THE ACTION OF THE TOXIN OF CROTALUS ADAMANTEUS ON BLOOD

A Thesis by

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The subject of snake venoms has received a great deal of attention. Weir Mitchell and Reichert have published an extended number of researches upon the venoms of poisonous serpents through the Smithsonian Institute (1). Noguchi, in 1909, in a monograph of the Carnegie Institute of Washington brought our knowledge of the subject up to date in a very extensive form.

Crotalus toxin as described by Mitchell is a pale yellow somewhat viscid fluid, the specific gravity of which is between 1.06 and 1.07. It is invariably acid to litmus. The venom dries quickly at low temperatures to a brittle mass resembling dried serum. The solution of dried venom undergoes putrifactive decomposition and gives an unpleasant odor. The toxic properties disappear from such decomposed solutions.

From a chemical analysis Mitchell believes the venom to contain:

1. Albuminoid body not coagulable by heat at 100° C.
2. Albuminoid coagulable at 100° C.
3. A coloring matter and an undetermined substance both soluble in alcohol.
4. A trace of fatty matter.
5. Salts (chlorides and phosphates).

The proteins which are the main component have been shown by Mitchell to be globulins and peptones. Flexner (2) called attention to the close resemblance between some forms of tox-albuminoids and snake venom poisoning and the preparation of anti-venin by Calmette (3) which confirmed their toxic-like nature, has fostered a belief that the protein molecule is the essential poison.

Strong evidence exists, however, that it is possible to separate pure toxic agents from the protein molecule to which they attack themselves. The work of Jacoby (4) especially showed that peptic digestion removes much of the protein but leaves the toxicity well preserved. Mitchell prepared an active substance, crotalin, which is an albuminoid body not coagulated at 100° C and free from globulin and most of the other foreign matter. More recently Faust (5) has prepared from the venom of C. adamanteus a non-nitrogenous compound which he called crotalo-toxin which he considers the active constituent that causes paralysis of the respiratory centers. He also claims it to have the cytolytic, hemolytic and hemorrhagic principles. He assigned to it the emperical formula $C_{17}\ H_{26}\ O_{10} + 1\frac{1}{2}\ H_2\ O$. From his pharmacological results the most prominent characteristic is the paralysis of the central nervous system and respiratory center. Flexner and Noguchi (6), however, pointed out that the hemorrhagic principle was the most important in the venom of crotalus and it is possible that while this non-protein principle contains a considerable degree of activity it does not represent all of the polytropic action

of the venom. Welker (7) has recently taken up the work of Marshall (8), which was begun in 1904 on the separation of the toxic and non-toxic fractions of rattlesnake venom and has succeeded in repeating Marshall's work. He is at the present time engaged in further work on the toxic fraction with a view to studying the nature of the toxic substances in the venom.

Since the work of Fontana in 1787 it has been known that the blood of animals dying of viper bite remained fluid. Brainard (9) showed that when an animal, bitten by a rattlesnake, died quickly, the blood on autopsy was coagulated, but if the bitten animal died slowly the blood remained fluid in the vessels. Weir Mitchell (10) confirmed Brainard's finding and later Mitchell and Reichert (1) demonstrated the anti-coagulating action of the crotalus venom in vitro.

Martin (11) in working on the venom of the Australian black snake (pseudechis porphyriacus) found that when insufficient venom was injected to cause a coagulation of the blood and death from thrombosis, there followed a period of non-coagubality of the blood. During this condition the blood could not be caused to clot with larger doses of the venom than would originally have produced extensive thrombosis. Martin, therefore, drew a parallelism between this negative phase phenomena and that produced by Wooldridge's tissue fibrinogen. This, however, he withdrew in 1905 (12).

In the case of poisoning in man by crotalus venom, it is not the thrombic action but rather the anticoagulant action that is essential. Flexner and Noguchi (13) have fixed the amount of venom in dry form, which can be obtained by a single extraction of both glands at 0.179 gm. to 0.309 gm. In the smaller laboratory animals this amount is sufficient to produce a thrombus, but is seldom sufficient in man.

Arthus (14) in working on venoms both in vivo and in vitro divides the coagulant venoms into two groups: Those of the thrombin type and the venoms of the type of organ extracts. Houssay & Sordelli (15), however, point out that in 14 venoms studied by them, all that coagulate in vivo were found to be thrombic in vitro. The apparent exception to this general rule that only venoms with the action of thrombin are capable of clotting in vivo is the venom of crotalus adamanteus. Because this venom produces thromboses only when injected in large doses, they conclude that it has only a very feeble thrombin power.

Houssay and Negrete (16) have shown that crotalus has a strong proteolytic action. It rapidly alters blood fibrinogen. From this Houssay and Sordelli conclude that in vitro the fibrinogen is destroyed before it can be clotted by the weak thrombic action. It was shown as early as 1904 by Morawitz and confirmed in 1909 by Mellanby (17) and by Hirschfeld and Klinger (18) that cytozyme is destroyed by the anti-coagulant venoms especially that of the cobra. Houssay and Sordelli include

crotalus adamanteus as having this property. They further believe that it is by lipolysis that this destruction is accomplished. When these venoms are injected in vivo, they produce incoagulability because they destroy cytozyme. That the destruction of cytozyme is accomplished by the toxin of crotalus with the liberation of fatty acids we have confirmed. But we do not believe that the total cause of this incoagulability is due to the cytozyme destruction. At the outset of this work we had attempted to find out whether or not the hemorrhagic condition produced by crotalus venom could be controlled by purified tissue fibrinogen which was made by Mills (19). Mills and Matthews (20) have shown that there is a dual mechanism for the clotting of blood, one in which cytozyme or cephalin unites with serozyme to form thrombin. This in turn unites with the fibrinogen of the blood to form fibrin. This thrombin contains the necessary calcium for blood coagulation. It will, therefore, clot citrated or decalcified plasma without recalcification. The other clotting occurs as was shown by Mills (21) when tissue fibrinogen unites through calcium with blood fibrinogen to form fibrin. In this method of clotting decalcified plasma by tissue fibrinogen, calcium must be supplied before clotting can take place. If, according to Houssay, the anti-coagulating action of crotalus toxin is produced alone by the destruction of cytozyme, the blood should be capable of clotting by tissue fibrinogen. The purpose of this paper is to show that such

is not the case, but rather that the almost complete and rapid destruction of blood fibrinogen is the cause of the non-coagulating condition of the blood.

EXPERIMENTAL

The venom used in this work was obtained in dry form from the diamond-back rattler of Southern Texas. Strictly speaking we do not know whether the venom was obtained from crotalus adamanteus or from crotalus atrox. There is very little difference in the two species except in the coloring of the tail. According to Noguchi the adamanteus has its habitat at more especially in the southeastern part of the United States while the atrox is found in the sub-arid and desert regions of Texas and the western United States. It is possible that a great deal of the venom called adamanteus is in reality from the atrox, but their mode of action is in all probability analagous.

I. THE MINIMUM LETHAL DOSE OF C. ADAMANTEUS VENOM FOR ALBINO RATS

A solution of the dried venom in 0.9 % NaCl solution was made by grinding in a small amount of salt solution and then diluting to volume. After shaking for several minutes the clear solution was filtered from the venom debris. Injections were made direct into the peritoneal cavity.

Insert Table I.

From the results as shown in Table I. the M.L.D. is 25×10^{-6} grams per gram of body weight. This is equivalent

to about 1/2 cc of a one percent solution of the venom for a 200 gram rat.

An interesting point in this connection is the high resistance of the white rat to crotalus venom as compared to the guinea pig and the rabbit. For the former Noguchi (22) has found that the M.L.D. on intraperitoneal injection to be 0.002 gram per kilogram of body weight while the latter requires, by the same method of injection, 0.0004 grams per kilo. Noguchi also comments on the resistance of the Danish white rat. This agrees very well with our knowledge of the resistance of the rat to infection and to anaphylaxis.

II. THE ACTION OF C. ADAMANTEUS VENOM ON CITRATED HORSE PLASMA.

Houssay has shown that this venom does not have a blood coagulant action in vitro, which he explains by the fact that the venom destroys the blood fibrinogen before the weak thrombic action can take place.

Since we now know that there are two distinct mechanisms of blood clotting, it seems advisable to test for the clotting principle of the venom by both methods.

Fresh horse blood was allowed to flow into sodium citrate to give a final concentration of 0.5 % citrate. The plasma was then freed from corpuscles by centrifuging. All experiments were made in a constant temperature water bath at 40° C. ~~0.02~~.

Insert Table II

In Experiment 6 no venom was added. In its place 1/2 cc of freshly prepared thrombin, made from fresh serum and cytozyme was used. This shows that the plasma can be rapidly clotted by thrombin. From the fact that doses of crotalus venom varying from 1/10 milligram to 1/10 gram did not produce clotting, we may reasonably conclude that the venom has no thrombic action in vitro.

After the time shown in Table II at which there was no clotting, 0.15 cc. of tissue fibrinogen, 1 ½ % solution, and 0.2 cc. of 1 % $CaCl_2$ were added to each experiment. No clotting took place in 16 hours although in Experiment 1 there was a very slight amount of fibrin precipitated. The amount was insufficient to form even a weak clot.

In order to test directly for the tissue fibrinogen method of clotting in vitro the experiments in Table III were made.

Insert Table III

In Experiment 9 the venom solution was replaced by like amount of 0.6 % solution of tissue fibrinogen prepared according to the method of Mills (12). It is evident that the venom has no such action in vitro, but rather a decided anti-coagulant effect which is produced in proportion to the amount of venom present.

III. THE ACTION OF C. ADAMANTEUS VENOM ON BLOOD FIBRINOGEN.

Houssay has reported that blood fibrinogen is rapidly destroyed by this venom. The results of the experiments given in Table II would also indicate this.

It was noticed that when the venom was allowed to act on the weak salt solution of blood fibrinogen for a short time, the amount of coagulum thrown out by half saturating the NaCl was apparently smaller in volume than from the concentrated solutions of fibrinogen.

Insert Table IV

Table IV shows quantitatively the result of this action. Blood fibrinogen was precipitated from citrated horse plasma by the addition of an equal volume of saturated NaCl. The precipitate was separated and washed three times with one half saturated NaCl and then dissolved in dilute salt. Reprecipitation with salt was effected and the precipitate again washed three times at half saturations. In these experiments the venom was allowed to act on the fibrinogen solution at 40° C. for different lengths of time. To the solutions were then added an equal volume of saturated NaCl. They were allowed to stand for several minutes, filtered, washed with one half saturated salt solution, dissolved in dilute salt and boiled. The resulting coagulum was filtered, washed free from chlorides and dried to constant weight.

There is little doubt from this result that blood fibrinogen is destroyed by the venom. The action is most rapid at the start and from the nature of the curve appears to be enzymatic.

In order to study further the nature of this action, the experiments in Table V were performed.

Insert Table V.

These three tests were made simultaneously using the same solutions and the same water bath for coagulation, therefore the same rate of heating.

At 56° C. the blood fibrinogen alone threw out a much heavier precipitate than that treated with the venom while the venom alone showed no coagulation. The temperature of the coagulating bath was maintained at 56° - 57° for 5 minutes after which the two cloudy tubes were filtered and again put in the bath. The temperature was brought back to 56° rapidly after which the heating proceeded more slowly. A slight haze again appeared in Experiment 1 at slightly over 56° C; but no coagulation took place. At 82° C. there was considerable coagulum thrown out. The solution of venom remained clear at both points. Finally all of the tubes were taken to 100° C. and maintained at that temperature for 10 minutes, after which they were filtered and the biuret test made on the clear filtrates. The crotalus solution was too weak in protein to give a positive reaction, while Experiment 2 (the mixture of venom and fibrinogen) gave the reddish coloration which is more pronounced with protein split products. The fact that this material is non-coagulable at 100° C. and yet gives a strong biuret reaction leads us to believe that it is either an albumose or a peptone. Since this

solution gives a precipitate by three quarter saturation with ammonium sulphate, we are inclined to believe that it is an albumose. We did not test the dialyzing property and are therefore not prepared to state definitely which it is. To find the effects of various amounts of venom on fibrinogen the experiments in Table VI were made.

Insert Table VI

These experiments were performed by allowing varying amounts of venom to act on a solution of fibrinogen for ten minutes. After the action the tubes were boiled thus giving both the unaltered fibrinogen and the protein coagulable at 82° C. together. The precipitates were washed with water and dried to constant weight. It serves only to give an idea of the concentration required to effect splitting of the protein.

In Table VII experiments are given which show the rate of fibrinogen splitting into the protein coagulated at 82° C. and also the non-coagulable protein which is found by difference.

Insert Table VIII

In these experiments the NaCl precipitate was obtained and treated as in Table IV. The filtrate from the washing with one-half saturated sodium chloride was boiled and this portion determined as in Table VI. The coagulable matter is given by difference. From these experiments we must conclude that the venom of C. adamanteus has a proteolytic action on blood fibrinogen, splitting it into a protein having a higher coagulating temperature, more soluble in sodium chloride and also forming a non-coagulable

albumose or peptone. (Is the coagulable protein serum albumin or serum globulin or both

IV. THE ACTION OF VENOM ON SERUM GLOBULIN AND SERUM ALBUMIN.

Serum was obtained from defibrinated blood and the globulin precipitated by 1/2 saturation of ammonium sulphate. After filtering, the globulin was freed from ammonium sulphate by dialysis. When the solution of globulin thus prepared is acted upon by crotalus venom, there appears to be no difference in the amount of globulin which can be coagulated by boiling. These results are shown in Table VIII. The increase in weight of the precipitate formed after treatment with the venom may possibly be due to the two milligrams of venom added. Treatment of the precipitate with boiling benzene failed to remove any soluble products. We are inclined to believe that the venom is without action on globulin within the time limits of this experiment. This also leads us to believe that the action on serozyme would be very minute, if at all, thus, in a measure, confirming Houssay's finding of the action of the venom on serozyme. Similarly the action of the venom was tried on serum albumin, which was obtained from defibrinated plasma. The globulin was removed from solution by half saturated ammonium sulphate. The filtrate containing the serum albumin was dialyzed to remove the sulphate. The albumin, when thus obtained, was acted on by crotalus venom for one hour and then coagulated by boiling. It was washed with boiling water and dried to constant weight. We experienced a great deal of difficulty in drying this precipitate to constant weight because

the loss in weight was extremely slow near the end of the drying. Results given in Table IX show that serum albumin is not acted upon within the time limit of the experiment.

Insert Table IX

V. THE EFFECT OF THE VENOM OF C ADAMANTEUS ON SYNERESIS OF FIBRIN CLOT.

In studying this effect 1/2 cc of citrated horse plasma was mixed with calcium chloride and maintained at 40° C. until coagulation was ready to occur. This point has been predetermined for the particular plasma used.

Just before the clotting took place equal volumes of solutions containing varying amounts of venom were added. The clotting would then take place normally and the amount of liquefaction was noted over a period of time. The results are shown in Table X.

Insert Table X

It is evident from this that small amounts of venom increase while larger amounts inhibit syneresis. That the fibrin clot was not rapidly, if at all, acted upon by the venom was observed by the amount of clot left at the end of six hours and furthermore by the fact that there was less solid in the control tube than in those to which the venom was added.

It is quite conceivable that clotting takes place in two distinct steps. The first is the formative step in which the blood fibrinogen is converted into fibrin; the second is the

precipitation of the fibrin thus formed. Since crotalus venom acts rapidly on blood fibrinogen and as we will show later it also acts on cytozyme, but not on fibrin it is possible to conceive that the point of combination of the blood fibrinogen with the thrombin, and the point of combination of the cytozyme with serozyme or calcium, is the point of attack on these substances by crotalus venom. For while these substances are readily attacked in their uncombined state, they are not attacked, at least to any great extent, when they are bound to another substance in the formation of fibrin.

VI. THE ACTION OF C. ADAMANTEUS ON CYTOZYME

That cytozyme or cephalin is acted upon by the venom of C. Adamanteus has been pointed out by Houssay, who believed the action to be a lipolytic one. In fact Houssay contends that the anti-coagulant action of crotalus toxin depends principally upon its cytozymatic action. With the refinement of the method for testing cephalin by Mills and Mat/hews, it was decided to test for the destruction of cephalin in this manner. When freshly prepared serum is added to decalcified citrated plasma it will cause clotting to take place without further addition of calcium salts, because of the thrombin remaining in the serum. This action is lost fairly rapidly as the thrombin is changed over into meta thrombin. If, however, a small amount of cephalin is added to this serum it is greatly activated due to the formation of thrombin with the serozyme present. If now this cephalin emulsion is acted upon by crotalus venom it should lose the power of activating serum to clot citrated plasma without recalcification. The results shown in Table XI confirm the fact that cephalin loses its power to participate in blood coagulation. Experiments 1 and 2 show the relative action of serum alone to serum which has been activated by cephalin. Experiment 3 shows that when venom is added to the cephalin, the mixture of cephalin serum venom became anti-coagulating in its effect.

Experiments 4, 5 and 6 were performed simultaneously. Experiment 4 shows that when cephalin emulsion is made acid with

HCl to 0.8% boiled 5 minutes and then neutralized, it still has the power of forming thrombin and thus activates serum. Experiment 5 shows that when cephalin emulsion is acted on by the venom for 10 minutes and then given the same acid heat treatment as the cephalin alone in Experiment 4, to destroy the venom action, the cephalin has lost its activating power and by comparison to the clotting time in the control Experiment 6, it is evident that the activating principle of the cephalin has been completely destroyed.

That the venom action on coagulation was inhibited by the acid heat treatment is evident from Experiments 7, 8 and 9. In these the venom was heated to 100°C with 0.8% HCl boiled 5 minutes and then neutralized. Cephalin emulsion alone is shown again to activate the serum and the cephalin plus treated venom has the same activity. Crotalus venom acts on cephalin with the liberation of fatty acids. This is shown in Experiment XII.

Insert Experiment XII

When cephalin emulsion is acted on by the venom at 40°C for 1/2 hour and the reaction mass evaporated carefully to dryness a part of the residue is soluble in 95% alcohol. This alcoholic solution is acid and on neutralization by NaOH and evaporation to dryness yielded a soap. We, therefore, believe that the venom splits out of cephalin a fatty acid. The identity of the acid was not proved.

VII. THE ACTION OF CROTALUS VENOM ON TISSUE FIBRINOGEN

The action of the venom of C. adamanteus has presented some extremely interesting results. In order to determine whether or not the venom effect was neutralized in vivo the following experiments were made.

1. A white rat, weight 250 grams, was given an injection of 0.62 cc. of 1% venom. This is equivalent to 25 x 10^{-6} gms. per gm. of body weight, or 1 M.L.D. Immediately after this injection, which was intraperitoneal, 1/4 cc. of tissue fibrinogen 1.5% solution was injected by the same route. The animal died in 1 hour 40 minutes, whereas the control, a 260 gram rat which was given 0.65 one per cent solution or 1 M.L.D. died between 7 and 23 hours later. The results were so unexpected that another rat, weight 300 grams, was given 0.25 cc. 1.5% tissue fibrinogen and one hour later this same rat was given 0.75 cc. 1% venom. This animal died in 20 minutes after the intraperitoneal injection of the venom. It was thus evident that tissue fibrinogen would not reduce the toxicity of the venom. But greatly increases it These experiments were repeated several times with the same results.

Experiments were then made in vitro as shown in Table XIII to determine the effect on the clotting of recalcified citrated plasma by a mixture of venom with tissue fibrinogen solution.

Insert Table XIII

These results are difficult to explain. There is at first a reduction in the clotting time in which it is evident

that tissue fibrinogen is predominating. After 35 minutes the clotting time of the plasma returns to normal and at the end of 1 hour the mixture shows the typical anti-coagulating effect of the venom alone. It is possible, from these results, that there is a relation in vivo between the length of time that the venom has acted on the tissue fibrinogen and the action of the mixture.

The following experiments were performed to demonstrate this point. A mixture of 1 cc. each of a 1.5% solution of tissue fibrinogen and 1% venom was made. 0.62 cc. of this mixture when first prepared, containing 1/2 M.L.D. of the venom were injected into the femoral vein of a 250 gram rat. Death resulted in 16 mimutes. Autopsy revealed intestinal hemorrhage with laking in the peritoneum, the blood was rendered non-coagulable, showing that the effect of crotalus toxin had predominated and that death was not due to intravascular clotting, which occurs following the injection of tissue fibrinogen alone.

A second rat, weight 350 grams, was injected with 0.46 cc. of the mixture containing 1/4 M.L.D. of venom. This, when injected immediately after mixing, caused death in one hour and 12 minutes. Controls with intravenous injection were now made by injecting M.L.D. crotalin toxin alone, which produced death in between five to seventeen hours. A second control injected intravenously with 3/4 M.L.D. recovered.

Two hours later a 260 gram rat was given 0.65 cc., containing 1/2 M.L.D., of venom, but which had been allowed to stand in contact with tissue fibrinogen during this time at 25°C. The animal survived. 0.48 cc. of this two hour old mixture was in-

jected into the femoral vein of a 380 gram rat. This rat thus received 1/4 of an M.L.D. This animal also survived as would be expected. To determine if the toxicity of the venom had been destroyed in this treatment with tissue fibrinogen, a mixture was made from equal parts of 3% tissue fibrinogen and 2% crotalus toxin. 0.78 cc. of this mixture when it had been allowed to stand for 2 hours at 25°C was injected into the femoral vein of a 260 gram rat. The dose here was 30 x 10^{-6} gms. per gm. of body weight, or slightly more than the M.L.D. The animal died in three hours, thus showing that the toxicity of the venom had not been destroyed. Since crotalus toxin is reported to cause intravascular clotting when given in large doses, we thought perhaps these deaths could have been due to the thrombic action of the venom augmented by the power of tissue fibrinogen to cause clotting. However, we did not fine any blood clots in the pulmonary artery or the right auricle of the heart. It is hard to find the thrombus on an animal as small as a rat. We decided to continue these experiments with rabbits.

A 570 gram rabbit was injected with 0.5 cc. of 1% venom. The injection was made into the marginal ear vein. Death occurred in five minutes. There was paralysis of the forelegs, loss of eye reflex before death. At death the body was perfectly limp. An autopsy there was no hemorrhagic condition in the peritoneum, nor could we find any thrombus in the venous system. The pulmonary artery and the right auricle were examined especially close. The blood clotted normally when withdrawn. The heart continued to beat after respiration had ceased.

A rabbit, weight 600 grams, was injected with 1 cc. of 6% venom into the marginal ear vein. The animal jumped into the air several times - no sign of paralysis. The symptoms were immediate. It became prostrated in one-half minute, respiration was difficult and ceased before the heart had stopped beating. On autopsy an extensive thrombus was found in the right auricle, ventricle and pulmonary artery. Elsewhere the blood was not coagulated, nor did it coagulate in 30 minutes.

An 800 gram rabbit was given 1 cc. of 5% venom into the marginal ear vein. The symptoms were like the previous case; the animal was unconscious for 45 seconds, respiration ceased in five minutes. There was a thrombus in the pulmonary circulation and the heart blood from other veins had not clotted, nor did they clot within one-half hour.

A 700 gram rabbit was injected into the marginal ear vein with 1cc. of 1% venom. There was paralysis of the forelegs. The rabbit uttered several squeals before death, which occurred in 20 minutes. No thrombus could be found on autopsy; the blood was non-coagulable.

No thrombus could be found. The blood which did not clot was centrifuged and the clear plasma obtained made half saturated with sodium chloride. No precipitate was formed. There was no coagulation of this plasma at 56°C., although there was an abundant coagulum produced at 62°C. This plasma could not be clotted by freshly prepared thrombin. It is evident that the blood fibrinogen had been destroyed.

From this work we know that doses below 1 cc. of 1% venom for a 700 gram rabbit will not produce intravascular clotting, while 1 cc. of a 5% solution or more will produce a thrombus. A solution of tissue fibrinogen, 1.5%, was prepared and 1.5 cc. of this preparation was injected into the marginal ear vein of a 600 gram rabbit. Death was rapid. There were no signs of paralysis, nor did the rabbit squeal as was the case when venom was injected. On autopsy heavy clotting was found, especially in the heart and pulmonary system.

Another 600 gram rabbit was injected with 0.3 cc. of the same solution of tissue fibrinogen. The animal quickly became unconscious and death followed a few minutes later. The heart continued to beat after respiration had ceased. On autopsy a thrombus was found in the right heart and elsewhere. Death resulting from large doses of crotalus venom was very similar to the death caused by tissue fibrinogen. The clotting was not so extensive, however, from a large dose of venom as from a small dose of tissue fibrinogen.

In studying the nature of the increased toxicity of a mixture of tissue fibrinogen and venom, we attempted to find out if the tissue fibrinogen was an additive factor to the weak thrombic action of the latter. A solution was prepared of equal parts of 1% venom and 1.5% of tissue fibrinogen. One cc. of this freshly prepared mixture was injected into the marginal ear vein of a 550 gram rabbit. There was marked paralysis of the forelegs, the rabbit squealed violently before death, which occurred in three minutes. No thrombus was found and the blood was coagulable.

This experiment was repeated by injecting a 600 gram rabbit with a dose of 1 cc. of a freshly prepared mixture of equal parts of 1% venom and 1.5% tissue fibrinogen. The symptoms were identical with those in the former case; the animal died in six minutes and again no thrombus was found.

In these experiments we have a dose of venom which would cause death without intravascular clotting, mixed with a dose of tissue fibrinogen which would, if acting alone, produce quite an extensive thrombus. The effect, therefore, is not additive.

In another rabbit a mixture was made representing 1.25 cc. of 1% venom and ¼ cc. of 6% tissue fibrinogen (equivalent to 1 cc. of 1.5%). 1.5 cc. of this fresh mixture was then injected into the marginal ear vein of a 600 gram rabbit. There was paralysis of the forelegs and

squealing, followed by quick death. No intravascular clotting was found, even though three times as much tissue fibrinogen was given as would ordinarily cause marked intravascular clotting.

In the next experiment the ratio of the amount of venom to the amount of fibrinogen was decreased. A mixture was made representing 1cc. of 1.5% tissue fibrinogen and ½ cc. of 1% venon. 1.5 cc. of this mixture was then injected into the marginal ear vein of a 600 gram rabbit. Death was very rapid - there was loss of consciousness in a few seconds, and death followed in three minutes. Symptoms were typical of tissue fibrinogen clotting, and on autopsy there was heavy clotting in the pulmonary circulation and in the heart. Table XIV summarizes these results. We believe that tissue fibrinogen is acted upon by crotalus venom, destroying its coagulating power.

CONCLUSIONS

1. The minimum lethal dose of the dried venom of crotalus admanteus has been determined for white rats.
2. Crotalus venom shows neither a tissue fibrinogen nor a thrombin clotting action on citrated horse plasma.
3. The venom has a marked proteolytic action on blood fibrinogen which it converts into a more soluble protein accompanied by the splitting out of an albumose or a peptone.
4. The venom has no marked action on serum albumin or serum globulin.
5. The venon has a lipolytic action on cephalin, liberating a fatty acid and destroying its power of thrombin formation with serozyme.
6. The venom has no marked action on freshly prepared fibrin.
7. The venom destroys to some extent the power of tissue fibrinogen to cause intravascular clotting.
8. The fact that large doses in vivo cause clotting indicates that the venom has a weak thrombin action, but whyythis appears ~~ars~~ only in vivo and not in vitro remains to be investigated.

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TABLE NO. 1

M. L. D. OF CROTALUS TOXIN

FOR WHITE RATS

INTRAPERITONEAL INJECTIONS

Weight in grams	Gms. Crotalin Gm. Body Wt.	Strength Solution	Dose in Cc.	Dose in Gm.	Results
200	.000050	1%	1.0	.01	Death in 1 hour
50	.000050	1%	0.25	.0025	Death in 1-1/2 hour
335	.000045	1%	1.06	.0106	Death in 1-1/4 hours
295	.000043	1%	1.24	.0124	Death in 2-1/4 hours
130	.000035	1%	.46	.0046	Death in 2-1/2 hours
315	.000030	1%	.65	.0065	Death in 3 hours
220	.000030	1%	.66	.0066	Death in 3 hours
230	.000025	1%	.58	.0058	Death in 3 hours
295	.000025	1%	.74	.0074	Survived 24 hours Passed blood, autopsy
260	.000025	1%	.65	.0065	Death between 5 and 32 hours
200	.000020	1%	.40	.004	Survived severe symptoms
250	.000015	1%	.37	.0037	Survived, Symptoms
250	.000012	1%	.31	.0031	Survived. Slight Symptoms

M.L.D. equivalent to .000025 gram per gram body wt.

TABLE NO. II

THE THROMBIN ACTION OF

C. ADAMANTEUS VENOM

Experiment No.	Amount Plasma	Volume of Venom Solution		Grams of Venom	Clotting time in Minutes
1	1 Cc.	1/10 Cc.	1/10%	0.0001	No clot in 35
2	1	1/2	1/10%	0.0005	No clot in 35
3	1	1/10	1%	0.0010	No clot in 35
4	1	1/2	1%	0.0050	No clot in 33
5	1			0.1000	No clot in 22
6	1	Thrombin			0.8

TABLE NO. III

ACTION OF THE VENOM OF C. ADAMANTEUS

ON RECALCIFIED, CITRATED PLASMA

Experiment No.	Citrated Plasma	1% $CaCl_2$	Volume of Venom Solution	Final Concentration of Venom	Temp. 40°C. Clotting time in minutes
1	1 Cc.	0.35 Cc.	0.25 Cc.	None	6.50
2	1	0.35	0.25	1-40,000	7.00
3	1	0.35	0.25	1-30,000	8.00
4	1	0.35	0.25	1-20,000	10.00
5	1	0.35	0.25	1-10,000	30.00
6	1	0.35	0.25	1-1000	Above 30
7	1	0.35	0.25	1-100	" "
8	1	0.35	0.25	1-10	" "
9	1	0.35	0.25	Tissue Fibrinogen	0.05

TABLE NO. IV

ACTION OF THE VENOM OF C. ADAMANTEUS

ON THE NaCl PRECIPITATE OF BLOOD FIBRINOGEN

Temperature 40°C

Volume of Fibrinogen	Amount of Venom	Time of Action	Weight of Fibrinogen Recovered Unchanged
5 Cc.	None		0.085 Grams
5 Cc.	1/10 Cc. 1%	1 Min.	0.040 Grams
5 Cc.	1/10 Cc. 1%	30 Min.	0.015 Grams

TABLE NO. V

THE EFFECT OF THE VENOM OF C. ADAMANTEUS

ON THE HEAT COAGULATION OF BLOOD FIBRINOGEN

Experiment No.	Volume Fibrinogen Solution	Volume 1% Venom	Volume 0.9% NaCl	Coagulation at 56°C	Other Coagulation Point	Biuret Test After Boiling
1	10 Cc.	None	None	Heavy	None	Negative
2	10 Cc.	1 Drop	None	Slight	82°C	Positive
3	None	1 Drop	10 Cc.	None	None	Negative

TABLE NO. VI

ACTION OF VARYING AMOUNTS OF VENOM

OF C. ADAMANTEUS ON FIBRINOGEN DIGESTION

AT A TEMPERATURE OF 40°C

Volume of Fibrinogen Solution	Amount of Venom	Time of Action	Weight Coagulated By Boiling
5 Cc.	None	10 Min.	0.0244 Grams
5 Cc.	1/100c. 1%	10 Min.	0.0170 Grams
5 Cc.	1/100c. 1%	10 Min.	0.0162 Grams
5 Cc.	1/10 Cc. 1/100%	10 Min.	0.0142 Grams

TABLE VII

ACTION OF THE VENOM OF C. ADAMANTEUS ON BLOOD FIBRINOGEN

	Experiment No. 1	Experiment No. 2	Experiment No. 3
Volume of Fibrinogen Solution	5 Cc.	5 Cc.	5 Cc
Amount of Venom	None	1/10 Cc. 1%	1/10 Cc. 1%
Time of Action of Venom on Fibrinogen		10 Min.	60 Min.
Amount Precipitated by 1/2 Saturated NaCl	0.0595	0.0013	0.0007
Amount Coagulated at 100°C	0.0007	0.0369	0.0376
Total Salt and Heat Precipitate	0.0600	0.382	0.0369
Non-coagulable by heat (by difference)	None	0.318	0.0331

TABLE VIII

ACTION OF VENOM OF C. ADAMANTEUS ON SERUM GLOBULIN

Volume Globulin	Volume Venom	Grams Venom	Recovered by Coagulation
10 Cc.	None	None	0.0635 Gram
10 Cc.	0.2 Cc.	0.002	.0667 Gram

TABLE IX

ACTION OF VENOM OF C. ADAMANTEUS ON SERUM ALBUMIN

Volume of Albumin Solution	Volume Venom	Grams Venom	Recovered by Coagulation
5 Cc.	None	None	0.5425 Gram
5 Cc.	0.2 Cc.	0.002	0.5436 Gram

TABLE X

THE EFFECT OF THE VENOM OF C. ADAMANTEUS ON SYNERESIS

Plasma	1% $CaCl_2$	Final Concentration of Venom	Volume of Venom Solution			
1 Cc.	.30	None	0.25 Cc.	10 min.	30 min.	6 hrs.
1	.30	None	0.25 Cc.	—	—	— — —
1	.30	1-50.000	0.25 Cc.	— — —	— — —	— — —
1	.30	1-30.000	0.25 Cc.	—	— —	— —
1	.30	1- 5.000	0.25 Cc.	o	o	—
1	.30	1- 1.000	0.25 Cc.	o	o	—

The — indicates the relative amount of serum expressed by the contraction of the clot.

TABLE XI

THE EFFECT OF VENOM OF C. ADAMANTEUS ON THE ACTIVATION OF SERUM BY CEPHALIN

Expt. No.	Plasma Cc.	Serum Cc.	Serum Cephalin Cc	Serum Cephalin Venom Cc.	Clotting Time Minutes	Remarks
1	1	1/2			3.00	
2	1		1/2		0.70	
3	1			1/2	32.0	The cephalin venom mixture added directly to the serum
4	1		1/2		1.80	Cephalin made acid 8/10% 5 minutes at 100°C. Neutralized before adding to serum.
5	1			1/2	5.00	The cephalin venom mixture was treated as in No. 4
6	1	1/2			4.80	Experiments 4, 5 and 5 made simultaneously.
7	1		1/2		.18	Untreated cephalin added to serum.
8	1			1/2	.20	Venom treated as in 4 and 5; then added to cephalin. Mixture added 10 minutes later to the serum.
9	1	1/2			1.50	Experiments 7, 8 and 9 made simultaneously.

The serum cephalin mixture was made from 9 Cc. serum and 1 Cc. 1/2% cephalin emulsion.

The serum cephalin venom mixture was: Serum 9 Cc., 1/2% cephalin 1 Cc., 1% venom 1/10 Cc.

EXPERIMENT XII

20 cc. 1% cephalin emulsion require 1.8 cc. N/20 KOH for neutralization Phenolphthalein indicator.

20 cc. 1/10% venom require 0.4 cc. N/20 KOH.

100 cc. 1% cephalin and 10 cc. 1/10% venom were mixed and maintained at 40°C. Fifteen minutes later 20 cc. of this required 2.2 cc. N/20 KOH. This shows that 0.53 cc. additional were required to neutralize the excess acidity. Thirty minutes later the same volume of N/20 KOH was required, and one hour later 2.25 cc.,or an increase of 0.05 over the fifteen minute period.

In this experiment the ratio of venom to cephalin was 1 to 100.

When the ratio was changed to 1 to 1000,twenty cc. required at the end of fifteen minutes at 40°C. 2.25 cc. N/20 KOH,while the blank for this number was 1.78 cc. before the reaction.

The excess acidity is,therefore,equivalent to 0. 47 cc. N/20 KOH. Then a small amount of venom can accomplish about as much splitting as the larger. The reaction apparently is checked by the formation of acid.

TABLE XIII

THE EFFECT OF THE VENOM OF C. ADAMANTEUS COMBINED WITH TISSUE FIBRINOGEN ON THE CLOTTING OF PLASMA.

Citrated Plasma	$CaCl_2$ 1 %	Mixture	Time in minutes after mixing fibrinogen and crotalin	Temp. 40° C. Clotting Time
1 cc	0.35 cc	None	None	9.50
1	0.35	0.10 cc	2	8.00
1	0.35	0.10	11	6.00
1	0.35	0.10	19	5.75
1	0.35	0.10	25	5.75
1	0.35	0.10	33	7.00
1	0.35	0.10	41	11.00
1	0.35	0.10	54	11.00
1	0.35	0.10	68	12.00
1	0.35	0.10	81	11.00
1	0.35	0.10	94	12.00

The mixture was a solution containing tissue fibrinogen in a concentration of 1 in 1,000 and Crotalus Venom in a concentration of 1 in 20,000.

TABLE XIV

ACTION OF THE VENOM OF C. ADAMANTEUS ON TISSUE FIBRINOGEN IN VIVO

App. wt. rabbit	Amount of Venom		Amount of Tissue Fibrinogen		Total Dose Cc.	Time until death. Minutes.	Remarks
570	0.5 cc.	1%			0.5	5	No thrombose
600	1	6%			1	3	Small thrombose
800	1	5%			1	6	Slight thrombose
700	1	1%			1	20	No thrombose
600			1.5 cc.	1.5%	1.5	3	Extensive intravascular clotting.
650			0.3	1.5%	0.3	4	Thrombose produced
550	0.5	1%	0.5	1.5%	1	5	No thrombose
600	0.5	1%	0.5	1.5%	1	6	No thrombose
800	1.25	1%	0.25	6%	1.5	3	No thrombose
600	0.5	1%	1	1.5%	1.5	3	Thrombose produced

All injections into marginal ear vein.