fact that these venoms are a heterogeneous mixture of substances, the first 3 named are many times as effective in this respect as crystalline trypsin.

As in the case of trypsin, the activation of prothrombin by snake venoms occurs in the absence of ionized calcium and in the absence of any demonstrable platelet or tissue derivative (cephalin). Strongly citrated plasma was coagulated by *Notechis scutatus* venom, which had no effect on fibrinogen; and prothrombin containing as high as 1 per cent citrate was promptly converted to thrombin by any of the 5 active venoms. Similarly, prothrombin which contained no demonstrable tissue or platelet derivative, and which was unaffected by the addition of calcium alone, was nevertheless activable by these venoms. Moreover, the addition of cephalin, platelets, or tissue extracts had no significant effect, either on the rate of thrombin production, or the amount ultimately formed. It seems clear that the venoms acted on prothrombin directly, and that neither calcium nor cell derivative was necessary for the observed production of thrombin.

Unlike trypsin, these 5 venoms did not rapidly digest the formed thrombin under the conditions of the experiment. In consequence, there was no optimum concentration of venom for the activation of prothrombin, but a broad zone over which the maximum amount of thrombin produced was more or less constant. A minute amount of venom sufficed to cause a maximum production of thrombin, and even a hundredfold excess over and above this necessary minimum had no demonstrable effect other than to increase the rate of thrombin

production (Figs. 2 and 3). It is to be particularly noted that in the physiological coagulation of blood there is a similar relationship between the amount of the activating principle (platelet derivative) and the rate and degree of thrombin production (3).

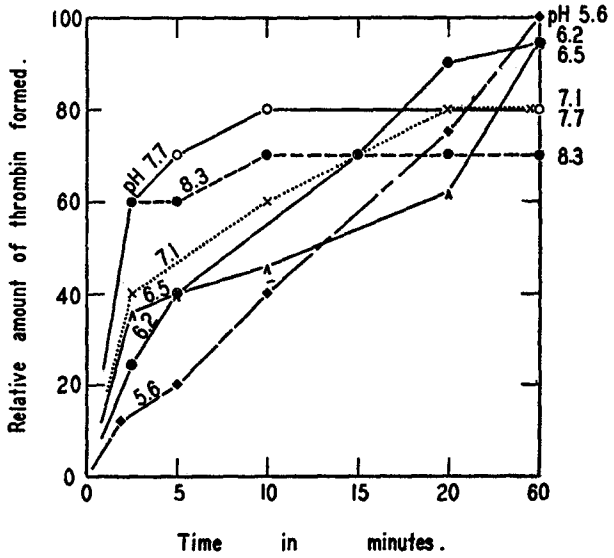

FIG. 4. The effect of pH on the rate and degree of thrombin production from prothrombin by *Bothrops atrox* venom.

To 0.8 cc. of purified prothrombin solution were added 0.8 cc. M/7 NaCl, 0.4 cc. M/10 phosphate buffer, and 0.4 cc. of a 1:50,000 dilution of *Bothrops atrox* venom. At the intervals indicated on the figure, varying samples were withdrawn and added to 0.4 cc. of fibrinogen and 0.1 cc. of the same phosphate buffer in a total volume of 0.8 cc.

The coagulation time served as the index of thrombin activity. It is to be noted that the reference curves on which these times were interpolated vary with the pH. Accordingly, a reference curve had to be constructed for each pH, based on data similar to those of Fig. 5.

The pH values given in Fig. 4 are based on a pK′ of 6.8 for the various buffer mixtures, and are in error to the extent that this pK′ deviates from 6.8 in M/60 phosphate buffer mixtures of varying ionic strength (*cf.* legend to Fig. 1).

One of three experiments to ascertain the effect of pH on the rate and degree of thrombin formation from prothrombin by *Bothrops atrox* venom is summarized in Fig. 4. As is there shown, over the pH range 5.6 to 8.3, increasing alkalinity caused a progressive increase in the

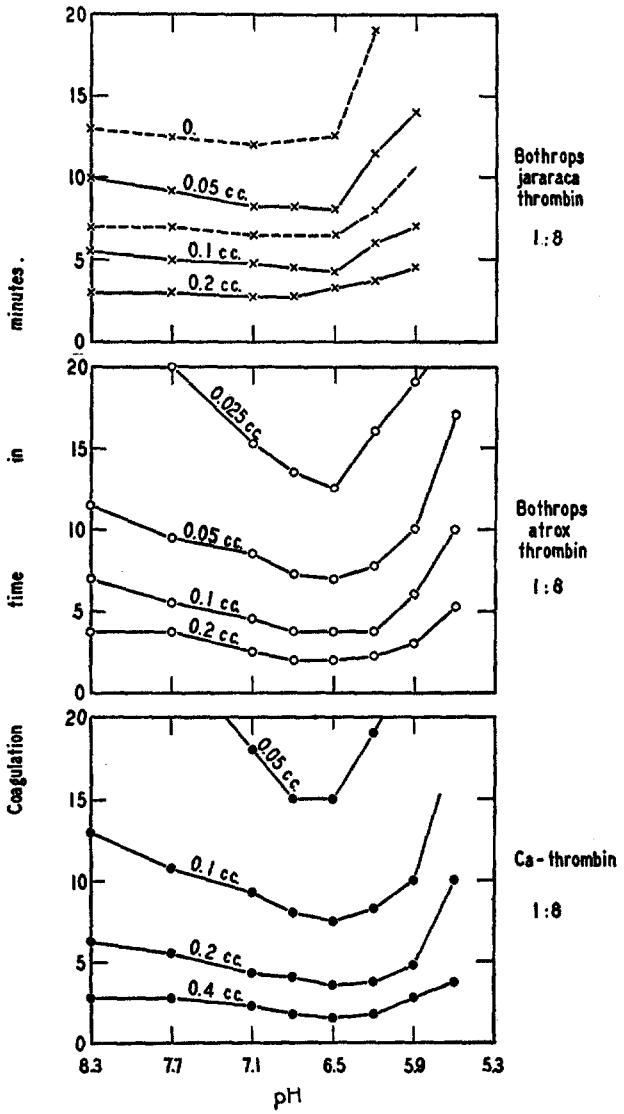

FIG. 5. The optimum pH for the action of various types of thrombin on fibrinogen.

To 0.8 cc. of purified horse prothrombin solution were added 1.2 cc. of M/7 NaCl and either (*a*) 0.4 cc. of 1:20,000 *Bothrops atrox* venom, (*b*) 0.4 cc. of a 1:8000 *Bothrops jararaca* venom, or (*c*) 0.2 cc. of 1 per cent $CaCl_2$ + 0.1 cc. of lung extract and 0.1 cc. N/7 NaCl. None of the solutions were buffered. After 1 hour

initial rate of thrombin production, but the amount ultimately formed steadily decreased. The difference between adjoining curves is no greater than the experimental error, but the consistency of the trend strongly suggests that these are real differences. The observation is as yet unexplained.

It is perhaps significant that the optimum pH for the coagulative action of the thrombin formed from prothrombin by *Bothrops atrox* or *Bothrops jararaca* venom, pH 6.5 (Fig. 5), coincides with that for the action of thrombin as formed from prothrombin physiologically by calcium and platelets (23), and coincides also with the optimum pH for the coagulative action of various venoms which act on fibrinogen directly (Fig. 1).

The question arises as to whether the conversion of prothrombin to thrombin by the 5 venoms found to be active in this respect can be ascribed to the proteolytic enzymes present in these venoms. The quantitative disproportion between the prothrombin and venom is of interest in this connection. A 1:2,000,000 dilution of *Bothrops atrox* often produced a complete activation of prothrombin to thrombin; and 1:25,000,000 dilutions had a definite, if partial, effect (Fig. 2; Table V). Since the protein concentration in the prothrombin solutions was approximately 0.05 per cent, the reacting proportions therefore varied between 1:1000 and 1:10,000. Granted that both the venom and the prothrombin solutions are crude preparations in which the active principle probably constitutes only a small and as yet indeterminate proportion of the total solid, this disproportion strongly suggests some reaction other than simple chemical combination.

In the second place, prothrombin is always associated with the

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at room temperature, varying amounts of each type of thrombin were added to 0.4 cc. of fibrinogen solution + 0.2 cc. M/10 phosphate buffer in a total volume of 1 cc., and the coagulation time noted.

The curves in the top section of the figure (*Bothrops jararaca* thrombin) represent two experiments with different prothrombin and fibrinogen preparations. The experiment summarized in the lower section of the figure (calcium-thrombin) was carried out with yet another batch of reagents. The absolute activities of the several thrombins are therefore not comparable. As is evident in the figure, the optimum pH is more clearly shown with the smaller amounts of thrombin, and centers at pH 6.3 to 6.7.

globulin fraction of plasma, and its known properties are wholly consistent with the thesis that it is itself a protein. It is destroyed by known proteolytic enzymes, such as trypsin and papain (5 *a*), and it was similarly digested by 9 out of the present series of 17 venoms (Table VII), including some only weakly proteolytic (Table IV). It is accordingly difficult to understand why the 5 venoms which did activate prothrombin to thrombin and which include some of the most

TABLE VII

*The Destruction of Prothrombin by Snake Venoms**

Venom used	Amount of 0.1 per cent solution added to 0.4 cc. prothrombin solution								Conclusion
	0.4 cc.	0.2 cc.	0.1 cc.	0.05 cc.	0.025 cc.	0.0125 cc.	0.0062 cc.	0	
	Coagulation time on adding $CaCl_2$, tissue extract, and, ½ hour later, 1.6 cc. fibrinogen in a total volume of 3.2 cc.								
	min.	*min.*	*min.*	*min.*	*min.*	*min.*	*min.*	*min.*	
Agkistrodon piscivorus	∞ †	∞	∞	∞	50	4	2¼	½	
Bitis arietans	∞	∞	∞	10	—	—	—	½	All these venoms
Crotalus atrox (1446)	∞	∞	∞	4	—	—	—	½	actively de-
(67248)	∞	∞	∞	∞	∞	∞	2	½	stroyed the
Crotalus horridus (1447)	∞	∞	∞	∞	∞	∞	6	½	prothrombin,
(1528)	∞	∞	∞	∞	∞	5	—	½	and rendered
Naia flava	90	5	5	4½	4	2½	1¾	¼	it non-activ-
Naia naia	∞	∞	5	2½	1	¾	¾	½	able by cal-
Sepedon haemachates	∞	∞	∞	4	—	—	—	½	cium + tissue
Vipera ammodytes	∞	∞	13	1½	¾	¾	¾	¾	extract
Vipera russellii	—	∞	12	2¼	1¾	¾	¾	¾	

* *Crotalus adamanteus*, *Crotalus terrificus terrificus*, and *Bothrops nummifera* could not be tested because the venom *per se* coagulated fibrinogen.

† No coagulation in 2 hours, *i.e.*, no significant production of thrombin.

actively proteolytic of this series (*Bothrops atrox*, *Bothrops jararaca*, *Crotalus basiliscus*), failed to digest the prothrombin, unless the proteolytic enzyme (or enzymes) was itself the cause of the transformation.

No explanation can be offered for the fact that some proteolytic venoms destroyed prothrombin, while others activated it to thrombin. In the analogous case of fibrinogen, it seemed fairly clear that the protein was affected only by venoms containing more than a certain

limiting concentration of proteolytic enzyme. Of those venoms which contained more than this necessary minimum, some digested the protein, and some coagulated it to form a fibrillar gel resembling fibrin. There was no correlation between the proteolytic activity as measured with gelatin and the type of effect produced on fibrinogen (Table IV). It was accordingly found necessary to assume that there were two types of venom enzymes as regards their effect on fibrinogen. In the case of prothrombin also, all these venoms affected the substance, but there was no apparent correlation between the magnitude of the proteolytic activity and the type of effect produced. The 5 venoms which activated prothrombin to thrombin include several of the most actively proteolytic (*Bothrops atrox, Bothrops jararaca*) and one of the least proteolytic (*Notechis scutatus*) of the present series. In this case also, we must assume that with respect to their effect on prothrombin there are two types of venom enzyme: one type which digests the substance and renders it wholly inactive, and a second type which activates it to thrombin.

An analogy is to be seen in the difference between trypsin and papain. In proper concentration, the former activates prothrombin and destroys fibrinogen, while the latter has exactly the opposite effect, *i.e.*, it destroys prothrombin and coagulates fibrinogen (5).

DISCUSSION

The present observations constitute additional evidence that the process of physiological coagulation involves two consecutive enzyme reactions.

Morawitz (19) originally suggested that platelets or tissue derivative constituted a kinase which, in the presence of calcium, activated prothrombin to thrombin. Although most subsequent workers in the field have not accepted this enzyme theory, it is nevertheless consistent with most of the known properties of the reaction (literature summarized in reference 4). It finds strong support also in the recent finding that crystalline trypsin may replace calcium and platelets, and is alone capable of transforming prothrombin to thrombin (5*a*).

As here reported, 5 of the 9 snake venoms found to coagulate plasma caused the conversion of prothrombin to thrombin. At least 9 of the remaining 12 non-coagulant venoms actively destroyed prothrombin.

For the reasons cited in the text, it is probable that the activation, like the destruction, is caused by proteolytic enzymes demonstrable in the venoms. It is particularly noteworthy that this activation proceeds in the absence of ionized calcium, and in the absence of cephalin, platelets, or tissue derivatives. As in the case of crystalline trypsin, these venoms therefore replace the physiological system calcium + platelet (or tissue) derivative in converting prothrombin to thrombin.

Given the observation that at least three systems may effect this transformation; that one of these systems (trypsin) is a crystalline proteolytic enzyme; and that a second system (snake venom) is a heterogeneous mixture of substances in which the active constituent is probably a proteolytic enzyme; it becomes an increasingly plausible working hypothesis that the third system, calcium and platelet (or tissue) derivative, also constitutes a proteolytic enzyme which activates prothrombin.[5] It is an open question whether this hypothetical enzyme hydrolyzes or combines with its substrate, prothrombin, to form the actual coagulant, thrombin.

Schmidt (20), one of the earliest of the modern workers on the coagulation problem, termed this thrombin a fibrin ferment, and believed it to be a proteolytic enzyme which split fibrinogen to form fibrin. Although most recent workers have, for reasons which are of debatable validity (4), discarded the enzyme theory of thrombin, the finding (5) that a proteolytic enzyme, papain, acts directly on fibrinogen to form a fibrillar gel resembling that produced by thrombin, constitutes cogent evidence in that direction.

In the present experiments 7 of 9 coagulant venoms were found to act directly on fibrinogen to form a fibrillar gel, and 5 of 9 non-coagulant venoms actively destroyed the protein. Most significant, the ability of the venoms either to digest or to coagulate fibrinogen was directly related to their proteolytic activity as tested with gelatin. It was therefore concluded that the coagulant and destructive action of these venoms on fibrinogen was in all probability due to their proteolytic enzyme content.

In view of the observations (*a*) that papain, a proteolytic enzyme, converts fibrinogen to a fibrillar gel, and (*b*) that the similar action of

[5] The one observation which seems difficult to reconcile with this hypothesis is the fact that a lipoidal tissue extract (cephalin?) can replace the tissue extractive.

certain snake venoms is probably due to their proteolytic enzyme content, it seems a valid working hypothesis that the physiological coagulant, thrombin, is also a proteolytic enzyme with a specific substrate, fibrinogen.

It is a well known observation that numerous bacteria, notably staphylococci, may cause the coagulation of decalcified blood or plasma. Since most bacteria elaborate proteolytic enzymes, one may well inquire whether this coagulative action may not be due to these enzymes, acting either on prothrombin to form thrombin, or directly on fibrinogen to form fibrin. Experiments in this direction are now in progress.

We may therefore tentatively view the coagulation phenomenon as the result of two consecutive enzyme reactions.

Physiological substrate		Activating enzyme	Product
1. Prothrombin	+	Calcium + platelets (tissue derivative; cephalin?) Trypsin Proteolytic snake venoms[6] Bacterial proteases?	Thrombin
2. Fibrinogen	+	Thrombin Papain Proteolytic snake venoms[7] Bacterial proteases?	Fibrin

As previously stated, this working hypothesis finds a complete analogy in the activation of chymotrypsinogen by a proteolytic enzyme, trypsin, to form a second enzyme, chymotrypsin (11).

SUMMARY

Nine of the 17 venoms here tested were found capable of coagulating citrated blood or plasma. As has been believed by most workers in the field, 7 of these 9 coagulant venoms convert fibrinogen to an

[6] *Notechis scutatus*, *Bothrops atrox*, *Bothrops jararaca*, *Crotalus basiliscus*, a mixed *Micrurus* venom: there are undoubtedly others not included in the present series.

[7] *Bothrops atrox*, *Bothrops jararaca*, *Bothrops nummifera*, *Crotalus adamanteus*, *Crotalus horridus*, *Crotalus terrificus basiliscus*, *Crotalus terrificus terrificus*: there are undoubtedly others not included in the present series.

insoluble modification resembling fibrin (*Bothrops atrox, Bothrops jararaca, Bothrops nummifera, Crotalus adamanteus, Crotalus horridus, Crotalus terrificus basiliscus, Crotalus terrificus terrificus*). The optimum pH for this coagulation was determined for 3 of these, and was found in each case to be approximately pH 6.5, the same as that for the action of thrombin on fibrinogen. Unlike thrombin, however, the fibrinogen-coagulating activity of the venoms was unaffected by the antithrombin elaborated in the course of anaphylactic shock.

In addition to coagulating fibrinogen directly, 3 of these venoms (*Bothrops atrox, Bothrops jararaca*, and to a less extent, *Crotalus terrificus basiliscus*) acted on prothrombin to convert it to thrombin, without the necessary intervention of either calcium or platelets. Finally, 2 venoms (*Notechis scutatus*, and to a slight extent, a mixed *Micrurus* venom), which had no demonstrable effect on purified fibrinogen, nevertheless converted prothrombin to thrombin.

Unlike the reaction between the venoms and fibrinogen, this activation of prothrombin has no definite pH optimum, but takes place over a wide zone (pH 5.6–8.3). In the case of *Bothrops atrox*, there was some indication that the initial velocity of the reaction increased with increasing alkalinity, but that the amount of thrombin ultimately formed decreased. Extraordinarily minute quantities of some of these venoms sufficed to produce a demonstrable activation of prothrombin. Thus, the fer de lance (*Bothrops atrox*) venom was active in a 1:25,000,000 dilution, and that of the Australian tiger snake (*Notechis scutatus*) was active in a 1:4,000,000 dilution.

The thrombin formed was indistinguishable from that produced by the action of calcium + platelets on prothrombin. Like the latter type of thrombin, and unlike venoms which act directly on fibrinogen, thrombin formed from prothrombin by venom was inhibited by antithrombin.

Every one of the 9 non-coagulant venoms in this series destroyed prothrombin; and 5 of these destroyed fibrinogen as well. As is discussed in the text, there is reason to believe that these several properties of the venoms (coagulation and destruction of fibrinogen; activation and destruction of prothrombin) depend on the proteolytic enzymes which they were found to contain.

These observations lend further support to the thesis that, in the

course of physiological coagulation, (*a*) calcium plus platelets (or tissue derivative) constitute an enzyme system which reacts with prothrombin to form thrombin, and which is thus analogous to trypsin and to several of the proteolytic venoms here discussed, and (*b*) the thrombin so formed is itself a proteolytic enzyme which, like papain and the majority of the coagulant and proteolytic snake venoms here studied, reacts with fibrinogen to form a fibrillar gel, fibrin.

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