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Received July 22, 1956. P.S.E.B.M., 1956, v93.

Fibrinolytic and Coagulant Activities of Certain Snake Venoms and Proteases.* (22647)

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In a search for an ideal agent for the intravascular dissolution of thrombi and emboli in man, investigators have studied heparin(1,2, 3), coumarins(4), trypsin(5), chymotrypsin (3), streptokinase(6), staphylokinase(7), and plasmin(8). Certain snake venoms have long been known to be fibrinolytic *in vitro*. In addition, many of these are also known to contain other proteins with coagulant, hemolytic, neurotoxic or other properties which would contraindicate their clinical use. A survey of available snake venoms was undertaken to compare their *in vitro* lytic actions against fibrinogen, fibrin, formed fibrin and formed whole blood clots with various other proteolytic agents. Coagulant and hemolytic properties were also studied.

Materials. Bovine fibrinogen was a 1% solution of Armour's Fraction I. Human fibrinogen was a crude fraction prepared by 25% saturation of human plasma with ammonium sulfate, collection of the precipitate, dialysis, and adjustment of the clottable protein concentration to 0.5%. Plasmin was a spontaneously activated dog serum unglobulin fraction(9). Plasminogen was prepared from human barium sulfate adsorbed plasma by 20-fold dilution, adjustment of pH to 5.3, collection of precipitate and dissolution in a volume of 0.85% NaCl equal to 1/10 the original plasma volume. The fibrinogen was removed as fibrin after addition of a trace of thrombin (1 unit/10 ml). Streptokinase (Varidase, supplied through the courtesy of Lederle Laboratories) was dissolved in 0.85% NaCl to contain 200 units per ml. Uroprotease was prepared from human urine as described by Celander and Guest(10). Trypsin and chymotrypsin (crystalline, courtesy Armour Laboratories) were 0.1% solutions in 0.85% NaCl. Ficin (Delta), bromelain (courtesy Pineapple Research Institute of Hawaii), papain (Worthington), and pepsin (Worthington) were prepared as 1% solutions in 0.85% NaCl. Snake venoms (supplied through the courtesy of Wyeth and Co.; Ross Allen's Reptile Institute; Burroughs, Wellcome; and Hynson, Wescott and Dunning, Inc.) were dissolved as 1% solutions in 0.85% NaCl. *Stypven* (Burroughs, Wellcome) was used as a 0.2% solution. Thrombin (Thrombin, Topical, Parke Davis) was prepared to contain 100 units per ml.

Methods. Fibrinolysis was estimated by observing lysis time in a clot containing 0.2 ml material to be tested, 0.2 ml fibrinogen, 0.2 ml borate buffer (pH 7.6) and 0.05 ml thrombin. Intimate mixing of the lytic agent and fibrin was obtained by a 2-tube mixing method(11), in which the fibrinogen and buffer were poured into the lytic agent and thrombin, at zero time, and subsequently poured back and forth three times. *Formed clot lysis. Fibrin*: Human fibrinogen clots

* This investigation was supported in part by a research grant from the Division of Research Grants and Fellowships of the National Institute of Health, U. S. Public Health Service.

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were prepared by adding 0.05 ml thrombin to 0.2 ml fibrinogen, mixing and allowing the clot to stand 15 min. The clot was freed from the tube by a sharp rap and suspended in 0.2 ml material plus 0.2 ml buffer. Lysis time was measured from time of addition of material to time of complete disappearance of the clot. *Whole blood*: 0.2 ml of fresh human blood was pipetted into each of a series of tubes, clotting and retraction allowed to proceed for 1 hour at 37°C and 0.2 ml of the test material added. *Fibrinogenolysis* was tested by incubating 1.0 ml material with 1.0 ml human fibrinogen and testing at intervals for residual fibrinogen by adding 0.2 ml aliquots to 0.05 ml thrombin. The time at which no clot formed was taken as the fibrinogenolysis time.

Thrombic activity was measured by determining the clotting time after addition of 0.1 ml material to 0.1 ml human fibrinogen. *Thromboplastic* activity was determined, in materials which were not thrombic, by substituting 0.1 ml material for thromboplastin in a prothrombin time test. *Hemolytic* and *hemagglutinating* activities were observed in tubes containing 0.2 ml material and 0.2 ml 1% dilution of human whole blood. All procedures were carried out at 37°C.

Results. Actual lysis times observed in the various fibrinolytic systems are shown in Table I. Venoms of the Crotalus, Bothrops and Agkistrodon groups were fibrinolytic, while those of the Cobras (crude Cobra, *N. naja, N. flava*) were not. *V. russellii* was fibrinolytic but inactive against the formed clots. *Stypven*, made from *V. russellii*, was inactive. All of the other agents were fibrinolytic except pepsin, plasminogen alone, and streptokinase in the bovine system. Ficin, bromelain and papain were the only ones with significant activity in the whole blood clots. When trypsin and chymotrypsin were tested as 1% rather than 0.1% solutions, no clots were formed in the fibrinolytic systems and the whole blood clots were lysed in 1 to 2 hours.

Inability of some of the preparations to lyse whole blood clots might depend upon the sensitivity of the enzymes to the serum antiprotease present in the whole blood system.

TABLE I. Fibrinolytic Activities.

	Fibrinolysis (min.)		Clot lysis (min.)	
	Bovine fibrin	Human fibrin	Human fibrin	Human blood
C. basiliscus	<1	5	48	180
C. atrox	3	5	90	62
C. horridus	5	19	151	480
C. adamanteus	15	21	280	480
C. hellerii	NC	8	480	480
C. terrificus	69	>120	1080	>1080
B. neuweidii	4	3	48	68
B. neuweidii 0.1%				>1080
B. jararaca	5	4	65	66
B. atrox	7	7	90	180
B. alternatus	20	21	155	480
A. piscivorus	4	8	65	65
A. mokasen	6	7	65	180
Crude Cobra	>120	>120	>1080	>1080
N. naja	>120	>120	>1080	>1080
N. flava	>120	>120	>1080	>1080
V. russellii	<1	5	>1080	>1080
'Stypven' 0.2%	>120	>120	>1080	>1080
Plasmin (dog)	4	5	148	>1080
Plasminogen (human)	>120	>120	>1080	>1080
Streptokinase (SK)	>120	5	142	>1080
Uroprotease (UP)	8	10	92	>1080
Plasminogen + SK	2	2	57	>1080
Plasminogen + UP	12	9	84	>1080
Trypsin 1 %	<1	<1	25	72
" .1%	<1	<1	28	>1080
Chymotrypsin 1 %	1	1	25	120
" .1%	1	2	32	1080
Ficin	5	31	1080	240
Bromelain	<1	2	280	540
Papain	2	2	900	160
Pepsin	>120	>120	>1080	>1080
Saline	>120	>120	>1080	>1080

Therefore, additional tests were performed by incubating the various agents with human serum and measuring residual lytic activity. The active snake venoms, ficin, bromelain and papain were inhibited far less than plasmin, uroprotease, and streptokinase-plasminogen. Trypsin and chymotrypsin showed variable activity: either increased or decreased, depending upon time of incubation and strengths of serum and enzyme, suggesting that two processes were occurring simultaneously: inhibition and activation of the serum plasminogen.

Thrombic activity, *i.e.* ability to gel fibrinogen, was found in 8 venoms and papain (Table II). Only trypsin, *V. russellii*, and

TABLE II. Toxic Activities.

Agent	Thrombic (sec.)	Thrombo-plastic (sec.)	Fibrino-genolytic (min.)	Hemolytic	Hemagglu-tinating
C. basiliscus	>600	>600	5	0	0
C. atrox	>600	>600	5	0	+
C. horridus	21.0	—	—	0	0
C. adamanteus	10.0	—	—	0	0
C. hellerii	45.0	—	—	0	0
C. terrificus	16.6	—	—	0	0
B. neuweidii	10.8	—	—	0	+
B. jararaca	18.4	—	—	0	0
B. atrox	11	—	—	0	0
B. alternatus	21.4	—	—	0	0
A. piscivorus	>600	>600	15	0	+
A. mokasen	>600	>600	10	0	+
Crude cobra	>600	>600	10	+	0
N. naja	>600	>600	10	+	0
N. flava	>600	>600	5	+	0
V. russellii	>600	30	25	0	0
"Stypven"	>600	19.0	>60	0	0
Plasmin (dog)	>600	100	>60	0	0
Plasminogen (human)	>600	56.5	>60	0	0
Streptokinase (SK)	>600	100	26	0	0
Uroprotease (UP)	>600	100	>60	0	0
Plasminogen + SK	>600	100	25	0	0
" + UP	>600	100	60	0	0
Trypsin .1%	>600	12.8	<¼	0	0
Chymotrypsin .1%	>600	68	<¼	0	0
Ficin	>600	63	¾	+	0
Bromelain	>600	>600	15	+	0
Papain	25.0	—	—	0	0
Pepsin	>600	100	>60	+	0
.85% NaCl	>600	95	>60	0	0

its derivative *Stypven* were thromboplastic. Certain snake venoms appeared anti-coagulant, *i.e.* thromboplastin time longer than that of saline. In each case this appeared due to fibrinogenolysis.

All of the snake venoms that could be tested (those which were not thrombic) were fibrinogenolytic, as were the other fibrinolytic enzymes. The cobra venoms were remarkable in being fibrinogenolytic but not fibrinolytic. Hemolysis was produced by the cobra venoms, ficin, bromelain and pepsin. Agglutination of human red cells, seen grossly and confirmed microscopically, was caused by *C. atrox, B. neuweidii, A. piscivorus* and *A. mokasen.*

The possibility that strongly fibrinogenolytic venoms showing no thrombic activity might actually be thrombic as well at a lower concentration was investigated. Fibrinogen was incubated with various strengths of three such venoms (*A. mokasen, A. piscivorus, C. basiliscus*) from 1% down to .00002%, the latter figure being far below the concentration necessary for fibrinogenolysis to be evident. At none of these strengths was thrombic activity apparent.

Discussion. Fontana, in 1787(12), noted the blood to remain fluid in animals dead of viper bite. Since then various venoms have been found to exhibit various activities on the coagulation and fibrinolytic mechanisms(13, 14,15). Therapeutic use in hemorrhagic diseases has not been encouraging. Other properties of venoms, including hemolysins(16), neurotoxins(17) and hyaluronidase(18) have been the subject of many investigations. The data here presented indicate that a number of snake venoms actively lyse human blood clots and appear to have a certain theoretical advantage over the more common fibrinolytic agents in that they are less inhibited by human serum antiprotease. However, these venoms are all either fibrinogenolytic, thrombic,

thromboplastic, hemolytic or hemagglutinating, thus contraindicating their clinical use in crude form. Fractionation of whole venoms has been reported(19,20) and it seems conceivable that a pure fibrinolytic fraction, devoid of toxic activities, might be separated and prove of therapeutic value.

Summary. Of the 16 snake venoms studied, 11 actively lysed human blood clots. However, only one of these, *C. basiliscus,* was devoid of thrombic, hemolytic, and hemagglutinating properties. This venom was fibrinogenolytic as well as fibrinolytic. The possible therapeutic use of certain venoms as dissolving agents for intravascular clots presents a theoretical advantage over most other fibrinolytic agents in that their fibrinolytic activity is not readily inhibited by human serum. Fractionation of a pure fibrinolytic principle may be possible.

Acknowledgement is gratefully made of the valuable bibliographic assistance of Miss Iris Kiem, Department of Epidemiology, University of Pittsburgh Graduate School of Public Health.

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Received July 23, 1956. P.S.E.B.M., 1956, v93.

Effect of Thyroxine on Liver Tyrosine-glutamic Acid Transaminase. (22648)

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In the course of studying the effects of different hormonal states and hormone administration upon activity of several rat liver enzymes it was observed that dietary treatment with 1% thyroid for 8 days or more resulted in marked inhibition of liver tyrosine oxidation(1). Furthermore, the degree of inhibition was equally significant in normal animals and in animals under conditions of varying hormonal state, whereas certain of the other enzymes were affected by thyroid only under restricted conditions. This suggested a direct effect of the hormone* upon the tyrosine oxidase system. Since low dietary protein decreased activity of homogenate preparations (1) and inanition has been reported to enhance tyrosine oxidation in soluble preparations(2) this effect was further investigated

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* Reference to thyroxine hormone means thyroxine itself or whatever active form to which it may be converted.