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I hereby recommend that the thesis prepared under my supervision by Edwin E. Dunn Jr.

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THE SEPARATION OF THE ENZYMES AND TOXIC PRINCIPLES FROM THE VENOM OF THE RATTLESNAKE

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THE SEPARATION OF THE ENZYMES AND TOXIC PRINCIPLES FROM THE VENOM OF THE RATTLESNAKE.

by

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I. Introduction.

The physiological action of the venoms of poisonous snakes has been a subject of continued interest for a great many years, on account of the multiplicity of the manifestations accompanying poisoning by the venoms.

The subject has been extensively investigated by S. Weir Mitchell and E. T. Reichert(1), Noguchi(2), Arthus(3), Houssay and his co-workers(4), and recently by Markowitz, Essex and Mann(5).

Arthus(6) pointed out the close resemblance between the effects following the injection of cobra and rattlesnake venoms, and those accompanying anaphylactic shock, espedially the fall in blood pressure, and dyspnea, followed by respiratory failure. This resemblance was also observed by Markowitz and Essex(7) in studying the venom of Crotalus Atrox. These authors pointed out the resemblance between poisoning by Crotalus venom and histamine

poisoning, though they regard the suggestion that the effects of Crotalus venom may be due to histamine as untenable. The reddening and production of a wheal when a small amount of Crotalus venom is injected intradermally is also similar to that produced by histamine, and by the injection of a protein antigen under the skin of a sensitized animal. When larger doses of venom are administered by this path, there occurs a distinct destruction of tissue around the site of injection.

Injection of Crotalus venom into the peritoneum is followed by production of areas of hemorrhage on the peritoneum and within the lungs. It is stated by Preioni(8) that venoms activate the digestive enzymes within the pancreas, causing the dissolution of tissue and hemorrhage.

The changes produced in the blood by venoms are of great physiological importance. The agglutination and lysis of erythrocytes and leucocytes by Crotalus venom was studied by Flexner and Noguchi(9,10), who concluded that agglutination, lysis of erythrocytes, and hemolysis were all due to separate substances. They reported that a temperature of 75-80^{o}C. maintained for thirty minutes does not decrease the hemolytic power of Crotalus venom, but that this property is markedly diminshed at a temperature of 90-96^{o} C.

McFarland and Weston(11) reported the agglutinin destroyed by heating to 75-80°C.

Essex and Markowitz(12) found that injection of Crotalus venom caused an increase in the viscosity of the blood of the dog, and an increase in the ratio of erythrocytes to plasma, due to the erythrocytes swelling and assuming a spherical shape. This swelling occurs prior to, and is closely associated with, hemolysis. Neither agglutination nor swelling and hemolysis occur when the blood is oxalated, or when the corpuscles are washed free from plasma. This confirms the observation of Flexner and Noguchi(9).

It has been suggested by Belfanti(13) that poisoning by venoms is due to their action in converting lecithin of the tissues of the poisoned organism into lysolecithin, and that lysolecithin in the organism is the real poison, since it is intensely hemolytic, and, as shown by Guerrini(14), causes capillary hemorrhage in the kidney when injected into the renal artery.

Snake venoms produce very marked effects on the clotting of blood. The venoms of Crotalus Adamanteus and Naja Tripudians(the Indian Cobra) are anticoagulant in effect, while those of Crotalus Terrificus and Daboia Russelli are strongly coagulant. It is true that the venom of Crotalus Adamanteus, when injected intravenously in large doses into small

animals(50 mg. per kg. in the rabbit)(17) produces thrombosis. However, in poisoning of man and the larger animals, the anticoagulant effect is the important one. Of the fourteen venoms studied by Houssay and Sordelli(15) that of Crotalus Adamanteus was the only exception to the rule that venoms coagulant in vivo are also coagulant in vitro. Perhaps this difference is only apparent, they suggest, and the thrombin action is masked by the rapid destruction of fibrinogen.

It was shown by Morawitz and confirmed by Mellanby(16) that the anticoagulant venoms, expecially that of the cobra, destroy cytozyme(cephalin). Crotalus Adamanteus is included in this list by Houssay and Sordelli(15) who, though cognizant of the rapid destruction of fibrinogen, attribute the anticoagulant action of the venom of Crotalus Adamanteus to the destruction of cytozyme. It was shown by Billing(17) and confirmed by Mathews(18), that though the venom of Crotalus Adamanteus changes cephalin, rendering it incapable of converting prothrombin into thrombin, the principal cause of the failure to clot is the rapid destruction of fibrinogen. It has been shown by Mills and Mathews(19) that the mechanism of clotting of blood by tissue fibrinogen is separate and distinct from that of thrombin clotting. It seemed possible, since tissue fibrinogen clotting does not require

free cephalin, that tissue fibrinogen might neutralize the hemorrhagic and anticoagulant effects of Crotalus venom. It was found however,(17,20) that, while Crotalus venom counteracts the ability of tissue fibrinogen to cause intravascular clotting, injection of tissue fibrinogen simultaneously with the Crotalus venom increases the toxicity of the venom. The toxicity of Crotalus venom has been successfully neutralized by solutions of sodium ricinoleate.(Carmichael) (21). After mixing the venom solution with the soap, as much as five times the lethal dose could be injected into rabbits without producing death. Preliminary experiments indicated that Crotalus venom could be similarly detoxified in vivo.

Since enzymes have been shown by recent work particularly that of Waldschmidt-Leitz and his co-workers,(22) to be remarkably specific in their action on substrates, it would naturally be anticipated that the splitting of the unsaturated fatty acid from the cephalin molecule and a lysis of fibrinogen should be due to the activities of different enzymes. Following the suggestion of Dr. Mathews, I undertook to separate from each other and from the associated material the substances responsible for the digestion of protein and the splitting of cephalin.

Separation of the toxic constituents of Crotalus venom has been attempted by a number of workers. Flexner and Noguchi (10) concluded from their observations that the neurotoxic, hemorrhagic, cytolytic, and agglutinating properties of the venom were each due to separate constituents of the venom.

Marshall(23) was able, by fractional precipitation of the proteins of the venom, to separate the very toxic albumin fraction from the nontoxic globulins. This work was repeated and verified by Welker(24), who found but slight toxicity in the fraction precipiatated by 33-46% ammonium sulfate, while that fraction precipitated by 46-64% saturation was very toxic. Welker found, as did Essex and Markowitz(25), who recently repeated the ammonium sulfate precipitation and obtained the same results, that repeated precipitation resulted in very marked loss of toxicity.

Mitchell(1) described a toxic substance, acting principally on the nervous system and heart. This substance, which he named "crotalin" was an albumose, not destroyed by boiling, and precipitated by alcohol.

Faust(26) reported the isolation of an alcohol-insoluble nitrogen-free "crotalotoxin" from the venom of Crotalus Adamanteus. Proteins were removed by precipitation with alkaline copper acetate or colloidal iron, or by heat coagulation at 85^{0}C., and albumoses were precipitated from the

filtrate by meta-phosphoric acid. To the product he asigned the formula $C_{34}H_{54}O_{21}$, and concluded his "crotalotoxin" to be identical with hemorrhagin, cytolysin, cytotoxin, and hemolysin, and similar in action to sapotoxin. He states, however, that he observed no effect on blood clotting either in vivo or in vitro.

Michel(27) reported separation of the toxin, hemolysin, and agglutinin of venoms by ultrafiltration. Houssay(28) has separated the hemolytic substances of snake venoms by adsorption from solution by animal charcoal.

II. Experimental.

The Crotalus venom used in this work was obtained from the same source as that used by Billing(17) and was the sample used by Carmichael(21) and by Professor Mathews(18) The dried venom had been preserved at the temperature of the laboratory fro at least six years, but had been kept protected from direct light. The venom was dissolved in 1.0% sodium chloride solution, and the undissolved material(which constitutes 4.4% of the original dried venom) removed by filtering, leaving a clear yellow solution. These solutions were invariably acid, the hydrogen-ion concentration as determined by use of the quinhydrone electroded being 5.6-5.9 (pH). Solutions were kept at 2°C., and no solution was used more than three days after being made up.

1. Digestion of Proteins by Crotalus Venom.

The work of Siebert(29) has shown that trichloracetic acid added to a solution containing proteins and their decomposition products, precipitates only the whole proteins, leaving albumoses and simpler decomposition products of the protein in the filtrate. In order for this separation to be complete. the concentration of protein in the solution must not be more than 1.0% and a final concentration of trichloracetic acid of 16% is necessary. In the same investigation, phosphotungstic acid was used to precipitate the intermediary protein decomposition products including the diamino acids, and the difference between the nitrogen of the trichloracetic acid filtrate and the nitrogen of the phosphotungstic acid filtrate was taken as albumose nitrogen.

This method of separation of whole protein from the products of digestion was used in the experiments which follow. Experiments were performed in the following manner: Duplicate samples of the protein solution were incubated at 38°C. with a small amount of the venom solution, neutralized to pH 7.4. Control samples containing no venom were incubated at the same time. Each sample was diluted to give a protein concentration not greater than 1 %.

After dilution, four volumes of a 20% solution of trichloracetic acid were added to one series, two volumes of phosphotungstic acid solution(5% phosphotungstic acid containing 30 cc. sulfuric acid (Sp. Gr. 1.84) per liter) to another series. To the control samples which had been incubated without addition of venom, trichloracetic or phosphotungstic acid was added, then an amount of venom equal to that contained in the samples incubated with venom. After mixing well and allowing the solution to stand at least fifteen minutes, the precipitated protein was filtered off, leaving a clear filtrate. Total nitrogen was determined on a portion of this filtrate, using the Kjeldahl procedure, modified as follows:

To a portion of the filtrate in a 300 cc. Pyrex Florence flask was added 3 cc. concentrated sulfuric acid, 0.3 cc. 2% coppersulfate solution, and 0.5 gram anhydrous sodium sulfate. The samples were heated on a sand bath until fifteen minutes after they became clear, usually two hours altogether. After addition of 75 cc. water and 10 cc. of 40% NaOH, the ammonia was distilled over into 25 cc. N/100 sulfuric acid, using an ordinary short Liebig condenser. The excess sulfuric acid was titrated with N/100 NaOH, using alizarine red as indicator.

The nitorgen content of the compounds in the venom which are not precipitated is included in the nitrogen figure obtained for the control samples incubated without venom. Therefore, subtraction of the nitrogen figure obtained for the control from that obtained for the sample incubated with the venom gives the nitrogen content of the compounds split off from the protein by the venom. The difference so obtained, the increase in soluble nitrogen produced by the action of the venom is the figure recorded in the tables.

Proteolytic Action of Crotalus Venom on Blood Plasma.

Beef blood collected from young, healthy animals, and containing three grams trisodium citrate per liter, was thoroughly centrifuged twice to remove all cells and the clear plasma so obtained was kept at $2^{\circ}C$.

Experiment 1.

3 cc. samples of this plasma were incubated with 0.5 cc. 1% venom, neutralized to pH 7.4, for thirty minutes. Samples and controls were then removed from the incubator, diluted, and the protein precipitated, and the nitrogen content of the filtrate determined as described above. The total nitrogen value of the incubated sample and control sample are given in this case, as well as their difference. The fibrinogen nitrogen of the plasma was determined by heating a 3 cc.

sample at 56°C. for fifteen minutes, separating the coagulat-fibrinogen by centrifuging, and washing three times with 5 cc. 1% NaCl. This washed coagulum was dissolved in 1% NaOH by heating, transferred to a flask, and the nitrogen determined.

Insert Table I.

The results given in Table I show that the proteins of the plasma are digested by a proteinase in the Crotalus venom, yielding hydrolytic products precipitated by phosphotungstic acid, but not by trichloracetic acid. No mono-amino acids were split off by this digestion.

Proteolytic Action of Crotalus Venom on Serum.

Fresh beef serum was used for this experiment. Blood was collected from young healthy animals and allowed to clot with out defibrinating. After standing four hours, the serum which had been pressed out from the clot was separated from corpuscles by repezted centrifuging.

Experiment 2.

This experiment was performed in the same manner as the experiment with plasma, using 3 cc. of th serum and 0.5 cc. of the neutralized 1% Crotalus venom solution.

Insert Table II

Table I.

Proteolytic Action of Crotalus Venom on Plasma.

	Mg Filtrate N.	
	Trichloracetic Filtrate	Phosphotungstic Filtrate
Plasma Venom	1.70	0.72
Plasma (control)	0.97	0.72
Difference	0.73	0.00
Total Fibrinogen Nitrogen		3.185 mg.

Table II.

Proteolytic Action of Crotalus Venom on Serum.

	Mg. Filtrate N.	
Time of Incubation	30 min.	2 hours.
Serum Venom	1.49	3.15
Serum (control)	0.88	0.82
Difference	0.61	2.33

Table II demonstrates that Crotalus venom digests the proteins of the serum. This digestion is especially marked after a two-hour incubation period.

Proteolytic Action on Serum Albumin.

Crystalline serum albumin was prepared from fresh horse serum according to a combination of the methods of Young and Adair and Robinson, used by Boyd(30). The final solution of the albumin after recrystallization was dialyzed for three days against distilled water in the refrigerator, toluene being added to prevent bacterial decomposition.

Experiment 3.

To 10 cc. of the dialyzed serum albumin solution was added 2 cc. venom solution and 3 cc. distilled water. This mixture was incubated for two hours at 38°C., along with controls containing no venom. Total nitrogen determination was also made on 5 cc. of the albumin solution.

Insert Table III.

This experiment shows that Crotalus venom has a slight but very definite effect in splitting serum albumin. Approximately 7.5% of the total nitrogen appears in the trichloracetic acid filtrate and very little in the phosphotungstic acid filtrate, indicating that the principal products of digestion are albumoses and peptones rather than mono-amino

Table III.

Proteolytic Action of Crotalus Venom on Serum Albumin.

	Mg. Nitrogen.
Total Nitrogen (5 cc.)	9.66
Trichloracetic Filtrate	0.72
Phosphotungstic Filtrate	0.08

acids. Billing(17) found no decrease in the amount of coagulated albumin obtained by heat coagulation after one hour incubation with the venom. However, purified crystalline serum albumin was not used in his experiments.

Proteolytic Action of Crotalus Venom on Fibrin.

In order to determine the extent of digestion of fibrin by Crotalus venom, dried fibrin was used. Centrifuged citrated plasma was recalcified and allowed to clot. The fibrin formed was washed thoroughly until perfectly while, then ground in a food chopper, and kept under distilled water at 2°C. for several days. After removal from the water, the fibrin was dried on a glass plate in a current of warm air, with frequent turning. The grayisn scales of the dried material were coarsely pulverized in a mortar and kept in a desiccator.

Experiment 4.

80 mg of the dried fibrin were suspended in 45 cc. of 1% NaCl in a 150 cc. Erlenmeyer flask, and allowed to stand two hours, At the end of this time, the fibrin particles had taken up water and regained their usual appearance. 5 cc. each of 1% venom was added to two of the samples, and the flasks were incubated at 38°C. for twleve hours. Each flask contained 0.1 cc. 10% thymol.

At the end of the incubation period, in the flasks containing no venom, the fibrin remained unchanged in appearance and the solution was clear, but in those containing venom the fibrin particles had disintegrated, leaving a finely divided residue on the bottom of the flask, and the solution was cloudy. 5 cc. of venom solution was added to each of the control samples and all were filtered immediately. 10 cc. of this filtrate were taken for nitrogen analysis. Other 10 cc. portions were precipitated by trichloracetic acid and phosphotungstic acid. A distinct protein precipitate was observed in all samples with both these precipitating agents. The nitrogen content of the filtrated was determined as in previous experiments, and values given in the table are for 20 mg. fibrin in all cases. 20 mg fibrin samples were taken for total nitrogen determination.

Insert Table IV.

The results given in this table show that the solid fibrin has been dissolved, with the splitting off of albumose or peptone, and a slight amount of mono-amino acids. The digestion is slow, however, since no change in the fibrin particles can be observed in less than six hours. These results confirm the observations of Professor Mathews on the slow rate of digestion of fibrin. Billing(17) reported that small amounts

Table IV.

Digestion of Fibrin by Crotalus Venom.

	Mg. N.	% total N.
1. Total N. (20 mg. Fibrin)	3.02	100.0
2. Filtrate N.	1.82	60.1
3. Trichloracetic Filtrate N.	1.46	48.4
4. Phosphotungstic Filtrate N.	0.28	9.4
5. Insoluble Protein N. (1 minus 2)	1.20	39.9
6. Soluble Protein N.--Ppted. by Trichloracetic. (2 minus 3)	0.36	11.7
7. Albumose, peptone, Di-amino acid N. (3 minus 4)	1.18	39.0

of venom added to recalcified citrated plasma immediately before clotting occured increased syneresis of the clot, while larger amounts decreased it. He observed no digestion of the clot up to six hours after clotting.

Proteolytic Action of Crotalus Venom on Casein.

The ability of Crotalus venom to clot milk is very weak, in comparison to its ability to cause rapid destruction of fibrinogen(Mathews)(18). The rennin action of the venom was compared with that of an extract of dried calves' stomach.

Experiment 5.

0.5 gram of dried gasolin extracted calves' stomach mucosa was extracted with water for thirty minutes. The extract was filtered through cotton to remove the solid material. In order to activate the rennin, sufficient hydrochloric acid was added to make the solution 0.10 N. acid. After fifteen minutes standing the solution was neutralized by adding 0.2N sodium carbonate. The total volume of solution was 50 cc.

The milk used was fresh market milk less than twenty-four hours old, kept in the refrigerator until used, The hydrogen-ion concentration of the milk was pH 6.50. After addition of the indicated quantity of rennin or venom solution was added the calcium chloride solution, and the

samples of milk were placed in a water bath at 40°C. The clotting time recorded was the time necessary to form a firm clot, breaking cleanly from the side of the tube.

Insert Table V.

These observation show that the rennin activity of the venom solution is approximately one-tenth that of the 1% extract of calves' stomach. The samples containing the venom were allowed tostand twenty-four hours at room temperature after clotting. While all samples showed shrinking of the clotted casein, no distinct sign of resolution or digestion could be detected.

Dissolved casein, according to Houssay and Negrete(31), is quickly transformed by the proteolytic venoms, so that it is no longer precipitated by acetic acid. In order to determine the proteolytic action of Crotalus venom on dissolved casein, the following experiment was performed.

Casein was prepared from fresh milk by the method of Van Slyke and Baker(32) by precipitating with normal lactic acid. Much less lactic acid was required than is given in their directions, 85 cc. being sufficient to coagulate the casein from 1500 cc. of milk. The precipitated casein was washed with alcohol and ether and air dried.

Table V.

Clotting of Milk by Crotalus Venom.

1% rennin cc.	1% venom cc.	1% $CaCl_2$ cc.	Milk cc.	Clotting Time Min.
1.00	----	0.05	3.0	1'40"
0.50	----	0.05	3.0	2'00"
0.20	----	0.05	3.0	3'15"
0.10	----	0.05	3.0	5'00"
0.06	----	0.05	3.0	6'30"
----	1.00	0.05	3.0	5'00"
----	1.00	0.00	3.0	7'00"
----	0.50	0.05	3.0	6'00"
----	0.25	0.05	3.0	13'20"
----	0.00	0.05	3.0	no clot

Experiment 6.

One gram dry casein was dissolved in 50 cc. 0.5 N. NaOH, the solution was neutralized to pH 7.1 by adding 30 cc. 10% acetic acid, and after adding 0.5 cc. 10% alcoholic thymol solution, the solution was made up to 200 cc. in volume. Total nitrogen determination were made on 10 cc. portions of this solution.

To 20 cc. portions of this solution were added 2.5 cc. each of 1% NaCl and 2.5 cc. of 1% Crotalus venom solution. All samples were incubated at 38°C. One series was removed for analysis at the end of two, the other at the end of twelve hours. Upon first adding the trichloracetic acid, no precipitate was produced, the solution remaining opalescent, but upon standing for four hours, a small amount of precipitate settled out, and could be filtered off, leaving the filtrate clear. Casein was precipitated immediately in the controls incubated without venom. In the samples precipitated with phosphotungstic acid solution, precipitate formed at once in all cases. The following table shows the increase in nitrogen content of the filtrates, calculated for 10 cc. of the original casein solution.

Insert Table VI.

Table VI.

Proteolytic Action of Crotalus Venom on Casein.

	Mg. N.		% total N.	
	2 hr.	12 hr.	2 hr.	12 hr.
1. Total N.	7.85	7.85	100.0	100.0
2. Trichloracetic Filtrate N.	5.62	6.09	71.6	77.7
3. Phosphotungstic Filtrate N.	1.43	2.34	18.2	29.8
4. Protein ppted. by Trichloracetic--N. (1 minus 2)	2.23	1.76	28.4	22.3
5. Albumose, Peptone Diamino Acid N. (2 minus 3)	4.19	3.75	53.4	47.9

These figures show that Crotalus venom digests casein readily, yielding a detectable amount of mono-amino acids, and a considerable amount of intermediate decomposition products. The results also show that increase of the time of incubation from two to twelve hours results in a relatively slight increase in nitrogen in the trichloracetic acid filtrate, but there is a marked increase in mono-amino acid nitrogen(phosphotungstic acid filtrate.)

2. Method of Determination of Proteinase and Cephalinase.

Determination of Proteinase Content of Preparations from Venom.

Billing(17) demonstrated that fibrinogen, but the action of Crotalus venom, is rapidly converted into an albumose and a protein coagulating at 82°C. The measurement of the amount of albumose formed upon incubation of plasma with the venom was used in the work reported here as a means of determining the proteinase content of the whole venom and of the fractions prepared from it. Plasma was used in preference to fibrinogen solution because of its greater stability. Since there is no increase in the nitrogen of the phosphotungstic acid filtrate when plasma is incubated with Crotalus venom for thirty minutes, the increase in nitrogen in the trichloracetic acid filtrate may be considered as the nitrogen of the albumose and peptone formed by the action of the Crotalus venom.

3 cc. samples of citrated beef plasma were incubated for thirty minutes with 0.5 cc. of the 1% venom solution, or with quantities of the solution to be tested in proportion to the expected activity. Trichloracetic acid filtrates were made and nitrogen determinations run according to the procedure given in detail above. The nitrogen content of the compounds split off from the plasma protein by the action of

the venom was taken as a measure of the proteinase activity of the venom or of the fraction being tested.

Determination of Cephalinase Content of Preparations from Venom.

Billing also demonstrated that, when an emulsion of cephalin is incuvated with Crotalus venom, the acidity of the solution increases, and yields a soap after neutralization, evaporation to dryness, and extraction of the residue with alsohol. This increase in acidity, due to the splitting off of the unsaturated fatty acid from the molecule, was used as a measure of the cephalinase content of the venom and preparates.

Purified cephalin was prepared from fresh sheep brain, following the procedure of Wadsworth and Maltaner(33), except that, in place of ether containing 3 % of added water, absolute ether was used for extraction and resolution. A somewhat larger yield was obtained than that reported for beef brain. The nitrogen content of the preparation was 1.58% and the phosphorus content, 3.56%. Phosphorus was determined by the method of Fiske and Subarrow, as modified by Schmidt(34). These figures agree closely with those obtained by Wadsworth and Maltaner. The cephalin, after purification, was kept under absolute alsohol at 2°C. to prevent the oxidation produced by exposure to air(35). When the cephalin was

to be used, it was separated from alcohol, dissolved in ether, and the ether evaporated under vacuum at room temperature, then the dry cephalin was emulsified in distilled water without heating. This 1% emulsion was preserved against bacterial action by the addition of an amount of 10% alcoholic thymol solution sufficient to five a concentration of 0.025% thymol. It has been shown(36) that thymol, in concentration of 0.014% is sufficient to inhibit growth of micro-organisms. Thymol does not inhibit the action of the venom cephalinase on the cephalin.

The cephalinase determination was first made as follows: To 20 cc. 1% cephalin emulsion in distilled water were added 2 cc. 1% venom solution or a portion of the preparate to be tested. After incubation, the mixture was diluted with 100 cc. distilled water, and titrated with N/100 NaOH, using phenolphthalein as indicator. Another 20 cc. portion of cephalin emulsion containing 2 cc. 1% NaCl was incubated and titrated at the same time, to give the blank titration value, and the amount of standard base necessary to neutralize the quantity of venom sample sued was also subtracted from the titration value of the incubated venom-cephalin sample. This method proved fairly satisfactory, but, due to the apalescence of

the solution, the end point of the titration was hard to observe accurately.

A more satisfactory procedure consists in separating the fatty acids from the unchanged cephalin and lysocephalin by extraction of the evaporated residue with acetone. According to Levene, Rolf and Simms(37) lysocephalin is insoluble in acetone and ether. The detailed procedure was as follows:

To 20 cc. 1% cephalin emulsion was added 2 cc. of the venom solution, and the mixture was incubated at 38°C. as before. The mixture in a 100 cc. beaker was evaporated to dryness on the steam bath. Usually about 1 1/2 hours was required to evaporate just to dryness. To the dry residue was added 30 cc. of acetone. After the residue had been loosened from the beaker with a stirring rod, the acetone was heated to boiling, allowed to cool five minutes, and filtered. This extraction was repeated three times more, and the combined extracts evaporated, leaving a small oily drop. This was dissolved in 50 cc. 95% alcohol, and the solution titrated to a faint pink with phenolphthalein. A blank sample containing 20 cc. cephalin emulsion and 2 cc. 1% Na Cl was carried through the same procedure. Subtraction of the titration value of the blank from that obtained with the venom sample gave the increase in acidity due to fatty acids split off from the cephalin by the venom. This figure taken as a measure of the cephalinase content of the venom preparates, is the one given in the tables.

3. Adsorption Behavior of the Enzymes of Crotalus Venom.

Adsorption procedures have been used with much sucess in the separation and purification of the constituents of toxins and of mixtures of digestive enzymes. Roux and Yersin(38) showed the adsorption of diphtheria toxin by calcium phosphate. Following the discovery of Flexner and Noguchi(9) that the brain tissue was capable of detoxifying venoms, Landsteiner and Raubitschek(39) showed that tetanus toxin, arachnolysin, and the neurotoxin and hemolysin of cobra venom could be adsorbed by cholesterol, stearic acid and palmitic acid and by protagon. Jaque and Zunz(40) demonstrated that tetanus and cobra hemolysins could be adsorbed by animal charcoal, kaolin, and barium sulfate.

Though the adsorption of digestive enzymes was discovered as long ago as 1861 by Brucke(41) who reported the adsorption of pepsin on calcium phosphate and cholesterol, the most important work on the separation of enzymes by adsorption has been accomplished by Willstaetter, Waldschmidt-Leitz, and their co-workers(42) inthe separation of the enaymes of the pancreatic juice by adsorption procedures involving the use of aluminum hydroxide, ferric hydroxide, and kaolin. In connection with this work, they have differentiated four different types of aluminum hydroxide suspension, differing from each other in their method

of preparation, chemical composition, and in specificity of adsorption behavior. The most useful of these is the variety designated by Willstaetter and Kraut(43) as C_γ. In addition to its use in separating proteolyticenzymes, this type has been used by Maschmann in the separation of tetanus spasmin from tetanolysin by adsorption procedures.(44)

Aluminum hydroxide C is prepared by precipitation from aluminum sulfate solution by dilute ammonia without prolonged heating. It corresponds in chemical composition to the formula $Al(OH)_3$. The compound obtained is very unstable. As Waldschmidt-Leitz says(22):

" The alumina gel C_α, which is obtained under the mildest conditions, is characterized by the ease with which it suffers alteration. In aqueous suspension it is changed after a short time, into a second compound, C_β, which while likewise unstable is more stable than the α-modification, and is then gradually transformed into the third modification C_γ. With these transformations, which are to be attributed to a chemical alteration of the molecule----there is connected an alteration of the basic and acid properties , and of the adsorption behavior of the gels, they are not to be explained by alterations in the colloidal state"------"Experiments show that this indifference of trypsin toward adsorption by alumina in acid solution holds only for the stable gel $Al(OH)_3$, designated as C_γ, whereas the trypsin is completely adsorbed by using an insufficiently aged alumina preparate, which contains varying quantities of the unstable β-modification".

Adsorption Behavior of Crotalus Venom Toward Aluminum Hydroxide Type C.

A similar variation in behavior toward the enzymes of Crotalus venom has been observed. The aluminum hydroxide type C was prepared April 14, 1931, according to the method

given by Willstaetter and Kraut(43). After freeing from sulfate and ammonia by many washings, water was added, making a thick suspension.

Experiment 7.

20 cc. of 1% Crotalus venom solution was mixed with 4 cc. of the aluminum hydroxide suspension(equivalent to 0.160 g. Al_2O_3), allowed to stand thirty minutes, then separated by centrifuging. The adsorption was repeated with another 4 cc. portion of the aluminum hydroxide suspension. After separation of the aluminum hydroxide suspension, the solution was then adjusted to a pH of 7.2 by adding N/100 NaOH, and the proteolytic activity of the solution after adsorption was determined as described above, using 0.5 cc. solution to 3 cc. plasma. The cephalinase activity was also determined, using 2.0 cc. of the adsorbed solution.

The first series of experiments was performed from one to three weeks after preparation of the aluminum hydroxide, the second after eight months aging, and the third after twelve months.

Insert Table VII.

Thses results show that the freshly prepared aluminum hydroxide suspension adsorbed both cephalinase and proteinase. After eight months aging, the cephalinase was completely

Table VII.

Adsorption by Aluminum Hydroxide C.

Sample	Date	Filt. N. Mg.	Cephal-inase cc. NaOH
1. Venom	4/31	0.70	2.85#
Adsorbed Sol.		0.00	0.00
2. Venom	12/31	0.78	4.10
Adsorbed Sol.		0.49	0.00
3. Venom	4/32	0.76	4.40
Adsorbed Sol.		0.42	3.50

cephalin sol. prepared from commercial cephalin.

adsorbed, leaving the major portion of the proteinase in the residual solution, and after twelve months neither the proteinase nor the cephalinase was adsorbed. This change in adsorption behavior would seem to be due to a change in the nature of the aluminum hydroxide, since it is parallel to that reported with trypsin.

Since the freshly prepared aluminum hydroxide adsorbs both cephalinase and proteinase, and all the proteins, leaving the separated solution free from materials giving the biuret test, it seemed that the toxic principles should also be removed. The following experiment demonstrates that the toxic principle is adsorbed.

Experiment 8.

To demonstrate the toxicity of the whole venom, 2 cc. of a 1% solution of dried venom were injected intraperitoneally into a female rat wieghing 195 grams. Paralysis and labored breathing were observed within twenty minutes and the animal died 1 3/4 hours after injection. Autopsy revealed profuse hemorrhage within the peritoneum, and the blood was incoagulable.

10 cc. of the same venom solution were shaken with 2 cc. of aluminum hydroxide C(0.080 g. Al_2O_3) for thirty minutes, the aluminum hydroxide separated by filtering and a second 2 cc. portion added, and the mixture again shaken. After

filtering, a cc. of this filtrate was injected intraperitoneally into a 200 gram female rat. The animal, though restless for fifteen minutes, appeared normal during the next two days, but was found dead 60 hours after injection. Autopsy revealed the blood clotted, and no signs of hemorrhage could be observed.

A 5 cc. portion of 2 % venom solution was shaken twice for thirty minutes with 1cc. suspension of Aluminum hydroxide C. and separated by filtering. This filtrate was injected intraperitoneally into a female rat weighing 325 g. The animal appeared uneasy for thirty minutes after injection, but showed no other effects, and appeared normal for fifteen days after the injection. A rat weighing 285 g. given 1 cc. of untreated venom(2%) died in three hours. Autopsy showed profuse hemorrhage and incoagulability of the blood.

These experiments demonstrate that the greater portion, if not all of the toxicity of the venom has been adsorbed by shaking with aluminum hydroxide, since 5 cc. of this filtrate corresponds to 50 mg. of the original venom, 10 mg of which is sufficient to kill a 250 gram rat in 7 1/4 hours, as will be seen from Table XIII.

As shown by series 3 in Table VII, aluminum hydroxide after one year's aging, adsorbed neither cephalinase nor proteinase at the normal hydrogen-ion concentration of the venom--pH 5.6-5.9. The next experiment was the determination

of its adsorption behavior toward solutions to which buffer solutions had been added. Citric acid-Na_2HPO_4 mixtures, prepared according to McIlvane(45) were used for pH 4.75 and 5.75. For pH 6.9 was used a NaH_2PO_4-NaOH buffer mixture given by Clark(46) substituting NaH_2PO_4 for KH_2PO_4.

Experiment 9.

To a 10 cc. portion of a filtered 1% venom solution were added 4 cc. buffer solution and 4 cc. of aluminum hydroxide C (0.160 g. Al_2O_3) aged twelve months. This mixture was allowed to stand thirty minutes. After separation from the aluminum hydroxide, the solution was brought ot pH 7.4 by addition of N/10 NaOH, and the proteinase and cephalinase content determined, using an amount of th solution equivalent to 0.5 cc. of the original venom solution.

Insert Table VIII.

As will be seen from the table, there is no specific adsorption under these conditions, both adsorbate and residual solution containing both cephalinase and proteinase.

Adsorption by Aluminum Hydroxide A.

Aluminum hydroxide type A is the type used by Willstaetter and Waldschmidt-Leitz to adsorb liapse from pancreatic juice. It is prepared by addition of an excess of ammonium hydroxide

Table VIII.

Adsorption by Aluminum Hydroxide Type C.
from Buffered Solutions.

pH	Filtrate N. Mg.	Cephalinase cc. NaOH.
Normal Venom	0.75	4.40
4.75	0.33	1.85
5.75	0.55	3.65
6.90	0.47	3.35

to aluminum sulfate solution, followed by long boiling, and corresponds in composition to the formula--Al_2O_3.1 1/2 H_2O. Two different lots of this material were made according to the method of Willstaetter and Kraut(43) and both were found to adsorb both cephalinase and proteinase from the Crotalus venom. Attempts to separate the tow enzymes from each other by elution and reapeated adsorption have not been successful. The following experiment illustrates the adsorption behavior of aluminum hydroxide type A.

Experiment 10.

40 cc. of a 1% venom solution were allowed to stand one hour with 10 cc. (0.525 g. Al_2O_3) of aluminum hydroxide A; then separated by centrifuging. The solution was mixed with another portion of aluminum hydroxide suspension(5 cc.) and allowed to stand for one hour, then separated. The solution remaining after separation is designated in the table as "residual solution". The two portions of aluminum hydroxide were combined and eluted with 40 cc. of the buffer 6.9 referred to in Experiment 9, allowed to stand for two hours, and separated by centrifuging. All these samples were adjusted to pH 7.4, and the activity tested as before, using the indicated quantities of the solution for the test.

Insert Table IX.

Table IX.

Adsorption by Aluminum Hydroxide Type A.

	Proteinase		Cephalinase	
Sample	cc. used	Filt. Mg.-N.	cc. used.	cc. NaOH
Venom	0.5	0.72	2.0	5.55
Residual Solution	2.0	0.00	4.0	0.10
Elution	0.5	0.47	2.0	0.90

Both enzymes are completely adsorbed by this treatment with aluminum hydroxide, and both amy be eluted by the neutral buffer solution, but this elution removes much more of the proteinase than of the cephalinase. This result indicated that a second adsorption and elution should yield an elution entirely free from cephalinase, yet with marked proteolytic activity.

Experiment 11.

40 cc. 1% venom solution were allowed to stand with 8 cc. (0.420 g. Al_2O_3) aluminum hydroxide suspension A for thirty minutes, then separated by centrifuging. The separated solution was mixed with another portion of aluminum hydroxide(4 cc.) and allowed to stand for thirty minutes. The aluminum hydroxide was separated and the combined portions of aluminum hydroxide were eluted with 20 cc. of a mixture containing 20 cc. glycerol and 80 cc. of the buffer solution 6.9. After thirty minutes, this elution was separated and a second 20 cc. portion added. These elutions were combined and dialyzed against distilled water for twelve hours to remove phosphates and glycerol before repeating the adsorption. The total volume of solution after dialyzing was 55 cc. This solution was made acid (pH 5.5) by adding 0.25 cc. 10% acetic acid, then mixed with 5.5 cc. (0.240 g. Al_2O_3) aluminum hydroxide type A. After thirty minutes standing the aluminum hydroxide was separated and eluted

twice with 20 cc. of the buffer-glycerol solution as before. This solution was again dialyzed for twelve hours in order to remove the glycerol before testing its activity.

Insert Table X.

The second elution still retains part of the cephalinase, and the separation of proteinase from cephalinase has not been rendered more complete by the repeated adsorption and elution. However, some degree of purification has been attained by this procedure, since the nitrogen content of the elution has been reduced to 0.203 mg. per cc. The nitrogen of the original venom solution was 1.245 mg. per cc.

Experiment 12.

To determine the toxicity of this elution, a female white rat weighing 180 grams was injected intraperitoneally with 5 cc. of the solution, equivalent to 37 mg. of the original venom. The animal showed distress after injection, sitting with fur ruffled for several hours. Death occurred between fifteen and one-half and twenty-two hours after injection, and autopsy revealed hemorrhage on the peritoneum around the site of injection and around the ribs. The blood was clotted, and the auricle of the heart was filled with a large clot. Another female rat, wieghing 80 grams was injected intraperitoneally at the same time with 2 cc. of the same solution. She showed the same signs

Table X.

Adsorption by Aluminum Hydroxide Type A.

Sample	Proteinase		Cephalinase	
	cc. used	Filt. N. Mg.	cc. used	cc. NaOH
Venom	0.5	0.70	2.0	3.90
Second Elution	0.7	0.33	4.0	1.20

of distress, and was in stupor for many hours, but after twenty-four hours appeared normal, and continued so as long as observed. This outcome indicated that some of the toxicity of the venom had been removed by the adsorption procedure , but that it still remained decidedly toxic. This is another indication that toxicity isnot due entirely to the production of lyso compounds from the phospholipids, as Belfanti (13) suggested, since the cephalinase content of this solution is very low.

Adsorption Behavior of Aluminum Hydroxide Types B and D.

Preliminary experiments with aluminum hydroxide types B and D showed no specific adsorption , either of cephalinase or of proteinase. The adsorbed solutions contained somewhat less both of cephalinase and of proteinase.

Adsorption Behavior of Lloyd's Alkaloidal Reagent.

Lloyd's alkaloidal reagent (hydrous aluminum silicate) designed for adsorption of alkaloid bases from solution, may also be used for adsorption of proteins. This property suggested that it might be of value in removal of proteins from the venom solution, or in adsorption of the active constituents. A preliminary experiment showed that the material had a high calcium content, which is partially extracted upon washing with water. In odder to avoid the presence of calcium in the solution after the adsorption process, the reagent was

suspended in distilled water, allowed to stand for some time, then separated. This was repeated until the solid material no longer separated completely from the water , and the wash water was free from calcium.

Experiment 13.

To a 20 cc. portion of 1% venom was added one gram (dry wieght) of the washed Lloyd's reagent. This was mixed and allowed to stand thirty minutes, then the reagent separated by centrifuging, and the procedure repeated with a second portion of the reagent. To another 20 cc. portion of 1% venom solution was added three grams of washed Lloyd's reagent, and the mixture allowed to stand for one hour. After centrifuging, the slightly opalescent liquid from each was tested for cephalinase and proteinase in the usual way. Both solutions gave a slight calcium test with ammonium oxalate.

Insert Table XI.

These results show that adsorption of cephalinase is complete and that of proteinase nearly so.

Adsorption Behavior of Diatomaceous Earth.

Experiment 14.

20 cc. of 1% venom solution were shaken with diatomaceous earth (2g.) and allowed to stand thirty minutes, then the diatomaceous earth removed by filtering. Cephalinase and proteinase were determined in the filtrate.

Insert Table XII.

Table XI.

Adsorption by Lloyd's Reagent.

Sample	Proteinase		Cephalinase	
	cc. used	Filt. N.-Mg.	cc. used	cc. NaOH
Venom	0.5	0.70	2.0	4.60
Ads. Once. (3 g.)	2.0	0.03	4.0	0.00
Ads. Twice (1g. each)	2.0	0.04	4.0	0.15

Table XII.

Adsorption by Diatomaceous Earth.

Sample	Proteinase		Cephalinase	
	cc. used.	Filt. N. Mg.	cc. used	cc. NaOH
Venom	0.5	0.75	2.0	4.05
Residual Sol. after Ads.	0.5	0.45	2.0	2.45

These figures indicate that approximately 40% of each of of the enzymes has been adsorbed. This is possibly due to removal of the protein from the solution, carrying with it some of the enzyme.

These attempts to separate the enzymes of Crotalus venom by adsorption procedures indicate that $Al(OH)_3$ type C. in its intermediary stage is a specific adsorbent for cephalinase and does not adsorb the proteinase. None other of the adsorption agents tried have shown specific adsorption behavior toward these enzymes.

4. Separation and Properties Of Cephalinase.

Faust(26), in reporting the isolation of crotalotoxin, showed that, when the slightly acidified venom solution was heated rapidly to 85°C., cooled, and filtered at once, the filtrate contained the major portion of the substance which acts upon the central nervous system and respiratory center, while the washed precipitate was inactive. This observation suggested that it might also be possible to separate in this manner the substances active in producing the incoagulability of the blood.

Experiment 15.

A 25 cc. portion of filtered 1% venom in a 8xl inch test-tube was acidified to pH 5.3 by adding 0.25 cc. 1% acetic acid, immersed in a beaker of boiling water, and heated with constant stirring just to 85°C. The tube was then removed, cooled rapidly under a stream of cold water, The precipitated protein was filtered off, and washed with 5 cc. 1% NaCl solution, the washing being added to the filtrate. Another portion was acidified, heated to 65°C., filtered and washed in a similar manner. After neutralization with N/10 NaOH, the cephalinase and proteinase of the filtrates were determined.

Insert Table XIII.

Table XIII.

Effect of Heat on Venom Enzymes.

	Proteinase		Cephalinase	
Temperature.	cc. used	Filt. N. Mg.	cc. used	cc. NaOH
Unheated Venom	0.5	0.72	2.0	3.90
65°C.	1.0	0.68	2.0	3.40
85°C.	1.0	0.00	2.0	3.20

After heating to 65°C., the filtrate has approximately half the proteolytic power of the original venom, while about 90% of the cephalinase is retained. Heating to 85°C., however, leaves the filtrate entirely free from proteinase, but causes little decrease in cephalinase content. It would appear therefore, that the proteinase is more closely associated with the protein than the cephalinase, since heating to 85°C. causes coagulation of an additional quantity of protein not coagulated at 65°C. This is in harmony with the theory postulated by Mathews and Glenn(47) in 1911, and by Willstaetter in 1922(48) that enzymes are composed of an active group attached to a colloid bearer of the same chemical nature as the substrate which they attack. Heating to 85°C. does not completely destroy the proteinase, since by extraction of the precipitated protein with 20% urea solution and dialysis of this extract, a slightly active solution is obtained.

In order to test the toxicity of the filtrate obtained after heating the venom to 85°C., a male white rat, weighing 133 grams, was injected intraperitoneally with 3 cc. of the neutralized filtrate, Slight distress due to the injection was observed at first, but this soon passed, and no other effects were observed. This result indicated that the major portion of the toxic substance had been removed or destroyed by heating to 85°C., since 2 cc. of the original venom

solution injected into a 200 gram rat caused severe shock after five minutes, and death in 1 3/4 hours.

In Faust's preparation of crotalotoxin, he found that albumoses could be removed by precipitation with metaphosphoric acid, without precipitating the physiologically active principles.

Experiment 16.

25 cc. of the venom solution was heated to 65°C. as described above, cooled, filtered and washed. To the filtrate was added 1 cc. 10% metaphosphoric acid, a slight excess over the amount necessary to cause complete precipitation After filtering off the precipitate, the filtrate was neutralized with n/10 NaOH and its activity tested. Both proteinase and cephalinase were found to be entirely absent.

It was found that, after heating the 1% venom solution to 85°C. and filtering off the precipitated protein, the addition of three volumes of 95% alcohol to the acid filtrate caused the formation of a flocculent precipitate. After this precipitate was removed and the alcohol evaporated from it in vacuo, the material dissolved readily in 1% NaCl and was active in splitting cephalin.

The final method used for the separation of the cephalinase fraction from the venom solution was as follows:

To 25 cc. 1% solution of Crotalus venom in 1% NaCl was added 0.25 cc. 1% acetic acid to bring the reaction to pH 5.2. This addition of acid was found necessary to insure complete coagulation of the protein, leaving a clear filtrate. The venom solution was then heated rapidly to 85°C., by immersion in a beaker of boiling water, then cooled to 20°C. and filtered, the heating and cooling requiring not more than three minutes. The precipitate was washed once with 5 cc. 1% NaCl, and the washings added to the filtrate. To the still acid solution were added three volumes of 95% ethyl alcohol. After standing thirty minutes the precipitated protein material was completely flocculated and could be separated by centrifuging. After separation, the precipitate was washed once with 95% alcohol, and the alcohol removed by placing in a vacuum desiccator under high vacuum for several hours. 1.00 gram of the original dry venom yielded 0.117 grams of pure white amorphous solid, which dissolved in 1% NaCl quite readily. Variation of the procedure of preparation by addition of four volumes of alcohol, or by allowing the precipitated material to stand under alcohol for forty-eight hours did not increase the yield of solid material, nor did it decrease its solubility in 1% NaCl, but after the longer time of contact with the alcohol, the material was less active, milligram for

milligram, than that which had been allowed to stand only thirty minutes under alcohol. A solution of the redissolved material gives biuret, Millon, and reduced sulfur tests and a copious precipitate by addition of picric acid. The solution is acid, a 0.2% solution having an approximate pH of 5.2. heating the solution to boiling produces no coagulation, The material consists principally of primary albumose, since a heavy precipitate is produced by the addition of nitric acid or acetic acid, and by half saturation of the solution with ammonium sulfate. Complete sautration of the solution after removal of the material precipitated by half saturation yields a slight precipitate. The presence of peptones could not be detected in the filtrate after removal of the precipitate.

The enzyme activity of the separated cephalinase fraction was compared with that of the original venom. A solution of the former was made up in 1% NaCl, and the cephalinase activity tested as usual , as well as the proteinase activity.

Insert Table XIV.

These results show that the cephalinase fraction has about five times the activity of the same weight of original venom, and that it has no proteolytic action on plasma. The apparently greater quantity of nitrogen in the cephalinase control sample

Table XIV.

Enzyme Content of Cephalinase.

	Proteinase		Cephalinase	
	Mg. used	Filt. N. Mg.	Mg. used.	cc. NaOH
Venom	5.0		20.0	
Sample		1.95		5.10
Control		1.01		1.20
Difference		0.94		3.90
Cephalinase	4.0		2.0	
Sample		0.80		3.25
Control		0.81		1.20
Difference		0.00		2.05

is without significance, since 0.10 cc. N/100 NaOH is equivalent to 0.017 mg. nitrogen under these conditions.

Experiment 17.

It was discovered by Mitchell(1) that venom lecithinase is destroyed by boiling. This was also found to be true of the cephalinase just described. A neutralized solution(0.5%) of cephalinase material in 1% NaCl was heated fifteen minutes in boiling water. The cephalinase content of the solution was determined in the usual manner, using 0.8 cc. of the boiled solution (equivalent to 4 mg. of the dry material) and 20 cc. of the 1% cephalin emulsion. The increase of acidity was equal to 0.20 cc. N/100 NaOH, while 2.15 cc. N/100 NaOH was required to neutralize the acid split off by the cephalinase in an equal quantity of the unboiled solution.

Toxicity of Cephalinase Material for White Rats.

The toxicity of the cephalinase solution was compared wtth that of the original venom. The results are given in the following table. Both venom andcephalinase were dissolved in 1% NaCl, filtered and neutralized to pH 7.2. then the indicated amount was injected intraperitoneally.

Insert Table XV.

Table XV.

Toxicity of Venom and Cephalinase.

Wt.	Sex	Venom cc.	Venom mg.	Cephalinase. cc.	Cephalinase. Mg.	Toxic Effects.
195	Female	2.0	20.0			Death in 1 3/4 hr. Hemorrhage.
290	Female	1.0	20.0			Death in 3 hr. Hemorrhage.
200	Male	1.0	10.0			Death in 10 hr. Hemorrhage.
250	Male	2.0	10.0			Death in 7 1/4 hr. Hemorrhage.
160	Female	1.0	5.0			Lived, in stupor 2 days. Hemorrhage at site of injection
240	Female			5.0	10.0	Death 35-46 hr. No Hemorrhage.
230	Male			5.0	25.0	Death in 12 hr. Some Hemorrhage.
190	Female			5.0	25.0	Death 24 1/2 hr. Hemorrhage. Blood clotted.
290	Male			5.0	25.0	Death 12 1/2 to 19 hr. no Hemorrhage Blood clotted.
180	Female			5.0	25.0	Death 10 hr. Hemorrhage in thorax. Blood clotted.

These observations show that, while the cephalinase material is still definitely toxic, it is less so than an equal wieght of the original venom. 25 mg of the cephalinase fraction is approximately as toxic as 10 mg. of the original venom. This shows definitely that the toxicity is not due primarily to the cephalinase, since the cephalinase material is five times as active in splitting cephalin as is the original venom. The production of hemorrhage is important in the poisoning by the whole venom, while the effect of the cephalinase fraction would seem to be predominantly on the central nervous system, since the aminals injected with it remain in stupor for many hours before death, and the hemorrhage observed is slight. This view is confirmed also by the results obtained on subcutaneous injection. A comparison of the activity of the cephalinase material with that of the original venom in producing local hemorrhage and lesion was made. The indicated quantity of material dissolved in 1% NaCl was injected subcutaneously in the abdominal wall.

Insert Table XVI.

With the exception of the animal numbered 21, in none of the animals, were other effects than the local ones noticed.

Table XVI.

Production of Local Lesions by Venom and Cephalinase.

Wt.	Sex	Venom cc.	Venom mg.	Cephalinase cc.	Cephalinase mg.	Toxic Effects.
160	Female	1.0	5.0			Hematoma 1 hr. Bleeding 5hr. Lesion closed 18 hr. healed 10 days.
175	Female	1.0	2.5			Hematoma 30 min. Bleeding 1 hr. lesion closed 8 hr. Healed 8 days.
80	Male	1.0	1.0			Moderate shock Normal 24 hr. No lesion.
68	Male			1.0	5.0	slight distress# No lesion.
163	Female			2.0	10.0	Hematoma 2 hr. slight hematoma 18 hr. No other effects.
80	Male			1.0	5.0	Slight distress slight hematoma 4 hr. no other effects.

Part of solution leaked through hole made by needle.

These results show that cephalinase has little effect in producting local lesions in the abdominal wall.

Experiment 18.

The toxicity of the boiled cephalinase solution was determined in connection with Experiment 17. 5 cc. (equivalent to 25 mg.) of the neutralized solution which had been boiled for fifteen minutes was injected into the peritoneum of a female rat weighing 175 grams. Slight distress was observed immediately after the injection, but after one hour, behavior was normal and recovery appeared complete the next day. A 180 gram rat given 5 cc. of the neutralized unboiled cephalinase solution intraperitoneally showed distress, appeared in stupor for several hours, growing gradually weaker and died ten hours after injection. The blood was clotted, though there was hemorrhage in the thorax and around the site of injection. This demonstrates that the toxicity of the cephalinase fraction is diminished or entirely destroyed by boiling for fifteen minutes.

5. Hemolysis by Venom and Cephalinase.

It was found by Flexner and Noguchi (10) that Crotalus venom had no power to hemolyze completely washed erythrocytes unless normal serum or plasma were added. This was also confirmed by Essex and Markowitz, (12) who observed that hemolysis was preceded by a swelling of the cells, and that neither the increase in volume nor hemolysis occurred with thoroughly washed erythrocytes. Kyes and Sachs(49) have shown that the substance which enable the venom to hemolyze the corpuscles was extractable from blood by alcohol, and that lecithin alone of all the alcohol extractable substances in blood enabled the venom to hemolyze the erythrocytes. Since "lecithin" at that time (1907) was a mixture of lecithin and cephalin, it seemed possible that pure cephalin should be able to accomplish this action also.

Experiment 19.

The erythrocytes were separated from citrated beef blood by thorough centrifuging, and washed three times with 1% NaCl to free from plasma. 4 cc. of the compact mass of centrifuged corpuscles was made to 100 cc. with 0.85% NaCl. In each tube was placed 1 cc. of this suspension, and 1 cc. of the venom solution of the dilution indicated. The control tube contained 1 cc. 0.85 % NaCl. To one series of tubes was added 0.05 cc.

each of 1to 1000 cephalin solution, the other series contained only venom and corpuscles. The samples were thoroughly mixed and placed in the incubator at 38°C. The extent of hemolysis was observed at the indicated intervals.

Insert Table XVII.

These observations show clearly that the addition of cephalin, giving a total concentration of cephalin less than 1 part in 45,000 is sufficient to enable the Crotalus venom to hemolyze the erythrocytes, and hemolysis does not occur at all without this addition.

The fraction of the venom containing the cephalinase, but free from proteolytic activity, has the power to hemolyze erythrocytes upon addition of cephalin, as is shown by the following table. Experiments were performed in the same manner as in the preceding experiment, using the solution of the cephalinase material whose preparation is described above.

Insert Table XVIII.

This production of hemolysis by the cephalinase material raises the question--Is cephalin the hemolysin, and is the

Table XVII.

Hemolysis by Venom.

C. H.---Complete Hemolysis

M. H.---Marked Hemolysis

H.---Definite Hemolysis

S. H.-Slight Hemolysis

V. S. H.-Very Slight Hemolysis.

0--No Hemolysis

	Time of Incubation.							
	1 hour		2 hours		4 hours		6 hours	
Venom Dilution	venom	venom ceph.	venom	venom cephal.	venom	venom ceph.	venom	venom ceph.
1-100	0	S.H.	0	S.H.#	0	S.H.#	0	M.H.#
1-1000	0	S.H.	0	S.H.	0	S.H.	0	0
1-10000	0	V.S.H.	0	S.H.	0	S.H.	0	0
1-50000	0	0	0	0	0	0	0	0
1-100000	0	0	0	0	0	0	0	0
0	0	0	0	0	0	0	0	0

methemoglobin.

Table XVIII.

Hemolysis by Cephalinase.

Dilution	Time of Incubation.							
	1 hour		2 hours		4 hours		6 hours	
	C.#	C. ceph.	C.	C. ceph.	C.	C. ceph.	C.	C. ceph.
1-500	0	0	0	V.S.H.	0	H.	0	M.H.
1-1000	0	0	0	0	0	H.	0	H.
1-10000	0	0	0	0	0	V.S.H.	0	H.
1-50000	0	0	0	0	0	0	0	H.
1-100000	0	0	0	0	0	0	0	V. S.H.
0	0	0	0	0	0	0	0	0

#C.--Cephalinase.

hemolytic effect due as Belfanti(13) suggests, to a lysocephalin produced by the action of Crotalus venom on cephalin, or is the hemolysin an entirely separate substance, associated with the cephalinase? It has been definitely shown by Levene and Noguchi(37) that lysocephalin is markedly hemolytic, sothe former explanation may be the true one. If this be the case, then hemolysis should be produced by the products of the action of the cephalinase on the cephalin, after removal of the unchanged cephalin and cephalinase.

Experiment 20.

20 cc. 1% cephalin emulsion and 2 cc. solution containing 4 mg. of the cephalinase preparation were incubated together for two hours, the mixture was evaporated to dryness and the dry residue extracted four times with 20 cc. portions of hot 95% alcohol, in order to dissolve the products of the action of the cephalinase, leaving the unchanged cephalin and cephalinase undissolved. The extract was filtered hot, and the filtrate evaporated to dryness. 165 mg of the original cephalin yielded 100 mg. extractable by theaalcohol. After evaporating the alcohol, a solution of this residue designated in the table as A. S. M. was made up in 1% NaCl, and the hemolytic activity of the solution tested as was the venom solution. Since cephalin itself is somewhat hemolytic, the hemolytic action of a cephalin emulsion was tested at

the same time for comparison.

Insert Table XIX

Table XIX shows little difference between the hemolytic action of the untreated cephalin, and that of the alcohol soluble material produced by the action of the cephalinase on the cephalin. If the ehemolysis were due to this latter substance alone, distinct hemolysis should be produced in six hours with a dilution of 1-45,000. As a matter of fact, there is no hemolysis with a dilution of 1-10000. This indicates that hemolysis is not due to lysocephalin or similar products, but is due to a substance associated with the cephalinase. This preparation was made by Mr. D. Irish in connection with some other work(50) and it was thought that the test of its hemolytic activity might give interesting results.

The preparation was made following the procedure of Levene and Rolf(51) for separation of lysocephalin. 100 g. pure cephalin was emulsified in water along with 4 g. of the Crotalus venom, and the total volume made up to 1500 cc. After this mixture had been incubated 24 hours at 40°C., then three volumes of acetone were added to the solution to precipitate the lipoid material. The precipitate was washed with acetone, then with ether, dissolved in warmed absolute

Table XIX.

Hemolytic Activity of Cephalin and its Split Products.

	Time of Incubation.							
	1 hour		2 hours		4 hours		6 hours	
Dilution	ASM	ceph.	ASM	ceph.	ASM	ceph.	ASM	ceph.
1-50	0	--	H.	--	M.H.	--	M.H.	--
1-100	0	S.H.	S.H.	M.H.	M.H.	C.H.	M.H.	C.H.
1-1000	0	0	0	0	H.	V. S.H.	H.	V.S.H.
1-10000	0	0	0	0	0	0	0	V.S.H.
1-100000	0	0	0	0	0	0	0	0
0	0	0	0	0	0	0	0	0

#A.S.M.--Alcohol soluble Material.

alcohol, precipitated with five volumes of absolute ether, re-dissolved in absolute alcohol and again precipitated with ether. Approximately eight grams of pure white material was obtained, containing 2.52% nitrogen and 4.05% phosphorus.

Experiment 21.

An emulsion of this material in 0.85% NaCl was made, and its hemolytic activity was tested as before.

Insert Table XX.

Hemolysis is produced by this material with surprising rapidity. The greater activity of the 1 to 1000 dilution is probably to be explained by the high viscosity of the 1-50 and 1-100 dilution, Therefore Crotalus venom produces by its action on cephalin, a substance having strong hemolytic powers, but it is doubtful if hemolysis by the venom can be ascribed wholly to this substance, since it is not probable that a sufficient quantity would be formed in the samples in Tables XVII and XVIII where only one part of cephalin is present in 45,000 of the s lution. Further investigation is required before it can be ascertained whether hemolysis is due to compounds formed from the phospholipids by the action of the venom, or whether the phospholipid functions as complement.

Table XX.

Hemolysis by Purified Lyso Compound.

Dilution	Time of Incubation.				
	0	15 min.	30 min.	1 hour	6 hour
1-50	M.H.	M.H.	C.H.	C.H.	C.H.
1-100	M.H.	M.H.	C.H.	C.H.	C.H.
1-1000	C.H.	C.H.	C.H.	C.H.	C.H.
1-10000	0	H.	M.H.	C.H.	C.H.
1-100000	0	0	0	0	0
0	0	0	0	0	0

6. Transformation of Hemoglobin to Methemoglobin.

It was found in testing the proteolytic activity of the Crotalus venom that, when plasma containing a small amount of hemoglobin was incubated with the whole venom, a noticeable change in color from the bright red of hemoglobin to a chocolate brown was observed at the end of the half hour incubation period. This change in appearance was also observed in the hemolysis experiments reported in Table XVII. with a 1 to 100 concentration of venom. In connection with experiments on the hemolytic action of his crotalotoxin, Faust(26) reported the presence of methemoglobin in the samples after three or four hours incubation with a concentration of 1 to 100 of the crotalotoxin.

Experiment 22.

Erythrocytes were separated from fresh beef blood by centrifuging, washed twice with 1% NaCl, and laked by diluting to ten volumes with distilled water. To 5 cc. of this solution was added 0.05 cc. of 1% venom, and the mixture incubated at 38°C. for sixteen hours. The sample showed a distinct darkening of color at the end of that time, and upon dilution and examination of the spectrum by means of a direct vision spectroscope, the two adsorption bands in the greenpart of the spectrum which are characteristic of

oxyhemoglobin were seen to be absent, while the control samples containing no venom showed no change in color, and the absorption bands of oxyhemoglobin could be seen plainly. The same results were obtained with samples to which toluene had been added to prevent bacterial action.

A solution of hemoglobin was prepared by the method of Welker and Williamson(52). 500 cc. of corpuscles freed from plasma by washing with 1% NaCl were laked with 300 cc. of distilled water. 800cc. of aluminum hydroxide suspension prepared according to the method of Marshall and Welker(53) were added to remove the proteins of the stroma. The mixture was allowed to stand three hours, then centrifuged to remove the aluminum hydroxide, and filtered. The final volume was approximately 1200 cc.

Experiment 23.

To 5 cc. of this solution were added the indicated quantities of 1% Crotalus venom solution, sufficient 1% NaCl to make a total volume of 6 cc. and 1 cc. toluene. All samples were incubated for twelve hours at 38°C. The hemoglobin content of the samples was determined by measuring the oxygen-combining capacity of a 1cc sample. The procedure used was that of Sendroy(54) except that

saponin was omitted from the ferricyanide solution, since, with hemoglobin solution, no hemolytic agent was needed. Hemoglobin values are given in milligrams per 100 cc. of the hemoglobin solution.

Insert Table XXI.

These results show that hemoglobin completely loses its oxygen combining power when incubated with Crotalus venom. This transformation does not occur immediately upon addition of the venom to the hemoglobin solution as is shown by the last sample in the Table. In this case, the venom had only three minutes(the aeration period) to act upon the hemoglobin.

Measurements were made of the absorption spectrum of a hemoglobin solution which had been incubated with one-tenth its volume of 1% Crotalus venom, and these were compared with those of a methemoglobin solution of equal dilution prepared by the addition of potassium ferricyanide to hemoglobin solution. In both a distinct absorption band in the red portion of the spectrum was observed extending from 625 to 640μμ. The absorption band of alkaline hematin in this part of the spectrum extends from 590 to 620μμ, so it is clearly methemoglobin and not alkaline hematin which is produced. Reduction

Table XXI.

Venom--cc.	Hemoglobin Mg./100 cc.
0.00	6.7
0.05	4.1
0.10	1.9
0.20	1.4
0.50	0.0
0.50#	6.2

#--Added just before analysis.

of alkaline hematin by ammonium sulfide produces hemochromogen, while reduction of methemoglobin by this reagent produces reduced hemoglobin, which, when aerated, gives the characteristic spectrum of oxyhemoglobin. Ammonium sulfide was added to a sample of the incubated hemoglobin-venom mixture, and the sample thoroughly shaken. Examination showed absorption bands at 570-585 , and 533-547μμ corresponding exactly with those of oxyhemoglobin(575-585μμand 532-550μμ). The corresponding bands of hemochromogen are located at 551-565 and 523-535μμ.

These observations show definitely that Crotalus venom has the power of slowly transforming hemoglobin to methemoglobin. The substance responsible for this action is readily adsrbed from the venom by aluminum hydroxides types A and C., ferric hydroxide, diatomaceous earth, and Lloyd's alkaloidal reagent, and is not eluted by phosphate buffer solution, N/100 Na_2CO_3, N/50 NH_3, or by M/30 Na_2HPO_4.

III. Summary and Conclusions.

1. The ability of Crotalus venom to destroy cephalin (cytozyme), and its ability to digest proteins are due to separate substances in the venom.

2. Crotalus venom contains one or more proteolytic enzymes which digest proteins of plasma and serum and crystalline serum albumin, fibrin and dissolved casein. The proteinase studied here is probably different from that which acts with such speed on fibrinogen.

3. Results indicate that the separation of the proteolytic enzymes from cephalinase from cephalinase may be accomplished by adsorption with aluminum hydroxide type C, which has been aged eight months.

4. The toxic principles, enzymes and proteins of the venom are all adsorbed by the freshly prepared aluminum hydroxide type C.

5. There has been prepared from the venom an albumose fraction which contains the cephalinase, but is free from proteolytic activity. This fraction is free from heat coagulable protein, and the substance which imparts the yellow

color to the venom. This material cannot be positively identified with any of the compounds or fractions previously isolated from Crotalus venom.

6. A portion of the toxicity of the original venom is retained by the cephalinase fraction, but the toxicity does not go parallel to the cephalinase content. This is not in accord with the hypothesis of Belfanti that the toxicity is due to the lysolecithin and lysocephalin produced by their action.

7. The fraction containing the cephalinase has hemolytic powers of the same order as the original venom. This hemolytic activity does not appear to be due to the cephalinase itself, though the venom is capable of producing intensely hemolytic substances by its action on cephalin.

8. Crotalus venom is able to transform hemoglobin into methemoglobin, both in laked corpuscles, and in hemoglobin solution. This transformation is due neither to the cephalinase, nor to the proteinase.

I am greatly indebted to Professor A. P. Mathews, for his kindly interest and helpful criticism during this investigation, and to Mr. Charles G. Merrell, whose generosity has made this work possible.

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IS THROMBIN AN AMINO-PROTEINASE?

I. Introduction.

The addition of formaldehyde to blood plasma prevents clotting by thrombin, due to combination of the formaldehyde with the free amino groups of the fibrinogen(1). Destruction of thrombin, or of some of its constituents other than cephalin is also indicated in the experiments cited. However, since free formaldehyde was not removed before addition of the thrombin to the plasma, it was impossible to distinguish between the effects due to the presence of formaldehyde and those due to changes produced in the fibrinogen. It is the purpose of the experiments here reported to show that formaldehyde produces a permanent transformation of the fibrinogen, and that inhibition of clotting is probably not alone due to the presence of formaldehyde.

II. Experimental.

In the experiments here reported, a purified fibrinogen solution was used in order to eliminate the possible influence of the other plasma proteins. The solution used was prepared from fresh beef plasma containing three grams tri-sodium citrate per liter, by a modification of the method suggested by McLean(2). The preparation of most of the solutions was completed as far as the final re-precipitation on the same day that the blood was collected. 112 g. of solid ammonium sulfate was dissolved in 800 cc. plasma, making the solution one-fifth saturated with ammonium sulfate. The precipitated fibrinogen was separated by centrifiging, and washed once with one-third saturated ammonium sulfate solution, and then re-dissolved in 800 cc. 2% NaCl. 100 cc. of a thick paste of freshly precipitated tri-calcium phosphate was stirred into the solution, and the mixture allowed to stand for three hours in order that the calcium phosphate might adsorb any thrombin present. After separating the calcium phosphate by centrifuging, the fibrinogen was precipitated by adding 144 grams solid NaCl. This precipitate was re-dissolved in 800 cc. 2% NaCl, re-precipitated, and finally dissolved in 800cc. 0.5% NaCl. I have found that initial precipitation by one-fifth satur-

ation of beef plasma with ammonium sulfate gives a precipitate more easily separated and re-dissolved, and a larger yield of fibrinogen than when sodium chloride is used. The opposite is true of horse plasma. It is not advisable to use ammonium sulfate for re-precipitation, however, since other protein material which is not clotted by thrombin is precipitated along with the fibrinogen. The following experiment demonstrates this:

Ammonium sulfate was added to plasma to one-fifth saturation, and the precipitate washed and re-dissolved as above. Fibrinogen was precipitated from half of this solution by one-half saturation with sodium chloride, and from the other half by one-fifth saturation with ammonium sulfate, and each precipitate again re-dissolved in 1% sodium chloride. 10 cc. of each solution was clotted by the addition of 2 cc. of the thrombin solution prepared as described below. The liquid was pressed from the clot, filtered, acidified with acetic acid, and heated to boiling. The liquid obtained after clotting from the solution precipitated with sodium chloride showed only slight opalescence upon boiling, while from the other a heavy coagulum was obtained.

The thrombin was made immediately before use from "prothrombase" solution prepared from citrated beef plasma by the mthod given by Mellanby(3). To 90 cc. citrated beef plasma were added 800 cc. distilled water and 70 cc. 1% acetic acid After two hours standing, the precipitate was centrifuged off, and re-dissolved in 80 cc. 0.1% sodium carbonate. The solution was brought to pH 7.0 by addition of 1% acetic acid, allowed to stand until the re-precipitated globulin had settled out, then filtered, yielding a slightly opalescent, colorless solution. This prothrombin was kept in the refrigerator until one hour before using. The prothrombin was converted into thrombin by adding to each 10 cc. of the solution 0.25 cc. M/50 calcium chloride, and 0.25 cc. 0.1% cephalin emulsion in water. After fifteen minutes standing, the solution reached its full thrombin activity.

The Effect of Formaldehyde on the Clotting of Fibrinogen.

The effect of formaldehyde on clotting of fibrinogen was compared with its effect on the clotting of the whole plasma, formaldehyde being present during the clotting tests in both cases.

Experiment 1.

To 10 cc. portions of 0.1% fibrinogen solution were added 0.07 to 1.40 cc. neutralized formalin solution(4.4%) giving

the formaldehyde concentration indicated. After standing the indicated length of time, a 1cc. portion was removed and 1 cc. of thrombin solution added. Samples were placed in a water bath at 40°C., and the clotting time observed.

Insert Table I.

This table shows that formaldehyde, when added to fibrinogen solution and allowed to ramain in contact with it for a sufficient length of time, inhibits its clotting by thrombin in the same manner as it does when added to plasma. It was expected that a much smaller concentration of formaldehyde would be required to completely inhibit the clotting by thrombin. This is not the case, on the contrary, a distinctly greater concentration is required in the fibrinogen solution for complete inhibition of clotting. With two hours contact, 0.19% completely inhibits clotting in plasma, while in fibrinogen solution, the clotting time is increased only from 40 to 85 seconds. With eight hours contact, plasma containing 0.07% formaldehyde is not clotted by thrombin, while under the same conditions, the clotting time of fibrinogen solution in increased from 40 to 60 seconds.

Table I.

Effect of Formaldhyde on Fibrinogen

Final Formaldehyde Conc.	Clotting Time in Seconds					
	Time Exposed to Formaldehyde Before Thrombin					
	0	30 min	1 hr.	2 hr.	4 hr.	8 hr.
0.00%	40	40	40	40	40	40
0.03%	40	45	45	40	40	45
0.07%	45	45	45	40	60	60
0.13%	50	55	60	60	75	100w
0.19%	65	65	65	85	90	no clot
0.24%	75	75	100	135w	no clot	no clot
0.30%	100	230w	210w	no clot	no clot	no clot
0.34%	100w	no clot	no clot	no clot	no clot	no clot
0.41%	105w	no clot	no clot	no clot	no clot	no clot
0.52%	no clot	no clot	no clot	no clot	no clot	no clot

w--weak clot.

Removal of Formaldehyde.

In order to show clearly the action of the formaldehyde on fibrinogen, it was necessary to remove the formaldehyde or to change it to an inactive compound, before the addition of the thrombin. Neutralization by means of ammonia and ammonium chloride is not satisfactory, as will be seen by inspection of the experiments cited later.

In order to determine the residual concentration of formaldehyde in the fibrinogen samples after partial removal, the following test was used(4):

To 1 cc. of the solution to be tested, add 0.05 cc. of 0.05% resorcinol solution and pour concentrated sulfuric acid down the side of the tube to form a layer underneath. A violet or purple ring is produced at the junction of the two layers, if formaldehyde is present. As determined by comparison with standards containing known amounts of formaldehyde, 0.01% gives a faint violet color, 0.005% gives no color. Leach's Fe_2Cl_6-HCl reaction was found to be too sensitive for use under these circumstances, since a distinct color is produced with 0.00001% formaldehyde.

An unsuccessful attempt was made to remove the formaldehyde from plasma by dialysis. Samples of plasma containing 0.12 to 0.28% formaldehyde were allowed to stand for three

hours, then dialyzed against running tap water for sixteen hours, NaCl was added to each to prevent precipitation of globulins. After dialysis, all samples were found to contain at least 0.1% formaldehyde.

It was found that fibrinogen could be freed from formaldehyde by precipitating by adding ammonium sulfate to the solution to one-fifth saturation, washing the precipitated fibrinogen with one-third saturated ammonium sulfate solution, then re-dissolving in 1% sodium choride. This method was used in the following experiments.

Clotting of Fibrinogen Freed from Formaldehyde.

Experiment 2.

A fibrinogen solution containing 0.8 mg. per cc. was prepared from beef plasma as described above. To 25 cc. portions of this solution were added varying amounts of neutralized fromaldehyde (38%) to give the concentration indicated, and an amount of 1% sodium chloride sufficient to bring the total volume of each to 26 cc. At intervals of 1, 2, 4, and 8 hours, a 4 cc. portion of each solution was removed and 1 cc. saturated ammonium sulfate was added. The precipitate was washed three times with one-third saturated ammonium sulfate, and re-dissolved in 4 cc. 1% sodium chloride containing 0.1% Na_2CO_3. All samples redissolved completely (except when a formaldehyde concentration greater that 1% was used) and after being redissolved, gave a precipitate

when the solution was half saturated with sodium chloride. Each sample was tested for formaldehyde by the test given above, and comparison with a known solution showed that not more than 0.005% formaldehyde was present in any sample. The control samples containing no formaldehyde were precipitated, washed and re-dissolved in the same manner as those containing formaldehyde. To one cc. of the redissolved fibrinogen solution was added 0.2 cc. thrombin solution. The clotting time was observed in a water bath at 40° C.

Insert Table II.

The results in Table II. show clearly that fibrinogen has been changed into a form which cannot be clotted by thrombin, probably by the reaction with the free amino groups. The transformation is gradual, and the amount of fibrinogen changed increases with increase of concentration of formaldehyde, and with increasing length of time of contact. This increase is shown by the progressive diminution of the amount of fibrin fromed by the action of thrombin, as well as by the increased clotting time.

The work of Hirose(5) has demonstrated that the action of thrombin on fibrinogen is that of a proteolytic enzyme. Thrombin first unites with fibrinogen to from fibrin, which it hydrolyses further, forming a protein heat coagulable at 78° and an albumose. Thrombin may be recovered from the fibrin

Table II.

Effect of Formaldehyde on Fibrinogen

Formaldehyde Removed.

Final Formaldehyde Concentration	Clotting Time in Seconds. Time Exposed to Formaldehyde. 1 hour	2 hours	4 hours	8 hours
0.00%	40	40	40	40
0.25%	40	60	95w	t-no clot
0.50%	40	100	#-no clot	#-no clot
0.75%	t-no clot	t-no clot	#-no clot	no clot
1.00%	#-no clot	#-no clot	#-no clot	no clot

w--weak clot
t--Fibrin thread
#--Precipitate after 4 min.

solution in the form of its inactive precursor, prothrombin. Since fibrinogen which has been methylenated by the action of formaldehyde is no longer clotted by thrombin, it appears that free amino groups must be present in the fibrinogen molecule to enable thrombin to unite with it, and that thrombin is, therefore, an amino-proteinase.

Comparison of the results given in Table II with those of Table I show that, with the same time of contact with formaldehyde, a much greater concentration of formaldehyde is necessary to inhibit clotting when formaldehyde is removed before thrombin is added, than when it is allowed to remain in the solution. This may possibly be due to a reversal of the reaction between the formaldehyde and the fibrinogen upon removal of the free formaldehyde. Results obtained by Benedicenti(6) would indicate, however, that the reaction is not so readily reversible. Solid casein and fibrin was allowed to remain in formaldehyde solution for 14 days, then filtered off and washed with water until the washings were free from formaldehyde. By steam distillation of a suspension of the washed protein was obtained a constant amount of formaldehyde which had been bound by the protein.

While formaldehyde in sufficient concentration renders fibrinogen incoagulable by thrombin, it would appear that it has some additional inhibitory effect, that it either destroys

prothrombin, or prevents the formation of thrombin. This conclusion is strengthened by the observation(1) that 0.1% formaldehyde is sufficient to prevent completely the clotting of recalcified plasma, and that a formaldehyde concentration of 0.53% is sufficient to inactivate in less than thirty minutes, a very active thrombin solution which normally would remain active for six hours.

The effect of formaldehyde on thrombin formation is not due to action on cephalin. A solution containing 0.5 % cephalin and 0.6% fromaldehyde was allowed to stand for 14 days. 0.5 cc. of thissolution and 0.5 cc. 2.5% $CaCl_2$ were added to 6 cc. prothrombin solution(7). After thirty minutes standing, addition of 0.5 cc. of this thrombin solution to 1 cc. plasma resulted in a solid clot in thirty seconds. Thrombin made by adding the same amount of normal untreated cephalin to prothrombin also produced a clot in thirty seconds.

Removal of Formaldehyde from Thrombin.

In order to decide definitely concerning the action of formaldehyde on thrombin it is clearly necessary to remove the formaldehyde from the thrombin solution or to neutralize it before adding the thrombin to the fibrinogen solution. Since formaldehydereacts readily with ammonia and ammonium chloride to produce hexamethylene tetramine, it seemed possible to render the formaldehyde inactive in this way. If

the inhibitory action of formaldehyde on clotting can be neutralized by the addition of ammonia or ammonium chloride, then an equivalent mixture of the two compounds should be without inhibitory effect. The following experiments, which show that such is not the case, were performed in connection with the work before reported(1), but were not then given in detail.

Experiment 3.

2.5cc. formaldehyde(4%) and 2.5 cc. 4.8% ammonium chloride were mixed and allowed to stand fifteen minutes. The indicated quantities of this mixture were added to active thrombin solution, made by adding to 6 cc. of prothrombin solution prepared by the method of Mills(7) 0.5 cc. 2.5% calcium chloride solution, and 0.5 cc. 0.5% cephalin emulsion. The thrombin was shown to be active before addition of the formaldehyde or ammonium chloride-formaldehyde mixture.

Insert Table III.

Observations given in Table III show that the inhibiting action of the formaldehyde-ammonium chloride solution is less than that of the formaldehyde alone, but it is evident that the addition of ammonium chloride is not a satisfactory method for neutralization of formaldehyde. The use of ammonium

Table III.

Effect of Formaldehyde-NH_4Cl on Clotting.

Time After Addition	CLOTTING TIME IN SECONDS.				
	0 form. 0 NH_4Cl	0.30% form.	0.30% form-NH_4Cl	0.62% Form.	0.62% Form.NH_4
Before	30	30	30	30	30
0	30	105	60	240	45
2 hours	105	420	300	no clot	no clot
3 hours	135	900	390	no clot	no clot
4 hours	135	no clot	480	no clot	no clot

chloride is open to objection on account of the possibility of a change in hydrogen-ion concentration, due to liberation of hydrochloric acid. Use of sodium acid sulfite is not satisfactory on account of the liberation of sodium hydroxide by reaction with formaldehyde.

Similar experiments were performed with an equivalent mixture of ammonia and formaldehyde. Quantities of this faintly alkaline mixture(pH 7.5) were added to the active thrombin solution, giving a total concentration of formaldehyde varying from 0.15 to 1.10% . There was some diminution of the inhibitory effect of formaldehyde by reaction with ammonia, but the action of the mixture was still definitely inhibitory.

Table IV shows the result of the trial of neutralization by addition of ammonia to the thrombin solution after addition of formaldehyde. The normal clotting time of the thrombin was observed, formaldehyde added, the samples allowed to stand fifteen minutes, ammonia added and the clotting time observed again.

Insert Table IV.

Table IV.

Effect of Formaldehyde-NH_3 on Clotting.

Time of Thrombin Test	Clotting Time in Seconds.				
	control 0 form. 0 NH_3	0.31% form. no NH_3	0.31% form. 0.10 cc. NH_3	0.31% form. 0.25 cc NH_3	0.31% form. 0.5 cc NH_3
Before	45	45	45	45	45
After Form.	45	150	150	135	150
15 min.	45	300	270	270	255
After NH_3	45	195	195	no clot	no clot
15 min.	45	285	20 min.	no clot	no clot
30 min.	45	345	no clot	no clot	no clot

Table IV shows that the inhibition of clotting is increased rather than diminished by the addition of ammonia, even when one-fifth of the amount equivalent to the formaldehyde present is used.

In connection with the experiments given in Tables I and II using the Mellanby method of preparation of prothrombin, attempts were made to remove formaldehyde by a method similar to that used in removing the formaldehyde from fibrinogen solution. However, upon precipitation of prothrombin by acetic acid, and washing the precipitate, much of the prothrombin activity was found in the wash water, even when half or fully saturated ammonium sulfate was used for washing. Washing with acetone, alcohol, or ether yielded an inactive preparation.

The action of formaldehyde upon prothrombin is being investigated further.

III. Summary and Conclusions.

1. When added to plasma or fibrinogen solution, formaldehyde partially or completely inhibits clotting of fibrinogen by thrombin. This partial or complete inhibition continues after removal of free formaldehyde from the solution.

2. The inhibition of clotting, due to the union of formaldehyde with the free amino groups of fibrinogen, increases in magnitude with increase of concentration, and with increasing length of exposure. This is an indication that thrombin in clotting blood acts as an amino -proteinase, and unites with the free amino groups present in fibrinogen.

3. Formaldehyde added to cephalin emulsion does not decrease its ability to convert prothrombin into thrombin in the presence of calcium chloride. This shows that the amino group of the cephalin does not enter into the clotting reaction, and indicates that cephalin participates by some other group, such as the phosphoric acid or unsaturated fatty acid.

4. The inhibitory action of ofrmaldehyde on blood clotting is not alone due to union of formaldehyde with the amino groups of fibrinogen, but observations indicate that formaldehyde destroys thrombin or some of its constituents, probably prothrombin, since there is no effect on the power of cephalin to produce active thrombin.

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