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UNIVERSITY OF CINCINNATI

May - 7 - 1932

I hereby recommend that the thesis prepared under my supervision by Ruby Hirose *entitled* Nature of Thrombin and its Action.

be accepted as fulfilling this part of the requirements for the degree of Doctor of Philosophy

Approved by:

C. A. Mills
A. P. Mathews
Shiro Tashiro.

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THE NATURE OF THROMBIN AND IT'S MANNER OF ACTION.

A dissertation submitted to the

Graduate School
of the University of Cincinnati

in partial fulfillment of the
requirements for the degree of

DOCTOR of PHILOSOPHY

1931

by

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Nature of Thrombin and it's Manner of Action.

Professor C. A. Mills suggested to me that I endeavor to secure further information concerning the chemical nature of thrombin and of its manner of action on fibrinogen to produce fibrin. Particularly he wished me to see whether thrombin was a specific proteolytic enzyme acting on fibrinogen, as has been frequently suggested but also questioned. His results recent indicated that it was such an enzyme. I was to study also the further hydrolytic cleavage or dissolution of the fibrin which usually occurs after a clot is formed to discover whether this hydrolysis was due to a slower but continued action of thrombin, which had its main action in the first cleavage leading to the fibrin formation, but which continued to act hydrolytically, though at a slow rate, on the fibrin thus formed.

My work has been guided throughout by constant consultation with Professor Mills and I wish to express here my deep obligation to him. I have also had the advantage of suggestions from Professor A. P. Mathews, and I am particularly indebted to him for criticism, and for his aid in putting the results together.

The general result of the study is to answer in the affirmative the question proposed. Thrombin is an enzyme, although with a very high heat resistance. Its principal

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action is a quick change of fibrinogen into a more insoluble simple, coagulable protein, fibrin, but it apparently continues its action leading to the formation out of the fibrin one or more soluble coagulable proteins, of which the principal one resembles serum albumin and is coagulated by heat at 78-80°C. and an albumose. It appears to be a specific enzyme for fibrinogen. Its clotting action is closely parallel to that of rennin, which transforms soluble caseinogen into insoluble casein; but thrombin will not clot milk. It resembles in some ways the specific fibrinogenase of venom of Crotalus adamenteus, studied by Dr. Billings and Professor Mathews. (1)

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Experimental.

Preparation of Fibrinogen, Prothrombin (serozyme) and Thrombin.

The method of obtaining prothrombin was that based on Bordet's work as developed by Mills (2). It consists in separating prothrombin or serozyme as it is called in Bordet's terminology, from blood plasma, purifying it, and then converting it to thrombin shortly before its use, by the addition of calcium chloride and a little cephalin.

The method more in detail was the following:

(1) Fibrinogen.

Horse plasma was secured by centrifugation of oxalated horse blood; this was freed from fibrinogen by the addition of an equal volume of a saturated solution of sodium chloride. The precipitated fibrinogen was removed with a stirring rod into another beaker; carefully washed with 15 per cent sodium chloride solution containing 0.5 per cent sodium citrate; redissolved in distilled water, since there was enough salt present to facilitate the solution of fibrinogen; and purified by further precipitations until the final solution was colorless. The purified fibrinogen was dissolved in distilled water equal in volume to one third of the original plasma. This was passed through a sterile Berkefeld filter into a sterile flask and this constituted the stock fibrinogen solution used in these experiments. The plasma after removal of

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the fibrinogen was used for the preparation of prothrombin.

The solution of fibrinogen coagulated at the temperature of 52-56°C. If the coagulated protein was filtered off and the filtrate heated again another coagulum was formed at a higher temperature of 72-75°C. No matter how many times the fibrinogen was precipitated there was a slight amount of this second heat coagulable protein present in the solution. I have found that this protein had a tendency to decrease the stability of fibrinogen.

Solution of fibrinogen gives a Biuret, Millon's and xanthoprotein reactions. Fibrinogen constitutes 0.4 to 1.087 per cent of the total plasma proteins.

(2) Prothrombin.

To the filtered plasma freed from its fibrinogen, and at room temperature of about 22°C. There was then added pure crystalline ammonium sulphate to the amount of sixteen grams for each one hundred cubic centimeters of plasma filtrate. This precipitated the prothrombin together with some serum globulin. After the precipitate had become flocculent and the precipitation was complete it was filtered and the collected precipitate carefully washed with a 16 per cent solution of ammonium sulphate to free it as much as possible from extraneous proteins. It was then lifted from the paper by a spatula and dissolved in distilled water and filtered. To the filtrate ammonium sulphate was then added in the same proportion of 16 grams to each one hundred cubic centimeters.

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Again filtered after brief standing; the prothrombin being again dissolved in distilled water. This process of purification was repeated. The final precipitate was dissolved in water equal in volume to one-third of the original plasma. This solution of prothrombin which was perfectly clear and colorless, was then placed in a parchment paper tube and dialysed against running tap water for twenty-four hours, when it was almost completely freed from sulphate and sodium chloride. On dialysis there appeared in the tube a precipitate presumably of a globulin nature, and which on testing proved to have little thrombic activity, on the addition of calcium and cephalin. It was accordingly discarded by filtration through a sterile Berkefeld filter. The clear salt-free prothrombin in a fairly concentrated form was found to possess a faint blue fluorescence.

The prothrombin thus prepared is a globulin, of a rather coarse dispersion, or large molecular size, since it is more easily precipitated by ammonium sulphate than is serum globulin, but not so easily as fibrinogen.

Prothrombin prepared in this way gave very little coagulum on heating to 56°C., but a heavy precipitate was formed when it was heated to 72°C. The coagulating temperature found by Bordet[3] and Mellanby[4] was 56°C. The solution of prothrombin gave a positive Biuret, tryptophane, Millon's, xanthoproteic, glyoxalic and faint ninhydrin and sulphur reactions. Molisch test was negative showing the absence of carbohydrate.

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Prothrombin constitutes about 1.176 to [illegible].95 per cent of the total plasma proteins. Prothrombin belongs to the psuedo-globulin class.

The chemical composition of this prothrombin will be considered more in detail in connection with the thrombin formed from it. It may be said here, however, that there are indications that the active principle is not the globulin which makes so large a part of the prothrombin, but is some smaller molecule attached to this.

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(a). Conversion of Prothrombin to Thrombin.

The prothrombin solution thus obtained has no clotting action on a solution of purified fibrinogen. Nor will it clot oxalated or citrated plasma, or plasma in any way deprived of its calcium salts. Neither will it clot a fibrinogen solution to which a small amount of calcium chloride has been added. Since thrombin will clot such a fibrinogen solution and even in the presence of sodium oxalate, it is clear that prothrombin is not yet thrombin.

The next question I considered was the nature of the change of prothrombin to thrombin, a substance produced by the activation of prothrombin of plasma with calcium and cephalin, and capable of clotting fibrinogen in the presence of excess oxalate.

There has been much controversy as to the nature of this change. Pekelharing (5) one of the first to study it, believed that it was due to calcium salts, and he suggested that thrombin acted really as a carrier of calcium. He thought that for some reason fibrinogen could not unite with calcium to form an insoluble calcium salt, fibrin. It was necessary for a third body, thrombin, to intervene as a calcium carrier. This ingenious conception of the role of thrombin was, however, rendered very improbable by the careful analyses of the calcium content of fibrinogen and fibrin by Hammarsten (6).

Hammarsten found that fibrin and fibrinogen each

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contained the same quantity of calcium, that is about 0.05 per cent. The presence of soluble calcium salt is not a positive condition for the formation of fibrin, because thrombin can transform fibrinogen into fibrin in the absence of lime salts. Fibrin is not richer in calcium than the fibrinogen used in the preparation of the fibrin and thrombin solution which was used contained 0.0007 gms per million as calcium oxide. The relation of calcium to blood clotting will be discussed later in this paper.

That phospholipid is an important element in blood clotting was discovered by Wooldridge (7) who showed that this phsopho-lipid which he obtained from the leucocytes, erythrocyt and brain was different from lecithin. Howell (8) discovered that the active phospholipid was actually cephalin, a brain and tissue phospholipid containing amino-ethyl alcohol in the place of choline of lecithin.

Concerning the method by which a little cephalin causes clotting has been uncertain. Howell has presented experimental evidence that favors the interpretation that it unites with or counteracts an anti-thrombin.

But Bordet (3) has shown that cephalin is necessary to convert prothrombin to thrombin. At any rate prothrombin (serozyme) alone will not clot a fibrinogen solution containing a soluble calcium salt in addition to itself. Nor will it clot a fibrinogen solution containing a little added emulsion of cephalin but no calcium. But if the prothrombin be added to a solution of fibrinogen containing both calcium and cephalin, then clotting occurs.

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Moreover, if the calcium and the cephalin be added first to the prothrombin solution, and then after a few minutes standing to permit interaction this solution be added to a fibrinogen solution containing a little sodium oxalate, the fibrinogen solution is coagulated as rapidly as though the oxalate were not there.

This experiment shows that calcium ions are certainly necessary for the conversion of prothrombin to thrombin, as Howell (8) supposes, but are not necessary for the conversion of fibrinogen to fibrin. Furthermore Mills, on reinvestigating the problem, found that the prothrombin solution as prepared by Howell (9) always contained free cephalin. And he thus supported Bordet's conclusion that both calcium ions and cephalin were necessary to convert prothrombin to thrombin.

Recent work done by Maltaner and Maltaner (10) sheds perhaps a new light on the importance of cephalin. Their investigation on the nature of cephalin was shown that active lipoidal substance combines with calcium of calcium chloride with the liberation of hydrochloric acid in the presence of cephalin. The chemical action of cephalin is associated with the freely ionizable H atom which combines with chlorine of calcium chloride to form the acid. In the presence of this acid lipoids form with protein an insoluble complex fibrin, which the lipoid by itself possesses no precipitating action upon protein with cephalin.

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Cephalin prepared in the laboratory gave an acid reaction to methyl orange indicator (pH. 311-4.4). Also the following combinations gave an acid reactions: Cephalin and prothrombin, cephalin and calcium chloride, and prothrombin-cephalin-calcium chloride (thrombin). Whether this acidity was due to cephalin alone cannot be said here.

The cephalin was obtained from calf brain. It was separated from lecithin so far as possible by repeated solution in ether and then allowing it to stand over-night in the ice-box; the sediment filtered off and 95 per cent alcohol added to precipitate cephalin. The precipitations were repeated five or six times until no more white precipitate was formed by setting the ether solution in the ice-box and the yellow color had disappeared. The purified product contained 1.41 per cent nitrogen and 3.55 per cent phosphorus, which gives an atomic ratio of N:P: :1:1.07 instead of 1:1, both the nitrogen and phosphorus are somewhat lower than the usual figures for cephalin of 1.84 per cent nitrogen and about 4.07 per cent phosphorus (11). (Levene N - 1.84 per cent and P - 3.65 per cent.) Whether this is due to my cephalin containing fatty acids of longer chain than C_{18}. such as arachidonic acid or whether the cephalin contained some water, and was not entirely dry, is uncertain. The analyses would fit a cephalin with two C_{24} acids.

The activity of this cephalin was marked as is shown by the following example.

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I added to the prothrombin solution as prepared, containing 2.95 mgs of prothrombin per cubic centimeter, one-half cubic centimeter, one- half cubic centimeter of 1 per cent calcium chloride solution and allowed it to stand thirty minutes at room temperature to secure the maximum activity of thrombin. One-half cubic centimeter of this mixture was then added to 2.0 cc of a fibrinogen solution, prepared by precipitation from oxalate horse plasma, as described, and placed in a constant temperature water-bath at 38°C. There was no clot in 24 hours, when the tube was examined.

This experiment proves that calcium alone is not sufficient to activate prothrombin as prepared by this method.

To a second 0.5 cc of calcium and prothrombin mixture was then added 0.5 cc. of an aqueous cephalin emulsion (2.0 mgms) and after mixing and standing for thirty minutes, one-half cubic centimeter of this was added to 2 cc. of the fibrinogen solution.

This clotted in 20 seconds. To a third 0.5 cc. of prothrombin and calcium mixture 0.5 cc. of a 1 per cent lecithin emulsion prepared from egg, and after 30 minutes added to 2 cc. of fibrinogen solution. This clotted in 205 seconds. This was due, probably to some cephalin being still in the egg lecithin, as it is very difficult to completely separate them. And to a fourth 0.5 cc. of prothrom-

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bin and calcium mixture 0.5 cc. of 1 per cent emulsion of commercial lecithin was added, and after 30 minutes all was poured into 2 cc. of fibrinogen solution. There was no clot in 24 hours.

Cephalin is, hence, necessary for conversion of prothrombin to thrombin just as is calcium, and as Bordet proved. This experiment clearly demonstrates that cephalin is not acting to neutralize an anti-thrombin, for there is no reason to suppose that the fibrinogen solution thus purified contains any anti-thrombin since it clots promptly on the addition of thrombin.

My first experiments are to show that thrombin unites to fibrinogen to form fibrin, then this subsequently hydrolyses giving active thrombin and a new protein which is coagulated by heat 65°C. If this fibrin solution is allowed to stand for several days albumose appears with the disappearance of thrombin.

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II. The Manner of Thrombin Action.

(a) Thrombin unites with fibrinogen to form fibrin.

Thrombin is a compound of a protein (serozyme or prothrombin), cephalin, and presumably also calcium. Direct determination of the calcium content were made on thrombin and it was found to be present in the easily ionizable from, since it was all precipitated by oxalate. (This is clearly presented in the latter part of this paper). Thrombin that was once formed, acts on fibrinogen even in the presence of excess oxalate or in fairly low amount of calcium; however, the speed of coagulation is not the same as if calcium was present.

Experimental evidence has been presented by Rettger (12) that thrombin enters into a chemical union with fibrinogen to form fibrin. To elucidate this particular point the following investigation was made.

The first point which I wish to show is that thrombin solution successive portion of fibrinogen, and removing each time the fibrin formed. The total amount of fibrinogen used was always in excess to utilize all the thrombin. This amount was predetermined for each sample of thrombin. When fibrin was not formed in one to two hours after the removal of the last clot from the first addition of fibrinogen, an additional quantity of fibrinogen was then added. This fibrin formation continued as long as thrombin was present. If thrombin became slightly inactivated the addition of small

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quantity of cephalin to this revived the activity of the prothrombin, so that it would coagulate more fibrinogen. (It seemed rather difficult to add sufficient quantity of cephalin to saturate all the prothrombin molecules). Finally the addition of cephalin to this inactive thrombin had no effect, indicating that the prothrombin had been all utilized. Now the addition of prothrombin to this inactive thrombin solution restored the clotting power slightly. This activation was due to the combination of prothrombin with the excess cephalin that was present in the tube. It is evident from this experiment that the thrombic activity is dependent upon the presence of cephalin and prothrombin and some calcium. Thrombin activity gradually decreased with the amount of fibrin formed.

As the quantity of thrombin decreased the amount of fibrinogen converted to fibrin was also decreased in proportion and any excess fibrinogen was left in the tube. Addition of few drops of the active thrombin immediately coagulated this excess fibrinogen to fibrin. Clotting time was found to be proportional to the amount of thrombin present.

For example, the following experiment displays clearly how fibrin formation is dependent upon the amount of thrombin present.

Fibrinogen solution was divided into three aliquot parts of 35 cc. (135 mgms), and one part was added to 10 cc. of active thrombin (18 mgms) and fibrin as it was formed was

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immediately removed and kept separately. One hour was allowed to elapse after the removal of the last clot before the second and third additions of fibrinogen were made, to insure that all the fibrinogen added were used.

The average fibrin weights obtained from the successive additions of fibrinogen to thrombin were as follows:

First addition: clot in 5 minutes-weight of washed and dried fibrin, 146 mgms.

Second addition: clot in 8 minutes-weight of washed and dried fibrin, 91 mgms.

Third addition: clot in 20 minutes weight of washed and dried fibrin, 40.55 mgms.

The results as shown have been obtained repeatedly with consistency with this sample of thrombin and fibrinogen. Clotting of fibrinogen is dependent upon the thrombin present as shown by the weights of fibrin produced. It was found that successive amounts of fibrin formed gradually decreased when fibrinogen solution was repeatedly added to the same thrombin solution. It can be said that thrombin was being removed in small quantities by fibrinogen. This removal of thrombin was not the result of adsorption to fibrin, since frequent washing of fibrin with water did not remove all the thrombin nor did any change in weight of fibrin occur.

To determine more specifically that thrombin unites with fibrinogen another experiment was tried where there was an excess of thrombin in the tube and the amount of fibrinogen

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was varied. It was found that the weight of fibrin obtained was larger than the weight of fibrinogen that was added to thrombin. This increase in the weight of fibrin cannot be changed by frequent washing with distilled water and by drying in the oven. Therefore the increase in weight is presumably due to union of thrombin to fibrinogen.

The results in Table I are averages obtained from different samples of fibrinogen and thrombin. These results are not in harmony with those of Mills, who has shown that one part of active thrombin can convert over one thousand parts of fibrinogen (13). I was never able to obtain that result when I continually removed my formed fibrin from the tube, the serum on standing for 24 hours did not show any evidence of clotting. However, it is seen then that a minimal amount of thrombin does not convert an unlimited amount of fibrinogen. However, it is true that a small amount of thrombin will seemingly change a large proportion of fibrinogen provided that formed fibrin (gel) was allowed to stand for a time without any interference. Gelation will take place solidly throughout the vessel, but when that gel was disturbed with a glass rod to remove the formed fibrin, it was found that only a few miligrams of fibrin had been formed and a large quantity of water was squeezed out. Excess fibrinogen was left in the vessel unused. This was shown by the fact, that another crop of fibrin was formed when thrombin was added.

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There is some definite relationship between fibrinogen and thrombin to form fibrin, even though this relation is irregular for the different samples. This increase is only valid if nothing else unites with the fibrinogen than thrombin and not water. The latter point will be shown definitely not to be the case despite the discrepancy in the weight of fibrin formed.

Insert Table I.

It is seen from this table that all of the added weight of fibrinogen has been accounted for in the weight of fibrin obtained in the experiment. There is an increase in the weight of fibrin over the original weight of fibrinogen used due to the union of thrombin to fibrinogen. In the first experimental tube 90 mgms of fibrinogen were added to 40.7 mgms of thrombin. The fibrin obtained weighted 105 mgms; this was the weight of washed and dried fibrin. An increase in weight of 16.6 per cent over the weight of fibrinogen added, the difference of fibrin formed and fibrinogen added gave the amount of thrombin united to the fibrinogen. In this, one part of thrombin converted 16 parts of fibrinogen.

Insert Table II.

Table II presents this fact of thrombin and fibrinogen union a little more clearer. In the first tube, 291 mgms of fibrinogen were added to 602 mgms of thrombin; 397 mgms of

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Table I

Increase in the Weight of Fibrin due to union of Thrombin to Fibrinogen

Weight of Thrombin mg	Weight of Fibrinogen added mg	Weight of washed Fibrin mg	+ Weight of Thrombin used mg	Increase in Weight of Fibrin %	Thrombin used / Fibrinogen
40.7	90.0	105.0	15.0	16.6	1:6
40.7	90.0	112.0	22.0	24.44	1:4.09
21.2	51.5	57.6	6.1	11.94	1:8.36
21.5	51.5	57.6	6.1	11.84	1:8.43
30.4	308.2	331.9	23.7	7.67	1:13
30.4	308.2	332.0	23.8	7.67	1:13
14.5	260.0	270.0	10.0	3.84	1:26
14.5	260.0	271.0	10.0	3.84	1:26
63.0	285.0	296.2	11.2	3.93	1:25.4
63.0	285.0	296.2	11.2	3.92	1:25.4

Thrombin is present in excess to convert all the fibrinogen.
+ Weight of Thrombin used = Weight of Fibrin - Weight of fibrinogen.

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Table II

Amount of Thrombin and Fibrinogen used in the formation of Fibrin.

Exp No.	Weight of Fibrinogen (Dried and washed) mg	Weight of Fibrin formed mg	Weight of Residue after clotting mg	Weight of Loss by washing (Wash-water dried) mg	Weight of Thrombin added. mg	+ Thrombin used to form fibrin mg	Increase weight of Fibrin. %
1	291.9	397.2	172.0	219.6	602.9	105.3	36.07
2	56.65	148.5	53.6	180.49	324.94	91.85	162.1
3	46.35	164.3	192.5	41.03	351.48	117.95	254.5
4	49.0	141.92	62.9	27.32	429.0	92.9	189.5
5	49.0	119.9	62.9	70.00	214.5	70.90	131.0

Thrombin is present in excess.

+ Thrombin united to fibrinogen: weight of fibrin - Weight of fibrinogen.

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fibrin was formed, an increase in weight of 36.07 per cent over the original weight of the fibrinogen added; 219.6 mgms of material was recovered in the wash water and 172 mgms of material was left in the tube after clotting, which was dried after there was no evidence of clot in 6-8 hours of standing at room temperature. From this the amount of thrombin used in the formation of fibrin is the difference of the weight of fibrin and the weight of fibrinogen used in the experiment.

The evidence for the probable union of thrombin and fibrinogen is given by the fact that during synthesis of fibrin by thrombin free amino groups determined indirectly by the decrease in the carboxyl groups, this group progressively decreased. And that thrombin was present as such in the washed fibrin solution and it can be recovered from the solution as prothrombin. This will be presented later in this paper.

Varying weights of fibrin were taken to determine the amount of free carboxyls it had in the molecule. These samples of fibrin were removed from the tube and carefully pressed free of water between filter papers. Drying in the oven was avoided for the particular reason that some chemical change might take place in the fibrin molecule. I did not wish any intramolecular change to take place causing the decrease in these groups.

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Insert Table III.

Table III represents the results for this determination, the carboxyl groups were determined by two methods, the Sorensen and the Willstatter method. It will be noticed that where was a gradual decrease in the number of groups showing that some union perhaps with the amino groups may have taken place. Carboxyls groups have decreased from 1.36 and 0.95 to 0.2 to zero. The results for the Sorensen ran a little higher for the reason that titration was in the aqueous medium, the alkali added in the titration caused some hydrolysis of the proteins increasing the number of carboxyls or the failure of formaldehyde to completely suppress the free amino groups.

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Table III

Analyses of Fibrin made from Thrombin containing varying amounts of Cephalin and Calcium chloride. Carboxyl group content.

Carboxyl groups.						Carboxyl groups.						
Exp No.	Weight of fibrin	Sorensen titration n/100 NaOH		Willstätter Peptide 50% alcohol	Willstätter Amino acid 95% alcohol	Sample	Weight Sample	Sorensen n/100 NaOH		Willstätter Peptide	Willstätter Amino acid	
	mg	cc		cc.	cc.		mg	cc.		cc.	cc.	
1	2.3	0.4		0.1	0.2	Fibrinogen	100	0.85		0.23	0	
2	2.5	0.2		0.1	0.1	Thrombin	100	8.47		1.8	0	
3	2.8	0.2		.05	.05							
4	2.6	0.1		0.0	0.05							
5	3.1	0.2		0.0	0.1							
6	3.4	0.0		0.1	0							
7	3.6	0.0		0.0	0.0							
8	3.7	0.0		0.0	0.0							
9	4.3	.2		0.0	0.0							
10	3.7	0.0		0.0	0.0							
11	3.6	0.1		0.0	0.0							
12	3.6	0.3		0.0	0.0							

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(b) Is syneresis a gradual solution of fibrin by thrombin.

In the preceding pages I have shown what goes to make the insoluble fibrin from soluble fibrinogen. It has been shown that definite quantity of thrombin converts only a given amount of fibrinogen and the increase in the weight of fibrin over fibrinogen is due to the union of thrombin to fibrinogen. It is a known fact that fibrin thus formed under-goes a spontaneous lysis, when it is allowed to stand in the tubes in which clotting occured, or in distilled water. This lysis is probably similar to that which occurs naturally in blood clots. This re-solution of fibrin was first noticed by Denis (14). He saw that clots redissolved in dilute salt solution. This observation was confirmed by Dastre (15) in sterile solution. Nolf maintained that this autolysis of fibrin was due to the presence of fibrinolysien (16).

It is not known whether syneresis where the clot contracts with squeezing out of the liquid serum is a process of actual contraction of the clot, or one of gradual re-solution of fibrin as result of dissociated or undissociated thrombin with the breaking down of the fibrinogen molecule. Must the clot contract before it is capable of undergoing re-solution?. If synersis is merely a contraction of the clot then the weight of fibrin removed from the tubes should not change at the different advancing stages of synersis. If it is a gradual re-solution there should be a gradual decrease in the weight fibrin removed from the tubes. The following experiments were conducted to settle this point.

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I have given in this experiment, the results of which are tabulated in Table IV, a series of tubes containing fibrin formed from a definite quantity of fibrinogen and thrombin. The formed clots were allowed to remain in the tubes until syneresis was evident. At different stages of re-traction of the clot fibrin was removed from the tube by twirling it around with a glass rod gradually, squeezing out the serum. Fibrin was then washed in distilled water to remove all the serum and dried in the oven at 38°C for 24 hours. The collected fibrin samples were weighed. The wash water were added to the respective serum and dried in the oven and finally weighed.

Insert Table IV.

The clots were solid throughout the tube and no retraction were evident in all the tubes within 24 hours. On the second day the clot had retracted up from the bottom 1/10th the height of the clot, as measured by a milimeter ruler. On the third day retraction from the sides of the tubes were noticed and with 1/4th retraction from the bottom, this gradually increased to 1/2 and 3/4 on the fourth and on the fifth day respectively. On the fifth day the clot was disintegrated and solution was complete on the sixth day.

In the controls, fibrin formed was immediately removed from the tubes by twirling the fibrin around a glass rod, then

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Table IV

Is Clot Retraction a Re-solution?

Temperature 38°C.

Exp. no.	weight of Thrombin mg	weight of Fibrinogen mg.	clotting Time	weight of Fibrin 24 hrs. mg	weight of fibrin 2-days mg	weight of fibrin 4 days. mg	weight. of fibrin 6 days. mg	† weight of residue after clotting mg.
1	13.75	58.9	10 min	3.6 control				43.9
2	13.75	"	"	3.6 control				43.8
3	13.75	"	"	3.4				43.8
4	13.75	"	"	3.5				43.8
5	13.75	"	"		2.9			44.8
6	13.75	"	"		2.9			46.1
7	13.75	"	"			2.6		45.1
8	13.75	"	"			2.6		45.5
9	13.75	"	"			2.4		48.7
10	13.75	"	"				2.3	49.3
11	13.75	"	"				2.3	49.1

† This is weight of fluid after clotting when it was dried.
Re-solution complete in 6 days.

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it was carefully washed with distilled water to remove all extraneous protein matter and dried, the wash water was collected with the serum and dried. The weights of fibrin from the four control tubes were consistent, weighing 3.6 mgms. In the experimental tubes, the clots were removed every twenty-four hours, washed and dried. The daily loss in weight of fibrin due to syneresis was on the average of 0.3 mg. Syneresis is then a very gradual re-solution of fibrin as concluded from this experiment.

I have seen this similar phenomenon of rapid re-solution of the clot in cases of work done with whole blood clots. Generally the process requires several hours to days, but in several instances I have observed this complete dissolution of solid clots of whole blood occur within a course of an hour or less, the corpuscles have settled to the bottom and the blood was fluid again. As Clots made with fibrinogen and thrombin disentegrated in an hour or two if the concentration of cephalin in thrombin was low, this is shwon in an experiment included in the latter part of this paper.

It may be that this rapid re-solution of laboratory fibrin and the retraction of whole blood clots are two different phenomena.

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(c) Influences of gases on clotting time and in the re-solution of fibrin.

During the course of re-solution of fibrin there was noted that in cases of fibrin in stoppered tubes containing water re-solution progressed just so far and it was then retarded. The fibrin clots in unstoppered tubes, (that is those tubes which were plugged with sterile cotton) completely went into solution. This was concluded to be due to the influence of oxygen. This observation was confirmed by passing oxygen gas through water containing solid fibrin. This dissolved in two-thirds the time required for fibrin to dissolve if it was unaided by the presence of free oxygen.

About 6 mgms. of fibrin were placed into each tube in the series containing 5 cc. 10cc., and 15 cc. of water respectively. Oxygen gas was allowed to bubble into the water through a sterile cotton plugs for two to three minutes the tubes were then stoppered and set aside until complete solution of the fibrin had taken place at room temperature. Similar series of tubes of fibrin were set up substituting hydrogen. and carbon dioxide for oxygen. These experimental results showed that oxygen accelerated the re-solution of fibrin, while hydrogen and carbon dioxide tended to retard the re-solution.

Insert Table V.

Broth cultures were made of these solutions, they showed faint cloudiness after 24 hours. This entire experiment was

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Table V.

Re-solution of Fibrin aided by Gases.
Completion of Re-solution at 20°C.

Exp No.	Sample	water c.c.	Air control days.	oxygen atmosphere days	Hydrogen atmosphere days	Carbon dioxide atmosphere days.
1	Dried Fibrin	5.0	4	3	4-5	7-8
2	"	5.0	"	"	4-5	7-8
3	"	10.0	"	"	4	7
4	"	10.0	"	"	4	"
5	"	15.0	"	5	4	"
6	"	15.0	"	5	4	"

weight of fibrin - 3.9 mgms.

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repeated using sterile tubes and pipettes. Broth cultures of these showed no growth until after the 40th hour and then the growth was very, very faint. From this the process of re-solution cannot be attributed to the presence of bacteria, not mentioning the filtrable viruses, of course, since the rate of solution of fibrin were simultaneous with the first set of tubes.

Next I decided to find out what effect these gases had on these proteins before the starting of the clotting experiment. The results are given in Table VI. The gases were passed into series of tubes each containing about 5 cc. of the preparation of fibrinogen, prothrombin, cephalin and thrombin; clotting was performed with these samples after 48 hours of exposure to these gases at room temperature. It was found that such oxygenated solution of fibrinogen was coagulated by thrombin in 19 hours, fibrinogen treated with carbon dioxide in 5 hours, and by hydrogen in 1 hour and 55 minutes.

Thrombin was the least affected by these gases and cephalin was slightly inactivated by all three gases. The effect of oxygen seem to be to change the proteins and cephalin in such a way to delay coagulation.

Clotting was performed with fibrinogen, prothrombin, cephalin, and thrombin again after nine days of exposure. It was found that fibrinogen was incoagulable by thrombin, cephalin

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was completely inactivated. Thrombin was slightly inactivated in this experiment. Perhaps the cephalin was partially destroyed; it seems that cephlin was more stable in the presence of prothrombin and calcium.

Insert Table VI.

Broth cultures were made from these tubes for the presence of bacteria, after 24 hours incubation. Samples prepared with no sterile precautions showed bacterial growth in 24-36 hours, but those in sterile tubes showed no growth.

A As result of this investigation on the gaseous influence oxygen is the variable factor in the process of coagulation, its presence in the tube containing fibrin in water hastens the re-solution, its presence before the onset of coagulation inhibits the clotting of fibrinogen by thrombin.

The fluidity of the blood in the vascular system is perhaps, maintained in part by the presence of the excess oxygen, which is carried by the red blood corpuscles, to oxidize the cephalin liberated from time to time by the destruction of the platelets, or liberated from the plasma proteins. At the same time, fibrinogen is preserved in an inactive state by its union with some other proteins. Any fibrin that is formed is immediately re-dissolved by oxygen. Perhaps, oxidase, hematoporphyrin and vitamins or hormones also play some part in this solution phse of clotting. Boyd (17) (unpublished work) of this laboratory has found that fibrinogen exposed to ultra-violet rays for 6-8 hours was incoagulable and when hematoporphy-

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Table VI

Influence of gases on Blood Proteins before clotting. Clotting Time.

Exposure for 48 hours and 9 days. 20°C

	Control Clotting time	Oxygen	Hydrogen	Carbon dioxide	Exposure.
Fibrinogen A.	18 min.	19 hrs.	1 hr. 15 min	5 hrs.	48 hrs.
		no clot	over-night. 19 hrs.	19 hrs.	9 days.
Fibrinogen B		5 hrs	5 hrs.	6 hrs.	48 hrs
		no clot	no clot	19 hrs.	9 days.
Prothrombin		25 min	25 min	25 min	48 hrs.
		no clot	no clot	1 hr. 10 min	9 days.
Thrombin	8 min	30 min	30 min	20 min	48 hrs
		gel 1 hr. 25 min	1 hr. 25 min	1 hr. 25 min	9 days.
Cephalin		very weak gel 19 hrs.	solid 19 hrs	solid 19 hrs.	48 hrs.
		no clot.	no clot	no clot	9 days.

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Table VI b

Re-solution of Fibrin
Completion. 20°C.

	Control	oxygen	Hydrogen	Carbon Dioxide	Exposure
	days.	days.	days.	days.	
Fibrinogen A	3	2	3	3	48 hrs
"		—	3	4	9 days
Fibrinogen B		2	6	6	48 hrs
		—	—	—	9 days
Prothrombin		2	3	3	48 hrs
"		-	2-3	2-3	9 days
Thrombin		2	2	3	48 hrs
		4	4	4	9 days
Cephalin		2	3	3.	48 hrs.

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rin and oxygen were present fibrinogen became incoagulable in 2 hours by thrombin.

In circulation cephalin complex is only dissociated when there is a disturbance in blood protein equilibrium; the same is true of fibrinogen. In death, clotting is very slow in the vessels due to the presence of oxygen in a large amount, regardless of the cessation of its intake and from the gradual disfunctioning of the erythrocytes. The oxygen is gradually diminishing in quantity due to the continuation of metabolism in the body after death the carbon dioxide is slowly accumalating with the acids. The clotting will be the result of lack of oxygen and increase in carbon dioxide and metabolic products. Body blood clots when the optimum oxygen diminishes or it may be that prophyrins are decomposed.

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III. Analysis of Fibrin Solution.

Fibrin is now known to go into solution like the natural blood clots, and albumose and a new protein, fibrinose (fibrinose-globulin - Hammersten (6) are found with the unchanged prothrombin in the fibrin solution.

About 1 to 2 grams of fibrin were used in this experiment. This fibrin previously washed and dried was allowed to stand in a vessel plugged with cotton containing 200 cc of distilled water and 25 cc of 0.9 per cent sodium chloride. The re-solution was generally completed after 15 to 30 days standing in the ice box. The vessel containing the re-dissolved fibrin showed an insoluble fraction settled in the bottom of the tube.

A portion of this faintly yellowish colored insoluble substance in the bottom of fibrin solution was extracted with hot benzene and there was found to be some extractable matter present. The loss in weight after benzene extraction of the insoluble fraction was due to the lipid present. The fatty material which was extracted with water from the benzene extract, removed of benzene by drying the extract at room temperature, was found to be inactive in clotting. It failed to activate prothrombin to thrombin in the presence of calcium.

About 2 cc. of fibrin solution was heat coagulated at 80°C., the coagulated proteins were filtered off and this heated again to a little higher temperature, cooled and

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filtered again. The filtrate was tested for albumose with 5 per cent phosphotungstic acid. There was very faint cloudine an evidence of albumose present in a very small amount.

Broth cultures of fibrin solution showed no bacterial growth after 24 hours of incubation at 38°C. Samples of fibrin where precaution for sterility was omitted showed growth. Bacteria did not facilitate re-solution, since lysis took place in those tubes which were sterile at about the same rate.

Fibrin solution was fractioned with neutral salts like plasma to see if fibrinogen and prothrombin could be recovered. To 200 cc. of the fibrin solution, sodium chloride was added to one-half saturation. There was a slight opasescence. The precipitation was not heavy enough to collect the material for examination. This was filtered, and to the filtrate sodium chloride was added to saturation. A copious amount of precipitate was now formed. The globulin was collected on a filter paper. The precipitate was removed with a spatula and dissolved in 20 cc. of 1 per cent sodium chloride solution. After the removal of fibrinogen, ammonium sulphate was added to one-half saturation to precipitate the prothrombin from the filtrate. The precipitate was collected on a filter paper, dissolved in 20 cc. of water, and then dialysed by running tap water for 12-24 hours to remove the sulphate. Some globulin was precipitated in the prothrombin in the dialyzing tube.

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The fibrin used in one case was composed of 1350 mgms. of fibrinogen and 195 mgms. of prothrombin, after the re-solution the fractions recovered by the above process gave 1095 mgms. of protein coming down on saturation with sodium chloride and 205 mgms. of prothrombin. The difference in the two weights of fibrinogen was due to the hydrolysis. The increase in weight of prothrombin was due to lipids and albumose precipitated with prothrombin.

Fresh solution of fibrinogen was coagulated in 2 minutes by this recovered prothrombin which had been previously activated with cephalin and calcium to thrombin. An active thrombin solution did not coagulate the new globulin fraction. Fibrin formation is not a reversible chemical reaction, one of the constituents was changed, that is fibrinogen, into this new protein, which I have called fibrinose (fibrino-globulin-Hammarsten) (6) by the action of thrombin. However, thrombin could not be recovered as such but as prothrombin. Fibrinose was heat coagulated at 65°C. and prothrombin at 71°C., this is the same coagulation temperature as the original prothrombin used in the experiment.

Following series of tables and a graph shows the results of series of analyses of fibrinogen, prothrombin and thrombin before the coagulation precess and the results of series of analyses of fibrin solution, fibrinose and prothrombin fraction recovered from the fibrin solution.

Insert Tables VI, VII, VIII; Graph I.

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Table VIII

Analysis of Fibrin Solution.

	Total N %	Amino-N %	Calcium %	Phosphorus %	Extractable lipid %	Carboxyls % peptide	Carboxyls % amino-acid
Fibrinogen	16.04	2.83	0.0534	0.1902	—	5.59	2.1
Prothrombin	15.89	12.8	0.45	4.861	1.11	4.01	5.34
Thrombin	15.7	5.3	0.714	4.68	8.93	3.35	3.25
Fibrin Sol'n	14.8	4.0	2.8	6.886	.5.26	5.72	4.0
"	15.84	2.84	3.92	8.123	4.55	5.88	4.21
"	15.0	2.74	1.08	5.655	6.9	3.5	3.5
Proteins extracted from the above fibrin solution							
(recovered) fibrinase	13.24	7.54	0.446	0.97	0.49	5.0	5.0
"	15.37	2.41	1.035	3.041	1.23	3.16	6.32
"	15.07	3.59	1.74	1.318	1.33	3.9	2.01
recovered Prothrombin	14.09	8.6	5.5	6.42	4.1	5.0	5.0
"	10.64	10.1	6.47	6.3	2.3	8.0	2.6
"	15.67	11.5	5.51	10.6	3.1	2.2	2.2

Fibrinase precipitated from fibrin solution by saturation with sodium chloride.

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Table VIII

Analysis of Fibrin Solution.

	Total N %	Amino-N %	Calcium %	Phosphorus %	Extractable lipid %	Carboxyl Peptide	Carboxyl Amino-Acid
Fibrinogen	15.90	1.919	2.12	0.5422	—	3.53	3.53
Prothrombin	15.89	9.027	0.5348	3.208	6.48	4.325	5.34
Thrombin	14.53	3.355	4.524	5.55	13.25	4.47	5.59
Cephalin	1.41	—	—	3.55	—		
Fibrin Soln	15.89	8.62	2.57	7.12	1.59	4.31	2.15
"	15.84	14.44	3.98	12.36	5.55	3.73	4.98
"	15.89	7.056	0.968	11.31	1.59	2.16	2.16

Proteins extracted from above Fibrin Solution.

Fibrinose	14.56	3.593	1.588	3.444	1.435	2.65	0.45
"	15.87	10.11	2.48	9.673	—	8.27	1.21
"	15.84	6.059	3.34	4.019	1.75	5.56	1.39
Recovered Prothrombin	15.89	6.115	0.55	5.982	0.619	5.0	5.0
"	13.29	5.692	0.773	7.162	1.35	5.305	1.756
"	15.89	4.68	0.887	8.76	5.85	3.69	7.38

Fibrinose is precipitated from fibrin solution by saturating with sodium chloride.

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Table IV

Analysis of Fibrin Solution.

	Total-N %	Amino-N %	Calcium %	Phosphorus %	Extractable lipid- %	Carboxyl % Peptide	Carboxyl % Amino-acid
Fibrinogen	15.89	0.4904	0.1612	0.4442	—	0.4424	0.4424
Prothrombin	15.80	12.85	0.415	3.1091	1.35	0.806	1.075
Thrombin	14.79	2.023	1.593	8.93	17.42	1.185	1.980
Fibrin Sol'n	15.82	6.86	2.58	3.558	4.4	5.43	5.72
"	15.89	11.46	4.48	6.156	6.94	10.88	14.52
"	15.89	9.43	1.06	3.48	3.627	3.52	3.52

Proteins extracted from fibrin Solution of above.

Fibrinose	15.00	1.012	2.548	1.00	1.7	0.746	2.6
"	15.88	4.217	2.419	4.032	1.0	3.45	1.0
"	15.80	0.31	3.592	2.089	1.83	2.89	0.723
Recovered Prothrombin	15.80	6.00	4.0	6.0	1.325	9.13	9.13
"	15.90	4.39	2.846	5.9	3.03	4.41	1.47
"	15.89	5.79	2.7047	1.046	0.29	4.54	4.00

Fibrinose is precipitated from fibrin Solution by Saturating with sodium chloride.

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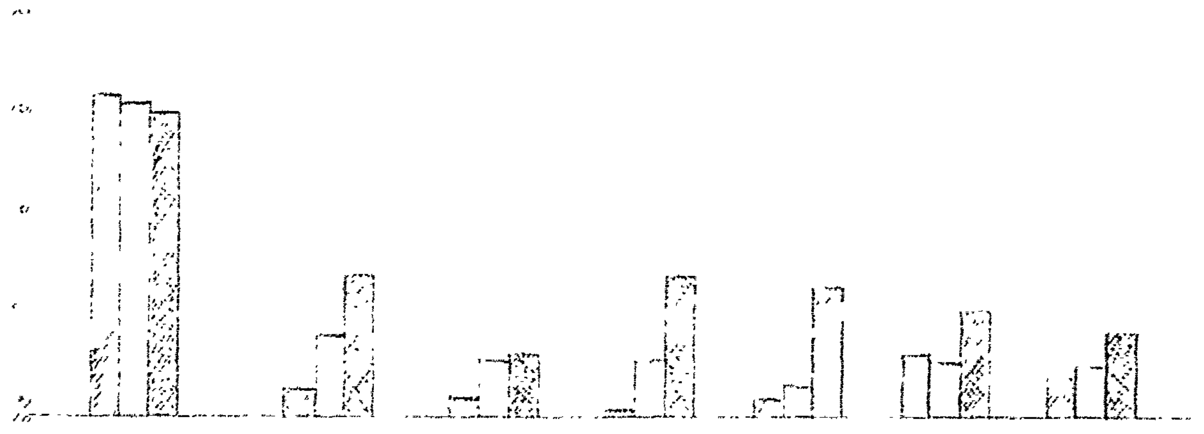

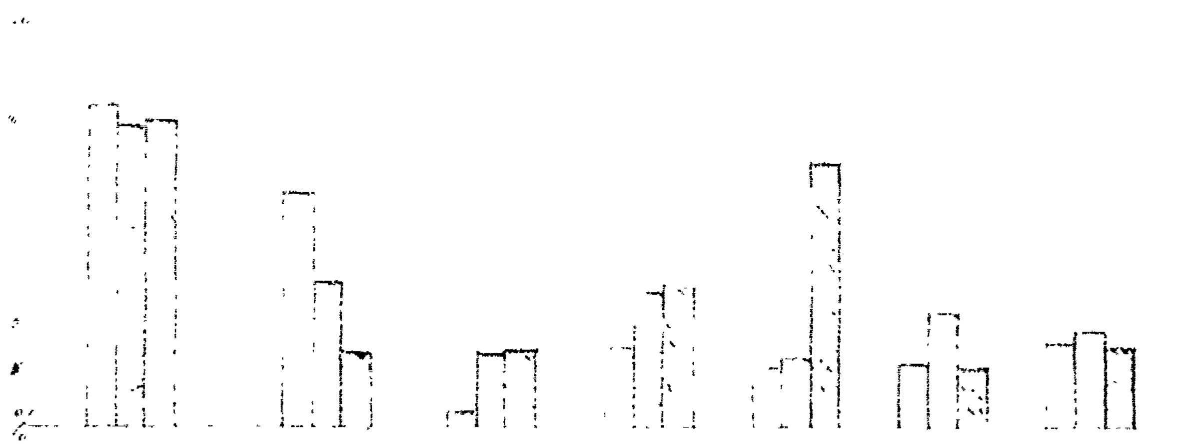

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It is interesting to note that the total nitrogen remained constant. No non-protein nitrogen was found. However, there was fluctuations in percentage of amino-nitrogen, phosphorous, the carboxyl groups, calcium and the extractable lipids from the original solution of fibrinogen and thrombin.

The amino-nitrogen, calcium, and phosphorus contents are increased in the fibrinose with the slight increase in the amino-acid carboxyls with a decrease in the peptide carboxyl groups. The large fibrinogen molecule seems to have been hydrolyzed into the next simpler product fibrinose and albumose by thrombin. This increase in the amino-nitrogen and the amino-acid carboxyls are an index to hydrolytic action of thrombin on fibrinogen. This is characteristic of protein hydrolysis, namely the transformation of non-amino nitrogen in the CONH linkage into free amino nitrogen and COOH as these linkings are hydrolysed with the formation of these groups.

This evidence for thrombin to break the fibrinogen molecule through peptide group is based on the fact that during hydrolysis of fibrin by thrombin amino groups and carboxyl groups are progressively liberated. Second, the positive biuret reaction is given by substances which contain this group and also by their partially hydrolyzed products (proteoses) which are liberated in equal amount, since there is no change in the acidity. Solution of fibrin is very, very faintly acid to methyl orange (pH 3.1 to 4.4) with transmitted light, there seems to be no apparent change in

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color by reflected light. The mixture of fibrinogen and thrombin also gives this slight indicator change before clotting.

Analyses of prothrombin recovered from fibrin solution showed quite a decrease in the amino-nitrogen and a slight increase in the two carboxyl groups, phosphorus and calcium.

Chemical analyses of fibrin solution showed an increase in the amino-nitrogen. This was due to the hydrolysis of the thrombin-fibrinogen union in the presence of water on standing with the subsequent formation of fibrinose and albumose. This disruption of the union does not take place in a short time, it takes from several days to several weeks.

Another finding of importance was the change in the heat coagulability of these fractioned protein from the fibrin solution. The original fibrinogen which was coagulated at 56°C. now changed to 65°C. The original prothrombin was coagulated by heat at 72°C. and the recovered prothrombin was coagulated by heat at the same temperature. As far as the heat coagulation was concerned, there is apparently no change in the physical property of the prothrombin from the original preparation. Fibrin solution was coagulated by heat at 80°C.

The tables shows that the thrombin molecule contains more amino groups and amino-acid carboxyls than the fibrinogen molecule. Hence, these groups are responsible in the union with the carboxyls and amino groups of fibrinogen to form fibrin. Even the presence of these reactive groups do not

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totally answer the question of the activity of thrombin, since thrombin convert more fibrinogen than the sum total of these groups present.

In the synthesis of proteins the end carboxyls and alpha amino groups are not the only groups responsible for an acid and base neutralizing power. Very small proportion of nitrogen is in the form of amino group (edestin 1.8 per cent as amino-nitrogen, fibrinogen 1.95 per cent and thrombin contains 3.33 per cent). the amide group CONH is responsible for peculiar conversion fibrinogen to fibrin. The breaking of this group must be laid to cephalin and calcium which activates prothrombin.

When cephalin is activated with cephalin and calcium the free amino-nitrogen of prothrombin are decreased to about one-half to one-third the original amount, the carboxyls remain the same. Cephalin, acid in reaction, combines with the free amino group, calcium then combines with the OH groups of the phosphoric acid unit in cephalin, the HCL formed unites with the other free amino group fo the protein to form the $R\text{-}NH_3CL$. This group is now more reactive than the free amino group and unites with the carboxyl groups in fibrinogen.

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(c) Presence of Albumose in Fibrin Solution.

It has been found that albumose was formed in the fibrin solution with another protein which is coagulated by heat at 65°C.

Hammarsten (6,) was the first to discover that substances found in the solution of fibrin were different from the substances entering into the clot. He called this splitting "deboublement" giving two new fractions in the solution, yielding one substance which coagulated at 56° and another at 64°C. Mills in his study of fibrin formation has found that re-solution of fibrin was associated with an ultimate change in the fibrinogen fraction which is coagulated by heat at 75°C. instead of at 56° like the original preparation. In his fibrin solution there was no non-protein nitrogen present, thus excluded the proteolytic action.

I have found a protein which was coagulated by heat at 65°C. and prothrombin was recovered practically unchanged and also some albumose. Since it was obtained by the hydrolytic action of thrombin on fibrinogen giving this substance and albumose. This heat coagulable protein was fairly noticed by Wooldridge and he called it fibrino-globulin.

Prothrombin and fibrinogen solutions used were free from albumose. It was tested in the following way:

Total protein was removed by first coagulating them at their respective temperatures; fibrin solution was heated to 80°C. This was cooled and filtered to remove the coagulum.

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For further analysis, the old fibrinogen solution was subjected to chemical analysis, the results are given in Table XI

Insert Table XI. Graph II.

It is clearly shown that aged fibrinogen by its precipitation with sodium chloride agrees very closely to the new protein from the fibrin solution. They are both coagulated by heat 62-65°C. The amount of free amino-nitrogen is the same in both fractions of fibrinogen. There is more increase in the carboxyl groups after fibrin formation that it is formed on standing. This is due to the thrombin action of the fibrinogen.

The change in fibrinogen from the active to the inactive stage may not be due only by the action of thrombin, but it may occur on standing. This change may occur by its exposure to air or by dilution with water. The amount of lipid present is less than that found in the recovered fibrinogen solution, this is tobe expected since there was no addition of surplus cephalin in the form of thrombin.

This denaturation of fibrinogen may be due to hydrolysis where another protein is loosened from the fibrinogen, the latter being more unstable when it is dissociated. There is no evidence as to whether one is dealing with a single protein, or with a mixture, or of a compound of 2 or more proteins when the proteins are precipitated from each other with salts. Under similar conditions of precipitation and reprecipitation, the

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Table XI

Comparative Analysis of Fibrinogen Solution.

Fibrinogen Solution	Heat Coagulation	Total-N %	Amino-N %	Calcium %	Carboxyl % Peptide	Carboxyl % Aminoacid
Inactive Sol'n.	62°C	15.12	7.21	1.612	1.53	0.152
" Sol'n.	62°C	15.10	7.20	1.625	1.583	0.158
Active Solution	56°C	14.86	9.41	0.706	0.39	0.819
"	56°C	14.86	9.417	0.706	0.397	0.818
"	56°C	14.86	9.70	0.706	0.39	0.80
Recovered Fibrinogen Sol'n.	65°C	[illegible]	7.59	0.446	5.0	5.0
" "	65°C	15.37	7.41	1.035	3.16	6.32

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Comparative Analysis of Fibrinogen Solutions

Graph II

Total N — Amino-N — Calcium — Carboxyl peptide — Amino acid

- Inactive fibrinogen solution.
- Active fibrinogen solution
- Fibrinoic solution.

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same product should be obtained theoretically. But it has been shown in cases of globulins, never two fractions have been separated purely from each other as shown by the presence of a protein heat coagulable at 75° in a solution of fibrin.

These combinations are soluble in H_2O or diluted salt as long as the soluble portions are in excess, but on standing, the soluble portion is split off by simple dissociation process, the remaining combination becomes richer and richer in the sparingly soluble fraction and lastly precipitates, like the precipitate formed in the denatured fibrinogen solution or in prothrombin solution, at first these sedimentations slightly affect the action of the preparation but soon as the amount increased fibrinogen and prothrombin solution became inactive, such sediments are also found in thrombin and fibrin solutions on standing.

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Part 11.

Evidence has been presented to show how thrombin transforms fibrinogen into fibrin and how it breaks fibrin down to fibrinose (fibrinoglobulin) and an albumose. Thrombin is found in the fibrin solution as shown by the fact that it can coagulate fresh solution of fibrinogen. This property was found to disappear quite rapidly on standing.

The following experimental facts are given to show what thrombin is - its properties and its constitution.

V. Properties of thrombin.

(a). Inactivation of the Thrombin Solution on Standing.

It is well known that thrombin solutions deteriorate rapidly on standing; but the nature of the change occurring in it is not still unknown. The reappearance of thrombin in old serum on the addition of dilute acids and alkali was found by Schmidt (19). The changed thrombin was called metathrombin by Morawitz (20). It was suggested by Bordet (21) and Mills and Mathews (22) that this inactivation of the thrombin or serum was due to a dissociation of thrombin into cephalin and serozyme. The change is known to be very rapid. This serum taken at once from a clot, if it be

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injected into the jugular vein of a rabbit, kills the rabbit by intravascular clotting, whereas it is allowed to stand fifteen minutes or more, large amounts of such serum may be injected without causing clotting. A similar loss of activity is shown with serum left after the action of fibrinogen. Both serums taken immediately from a clot will clot oxalated plasma almost instantly, whereas after it has stood 2-3 hours at room temperature it will clot such only after several minutes to several hours.

The thrombin prepared in a dry form by Collingwood (23) was very active in vitro, but produced no intravascular coagulation.

The nature of this inactivation of thrombin was studied. The first experiments were designed to see whether the purified thrombin prepared from prothrombin, calcium chloride and cephalin emulsion in the proportions of 2 cc. prothrombin, 1/2 cc. of 1 per cent calcium chloride solution and 1/2 cc. of cephalin emulsion containing a known amount of cephalin calculated in the dry weight, would lose activity on standing. It was found that it did and at a relatively rapid rate at the start, but a residual activity continued for a long time. Furthermore some solutions remained active for a long time in the ice box without any antiseptic being added; whereas others lost their activity quite rapidly.

In contrasting these solutions it was found that strong solutions preserved in larger proportions of their

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activity than did dilute solution. In fact, the more dilute the solution, the more rapid the deterioration. This is shown in the following experiments. The results are tabulated in Table XII.

Insert Table XII.

It is obvious from the table that greater the concentration of protein per cubic centimeter greater is the stability of the protein solutions. For instance, prothrombin solution containing 5.2 mgms. per cubic centimeter when it was activated with cephalin and calcium chloride on the third day after its preparation clotted fibrinogen in one hour and ten minutes, the sample containing 29.5 mgms. per cubic centimeter when it was activated to thrombin in the usual way at the end of 3 months standing in the ice box clotted fibrinogen in 5 minutes. Fibrinogen solutions containing 3.54 mgms. per cubic centimeter lost its coagulability in 2 days, while the sample containing 20.1 mgms. retained its coagulability till the 20th day. It was clotted by active thrombin in 15 minutes, the original time was 2 seconds. Fibrinogen on standing gradually lost its original activity, nevertheless, it shows clearly that concentration of protein in solution prevents rapid inactivation. Thrombin solution also loses its activity slowly on standing in the ice box, however, much slower than if it was allowed to stand at room temperature.

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Table XII

Stability of Blood Proteins Solutions.
Effect of Dilution on Standing in
Ice-box. (7°C)

Sample	weight (dried) mg	length of Storage	Addition $CaCl_2$ + cephalin	Fibrinogen* mg	Clotting Time standing Prothrombin	Clotting Time original
Prothrombin	29.5	3 mo.	.05 mg $CaCl_2$ & .37 mg cephalin	17.4	5 min	4 sec.
"	28.2	3 mo.	"	"	4 min	3+ sec
"	10.1	10 days	"	"	12 min	10 sec.
"	5.2	3 days	"	"	weak gel thr. 10 min	20 sec.
Fibrinogen	18.9	10 days	Thrombin added 13.75 mg	"	15 min	2 sec
"	20.1	10 days	"	"	13 min	2 sec
"	3.54	2 days	"	"	no clot	8 min
Thrombin	13.1 mg	2 days	none	"	8½ min	3 sec
"	7.32 mg.	2 days	none	"	28 min	50 sec
"	28 mg.	4 days	none	"	3½ min	2 sec

* addition of fibrinogen to activated prothrombin after 30 minutes standing.

clotting time - time of conversion of liquid state to a solid state.

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In seeking for the cause of this inactivation of the thrombin it was observed that although the original solutions were clear and sterile, yet on standing there slowly settled out a faintly yellow, or sometimes a white, gelatinous precipitate. The precipitate was examined for its activity, and contrasted with the activity of the supernatant clear filtered liquid.

The precipitate was washed on the filter with 1 per cent sodium chloride or in distilled water. It was, therefore, neither globulin nor albumin. After drying at 38° C. it yielded to successive extractions of ether and benzene between 2-5 per cent of its weight in different samples to these solvents. This material was apparently lipoid in character. It was insoluble in water, but emulsified with the water. It gave no Pettenkoger reaction, thus indicating that it contained neither oleic nor any other unsaturated fatty acid.

The addition of this emulsion to prothrombin solution together with little calcium chloride or without the latter, failed to clot the fibrinogen.

The protein residue after extraction with ether and benzene failed to clot a fibrinogen solution when added with a little calcium chloride and cephalin. The protein residue gave a biuret test.

The thrombin precipitate contained most of the

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activity, whereas the supernatant liquid was less active. Hence, most of the activity of thrombin was precipitated out by standing in the ice box (7°C.) in a sterile condition.

The relative activities of these materials and of the protein freed by benzene and ether of its extractable lipid were tested on 2 cc. lots of a fibrinogen solution of the usual strength. The sediment was tested by dispersing it through an amount of a 1 per cent sodium chloride solution equal in volume to that of the original sample.

Equal volumes of this suspension and of the supernatant liquid were added to 2 cc. portions of the fibrinogen solution, the clotting time was observed and the weight of fibrin determined. The results are shown in Table XIII.

Insert Table XIII.

An inspection of Table XIII shows that most of the activity was in the sediment. It clotted in 3 minutes and yielded 3.5 mgms. of fibrin (determined by direct weighing of the dry material) whereas the supernatant liquid clotted in 10 minutes and yielded 2.8 mgms. of fibrin. The mixture of the two in equal parts clotted fibrinogen in 5 minutes and yielded 3.0 mgms. of fibrin.

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Table XIII
Activity of Thrombin kept in Ice-box 2 days. (7°C).

Exp No.	Sample	weight dried. mg.	Total-N %	Fibrinogen	Clotting Time 38°C	weight of fibrin mg
1	Thrombin Ice-box	4.43	13-10	8	5 min	6
2	supernatant layer Thrombin	1.90	12.88	"	10 min	3.8
3*	Sediment washed Thrombin	2.20	11.63	"	3 min	5.5
4	Fresh-active Thrombin	9.2	13.61	"	3 min	10.5

* sediment from Thrombin solution washed with saline 3 times, suspended in saline volume ½ of original thrombin solution.

Exp No.	~~Lipid~~ Sample	Lipid-ether extracted dried	Retraction of clot 5 hrs. *
1	Ice-Box Thrombin	9.60	⅓
2	supernatant layer Thrombin	none	½
3	washed sediment from Thrombin	2.8.9	none
4	Freshly prepared Thrombin	13.25	very slight surface liquefaction

* comparative measurement of clot retraction with a millimeter ruler.

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The clot thus formed dissolved (or contracted) more rapidly in case of fibrin formed from supernatant liquid than with the sediment of thrombin solution. Fibrin made from thrombin sediment did not dissolve within 24 hours.

I next examined the composition of the sediment and compared this material with the supernatant fluid, with a sample of thrombin prepared by Schmidt's (24) method of alcohol precipitation from serum, and with an active solution of fresh thrombin made as usual by the addition of cephalin and calcium chloride to prothrombin.

Total nitrogen was determined by Micro-Keldahl method on a given volume of the solution containing a known amount of the substance calculated in the dry form, and amino-nitrogen by micro-Van Slyke method.

Free carboxyl groups were estimated by the method of Willstatter and Waldschmidt-Leitz (25). By this method the carboxyl groups of aliphatic amino acids may be titrated directly in alcohol medium. Alcohol suppresses the basic properties of the amino groups and develops the acidity of carboxyl groups even in the presence of the ammonium and the phosphates groups, thus it insures more exact results than the Sorensen method. This procedure differentiates between the proportions of amino acid carboxyls and pepitide carboxyls by the concentration of alcohol used. The method in detail is as follows:

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Two cubic centimeters of the protein suspension or solution is pippeted into a 50 cc. Erlemeyer flask containing enough alcohol to make 50 per cent, the peptides are precipitated with their free carboxyl groups, this is titrated with N/10 alcoholic KOH using thymolphthalein as the indicator. At the pH. of the indicator the protein goes into solution leaving no turbidity, this is the isoelectric point (appearance of the blue color) of the peptide. The amino acid carboxyls are titrated in 95 per cent alcohol, 25 cc. of it is added to the same flask after the titration of peptide and titrated again with the alkali. Blank reading for alcohol is subtracted from the total reading, and the result is given in cc. of N/10 alcoholic KOH used.

These results were compared with the clotting time and with the quantity of lipid extracted by ether and benzene from the dried residue, the results are shown in Table XIV and Graph 111.

Insert Table XIV and Graph 111.

From this table it will be seen that all of the thrombin contained from 11 to 13.5 per cent of total nitrogen based on the total dry substance. No narrow relation appears between the total nitrogen and the clotting power. Fresh thrombin with 4 minutes clotting time

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Table XIV

Sample dried 100mg	Total-N %	Amino-N "	Carboxyl Peptide	Carboxyl Amino-acid	lipid ether extracted
Thrombin ice-box	13.10	216.50	329.4	357.2	9.60
Supernatant Thrombin	12.88	191.79	307.00	591.00	none
Sediment washed Thrombin	11.63	254.46	495.00	103.00	28.9
Fresh, Active Thrombin	13.61	362.37	878.6	140.6	13.25
Alcoholic ppt serum (Schmidt)*	10.66	75.16	209.12	88.88	11.00

* 15-20 volumes 95% alcohol added to defibrinated serum, stand few months, filter, and dry. Extract with water

Sample dried	clotting time.	Product. clotting time x Amino-N	Product clotting time x Peptide COOH	Product clotting time x Amino-acid COOH
Thrombin ice-box	5 min	1082.5	1647.00	1786.00
Supernatant Thrombin	weak gel 6 min	1150.74	1842.00	3546.00
Sediment washed Thrombin	strong gel 3 min	1273.3	2475.00	515.00
Fresh, Active Thrombin	2 min	1087.11	2535.8	421.8
Alcoholic ppt serum (Schmidt)	20 min	1502.3	4189.4	1777.6

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Analysis of Thrombin kept in Ice-box. (7°C)

Graph III

% Total-N

% Amino-N

% Carboxyl Peptide

Aminoacid

% lipid.

Active Thrombin

Supernatant of Ice-box Thrombin

Sediment of Ice-box Thrombin

Alcoholic ppt. of Serum

Thrombin - ice-box

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contained material with 13.6 per cent nitrogen; this was the highest nitrogen possessed the greatest clotting power; the Schmidt's thrombin contained least nitrogen and had the weakest clotting power, but the supernatant substance with 12.88 per cent nitrogen clotted fibrinogen in 6 minutes while the sediment with 11.6 per cent nitrogen clotted fibrinogen in 5 minutes.

There was, however, a fairly close relation between free amino-nitrogen and the clotting time. The product of the number of grams of free amino-nitrogen by the clotting time was approximately a constant of the value of about 1.3 gram-minutes. The product of the peptide carboxyl by the clotting time was obviously not constant.

The percentage of lipid extractable from thrombin also shows no relation to clotting time. The material in the supernatant liquid had no extractable lipid, except for a trace. This clotted fibrinogen in 6 minutes, whereas Schmidt's thrombin contained 11.0 per cent extractable lipid, but it clotted fibrinogen only after 20 minutes. Freshly prepared thrombin that was very active had 13.25 per cent of extractable lipid. Of course, in these determinations the dry material was obtained by simply evaporating the solution to dryness; and this dried residue contained free as well as combined lipid and salt and proteins which was extracted with the lipid.

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The general result is to indicate a relationship between the number of free amino groups of the thrombin and its activity.

The conclusion which seems most probable is that the thrombin unites through this group with the fibrinogen. This is borne out by the fact that thrombin treated with formalin, which is known to unite with amino groups, and so to block them, will no longer clot fibrinogen, as shown by Dr. Dunn in our laboratory (26).

The fact that the supernatant liquid, which is not very active contains a protein and a trace of lipid extractable by ether and benzene, while the sediment which is more active, contains nearly 29 per cent of lipid so extractable, indicates that the change in the thrombin which occurs on standing is that suggested by Bordet and Gengou, and Mills namely, a dissociation into cephalin and serozyme (prothrombin). There are other facts pointing in the same direction, namely, the recovery of prothrombin from fibrin, as shown later. And also the fact that thrombin thus aged and inactive becomes active again on the addition of fresh cephalin, calcium is not necessary.

Thrombin thus resembles closely tissue fibrinogen, since, as Mills found, if the lipid be extracted from tissue fibrinogen, it loses its clotting powers and becomes inactive and even powerfully anti-coagulant. The

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clotting power is restored by re-addition of cephalin to the anti-coagulant. The difference between them is the much greater ease of dissociation of cephalin from thrombin than from tissue fibrinogen. The difference is that tissue fibrinogen clots fibrinogen only in the presence of calcium ions; whereas thrombin does not require calcium.

The relation of the clotting power to the amino nitrogen indicates that a part of the molecule of the thrombin is protein, or peptide, in nature.

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(b) Is Thrombin a Protein?

The preceding facts indicated that thrombin was a protein, or contained a peptide of some nature in its molecule in addition to cephalin and calcium.

If it is protein, it might be inactivated by digestion either with pepsin or trypsin. Schmidt (27) thought that thrombin itself was not a protein, since he obtained active thrombin solutions which gave a very feeble biuret reaction. But this reaction is very delicate. It might very well be that the enzyme itself was not protein, but was ordinarily united with protein, since it must combine readily with some proteins since it unites with fibrinogen.

If thrombin is digested by pepsin, it shows that it has one or more free amino groups as stated by Hugounenq and Loisleur (28). This will substantiate the evidence of the presence of amino groups in thrombin as shown in Table XV. Pepsin is considered an acid enzyme or it contains an acid group, hence it must attack the free amino groups.

To test the action of pepsin on it the following experiment was tried.

A. Peptic digestion of thrombin.

Standard pepsin solution was prepared as follows: 0.3 gm. of 1:3000 U.S.P. pepsin was added to 100 ccs. of 0.2 per cent HCl. To every one-half cubic centimeter of

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the 0.3 per cent pepsin solution 4.5 ccs. of 0.2 per cent HCl was added.

One cubic centimeter of this pepsin solution was added to each 25 ccs. of thrombin solution. These tubes were incubated at 38°C. for various lengths of time. Each tube was removed from the bath at 5 minute intervals. One cubic centimeter of this pepsin-thrombin solution was tested with fibrinogen for clotting property.

In order to prevent the pepsin from acting on fibrinogen, pepsin was destroyed by the addition of 0.3 cc. N/10 sodium carbonate to the thrombin solution to neutralization, before the addition of fibrinogen.

Table XV below presents the data. The activity of thrombin was destroyed by pepsin under these circumstances in 20 minutes.

For the control, 1 cc. pepsin solution was boiled and 0.3 cc. of N/10 sodium carbonate solution was added to neutralize the acid. The pepsin solution thus treated was neutralized exactly and was added to thrombin. After 20 minutes of incubation, 1/2 cubic centimeter of this was added to 2 cc. of fibrinogen, it was clotted in 5 minutes. The control containing no pepsin at the start coagulated fibrinogen in 6 minutes.

The results show that the thrombin activity was completely destroyed in 20 minutes by pepsin. This could not be reactivated by addition of fresh cephalin and calcium.

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Insert Table XV.

B. Tryptic action on thrombin.

Standard solution of trypsin was prepared by adding 3 cc. of 0.5 per cent HCl to 1 gm. of pancreatin (Merck). This was allowed to stand 30 minutes, then 0.1 cc. of 10 per cent sodium carbonate solution was added (red to methyl orange pH 4.4) and filtered. At this acidity trypsinogen is activated and trypsin is preserved, while lipase and amylase are destroyed. To the filtrate containing only trypsin 10 per cent sodium carbonate was added until there was a faint red color produced with methyl orange. Trypsin was stabilized at this acidity and this constituted the stock solution. It was preserved with toluene and kept in the ice box (7°C.).

For the experiment, 10 per cent sodium carbonate solution was added to 10 cc. of trypsin solution to make it alkaline to litmus for optimum activity (pH8).

To each 25 cc. of thrombin solution 1 cubic centimeter of trypsin solution was added. This was incubated at 38°C. for various lengths of time. Each tube was removed from the constant water bath at 5 minute interval. The enzyme was destroyed by heating it to 65°C. before the fibrinogen was added.

Insert Table XVI.

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Table XV

Proteolytic Action of Pepsin on Thrombin.

Exp. No.	Thrombin Solution	0.3 % Pepsin soln. (1-3000) HCl.	Incubation 38°C	neutralized with alkali $\frac{1}{10}$ Na_2CO_3	Hydrolyzed Thrombin	Blood Fibrinogen	Clotting Time
1	control ½ c.c.	none	none	none	none	2 c.c.	6 min
2	2.5 cc.	1 cc.	5 min	0.2 cc.	½ cc.	"	1 hr.
3	"	"	10 "	"	"	"	4 hrs
4	"	"	15 "	"	"	"	15 hrs
5	"	"	20 "	"	"	"	no clot 24 hrs
6	"	" boiled	20 "	"	"	"	5 min.

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Table XVI

Proteolytic Action of Pancreatin on Thrombin
Tryptic Action.

Exp. No.	Solution Thrombin	Pancreatin 1% solution	Incubation 38°C	neutralized with % HCl Heated to 65°C	Hydrolyzed Thrombin	Blood Fibrinogen	Clotting Time
1	control ½ cc.	none	none	none	none	2 cc.	15 sec.
2	45 cc	1 cc.	5 min	% HCl to [illegible] Heated 65°C	½ cc.	"	1 hr 20 min
3	"	"	10 "	"	"	"	3 hrs. 45 sec.
4	"	"	15 "	"	"	"	5 hrs.
5	"	"	20 "	"	"	"	no clot 24 hrs.

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At the end of first 5 minutes incubation this treated thrombin coagulated fibrinogen in 1 hour and 20 minutes; at the end of 10 minutes incubation, fibrinogen was coagulated in 3 hours and 45 minutes. At the end of 20 minutes of incubation the thrombin activity of the thrombin trypsin mixture was tested and it was found to be totally lost. There was no coagulation of fibrinogen at the end of 24 hours.

Thrombin was digested not only by pepsin, but also by trypsin. According to Willstatter and Waldschmidt-Leitz's interpretation of tryptic activity (29), thrombin must be combined with trypsin (basic) through the carboxyl groups of the substrate in order to be digested. Since thrombin is attached by trypsin, it must contain carboxyl groups as previously shown in Table XIV.

C. Action of Lipase on Thrombin.

I next tested to see if lipase would destroy the activity of thrombin by acting upon cephalin.

Lipase solution was prepared by adding a trace of bile (one to two drops) to 5 ccs. of 10 per cent water extract of fresh hog pancreas (neutral to litmus). One cubic centimeter of this preparation was added to 1 cc. of 1 per cent cephalin emulsion and after 2 hours of incubation at 38°C. 1/2 cubic centimeter of this was added to activate prothrombin calcium to thrombin. This coagulated fibrinogen in 14 hours.

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Insert Table XVII.

At the end of 4 hours of incubation lipase had completely hydrolyzed cephalin as indicated by its failure to activate prothrombin-calcium mixture to thrombin. Thrombin activity is then concluded to be destroyed by the proteolytic enzymes and by lipase in 2 hours.

It may be that the proteolytic enzymes were the cause; or it may be that there may have been specific cephalinase in each case. Billings and Professor Mathews have shown (1) that there is such a specific cephalinase in the venom of Crotalus adamenteus, and it may possibly be also in the pepsin and pancreatin I used. But this cephalinase will act on the combined cephalin of tissue fibrinogen and thrombin and not only on free cephalin. However, the action on tissue extract (Merrell's Fibrogen) cannot be said to be due to the combined cephalin since the commercial preparation contains 1/2 to 1 per cent of free cephalin, which if removed destroys the coagulative property of the tissue extract.

From these experiments it appears that the activity of thrombin is slowly destoryed by pepsin and trypsin but just how this was accomplished is obscure.

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Table XVII

Action of lipase and Venom of C. adamanteus. on Cephalin, Thrombin and Tissue fibrogen.

Exp No.	Cephalin Emulsion c.c.	lipase Solution c.c.	Incubation 38°C	Prothrombin + Ca Mixture c.c.	stand 20 min and use	Fibrinogen Solution cc	clotting Time
1	1.0	none	none	4.0	0.5 cc	2.0	3 min.
2	"	1.0	2 hrs.	"	"	"	14 hrs.
3	"	"	4 hrs	"	"	"	no clot 24 hrs
4	"	venom "	2 hrs	"	"	"	"
5	Thrombin control 1.0	none	none	none	"	"	3 min
6	"	1.0	2 hrs	none	"	"	no clot 24 hrs.
7	"	venom "	2 hrs	none	"	"	"
8	Tissue fibrogen control 1.0	none	none	none	"	"	14 min
9	"	1.0	4 hrs	none	"	"	no clot 24 hrs.
10	"	Venom 1.0	4 hrs	none	"	"	"

Prothrombin - Calcium Mixture = 1cc. Prothrombin Solution + 0.5 cc. (?) cephalin emulsion + 0.5 (1%) calcium chloride Solution.

Tissue fibrogen - Merrell. contained 0.5 of 1% free cephalin.

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(c) The Influence of additional cephalin in thrombin on clotting time. Weight of fibrin formed and in re-solution of fibrin.

It has been found that if the amount of cephalin was less than that required to bind all of the prothrombin to fibrinogen, a loosely combined fibrin was formed (gel) and any attempt to remove the fibrin thus formed with the glass rod was rendered impossible. The thread-like appearance of fibrin manifested by those which contained high cephalin was absent in the gel clot.

Table VIII shows that an increase in the cephalin content causes a decrease in the clotting time to a certain minimum point beyond which the addition of more cephalin has no effect. The solidity of the clot increased with the increase in concentration of cephalin.

Insert Table XVIII.

This experiment was tried to see what specific effect, if any, an increase in cephalin might have on the clotting time and also on the retraction or re-solution of the clot.

To an active thrombin solution already containing sufficient cephalin (0.37 mgm.) for full activity additional amounts of cephalin emulsion were added. Five minutes were allowed to elapse before fibrinogen solution was added.

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Table XVIII 76

Effect of Additional Cephalin in Thrombin on Clotting Time, Weight of fibrin, and Retraction of Clot.

Let this stand. 5 minutes.											
Exp. No.	dry Weight Thrombin mgs.	Water cc.	Cephalin calc. as dry mg.	Total wt. Thrombin mg	Fibrinogen Solution mg	Clotting Time. Control	Clotting Time. Exp.	Fibrin Weight mg. Control	Fibrin Weight mg. Exp.	Clot Retraction complete.	K = C.T *
1	13.75	0	0	13.75	38.9	3'0	-	3.1		v. slight 48 hrs.	41.25
2	"	1.0	0	13.75	"	3'40"	-	3.3		3 days.	46.75
3	"	0.1	3.33	17.08	"	5'25"	1'30'	3.3	6.0		39.28
4	"	0.2	2.96	16.71	"	4'36"	2'00"	3.4	5.05		50.06
5	"	0.3	2.59	16.34	"	4'20"	2'25"	3.1	5.1		39.24
6	"	0.4	2.22	15.97	"	4'30"	1'90"	3.3	4.0	4 days.	23.95
7	"	0.5	1.85	15.60	"	5'10"	1'20"	3.3	5.0		18.72
8	"	0.6	1.48	15.23	"	5'15"	1'90"	3.4	3.9		22.84
9	"	0.7	1.11	14.86	"	7'05"	1'90"	2.6	3.85		17.31
10	"	0.8	0.74	14.49	"	8'00	2'05"	3.6	3.8		29.7
11	"	0.9	0.37	14.12	"	5'50"	2'45"	3.2	3.7	5-6 days	35.39
12	"	1.0	0	0	"	6'30"	2'50"	3.5	3.8		34.37
13	"	0	4.07	17.81	"	6'30"	2'45"	3.2	6.3	6 days.	43.63
14	"	0	4.44	18.18	"	7'00	4'30"	3.4	5.9		78.17

Controls for each experiments conducted using water in place of cephalin.

* Konstant = Conc. enzyme × Time of clotting.

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The results in Table XVIII show that the addition to 5 cc. of active thrombin successive portions of cephalin emulsion equivalent up to 2.59 mgms. cephalin had a decreasing effect on the mean clotting time as compared to the control clotting time determined with each experiment. By the addition of 0.74 mgm. to 1.85 mgms. of cephalin the clotting time was reduced from 2 minutes and 5 seconds down to 1 minute and 20 seconds. The addition of cephalin above 1.85 mgms. gradually lengthened the mean clotting time.

These results are fairly significant.

Controls were determined for each experiment, since dilution of thrombin affects the clotting time of fibrinogen. The volume of control tubes were made up with 1 per cent saline solution equal to the volume in the experimental tubes. The mean clotting time was taken, that is the difference of clotting time between an experimental tube and a control tube.

It is obvious from the table that increase in dilution of control (tube #11) lengthened the clotting time from 3 minutes in the original undiluted control to 8 minutes in the tube containing 0.9 cc. of saline solution. This tube contained the same amount of cephalin, 0.37 mgm., as in tube #1.

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The weight of fibrin obtained seems to be dependent on the quantity of cephalin present in thrombin from results tabulated in Table XIX. It shows a series of tubes containing increasing quantities of cephalin in a given amount of preformed thrombin, which already contained some cephalin (0.37 mgm.). The weights of fibrin increased as the cephalin content was increased up to a certain point. The weight of fibrin in the tube containing only preformed thrombin, that is, the minimum amount of cephalin (0.37 mgm.) was 3.1 mgms.; this fibrin weight increased to 6.6 mgms. in tubes containing thrombin with 3.33 mgms. cephalin content. This constituted the maximum yield and any increase in cephalin above 3.33 mgms. did not increase the weight of fibrin.

Insert Table XIX.

According to results from the data found in Tables XVIII and XIX, the increase in cephalin in the preformed thrombin, first has a decreasing effect upon clotting to a certain limit (2.59 mgms.) and second, it has an increasing effect on the weight of fibrin up to a certain limit in the cephalin content (3.33 mgms.). This increase in weight is not proportional to the clotting time, since the maximum decrease in clotting time due to increase in additional cephalin content ceased when 2.59

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Table XIX

Amount of Fibrinogen and Thrombin in Fibrin.
Weight of Fibrin.

Control					Cephalin content varying in Thrombin.			
Exp No	wt of Fibrinogen + Thrombin	wt of Fibrin washed + dried. mg	wt after clotting mg.	wt loss through washing mg	wt of Fibrinogen + Thrombin + cephalin mg	wt. of Fibrin dry mg.	wt after clotting mg	wt loss thru washing mg
1	13.7 = F 52.6 = T	3.1	29.7	19.85	52.65	3.1	39.4	10.15
2	"	3.1	33.2	16.15	52.65	3.1	37.8	11.75
3	"	3.3	29.0	20.13	55.98	6.6	52.6	0.23
4	"	3.2	31.3	18.35	55.61	5.05	42.5	8.06
5	"	3.3	29.4	19.95	55.24	6.1	50.6	0.46
6	"	3.4	46.1	3.15	54.87	4.6	50.6	0.33
7	"	3.3	30.8	18.55	54.5	5.0	44.8	4.5
8	"	3.2	30.2	19.25	54.13	3.9	44.9	5.33
9	"	3.1	33.9	15.65	58.39	3.6	52.5	2.09
10	"	3.2	43.7	5.75	58.39	3.8	52.9	2.72
11	"	3.2	26.7	20.75	58.02	3.7	53.0	1.32

Weight of Fibrinogen for each experiment was 13.75 mgms.
Increase in weight of Thrombin due to additional cephalin.

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mgms. of cephalin was present, and the weight of fibrin increased up to concentration of 3.33 mgms. of cephalin, after which the weight of fibrin did not increase.

In addition to the increasing addition of cephalin content, constant quantity of 1 per cent calcium chloride solution was added. This experiment was performed to find if calcium has any synergistic action on cephalin in the clotting of fibrinogen.

Insert Table XX.

Table XX presents the following points; first, with the increase in quantity of cephalin and calcium in thrombin, there was noticed a gradual decrease in the clotting time; second, with a decrease in cephalin content and an increase in calcium the clotting time was not decreased to such an enormous extent from the tubes containing cephalin in varying quantities. Calcium does not exert a very marked decreasing effect upon the clotting time; third, the color of fibrin formed from thrombin containing more calcium were white like the colcr of chalk, while those formed from thrombin containing minimum amount of calcium were translucent.

The weights of fibrin did not increase as markedly as when cephalin alone was increased. For instance, the tube containing addition mgms. of cephalin in thrombin gave

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Table XX

Effect of Additional Calcium & Cephalin in Thrombin on clotting Time, weight of Fibrin, Retraction of Clot.

Thrombin mg	H_2O cc	Additional cephalin mg	Additional calcium mg	Stand 5 min add Fibrinogen mg	Clotting Time		weight of Fibrin mg		Retraction of clot + of complete	
					Control*	Exp.	control	Exp.	control	Exp.
1 12.75	0	0	0	36.1	5 min		2.1		2 days	
2 "	0.1	3.33	0.05	"	4'	1'	2.7	3.7		
3 "	0.2	2.96	"	"	3'45"	1'02"	3.4	4.3		
4 "	0.3	2.59	"	"	3'38"	1'	2.5	3.7	5 days	
5 "	0.4	2.22	"	"	4'05"	1'05"	2.2	3.6		8-9 days.
6 "	0.5	1.85	"	"	4'55"	1'	2.3	3.4		
7 "	0.6	1.48	"	"	6'	1'	2.7	3.1		
8 "	0.7	1.11	"	"	[illegible]'05"	2'10"	2.0	2.6	3 days	
9 "	0.8	0.74	"	"	13'50"	3'09"	2.2	2.8		5-6 days
10 "	0.9	0.37	"	"	15'45"	5'15"	2.2	2.5		
11 "	1.0	0	"	"	14'05"	6'50"	2.0	2.3	2 days	3 days
12 "	0.9	4.07	"	"	14'05"	1'05"	2.2	3.6	5-6 days.	5 days

* Control tubes contain no additional cephalin or calcium, H_2O is added to make up to equal volume.

+ Retraction of clot measured by millimeter ruler.

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a fibrin yield (dry weight) of 6.0 mgms. (Table XVIII) than in another tube containing both cephalin (3.33 mgms.) and calcium (0.05 mgm.) gave a fibrin weight of 4.3 mgms. I have obtained this decrease in fibrin weight containing calcium in excess.

Calcium is not absolutely necessary in the coagulation of fibrinogen, but it is essential in the conversion of prothrombin to thrombin in addition to cephalin, since prothrombin and cephalin mixture without calcium failed to clot fibrinogen. I shall mention in detail later about the initiation of clotting with thrombin containing no calcium (decalcified).

Another interesting result that was found in this experiment concerns the rate of re-solution of fibrin. The more solid the clot, which meant higher concentration of cephalin, the slower was the rate of its re-solution. This can only be explained by the fact that a firm union between thrombin and fibrinogen has been established at the expense of the cephalin molecule and perhaps calcium. The reason for slowed re-solution of fibrin containing more cephalin may be due to the fact that cephalin has a fairly strong affinity for water and this decreased the amount of free water present in the system to affect hydrolysis of fibrin.

1. Does cephalin and calcium increase the number of carboxyl or amino groups?

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In order to explain for the activity of thrombin when additional cephalin was added and also calcium, determinations for carboxyl groups were made, to find out whether or not there is an increase in the carboxyl groups. Sorensen test using formaldehyde was used against the Willstatter method for comparison of the number of carboxyl groups.

Insert Tables XXI and XXII.

It was found that there was a definite decrease in carboxyl groups with the decrease in the cephalin content. resence of calcium did not seem to decrease the number of carboxyls. However, in the Sorensen titration there was an increase in the carboxyl groups, which showed there was an hydrolysis of some protein brought about by the alkali in water, or it may be that cephalin united with the nitrogen of the aminde grouping.

Free amino nitrogen groups were not determined, so conclusions cannot be drawn from this experiment regarding the liberation of free amino group. If the carboxyls were decreased, then there should be a decrease in free amino groups, provided they were combined with the carboxyls. The activity of thrombin may be attributed to the presence of these groups.

These determinations do not seem very significant

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Table XXI

Carboxyl Content of Thrombin containing increasing amount of Cephalin

No. Exp.	Thrombin weight. dry mg. control	Sorensen cc. N/20 NaOH.	Carboxyl - cc. N/10 KOH.	
			Peptide	Amino Acid.
1	13.75	0.9	0.1	0.1
2	17.08	1.65	"	"
3	16.71	1.1	"	0.3
4	16.34	"	0.05	"
5	15.95	"	0	0.16
6	15.60	0.4	0.05	0.05
7	15.23	0	0	0.15
8	14.86	0.1	0	0.05
9	14.49	0.05	0	0
10	14.12	0	0.05	0.1
11	13.75 ~~17.81~~	1.1	0.1	0.05
12	17.81	0.3	0.2	0.05

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Table XXII

Carboxyl content of Thrombin containing Cephalin and Calcium in additional quantity.

No Exp.	Thrombin weight mg control	Sorensen cc. N/20 NaOH	Carboxyl	
			Peptide	Amino acid.
1	13.75	0.4	0.1	0.4
2	17.13	0.1	0.1	0.2
3	16.76	0	0.1	0.2
4	16.39	0.1	0.05	0.3
5	16.02	0	0.15	0.2
6	15.65	0.5	0.2	0.2
7	15.28	0.5	0.1	0.15
8	14.91	0.1	0.05	0.1
9	14.54	1.3	0.0	0.1
10	14.17	1.6	0.2	0.05
11	0.00	2.0	0.1	0.1
12	17.86	1.8	0.1	0.05

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as to the activity of thrombin and they are to be determined in another work.

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(d) Does thrombin carry cephalin to fibrinogen to make fibrin?

Pekekharing (5) suggested thrombin was a carrier of calcium to fibrinogen and the calcium fibrinogenate formed became fibrin. This view Hammarsten showed to be untenable (6). Prof. Mills suggested that perhaps cephalin was carried over to fibrinogen to make fibrin.

To determine this, I analyzed the phosphorus content of fibrinogen and fibrin. If cephalin was united to fibrinogen the phosphorus content of fibrin should be higher than that of fibrinogen, since cephalin contains 4 per cent of phosphorus.

The results embodied in Table XXIII indicate in some instances a slight increase of phosphorus in the fibrin. The amount, however, is within the limits of error of the method, and in some cases, there was a very slight change observed.

Therefore, that fibrin contains more phospholipid than fibrinogen is very slight. In these determinations neither the fibrin nor the fibrinogen were extracted with alcohol or ether before the determinations were made.

Insert Table XXIII.

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Table XXIII

Phosphorus Content in Fibrin.

Thrombin			Fibrinogen			Fibrin.		
Weight dry	P	P %	Weight dry	P	P	Weight dry	P	P
mg.	mg		mg.	mg	%	mg.	mg	%.
4.025	0.0235	0.[illegible]586	2.02	0.0124	0.6138	–	–	–
0.3825	0.0025	0.6563	10.02	0.0639	0.6264	6.53	0.0415	0.635
0.8010	0.0049	0.6132	10.2	0.0639	0.6264	9.60	0.0672	0.700
1.1505	0.0076	0.6667	14.9	0.0943	0.6664	14.08	0.100	0.71
1.5275	0.0099	0.6498	23.45	0.1448	0.6188	25.07	0.1651	0.6585
2.125	0.012	0.6023	33.65	0.2235	0.6124	35.1	0.2325	0.6624

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Fibrinogen when thus analyzed contained about an average of 0.64 per cent of phosphorus and fibrin about 0.671 per cent.

The extraction of the lipid from both fibrinogen and fibrin gave very irregular results, owing to the fact that free as well as combined cephalin was probably embraced in the solution, since cephalin had been added to the prothrombin to make thrombin. Very possibly an excess was present and it was precipitated with the fibrinogen. This result is given in Table XXIV.

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(e) Phosphorus Content of blood proteins after extraction with benzene.

I next decided to find out whether or not there were any increase of phosphorus in the molecule which could not be extracted with benzene and it has been found that some lipoid was attached directly to the fibrin molecule as shown by the phosphorus determination after the lipid extraction with benzene. This was not the case in the determination of lipid of thrombin.

Phosphorus was determined colormetrically by the Fiske and Subbarow (30) method. The phosphorus of the protein was converted to phosphoric acid by digestion with concentrated sulphuric acid. This phosphoric acid was transformed to phosphomolybdic acid by ammonium molybdate and it was then reduced by amino-naphtholsulphonic acid in the presence of sulphites with the production of a blue color.

Practically all of the lipid can be extracted from thrombin leaving only a trace of phosphorus as determined by the colorimetric method above. They could not be extracted from fibrin and prothrombin, also from recovered fibrinogen and recovered prothrombin obtained from the fibrin solution. The increase in the percentage of phosphorus determined after benzene extraction of the proteins was, therefore, due to the direct attachment of cephalin to the recovered fibrinogen and prothrombin, and

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fibrin. There was an evident irregularity in the extraction of the phospholipid with benzene. The amount of lipid extractable by benzene in thrombin is large, due to the mechanical attachment or by its loose union with prothrombin, as shown in Table XXIV.

Insert Table XXIV.

There was some extractable lipid in fibrinogen. It contained 0.867 per cent of phosphorus in the molecule. It was increased from 0.877 per cent to 13.20 per cent in the recovered fibrinogen, the extractable lipid of this fibrinogen was now increased from 0.102 to 1.425 per cent.

Phosphorus was increased in the recovered prothrombin fraction from fibrin solution from 0.80, to 3.38 per cent.

This phosphorus increase was due to the addition of cephalin to the fibrin through the thrombin, and its increase in fibrinogen and prothrombin showed that cephalin must have combined with some groups in the respective molecule. Just how this is accomplished cannot be stated.

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Table XXIV

Phosphorus Content of Blood Proteins after Benzene Extraction.

Sample	Benzene Extractable lipid %	P content after extraction mg	
Fibrinogen	0	1.867	
Fibrinose	1.435	0.877	
"	0.102	13.2	
"	1.75	1.20	
Prothrombin	1.325	0.8037	
Recovered Prothrombin	0.619	2.22	
"	1.35	2.123	
"	5.85	3.78	
Thrombin	13.25	0.895	
Fibrin	0.0581	1.59	
Fibrin	0.246	5.55	
"	0.0174	1.59	

Fibrinose is obtained from fibrin solution by precipitating it with sodium chloride on saturation.

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(f). The susceptibility of thrombin to heat.

Enzymes are usually destroyed by heating between 60°C and 70°C. Most enzymes are said to be very rapidly destroyed in aqueous solution below the boiling point, although for example, according to Miyake and Ito (31) Aspergillus oryzae amylase solution, suitably prepared retains some activity even after heating at 100°C. for 2 hours. Schmidt (32) has found that after 5 to 10 minutes boiling the thrombin action was entirely lost, but it was not totally lost on heating to 70°C. Wittich showed a similar result with pepsin. Rennin (33) derived from stomach resists boiling. Hammarsten had found that pepsin solution, which had been heated 48 hours at 40°C. to weaken the rennin action coagulated milk in 6 hours and 10 minutes. Prothrombin is coagulated and precipitated at 58°C. according to Bordet (3) and Mellanby (4), but prothrombin that I have prepared in the laboratory was coagulated at 68-71°C. Thrombin activity was partially or completely destroyed depending upon the length of time of heating and on the amount of proteins coagulated out from the solution. Dried preparations of enzymes are usually more stable, like thrombin, it was only slowly inactivated at 80°C. This point is shown when cephalin emulsion and 1 per cent calcium chloride solution are added to this heated prothrombin and then allowed to stand for 30 minutes

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or even 1 hour, there was no thrombin present, coagulation of fibrinogen was negative within 24 hours, or in some case there was a presence of a very weak gel.

It is, of course, well known that such coagulated particles may adsorb the enzyme and carry it down with them and thus lead to the erroneous conclusion that the enzyme has been destroyed. But this is not the case with prothrombin, for I added to the suspension of coagulated prothrombin both cephalin and calcium chloride, but I was never able to obtain any clotting action on fibrin gel. From this it is clear that prothrombin is very heat sensitive and destroyed by heating to 58°C. for 5 minutes.

Thrombin proved, on the contrary, to be extraordinarily resistent to heat when thus tested; but if the solution was filtered first it was removed with the coagulated protein.

Table XXV shows a series of tubes containing 5.0 ccs. of the active thrombin solution which were subjected to various temperatures as indicated for 5 minutes. The tubes were cooled immediately in cold water, and it was shaken to secure a uniform distribution of protein. The temperature of the thrombin solution was brought back to 38°C. and fibrinogen solution was added. Clotting time was noted.

Insert Table XXV. Graph IV.

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Table XXV

Effect of Temperature on Thrombin Activity.

Exp. No.	Weight Thrombin sec. dried	Thrombin Sol'n heated 5 min.	Amount Thrombin used heated	Fibrinogen Solution	Clotting Time.	Carboxyl		free amino	Clot retraction	
						Peptide	Amino-acid	nitrogen	24 hrs	complete
	mg	°C.	c.c.	cc.		%	%	%	%	
1	36.25	none	0.5	2.0	10 min	13.2	4.34	10.68	100	
2	"	62°	"	"	2 hrs	2.479	14.07	2.019	75	2 days
3	"	71°	"	"	4 hrs 30	8.79	15.39	1.7715	25%	2½ .
4	"	80°	"	"	19	2.47	13.2	1.6715	50%	"
5	"	90°	"	"	hrs	2.47	17.59	3.683	50%	3 days
6	"	100°	"	"	v. faint gel.	2.47	19.59	2.495	1 hrs 100	
7	"	100-110°	"	"	no clot 24 hrs	9.56	17.51	2.632		

Temperature rise was 4° per minute

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Influence of Temperature on the Stability of Thrombin. Graph IV.

5hrs

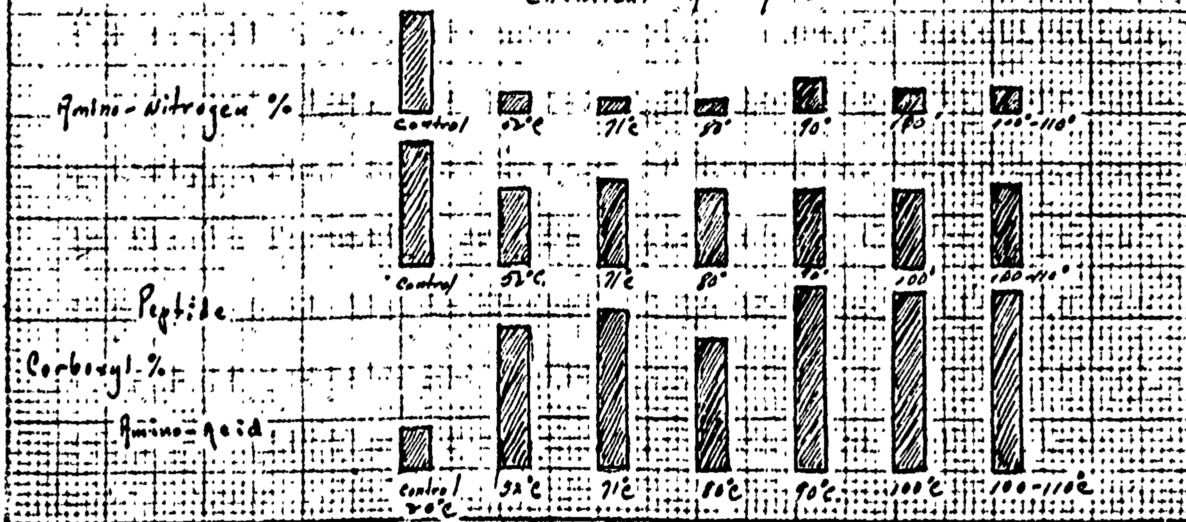
4

3

2

1 hr

30°C 40 50 60 70 80 90 100 110°



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There was apparently no coagulation of thrombin at 40°C., while a slight precipitation of protein was noticeable at 58°C. At 80°C. there was a fairly complete coagulation of thrombin, since its filtrate showed only a cloudiness upon further heating. Between the temperature of 100° and 110°C. the activity of thrombin was apparently completely lost. The filtrate from thrombin solution heated to 100°C. showed a very faint biuret test. Addition of fresh cephalin emulsion and calcium did not restore the activity of the thrombin.

In another test it was found that the washed coagulated protein from the thrombin solution alone did not clot fibrinogen. The protein has undergone a chemical change by heating.

The variation in the clotting time of the filtrate from heated thrombin, point out that the thrombin is changed by heating. It undergoes irreversible inactivation as indicated by the failure in the restoration of the initial activity of thrombin. As the temperature is raised, the reaction proceeds more rapidly towards inactivation, and in addition, the veloxity of clotting falls off more quickly, owing to the change of thrombin. This decrease in fibrin formation may be explained on the basis that thrombin activity is decreased in the proportion to the amount of protein precipitated out (which are inactive) from the solution of thrombin. Since not all of the

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proteins are precipitated at any one temperature.

Re-solution rate of fibrin formed from these samples of heated thrombin was determined in this experiment and it was found that the rate of re-solution was slower in tubes which contained thrombin inactivated at higher temperature. Thrombin heated to 90°C. showed no sign of re-solution in 2 hours. This further supports the chemical change in thrombin by heating.

This evidence of change is given in the shift in carboxyl groups and the free amino grousp, as shown in Table XXV.

The number of free amino nitrogen dropped immediately when thrombin was heated to 52°C. from 10.68 per cent down to 2.01 per cent, then gradually decreased at 80°C. and then there occurred a slight rise at 90° and 100°C. The peptide carboxyl groups were decreased from 13.2 per cent to 7.4 per cent on an average down to 3.68 and 2.49 per cent respectively, with an accompanying increase in the number of amino acid carboxyls from 14.39 per cent to 17.5 per cent. Whatever causes this shift in the reactive groups on heating cannot be explained here until further study on this point has been done.

The influence of heat on thrombin is of practically little significance. It really gives a rough idea of the stability of the enzyme preparation. The temperature

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of inactivation varies with the method of preparation of thrombin. Mellanby claims that his thrombin is destroyed by heat at 56°C.

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(g). Influence of salts in the inactivation of prothrombin and thrombin by heat.

In the previous experiment it has been shown that heating thrombin at different temperatures for a given length of time partially or completely inactivated it. It was found that prothrombin solution cannot be heated to 100°C. for 5 minutes and still be activated with cephalin and calcium to clot fibrinogen, but prothrombin in the presence of cephalin and calcium chloride is able to withstand heating to a certain point.

Clear solution of prothrombin was used to demonstrate this point in the experiment.

A. Prothrombin solution, 10 cc. of the solution heated to 56°C. for 1 minute showed a very faint cloudiness; this was filtered, and to the filtrate sodium chloride was added to 1 per cent concentration. This solution containing the salt was heated to boiling for 1 minute and filtered again to remove the coagulum; to 2 cc. of the cooled filtrate 1/2 cc. of 1 per cent calcium chloride were added. After 30 minutes standing to obtain maximum activity, 2 cc. of fibrinogen solution was added to 1/2 cc. of the activated filtrate. Clotting was noted in the tube after standing 15 hours at 38°C.

Prothrombin was heated to 80° and 100° and treated as above did not coagulate fibrinogen in 24 hours.

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Prothrombin was heated to 56° and 100°C. without the addition of salt. They were activated with cephalin and calcium to form thrombin. The thrombin made from prothrombin heated to 56° coagulated fibrinogen in one hour and while the latter formed no clot in 24 hours.

B. In this experiment prothrombin filtrate (previously heated to 56°) was activated with cephalin and calcium and after 30 minutes standing it was subjected to boiling for 1 minute and sodium chloride was added to 1 per cent concentration. The filtrate from this heated solution coagulated fibrinogen solution solidly in 5 hours and 45 minutes.

The following experiment was performed to settle the question of whether the presence of salt hastened the inactivation of thrombin by heat. Active thrombin solution was divided into 2 portions, A and B, of 10 cc. each.

1. Thrombin solution-sodium chloride was added to a concentration of 1 per cent and the contents of the tube were subjected to boiling over a free flame for 1 minute.

This heated thrombin was divided into portions (1) and (2). (1) was filtered and (2) was left unfiltered. Two cc. of fibrinogen solution was added to 1 cc. of thrombin solution (1 filtered and 2 unfiltered) respectively. The clot found in these tubes in 5 hours was solid in (2) and showed a very weak gel in (1).

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2. Thrombin solution, this was boiled for 1 minute and cooled, sodium chloride was added to a concentration of 1 per cent.

This sample of thrombin was divided into portions (1' - unfiltered and 2' - filtered). (1') gave a gel clot in 5 hours and (2') showed no clotting.

It is shown that addition of salt to thrombin before it was subjected to boiling hastened the inactivation, much quicker than if sodium chloride was adder after boiling. One per cent ammonium sulphate accelerated the inactivation of thrombin much faster than sodium chloride.

Thrombin can resist a high temperature for a short period of time provided the solution is fairly salt-free and while prothrombin was found to be totally inactivated, when it was heated to 80°C. for 5 minutes.

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VI. Is calcium necessary in the formation of fibrin?

An attempt was made to analyze thrombin as to the presence of calcium not precipitable by oxalate, which is claimed to be responsible in the formation of fibrin, according to Arthur (34). Calcium in thrombin was precipitated by the Tisdall method (35). To 10 cc. of the thrombin solution in a 15 cc. conical graduated centrifuge tube, 5 cc. of a saturated solution of ammonium oxalate was added. The contents of the tube were shaken and the mixture was allowed to stand at room temperature for 1/2 hour. This was centrifuged at about 1500 g.p.m. for 5 minutes. The supernatant fluid was removed by simply inverting the tube for a moment and allowing the fluid to flow out into another tube. This calcium-free thrombin which coagulated fibrinogen solution in 12 hours. This fibrinogen contained 0.15 per cent calcium.

Two per cent ammonium hydroxide was added to the 4 cc. mark on the tube containing calcium oxalate. The precipitate of calcium oxalate was thoroughly mixed and the tube was again centrifuged 5 minutes. This process was repeated once more.

The crystals of ammonium oxalate were then dissolved by the addition of 2 cc. of approximately N/1 sulphuric acid. The contents of the tube were heated in a boiling water bath for 1 minute and was titrated with

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0.01 N $KMnO_4$ until a pink color was obtained which persisted exactly for 1 minute. The thrombin solution bave 0.2 mgm. of calcium in the 2 cc. sample.

One-half of the quantity of the supernatant fluid (thrombin) was evaporated with nitric acid to dryness to destroy the organic matter and N/10 HCl was added to acidity and excess acid was neutralized and the calcium was precipitated in the usual way. No precipitate was found in the tube.

All the calcium was then precipitated. The calcium evidently was not necessary in the transformation of fibrinogen to fibrin after active thrombin was formed. The fibrinogen solution was not calcium free, this calcium may have induced the coagulation of fibrinogen when the cephalin concentration was kept constant in the thrombin. It was found that the clotting time was decreased in proportion to the amount of calcium precipitated out with sodium oxalate solution. The results are presented in Table XXVI.

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Table XXVI

Calcium in the Formation of Fibrin

Thrombin Solution	Ammonium oxalate 5% Sat. sol'n c.c.	Clotting Time 2 cc. Fibrinogen	Nature of Clot.
1.0 cc.	0	2 min.	Solid
"	0.1	9 "	"
"	0.2	" "	"
"	10.0	1 hr. 30 min	"
"	0.4	95 min	"
"	1.0	150 "	"
"	1.5	20 "	"
"	3.0	35 "	"

1/2 cc. Thrombin contains 8.3 mgs Ca.

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Discussion.

The general result of the study is that thrombin first unites with fibrinogen to form fibrin; and that it then hydrolyses the fibrin with the formation of a protein, which is coagulable by heat at 64-5°C. and an albumose. Thrombin is, therefore, a proteolytic enzyme of a specific nature, and remains combined with the fibrin it produces.

Experiments were performed with a definite amount of thrombin and a variable quantity of fibrinogen, the thrombin being present in an amount sufficient to convert all the fibrinogen that was added.

It was fount that the weight of fibrin formed, after washing and drying (38°C. for 24 hours) was larger than the weight of the added fibrinogen. This increase in weight did not change by frequent washing with distilled water, nor did it change on further drying. The fluid left after the removal of fibrin when united with the wash-water from the fibrin, was dried to constant weight, and was found to be less than the weight of fibrinogen or thrombin used. This showed that the fibrinogen had united with water or thrombin or both to make fibrin.

The fibrin thus formed, when it is carefully washed and dried at body temperature, redissolves slowly

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on long standing in distilled water; but much faster if the fibrin be placed in isotonic salt solution. This solution is termed hereafter "the fibrin solution". It contains an albumin coagulating on heating to 80°C. There was a small amount of coagulation at 62° - 65°; but most of the fibrin was represented by the protein coagulating at 80°. This fibrin solution was capable of coagulating fibrinogen solution when it was tested within 24 - 36 hours after the onset of lysis. This coagulating property disappeared within 6 days. This was evidence for the presence of some thrombin which had apparently dissociated from its union with the fibrin molecule. Albumose was not found in this fibrin solution, at the start, but a small amount was present after the third or fourth day. It was detected in the filtrate from the fibrin solution which had been heated to 80°C. and filtered to remove all the coagulable proteins. This albumose was precipitated with 5 per cent phosphotungstic acid in sulphuric acid (30 cc. in 1 liter). It was surprising that the amount of this albumose found in the filtrate did not vary in a fibrin solution 5 days from a solution 4 months old. Whether this albumose was formed from the hydrolysis of fibrinogen by a prolonged action of thrombin, or whether it is one of the constituents of the fibrinogen molecule, as is perhaps more probable, can

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not as yet be said.

That thrombin combines with fibrinogen is substantiated by 2 experimental facts; (1) the presence of prothrombin in fibrin solution previously carefully washed with water and dried; (2) there is an increase in the weight of fibrin over the weight of the fibrinogen added to thrombin; this weight cannot be changed by frequent washing with water. This is assumed that water does not unite in the fibrin molecule.

Fibrin solution was carefully analyzed and it was found to contain more free carboxyl and free amino nitrogen groups than the original mixture of fibrinogen and thrombin used in the experiment. This shows that a true hydrolysis had occurred.

Fibrin solution contained a protein which was precipitated by saturation with sodium chloride and it was coagulable by heat at 65°C. It was not precipitated, as is fibrinogen, by half saturation with sodium chloride. This fraction contained a larger proportion of free amino and carboxyl group and of calcium and phosphorus than fibrinogen. The total nitrogen was somewhat less than fibrinogen. It is probably a modified prothrombin. I have found that a fibrinogen solution standing in the ice box undergoes the same change which is obtained much more rapidly by the action of thrombin. The phosphorus content of this fibrinose or fibrinoglobulin is not as high as in the

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fraction obtained from the fibrin solution, since this fibrinogen hydrolysis was brought about in the absence of thrombin action.

Another constituent of the fibrin solution is another protein, which is precipitated by 1/2 saturation with ammonium sulphate. This substance precipitated in part when it was dissolved in water and dialyzed for 24 hours with running tap water and was coagulated by heat at 71-2°C. It was probably a serum globulin in part at least. A solution of this substance coagulated fibrinogen when it was activated by the addition of cephalin and calcium. There was a slight increase in amino-nitrogen and carboxyl groups over that of the original thrombin solution, but this change did not parallel the activity. It contains, therefore, most of the prothrombin perhaps attached to the globulin.

The action of thrombin appears, therefore, from the foregoing to be very analogous to that of rennin. Rennin is a specific proteinase which hydrolyzes caseinogen to form casein. Thrombin also resembles in a striking fashion a fibrinogenase of the venom of Crotalus adamenteus as studied by Billings and Mathews (1), in that both form a protein with fibrinogen which is heat coagulable at 80°C. and an albumose like substance.

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I have found that re-solution of fibrin by thrombin or some other protease is accelerated by the presence of oxygen. Fibrinogen solution exposed to an oxygen atmosphere for 19 hours was found to be completely incoagulable by thrombin, while the activity of the thrombin is only slightly diminished.

Evidence has been presented to show how thrombin transforms fibrinogen into fibrin and how it breaks fibrinogen into fibrin and ultimately redissolves the latter and splits off albumose. The following observations and data are offered to describe the properties of thrombin.

Thrombin is a substance produced by the activation of prothrombin (serozyme of Bordet) of blood plasma with cephalin and calcium and is capable of clotting blood fibrinogen.

1. Preparation of prothrombin and its conversion to thrombin. It has been found that the thrombin solution deteriorates on standing, just as does serum taken from a clot. A clear thrombin solution deposited a sediment rich in lipoid on standing in the ice box, which if it were removed and washed possessed a much enhanced coagulant activity compared with the supernatant portion of the solution. There was no relation found between the total nitrogen in this precipitate and the clotting power, but the

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product of the cl tting time by the number of free amino groups was fairly constant. The number of these groups in various samples of thrombin was not, however, constant. There was more lipid (cephalin) matter found in the thrombin sediment than in an equal quantity of the material left in solution in the supernatant fluid. The coagulant property of this sediment gradually decreased on standing, perhaps due to the oxidation of cephalin contained in the insoluble fraction, because when cephalin emulsion is exposed to air, it was found to lose its activity.

2. The activity of thrombin was decreased when exposed to action of pepsin and trypsin, and also lipase. Thrombin is not as thermolabile as one believes it to be.

3. The activity of a thrombin solution was decreased in proportion to the amount of protein precipitated out from a thrombin solution by heat. In other words, the inactivation of thrombin was proportional to the increase in temperature. But a thrombin solution heated to 100°C. for 5 minutes, but not filtered, was still able to clot fibrinogen, though at a rate much reduced from that of the same solution before heating. The presence of salts accelerated the inactivation of thrombin by heat. Prothrombin was found to be more sensitive to heat than thrombin. It was destroyed at temperateres between 70° and 80°.

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4. It has been found that the concentration of cephalin affects the clotting time and the nature of the clot. The clotting time was decreased in proportion to the increase in cephalin up to 2.59 mgms. per 13.75 mgms. of thrombin beyond which the addition of cephalin had no affect. In addition, the increase in cephalin up to 2.59 mgms. decreased the rate of re-solution of the fibrin and the weight of fibrin obtained increased up to a cephalin addition of 3.33 mgms. per 13.75 mgms. of thrombin (contains 0.37 mgm. cephalin).

Thrombin, after complete removal of its calcium by oxalate (this thrombin was ashed with nitric acid, this ash gave no oxalate of calcium) coagulated fibrinogen in 12 hours. The fibrinogen, however, was not calcium free (0.15 per cent calcium).

The gradual removal of calcium from the formed thrombin slowly lengthened the clotting time. If there is an excess calcium in thrombin, the color of fibrin is chalk white and fibrin formed from thrombin deficient in calcium is translucent.

Increase in cephalin in thrombin to a certain limit, that is 2.22 mgms. of cephalin for each 13.75 mgms. of thrombin, which is equivalent to 7.18 mg. of prothrombin, or maximum cephalin content of 2.79 mgms. (since 13.75 mgms. of thrombin contains 0.57 mgm. of cephalin)

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decrease the clotting time. Calcium slightly enhances the activity of cephalin in lowering the coagulation time, but its effect is not very significant. The fibrin yield is slightly less than if cephalin alone was added in excess tb thrombin.

Contrary to accepted facts that prothrombin is heat coagulable at 56°C., I do not believe that protein fraction, which is coagulable at 56°C. is totally responsible in the coagulation when it is converted to thrombin by cephalin and calcium, because I have found that prothrombin prepared in the laboratory is heat coagulable at 72°C. There is some protein in this prothrombin solution which is coagulated by heat at 56°C., if this was coagulated protein filtered off and the filtrate upon activation with cephalin and calcium was found to coagulate fibrinogen in 3 minutes. The control using the unheated prothrombin clotted fibrinogen in 2 minutes. This result is obtained provided that prothrombin is not heated at 56°C. for more than 1 minute.

I have found in flasks containing prothrombin solution, which had been standing in the ice box for some time,had a heavy precipitate of presumably insoluble globulin in the bottom of the flask. As this amount of precipitate increased in the flask, the protein which was

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coagulable at 56°C., was found to be proportionately decreased. If the activity of thrombin was dependent upon the protein fraction which was coagulated at 56°C., then the thrombin action should be decreased in proportion to the amount of the precipitated found in the flask containing prothrombin, but this was not found to be the case. The coagulation temperature by heat of such prothrombin was still 71°C. This activated prothrombin coagulated fibrinogen in 1-2 minutes.

Thrombin does not clot milk nor digest other proteins. Thrombin is then a specific hydrolase for fibrinogen, and it is produced by the activation of prothrombin of blood plasma with cephalin and calcium. It appears to be a protein or attached to one.

Thrombin from these observations and experiments has a three-fold purpose, first it unites with fibrinogen to form fibrin; second, it dissolves the fibrin in the presence of water into thrombin and a new protein, fibrinose or fibrinoglobulin (Hammarsten), which is not coagulable by active thrombin. This process is hastened by the presence of oxygen; third, thrombin hydrolyzes a portion of fibrinose into an albumose.

Attempt should be made to obtain crystalline fibrinogen and prothrombin so that for each sample of these plasma proteins one can secure a uniform number

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of amino and carboxyl groups, and thus study the chemical change with a little more certainty. Combustion analyses should be run for each sample to determine the chemical constitution of the substance, to see whether the substance is the same for every preparation of fibrinogen and prothrombin as well as fibrin. Until this can be done these results in this paper cannot be taken purely quantitatively, the variable are too numerous.

Conclusion.

In summary, then, of the experimental evidence presented in this paper, the following points have been found.

1. Thrombin is a specific enzyme for fibrinogen, it unites with fibrinogen to form fibrin, and then hydrolyse it with the formation of a protein coagulating at 64-65°C. by heat and an albumose.
2. Fibrin, when washed and dried will slowly go into solution in distilled water, although the solution is much faster in iso-tonic salt solution. This fibrin solution will clot fibrinogen. Thrombin is found in this solution 1-2 days after the onset of lysis and then it gradually disappears.

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3. Fibrin solution, which is coagulable by heat at 80°C., can be fractioned with sodium chloride and ammonium sulphate to give two proteins, one which is coagulated by heat at 65°C. and this is not coagulated by thrombin. The other protein is coagulated by heat at 71°C. and it can be activated with cephalin and calcium chloride to form thrombin to clot a fresh fibrinogen solution.

4. Thrombin can be heated to 100°C. for 5 minutes and still be able to coagulate fibrinogen. Inactivation by heat is hastened by the presence of salt in a small quantity.

5. Thrombin action is also destroyed by the action of pepsin, trypsin and lipase.

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