UNIVERSITY OF CINCINNATI

May 27, 1935.

I hereby recommend that the thesis prepared under my supervision by Alexander K. Presnell

entitled THROMBIN, A PROTEOLYTIC ENZYME.

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

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Form 668—G. S. and Ed.—1M—7-31

THROMBIN, A PROTEOLYTIC ENZYME.

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

1935

by

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A.B. Miami University 1932.

M.S. University of Cincinnati 1933.

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Thrombin, a Proteolytic Enzyme.

Introduction

The nature of the chemical changes involved in the transformation of fibrinogen into fibrin has remained a matter of controversy since the work of Buchanan (1&2) who, in 1835, showed that certain serous fluids could be clotted by the addition of leucocytes or serum. Earlier workers, notably Chaptal (3), believed that fibrin existed in the plasma and that clotting was due to a spontaneous solidification. In 1859 Denis (4) found that crude fibrinogen preparations clotted on standing, yielding fibrin and a protein left in solution. From this he concluded that the precursor of fibrin underwent cleavage in the act of clotting. Two years later Schmidt (5&6) prepared fibrinogen and serum globulin which he believed to unite to form fibrin. Fractionation of plasma with salts led Hammarstein (7) to the conclusion that not serum globulin, but some other substance in the blood was responsible for the clotting of fibrinogen. He confirmed the observations of Denis (4) and termed the soluble protein fibrino-globulin. In 1892 Schmidt (8) prepared thrombin and named it "fibrin ferment", thereby indicating his belief that it acted as an enzyme.

Many of the more recent workers have cast doubt upon the enzymic nature of thrombin. Kugelmass (9) concluded that coagulation was due to the adsorption of fibrinogen on particles of thrombin, but the results on which this hypothesis was based are open to criticism. Stuber and Sano (10) maintain that the action of thrombin is merely that of changing the degree of hydration of fibrinogen. A very different interpretation is offered by Hekma (11). He believes that thrombin is an agglutinin which produces clotting by the aggregation of the particles of fibrinogen. However, there are several differences between thrombin and agglutinins. Thus agglutination is quite rapid at 0° C. and is somewhat reduced as the temperature approaches 40° C., whereas thrombin has almost no activity at zero and is said to be most active at a little above body temperature. Furthermore, agglutinins in serum retain their activity for months while under the same conditions thrombin becomes inactive after standing a few hours. Lastly, autohaemagglutinins are rarely found in serum although thrombin is always present in fresh normal serum.

Hekma also states that coagulation may be brought about without the intervention of thrombin by the decomposition of platelets with a consequent diminution of alkalinity. This hypothesis is almost identical with

that of Wadsworth, Maltaner, and Maltaner (12) who say that calcium chloride reacts with cephalin with the liberation of hydrochloric acid. They believe the acid, cephalin, and fibrinogen react to produce fibrin. According to their theory thrombin does not exist. A similar theory has been postulated by Leiri (13). He states that upon being shed the hydrogen ion content of the blood increases to approximately the isoelectric point of fibrinogen at which point the latter substance flocculates upon the fragments of the platelets and thus becomes fibrin.

These three theories are open to several serious criticisms. In the first place they assume that the isoelectric point of fibrinogen is very near neutrality. Although Kugelmass (9) places this isoelectric point at pH 8.0, Mellanby (14) and Stuber and Funck (15) agree that it is between pH 5.0 and pH 6.0. Klinke and Ballowitz (16) say that it is at pH 4.4, and Howell places it between pH 4.0 and pH 5.0. Admittedly, blood never approaches such an acidity during normal clotting and seldom goes below a pH of 7.2. Furthermore the acidification of pure fibrinogen may cause it to precipitate but not to clot. The assumption of a purely physical change is not justified, as Abderhalden and Buadze (17) have shown that fibrinogen and fibrin give rise to different

specific protective enzymes. The appearance of fibrinoglobulin during the clotting of whole blood or of purified constituents is also proof of a chemical change. In regard to the belief that thrombin is not essential to the coagulation of fibrinogen, it has been shown by Mills and Mathews (18) and by Sumner (19) that it is practically impossible to clot specially purified fibrinogen without the addition of a specific coagulant.

Some of the conclusions of these authors are so irreconcilable with others that an explanation of the discrepancies is difficult. Many of the contradictions are doubtless due to the use of supposedly pure preparations which were contaminated with one or more substances. Thrombin, prothrombin (serozyme), tissue fibrinogen, and cephalin have frequently been misleading contaminants. The lack of exact methods of determination of clotting time, and consequently of the active participants in clotting, has added somewhat to the confusion. The instability of the clotting factors as usually prepared has proven a serious obstacle by making the duplication of results impossible in many cases. However, there are several apparently well founded observations which imply contradictions that cannot readily be explained by such disturbing factors. Thus Rettger (20) state that pure thrombin combines with fibrinogen in definite proportions

and therefore is not an enzyme. On the other hand Mills (21), Mellanby (22), and Eagle (23) have shown that their preparations of thrombin were capable of converting a thousand times their weights of fibrinogen into fibrin. It is of interest to note that other natural coagulants have been found by Nausenbach (24) and Grohmann (25) in yeast and certain fungi, by Loeb (26), Gratia (27, 28 and (29), and Cekada (30) in bacteria, and by Lamb (31), Martin (32), Barratt (33), and Collins (34) in the venoms of the snake Echis carinatus, Notechis sentatus, Crotulus terrificus, and Lanchesis lanceolatus. All of these substances are active in such small amounts that their enzymic nature can hardly be doubted. For example Barratt (33) states that the venom of Echis carinatus is active as a coagulant in a concentration of one part in three million.

Since there are several substances which are capable of clotting fibrinogen; since, in some of these, the properties of in vivo and in vitro coagulation have been shown to be distinct; and since Cekada (35) believes he has separated thrombin into two physically and chemically different but active substances; it is within the limits of possibility that blood may contain more than one thrombin-like substance, or that the thrombin molecule may under some conditions of preparation undergo a slight

denaturing effect without total destruction of its clotting function. If either of these possibilities occurs some of the recorded differences in the manner of action of thrombin may be explained.

The opponents of the theory that thrombin is an enzyme point out that the time of clotting does not follow enzyme laws at the extreme ranges of thrombin and fibrinogen concentrations although intermediate concentrations indicate enzyme action. This has been discussed by Mellanby (22) who believes that the discrepancies found when large amounts of thrombin are used are due to a time interval during which the thrombin unites with the fibrinogen before acting upon it. It is also possible that the chemical changes may take place with great rapidity although a few seconds more are required for the completion of the physical changes necessary for actual clotting. He explains excessively prolonged clotting with high dilutions of thrombin as due to the adsorption of the thrombin by the fibrin particles first formed.

Eagle (23) has shown that a disappearance of thrombin takes place at the moment of clotting. A large part of the chemical transformation of fibrinogen into fibrin takes place before this as is shown by the formation of fibrin threads immediately prior to solidification.

It thus seems probable that the disappearance of thrombin when clotting takes place is due, as Mellanby says, to the adsorption of thrombin by fibrin particles. This removal of thrombin, together with the impurity of his materials, might readily account for the conclusion of Rettger (20) that thrombin combined with fibrinogen in definite proportions.

Another objection to the theory that thrombin is an enzyme arises from the fact that thrombin prepared according to the method of Schmidt (8) is said by Rettger (20) not to be entirely destroyed by rapid heating to 100° C. Hirose (36) found the same thing true of thrombin made from prothrombin by the action of cephalin and calcium chloride. The prothrombin had been prepared by salting it out from plasma. However, purer preparations are more thermolabile, those of Morawitz (37) and Mellanby (38) being destroyed at 60° C. An even purer thrombin more recently prepared by Mellanby (22) is totally destroyed by five minutes' heating at a temperature of 50° C. Serum, or recalcified citrated plasma if heated to 60° C. for fifteen minutes exhibit no evidence of either thrombin or prothrombin. In the preparations said to be very resistant to heat it is possible that the large amount of cephalin present had a protective action.

The correlation between time of clotting and

temperature has been studied by Burker (39), Addis (40), Kawashima (41), and Mellanby (22), all of whom obtained the same type of curve and concluded that their results were highly indicative of enzyme action. Two disturbing factors were present however. In the first place at the high temperature the destruction of thrombin, rather than its mere inhibition by an other than optimum condition, might be responsible for a part of the increased time of coagulation. Secondly the calcium content, rather than the calcium ion concentration, was held constant in the experiments of the first three investigators with the introduction of a second variable factor as a result. This criticism does not seriously affect the work of Mellanby since the ionization of his purified thrombin is practically nil.

The recent work of Hsü and Wu (42) shows that fibrinogen loses weight upon conversion to fibrin and thus supports the hypothesis of hydrolysis. Fuchs and Zahrzewski (43) take the same viewpoint on the basis of their finding that an increase in non-protein nitrogen accompanies clotting. These changes are similar to those that take place as a result of the autolysis of fibrin and strengthen the conclusions of some investigators who believe that clotting and fibrinolysis are two different steps of the same process. That fibrinogen

can be clotted and subsequently lysed by the same substance is claimed by Gengou (44) who states that a single active principle secreted by certain staphylococci is capable of causing both these changes.

Haugardy (45) has reviewed irrefutable evidence that fibrinolysis is a phenomenon of chemical disintegration of the fibrin molecule into more simple products, among which have been distinguished two globulins, and traces of proteose, peptone, and amino acids. He states, but does not prove, that the proteolytic action of thrombin is responsible for this hydrolysis. Investigations of the globulin fraction of the autolysate have shown that in addition to the fibrino-globulin of Hammarsten (7) at least two other substances are present. Mills (46) has found a protein coagulable at 75° C., and Hirose (47) has shown a protein with the properties of prothrombin to be present. Welker, Gilman, and Hektoen (48) present rather uncertain evidence that pseudoglobulin and fibrinogen arise from the lysis of fibrin.

Waldschmidt-Leitz, Stadler, and Steigerwald (49) found that many substances digested by trypsin-kinase but not by erepsin have an inhibiting effect upon blood clotting, while substrates digested by erepsin but not by trypsin-kinase have no such effect. They also found that trypsin-kinase, but not trypsin, has an accelerating

effect upon blood clotting and concluded that thrombin is related to trypsin-kinase in its action. On the other hand Strughold and Wöhlisch (50) deny that trypsin-kinase accelerates clotting, state that the hydrolytic degradation of fibrinogen by thrombin is not demonstrable, and say that thrombin merely acts as a denaturing enzyme specific toward fibrinogen. This objection is so contrary to the findings of most workers that it does not invalidate the work of Waldschmidt-Leitz and his associates.

The belief that thrombin acts in a manner similar to that of trypsin, which Northrop and Kunitz (51) showed to coagulate fibrinogen, is rather hard to reconcile with the results obtained by Collingwood (52) and Hugounenq and Loisleur (53). The former found that the methylenation of the amino groups of thrombin and fibrinogen with formaldehyde destroyed the activity of these substances. The latter showed that although amino groups were necessary in pepsin substrates, they were not essential to the substrates of trypsin. The work of Collingwood has been recently extended by Dunn (54) who agrees that fibrinogen is totally destroyed by even relatively small amounts of formaldehyde, but shows that the partial blocking of the amino groups of thrombin causes a reduction, rather than a destruction, of its activity.

Many of the foregoing observers did not differentiate between the action of thrombin in clotting fibrinogen and that of tissue fibrinogen. It was shown by Mills and Mathews (55) and by Mills (56) that these substances clotted under slightly different conditions, namely: thrombin clots a fibrinogen solution containing oxalate; tissue fibrinogen will not do so since calcium ions are necessary for the exhibition of its clotting powers; thrombin decomposes very rapidly and requires reactivation by calcium and cephalin, while tissue fibrinogen requires no addition of cephalin at any time and does not decompose readily. On the other hand it was shown by Nakamura (57) that the fibrin formed by thrombin and by tissue fibrinogen was crystalline and apparently was the same in each case.

These facts together with those cited above indicate the possibility that many different proteolytic enzymes may clot fibrinogen, just as they will clot caseinogen but that thrombin and the tissue fibrinogen enzymes have a special activity on fibrinogen just as rennin has on caseinogen.

Indeed it seemed probable that fibrin was to be regarded as the "protean" of fibrinogen, just as casein is the protean of caseinogen and that as suggested by Osborne many years ago, the proteans are the first

hydrolytic cleavage products of the proteins.

At the suggestion of Professor Mathews the present work was undertaken in an effort to determine more certainly the mechanism of the action of thrombin upon fibrinogen with particular reference to the above hypothesis.

Experimental

Materials.

Horse blood was obtained by bleeding through a paraffined canula inserted in the carotid artery. The blood was received, with stirring, into sufficient 50% sodium citrate to give a final concentration of 0.5%. Rabbit blood was obtained by heart puncture with an oiled syringe containing the required amount of sodium citrate. The citrated blood was thoroughly mixed and transferred from the syringe to a centrifuge tube cooled with a salt-ice mixture. In both cases the corpuscles and most of the platelets were readily removed by centrifuging. In the preparation of the purified clotting factors the remaining platelets were removed by passing the plasma through a Berkefeld filter or a fiber filter pad.

Fibrinogen was prepared by precipitation from the plasma by an equal volume of neutral, saturated sodium chloride containing 0.1% sodium citrate. Saturated

solutions of many samples of sodium chloride are not quite neutral and give a less soluble precipitate. Re-solution was effected with distilled water, sufficient salt remaining in the precipitate to render it soluble. From three to six such precipitations and re-solutions were carried out. The fibrinogen so obtained contained minute quantities of prothrombin and cephalin since on recalcification it would usually form a weak clot within twenty-four hours in the incubator. Most such preparations became incoagulable in from three to seven days in the refrigerator, but when rabbit blood was used and all operations carried out in the cold the product was much more stable. One sample, kept at 5° C., showed no appreciable deterioration after four weeks and clotted fairly readily after the sixth week.

Prothrombin was prepared by the addition of one volume of saturated ammonium sulfate to four volumes of plasma from which the fibrinogen had been removed by precipitation with an equal volume of saturated sodium chloride. The prothrombin was collected by centrifuging, redissolved in distilled water and reprecipitated in the same manner. After the last, usually the third, such precipitation, the excess salt was pressed out with filter paper and the precipitate dialyzed over night against running water. Finally the prothrombin was redissolved

in 8% sodium chloride. This preparation, containing large amounts of globulin, gave a rather unstable product which contained some cephalin as was shown by its ability slowly to clot purified fibrinogen on the addition of calcium chloride.

The method of preparing cephalin was a slight modification of the unpublished method of D. D. Irish of this laboratory. Fresh sheep brains were ground thoroughly and refluxed several hours with each of three successive portions of acetone. The dried material was extracted with absolute ether and the extract concentrated in vacuo until precipitation began to take place. It was then placed in the refrigerator for two days, at the end of which time a rather large amount of very slightly active material had settled out. The precipitate was discarded by decantation and four volumes of absolute isopropanol were added to the supernatant liquid. The precipitated cephalin was redissolved in absolute ether and reprecipitated as before. When allowed to remain in a closed container under the ether-isopropanol mixture the product was extremely stable and retained most of its activity many months after being dried and stored in a desiccator.

Several methods were used for the preparation of thrombin. In the early part of the work it was made by

incubating the previously described prothrombin with cephalin and calcium chloride for half an hour at room temperature. Later this method was superseded by others which yielded a far more stable and a purer product.

The procedure of Mellanby (22) was found to give a very satisfactory product but was attended by certain difficulties. Beef plasma, kept fluid by the addition of 0.2% sodium oxalate, was diluted with 10 to 15 volumes of distilled water, the higher dilution being preferable. The solution was brought to pH 5.3 by the addition of 1% acetic acid (about 35 cc. of acid per liter of solution). The prothrombin, together with a large part of the fibrinogen, was precipitated. After two hours the supernatant fluid was poured off and the precipitate collected by centrifuging. The compact mass was re-suspended in an amount of distilled water equal to half the volume of the plasma used. A 0.168% calcium hydroxide solution was made by saturating water with calcium oxide at 16° C. and diluting tenfold. This was brought to pH 7.0 by cautious treatment with carbon dioxide. Equal volumes of this and the prothrombin suspension were mixed, allowed to stand ten minutes, and filtered as rapidly as possible. This treatment removes the prothrombin from the fibrinogen on which it was adsorbed. The clear filtrate was again

brought to pH 5.3 by the addition of 1% acetic acid and centrifuged. After 24 hours' standing the precipitated prothrombin was converted spontaneously into a highly active thrombin which, when dried with acetone, retained its activity well over a year.

Thrombin as prepared by Eagle (58) was also used. Citrated horse or rabbit plasma was diluted with from 10 to 15 volumes of cold distilled water and carbon dioxide bubbled through for five minutes. The precipitate was collected by centrifuging, redissolved in saline to the volume of the plasma used and made neutral to litmus with sodium bicarbonate. The solution was recalcified with one-twentieth its volume of 0.1 M calcium chloride and completely defibrinated by stirring. The resulting thrombin solution remained very active for several days when kept in the refrigerator.

The Clotting of Fibrinogen

The first question to be answered was whether or not the transformation of fibrinogen into fibrin was due to the hydrolysis of the former. To decide this point the total nitrogen and the free amino groups of equal amounts of fibrinogen before and after clotting were determined.

Equal amounts of rather dilute fibrinogen solution were placed in two tubes, one of which was heated to 58° C. for half an hour and the coagulum filtered and washed repeatedly with 8% sodium chloride. To the other tube a very small amount of Mellanby's thrombin was added and the contents completely defibrinated. The serum from this clotted additional fibrinogen in each case, proving the sufficiency of the amount of thrombin added. The fibrin removed was washed as had been the coagulum obtained at 58° C. and the two were analyzed for nitrogen. The heat coagulated fibrinogen was washed from the hardened filter paper into the digestion flask with ammonia-free water to eliminate analyses of the filter paper.

All determinations of total nitrogen were made by the gasometric micro-kjeldahl method of Van Slyke (59) with a modified procedure of digestion. To each 100 cc. flask containing the material to be analyzed were added 2 cc. of the digestion mixture which consisted of 100

parts of saturated potassium sulfate and 1 part of seleneium oxychloride by volume. Glass beads were added to reduce bumping and the digestion carried out over micro burners until the mixtures were perfectly clear and colorless. 10 cc. of ammonia-free distilled water were added to each flask and the solidified material dissolved. 5 drops of alizarin red were added to each digest and 40% sodium hydroxide was run in from a burette until the indicator turned pink. At the same time the flask was kept immersed in a beaker of cold water to prevent the loss of ammonia. 10% sulfuric acid was then added until the indicator turned yellow again. The contents of each flask were then washed into graduate cylinders and sufficient ammonia-free water added to make the volume to 25 cc. Before making a series of analyses, the Van Slyke apparatus, cleaned, and with the stopcocks well greased, was carefully tested to be sure no leaks existed. It was found best to use a stopcock grease with a high content of rubber and paraffin and a rather low content of vaseline. The ingredients were dissolved in toluol which was later evaporated at 110° C., this procedure eliminating the charring which accompanies melting of the ingredients at higher temperatures.

10 cc. of the digest to be analyzed was run into the extraction chamber and the external chamber was

washed with 2 cc. of distilled water, making a total volume of 12 cc. in the extraction chamber. The dissolved air was pumped out by lowering the mercury level within the extraction chamber to the 50 cc. mark and shaking for two minutes. The extracted gas was ejected and 1 cc. of alkaline sodium hypobromite was drawn into the extraction chamber. The external capillary orifice was sealed with mercury and the gas formed was pumped out of solution. After shaking the extraction chamber for three minutes all the ammonia had been converted into nitrogen by the action of the hypobromite. The liquid level within the chamber was then raised to the 2 cc. mark and the pressure, P_1, was read on the manometer scale. The extracted nitrogen was ejected and the vapor pressure of the liquid, P_2, was noted with the liquid at the 2 cc. level. The temperature of the water bath surrounding the extraction chamber was recorded. The quantity of nitrogen present was calculated according to the formula:

$$\text{mg. } N_2 = (P_1 - P_2 - c)f \quad \text{, where } c = P_1 - P_2$$

of a blank, and f is a factor varying with the temperature and tabulated by Van Slyke. 1 mm. on the manometer scale corresponds to about 0.003 mg. of nitrogen.

The results obtained by this procedure in a series of determinations are expressed in Table I and show that fibrinogen loses about 9.6% of its nitrogen upon conversion

to fibrin. These results are very similar to those of Hsüü and Wu (42) and indicate the hydrolysis of fibrinogen into fragments very dissimilar in size, rather than into two almost equal parts as Schmiedberg (60) believed.

(Insert Table I.)

A few workers have found that fibrinogen increases markedly in weight upon clotting. This may possibly have been due to the adsorption of impurities present in the thrombin or prothrombin solution used, or to the incomplete removal by washing of impurities surrounded by fibrin. That such an increase is not necessarily due to the union of thrombin with fibrinogen is shown by the previously cited statements of Mills (21), Mellanby (22), and Eagle (23) that fibrinogen can be clotted by one-tenth of one per cent of its own weight of thrombin. These observations have been confirmed by the present worker with Mellanby's thrombin and fibrinogen from which all prothrombin had been removed.

For the determination of free amino groups, the method of Van Slyke (61) was first employed with whole plasma, one sample being analyzed while citrated and the other being allowed to clot in the extraction chamber after recalcification. In all cases the values were impossibly high and indicated an increase of almost 50%

Table I.

Loss of nitrogen of fibrinogen upon conversion to fibrin.

Sample	Fibrinogen nitrogen mg.	Fibrin nitrogen mg.	% nitrogen lost
1	1.92	1.71	10.94
2	2.81	2.59	7.83
3	2.65	2.40	9.43
4	2.17	1.91	11.98
5	2.46	2.27	7.72
Average			9.58

after clotting. Beyond doubt these results were due to the excessive and unpreventable foaming in the extraction chanber which made it impossible to transfer the gas to the Hemple pipette accurately or within the required time limit. The large apparent increase in the number of amino groups after clotting may have been partly due to a slight decrease in foaming dependent upon the reduced amount of soluble protein. This would result in a more complete transfer of gas to the pipette.

Sørenson's titration method was also used and found to give better results than the gasometric procedure. However, when the determinations were made on whole plasma, the masking of the end-point by the original color of the solution and the increased percentage of error due to the presence of amino compounds other than those involved in clotting caused too large an error in the method. Because of this the determinations were run on purified samples of fibrinogen to which a small quantity of cephalin and prothrombin had been added. Dilute solutions of fibrinogen were used as it was found that in dilute samples that were recalcified and allowed to clot the fibrin could be broken up into less compact masses than could be obtained with concentrated solutions.

The titrations were carried out in small, covered flasks to reduce the influence of atmospheric carbon

dioxide. Since it was necessary to read the final, rather than the initial, end-point, both the clotted and the unclotted samples were allowed to stand about the same length of time before the final reading. 0.005 N sodium hydroxide and hydrochloric acid were used for titrating. Table II gives the results of a series of these determinations and shows that an increase of about 4 - 5% in free amino groups takes place when fibrinogen clots. Each titration value represents the average of two titrations on identical samples, the readings of which varied by not more than 0.25 cc.

(Insert Table II.)

That fibrinogen loses almost a tenth of its nitrogen content upon clotting and that when fibrinogen clots the number of amino groups increases, indicate hydrolysis. Since the rate of clotting depends upon the thrombin content, thrombin must be the catalytic agent or enzyme that is responsible for the hydrolysis.

Table II.

Sorensen titration values of fibrinogen before and after clotting.

Sample	Titration before clotting (cc. NaOH)	Titration after clotting (cc. NaOH)	% increase
1	24.32	25.94	6.25
2	21.00	22.39	6.21
3	34.17	35.43	3.56
4	23.64	24.58	3.83
5	32.79	34.20	4.12
Average			4.79

The Autolysis of Fibrin and of Fibrinogen

The re-solution of fibrin in dilute salt solutions was first observed by Denis (62). Dastre (63) confirmed the observation in sterile solution and called attention to the similarity of the spontaneous lysis of fibrin to its digestion by trypsin. As has been previously mentioned, Haugardy (45) shows fibrinolysis to be a hydrolytic process and believes thrombin to be the enzyme which causes it.

Fibrinogen also undergoes changes upon standing in sterile solution as is shown by its loss of clotting activity and the appearance of a more or less heavy precipitate. That such changes might be due to the presence of amounts of thrombin too small to cause clotting is entirely possible. In such a case the lytic products of both fibrin and fibrinogen might be expected to be very similar in nature whereas if the denaturation of fibrinogen were due to a different factor the products obtained would probably be different. To compare the products of autolysis several methods were used, the first of which was a study of the temperatures of coagulation.

Fibrinogen was prepared from horse blood as previously described, four precipitations being carried out. A part of this was further purified by a combination of the method of Mills and Mathews (18) and of Sumner (19).

The concentrated solution of fibrinogen was first treated with freshly precipitated tri-calcium phosphate which had been neutralized with carbonate-free sodium hydroxide. After shaking this suspension for fifteen minutes the precipitate was removed by centrifuging and ten grams of Norite were added to each hundred cubic centimeters of the supernatant fluid. After ten minutes' shaking the mixture was filtered and the charcoal washed with several portions of 1% sodium chloride the total volume of which was equal to that of the solution treated with charcoal. The washings were combined with the filtrate and contained most of the fibrinogen but no demonstrable prothrombin. The fibrinogen was finally reprecipitated with sodium chloride and redissolved in distilled water to concentrate it.

Re-suspension of the tri-calcium phosphate in distilled water followed by the passage of carbon dioxide through the mixture, resulted in the liberation of the adsorbed prothrombin. This was demonstrated by filtering the carbonated liquid, adding a little cephalin and calcium chloride to a small portion of the filtrate, and allowing it to stand thirty minutes at room temperature. At the end of this time it caused clotting in from five to fifteen minutes when added to an equal portion of purified fibrinogen. No satisfactory method was found for eluting the prothrombin from the charcoal, but when

a heavy suspension of the charcoal was incubated with calcium and cephalin the mixture found a weak clot, the serum from which clotted purified fibrinogen very slowly. This shows that nearly all of the prothrombin is adsorbed by the calcium phosphate and that the rest of it, as far as can be measured by clotting activity, is taken up by the charcoal. It also shows that some of the fibrinogen remains in the charcoal. However, it does not prove that prothrombin is the only substance removed by the procedure.

Fibrin was prepared by allowing a small quantity of Mellanby's thrombin to clot the ordinary preparation of fibrinogen. About one part of thrombin to eight hundred parts of fibrinogen, on the basis of dry weights, was used. Both preparations of fibrinogen gave heavy coagula at 56° C. and fainter ones at 66° C. The three preparations, - fibrin, fibrinogen, and purified fibrinogen were covered with toluene and placed in the incubator at 37° C. At intervals samples were removed for the determination of salt precipitation and heat coagulation. The disappearance of clotting activity was measured by incubating portions of the fibrinogen with Mellanby's thrombin for two hours. Absence of clotting within this period was interpreted as loss of activity. Salt precipitability was determined by adding an equal

volume of saturated sodium chloride. Opalescence without precipitation was not recorded. In the determination of heat coagulation temperatures the samples were slowly heated until coagulation occurred, and the temperature maintained at that point for twenty minutes since smaller time intervals were insufficient to cause complete coagulation at any given temperature. The material was then filtered and the process repeated with the filtrate.

The disappearance of thrombin coagulability of the ordinary preparation of fibrinogen was found to be complete in three days, whereas the purified preparation remained active until the sixth day. Precipitability with half-saturated sodium chloride and coagulation at 56° C. remained until the fifth day in the impure fibrinogen and until the ninth day in the purified material. This shows that the purified material is much more stable than that not so purified. The purification has apparently removed a proteolytic enzyme of some kind, possibly prothrombin.

Within thirty minutes of the time of formation of the fibrin the serum was found to contain substances coagulating at 62°, 66 - 67°, 72°, 75°, and 84° C. The second and fourth of the coagula were quite heavy while the others were very faint but definite. The same

substances were found in the digest of the impure fibrinogen but did not appear definitely for over a day and gradually became more pronounced as the material coagulable at 56° C. disappeared. In the purified fibrinogen the appearance of these substances required two days and the coagulum at 72° C. was never definitely present while that at 84° C. was extremely faint. The substance coagulating at 66 - 67° C. was first discovered by Hammarsten (7) and that coagulating at 75° C. was found by Mills (46).

As 72° C. is recognized by some workers as the temperature of coagulation of prothrombin, the absence of coagulation at this point in the purified fibrinogen is not suprizing. Rather, it tends to show that the method of purification removed only one heat coagulable substance, and that that was prothrombin. Furthermore it demonstrates that if the prothrombin is directly or indirectly responsible for the degradation of fibrinogen, which seems highly probable, extremely minute amounts of it are capable of bringing about the change. Whether prothrombin may have weak fibrinolytic properties, or whether its activity is dependent upon the formation of thrombin in very small amounts by cephalin and a trace of free calcium is not known, but in either case the minute quantity of active substance present in the purified fibrinogen

indicates that it is an enzyme.

As Hirose (36) had found rather large amounts of a substance that appeared to be an albumose in autolyzed fibrin solutions it appeared probable that the hydrolysis might go even further. The finding of Fuchs and Zahrzewski (43) that non-protein nitrogen increased during clotting strengthened this conclusion. It was therefore decided to analyze both fibrinogen and redissolved fibrin to determine the extent of the hydrolysis.

The fibrin and fibrinogen were prepared as previously described and allowed to stand in the refrigerator at 5° C. under toluol. After periods of one to three weeks samples were removed and examined but no substance simpler than metaprotein could be found. As the first stages of fibrinolysis had been found to be comparatively rapid even at low temperatures this was at first believed to be due to lack of further hydrolysis. However, similar samples were stored for longer periods of time and others were allowed to stand at higher temperatures. None of these samples contained, before storage, detectable amounts of nitrogen not precipitated by 2.0% trichloracetic acid.

After various periods of time the materials were analyzed by the method of Wastneys and Borsook (64). The complicated procedure may be very briefly outlined as

follows. Total nitrogen is determined, protein and metaprotein are precipitated with 2.0% trichloracetic acid and the acid destroyed and its products removed by boiling after filtering off the precipitate. Metaprotein is precipitated at its isoelectric point and filtered off. Protease is precipitated from the trichloracetic acid filtrate by saturation with sodium sulfate and removed by filtration. From this filtrate peptones are removed by acidified tannic acid and filtration. Alcohol and zinc chloride are used to precipitate the subpeptone nitrogen from this filtrate. The unprecipitated nitrogenous material consists of amino acids and simple peptides. Total nitrogen determinations are made on each filtrate and the various fractions determined by difference. The Van Slyke micro-kjeldahl method was used for the analyses.

Several such series of fractional analyses were made and the results found to be quite comparable considering the durations and the temperatures of the various incubations. In Tables III, IV, and V are given the results of three series of analyses. Each number is the average of two analytical values differing by not more than three per cent.

(Insert Tables III, IV, and V.)

Table III.

Nitrogen distribution in autolysates of fibrinogen and fibrin expressed as percentages.

Samples autolyzed 6 months and 3 weeks at 5° C.

	Fibrinogen	Fibrinogen	Fibrin
Protein	30.9	32.9	22.6
Metaprotein	32.0	30.4	11.7
Proteose	7.0	5.6	29.7
Peptone	9.1	9.1	10.2
Subpeptone	8.6	9.1	4.7
Amino and simple peptide	12.4	12.9	21.1

Table IV.

Nitrogen distribution in autolysates of fibrinogen and fibrin expressed as percentages.

Samples autolyzed three months at room temp.

	Fibrinogen	Fibrin
Protein	42.5	23.8
Metaprotein	10.9	11.8
Proteose	9.5	27.9
Peptone	12.4	11.2
Subpeptone	7.1	4.7
Amino and simple peptide	17.6	20.6

Table V.

Nitrogen distribution in autolysates of fibrinogen and fibrin expressed as percentages.

Samples autolyzed seven days at 37° C.

	Fibrinogen	Purified Fibrinogen	Fibrin
Protein	67.1	76.9	36.2
Metaprotein	17.3	19.4	23.1
Proteose	3.9	3.1	19.8
Peptone	4.0	0.6	7.8
Subpeptone	3.5	0.0	5.4
Amino and simple peptide	4.2	0.0	7.7

These results show very definitely that the breakdown of both fibrin and fibrinogen continues until very simple substances are formed. That this hydrolysis is caused by an enzyme is supported by the dependence of the speed of the reaction upon the temperature. That thrombin, or possibly prothrombin, is the substance which accelerates the reaction is strongly indicated by the fact that the velocity of the hydrolysis is dependent upon the amount present of some substance which is precipitated and adsorbed under the same conditions as is prothrombin.

It is of interest to note that none of the more highly digested products were detectable until long after the initial changes had been observed. This indicates that the first hydrolyses do not take place at or very near the terminal peptide linkages. Splitting at these points with the formation of small fragments takes place much more slowly.

It appears quite evident that the action of thrombin is one of hydrolysis, and that the formation of fibrin is only an intermediate step in the process. Formation of appreciable amounts of fibrin is usually the most rapid step in the hydrolysis, but may not occur even though the digestion proceeds much further providing sufficiently small amounts of thrombin are present. This

may possibly be due to a union of the small amount of thrombin present with the fibrinogen, which union is not broken until the undetectable amount of fibrin formed as a result of it has been further hydrolyzed. This hypothesis is rendered more probable by the work of Hirose (36), of Mellanby (22), and of Eagle (23) who present substantial evidence that fibrin possesses to a measurable extent the power of binding thrombin.

Whenever fibrin or fibrinogen is allowed to autolyze a more or less heavy flocculent precipitate is formed. In samples of fibrin this is usually not noticed until the bulk of the fibrin is greatly reduced, but if the solution containing the fibrin is gently shaken, the precipitate may readily be detected after the fresh fibrin preparation has been incubated for twenty-four hours at 37° C. In ordinary preparations of fibrinogen the precipitation appears to be complete after one day's incubation at that temperature, but in samples of fibrinogen which have been freed as far as possible from prothrombin the precipitate can be detected only after three to ten days incubation and remains very faint.

This observation suggested that the precipitate might be an altered form of prothrombin or of thrombin.

Examinations of the dried precipitates from solutions of fibrin and ordinary fibrinogen were made but not

enough of the material could be collected from the purified fibrinogen to analyze it. All the precipitates examined appeared to be identical. Between 40 and 50% of the material was ether soluble whereas only 25 to 35% was soluble in cold alcohol. The remaining material was fairly soluble in dilute sodium hydroxide and contained from 13 to 16% of nitrogen. It thus appears that the precipitate consists of a lipid and a protein. Neither the whole precipitate nor the fractions obtained by extracting it with ether showed the clotting activities of prothrombin, thrombin, or cephalin. However, it seems probable that one or more of these three substances, in an altered form, was present in the precipitate.

Is Serum Globulin or Serum Albumin Formed from Fibrinogen?

The origin of serum globulin and of serum albumin is not definitely known but there is some evidence that the former, and possibly the latter, is formed from fibrinogen. In embryonic development albumin is the first substance to appear, but serum globulin has never been found until after both fibrinogen and prothrombin have been formed. In many diseases an increase of fibrinogen is accompanied by an increase of serum globulin, and in some cases the serum albumin is also increased.

More direct evidence has been found by Welker, Gilman, and Hektoen (48). These workers state that fibrin, digested with pepsin or allowed to autolyze, gives rise to material possessing the precipitin reactions of pseudoglobulin of serum. No indication of the presence of serum albumin was found. However, their fibrin was prepared by washing blood clots and could hardly be expected to be pure. That serum albumin was absent may readily be understood in view of the finding of Kylin and Kristenson (65) that albumin, because of its smaller molecular size, passes more readily into serum than does globulin. When the material was

washed with water and the salt content thereby decreased, the difference of diffusion rates of the two would be even greater.

The chemical evidence is strongly against the transformation of globulin into albumin or vice versa although such changes have been stated to have occurred. The serological evidence, with the exception of the work of Welker and his associates, is equally opposed to the occurrence of such a change. However, there is no reason to believe that serum globulin or serum albumin might not arise from fibrinogen.

Both serum globulin and serum albumin are usually considered to coagulate at about 75° C., but Halliburton (66, 67, and 68) has found coagulation temperatures of the latter substance at 73°, 78°, and 85° C. It will be remembered that in the present work coagulation temperatures of fibrin digests have been found at 72°, 75°, and 84° C., and that small differences of salt or hydrogen ion concentration will cause appreciable differences in the temperatures of coagulation, as will also variations in heating time.

To determine whether or not serum globulin and serum albumin were among the products of fibrinolysis, salt precipitations, heat coagulations, and precipitin reactions were utilized.

Attempts to fractionate digests with ammonium sulfate proved unsatisfactory because of the difficulty of complete removal of the ammonia by dialysis. Removal of ammonia by magnesium oxide caused such violent bumping during the subsequent Kjeldahl digestion that it was not found practicable. Sodium sulfate was found to give fairly good results as a precipitant and the method of Howe (69), modified on the basis of later work by the same author (70), was adopted. A brief outline of the method is as follows:

To tubes containing 0.5 cc. each of the substance to be analyzed are added 15 cc. sodium sulfate of such concentrations that the final concentrations of the salt in the various tubes will be 10.6, 13.5, 17.4, and 21.5%. Fibrinogen is precipitated in the first of these while both fibrinogen and euglobulin are precipitated in the second. In the third, in addition to these substances, pseudoglobulin I was precipitated. All globulins are precipitated in the fourth. Proteins are completely removed with trichloracetic acid. Analyses of these filtrates, together with a total nitrogen determination enables the calculation of the amount of nitrogen in each fraction. Analyses were by the method of Van Slyke.

The original procedure involved the estimation of fibrinogen by converting it into fibrin and analyzing

it as such, and the modification of salting out the fibrinogen was found by Howe to give the same results when whole plasma was used. This was verified in the present work. It is therefore obvious that the quantitative differences between fresh serum and plasma divested of fibrinogen are too small to be determined by this method. To decrease the percentage of error caused by the presence of other proteins fibrinogen solutions were substituted for whole plasma.

To the usual preparation of fibrinogen, containing sufficient sodium citrate to prevent clotting, was added one-fiftieth its volume of prothrombin as prepared by Eagle (58). This was equivalent to one mg. of prothrombin to two hundred of fibrinogen. The solution was then divided into two portions one of which was recalcified and allowed to clot. Both were covered with toluol and incubated for three hours at 37° C. At the end of this period samples of the fibrinogen and of serum were removed and analyzed by Howe's method. The results of a typical series of analyses are given in Table VI. Each number represents the average of two analyses not varying by more than three per cent.

(Insert Table VI.)

Table VI.

Nitrogen distribution in protein fractions of fibrinogen and serum from fibrin, expressed as percentages.

Determinations by salt precipitation.

Samples autolyzed 3 hours at 37° C.

	Fibrinogen	Serum from fibrin
Fibrinogen	96.0	0.0
Euglobulin	2.7	55.3
Pseudoglobulin I	1.0	29.6
Pseudoglobulin II	0.0	0.0
Non-globulin protein	0.3	9.7
Non-protein	0.0	5.4

It is seen from this table that practically all of the nitrogen of the fibrinogen remains precipitable by 10.6% sodium sulfate, whereas about 55% of the nitrogen of the serum is precipitated in the euglobulin fraction and 28% in the pseudoglobulin I fraction. The apparent increase in non-protein nitrogen after clotting is interesting because of the similar finding of Fuchs and Zahrzewski (43), but is too small to be significant.

The method of salt precipitation is quite useful but merely shows the class to which a protein belongs. In order to determine other characteristics of the proteins found, to corroborate the salt precipitation data, and to ascertain whether or not albumin was present, similar analyses were made by means of heat coagulation.

Since concentrated solutions of fibrinogen were found frequently to give unfilterable, gelatinous solutions upon heating, the solutions were diluted before coagulation. Fibrinogen preparations containing one-fiftieth volume of Eagle's prothrombin were divided into two portions. One portion was recalcified and allowed to clot, both were incubated three hours at 37° C., and samples of the unclotted material and of the expressed serum were taken for analysis. To each of several test tubes graduated at 10 cc. were added 0.5 cc. of the fibrinogen, or of the serum, and 9.5 cc. of ammonia-free

isotonic sodium chloride solution. The tubes were then heated one to two degrees above the various coagulation temperatures previously established. In each case the temperature was maintained for half an hour to insure complete coagulation. At the end of this time the tubes were cooled and the water lost by evaporation replaced. The contents were then filtered through dry filters and 1.0 cc. samples of the filtrates analyzed for nitrogen. The total nitrogen and the non-protein nitrogen were determined as in Howe's procedure. Unfortunately, in many instances a portion of the coagulum passed through the filter regardless of precautions. This was always found true of the fraction coagulating at 62° C. although the next higher fraction usually gave a clear filtrate. For this reason these two fractions were determined together. In other cases in which the filtrates were not perfectly clear it is believed that the error so incurred was not great enough to affect the results seriously. Table VII gives the data obtained from a typical series of analyses.

(Insert Table VII.)

A comparison of this table with Table VI shows that somewhat less of the fibrinogen is coagulated at 56° C. than is precipitated by 10.6% sodium sulfate. It is also seen that the heavy coagula formed at 66 - 67° and

Table VII.

Nitrogen distribution in protein fractions of fibrinogen and serum from fibrin, expressed as percentages.

Determined by heat coagulation.

Samples autolyzed 3 hours at 37° C.

Coagulation temperature	Fibrinogen	Serum from fibrin
56° C.	88.8	0.0
62° and 67°	6.1	49.4
72°	1.5	9.7
75°	2.9	24.1
84°	0.0	1.7
Non-coagulable protein	0.7	8.5
Non-protein	0.0	6.6

at 75° C. correspond roughly, but not necessarily respectively, to the euglobulin and pseudoglobulin I fractions. This indicates that serum globulin might be one of the products. That nearly all of the non-globulin protein is incoagulable by heat indicates the absence of any but small amounts of albumin. The unidentified fraction possibly consists largely of the albumose found by Hirose (36).

Although the preceding findings are suggestive of the formation of serum globulin as a result of the action of thrombin on fibrinogen they by no means prove it. In order to settle this question precipitin reactions were employed. For this purpose horse fibrinogen was prepared and purified by six precipitations with sodium chloride. This procedure should, by dilution, reduce the serum globulin present to less than a billionth of its original concentration. This preparation was clotted with Mellanby's thrombin and allowed to autolyze two weeks under toluol at room temperature, practically all of the fibrin being dissolved within that time. The material was filtered, and to the filtrate was added an equal volume of saturated ammonium sulfate. The precipitate was collected by centrifuging and redissolved in dilute salt solution. Three such precipitations were carried out, the final solution being one-fourth the

volume of the plasma from which the fibrinogen was made. Each of four normal male guinea pigs was injected intraperitoneally at four day intervals with three successive 0.5 cc. doses of this globulin. Serum globulin was similarly prepared from horse plasma, the fibrinogen of which had been previously removed with sodium chloride.

Four weeks after the first injections blood samples were taken from each of the guinea pigs and the serums tested, by means of precipitin reactions, against the injected globulin solution to be certain that immunity had developed. As controls, blood samples from four non-immunized guinea pigs were similarly tested. Samples of serum from all eight animals were then tested against the preparation of horse serum globulin. Table VIII gives the results. Although there is an indication

(Insert Table VIII.)

of the presence of the same substance in both the horse serum globulin and the globulin recovered from the fibrin autolysate, not much of the substance could have been present unless it was somewhat denatured. Since the globulins used were purified only by precipitation it is quite possible that the horse serum globulin may have been contaminated with traces of autolytic products of fibrinogen formed before the removal of this substance.

Table VIII.

Dilutions at which precipitin reactions could be detected with normal guinea-pigs and with those immunized against the globulin recovered from autolyzed fibrin.

Substance tested	Immunized animals				Normal animals			
	1	2	3	4	1	2	3	4
Recovered globulin	1:300	1:500	1:400	1:400	1:10	1:10	1:20	1:10
Horse serum globulin	1:10	1:20	1:50	1:20	1:10	1:10	1:20	1:10

Each of the four animals sensitized to the globulin derived from fibrinogen as stated above was injected intraperitoneally with an amount of the horse serum globulin representing 2 cc. of plasma. The second animal of the series developed symptoms of shock and died in 25 minutes, but the others appeared unaffected.

A similar procedure was made use of to determine the presence of serum albumin in the fibrin autolysate. In this case the "albumin" was prepared by removing the globulin from the autolysate by the addition of sufficient solid ammonium sulfate to make the solution half saturated, and centrifuging. The supernatant liquid was dialyzed under toluol for ten hours against running water to eliminate excess salt. 10 cc. of this liquid were used for each of the three injections. Crystalline horse serum albumin, made according to the methods of Young (71) and Adair and Robinson (72) was used as the known protein. The serum from the guinea pigs sensitized to the albumin prepared from the fibrin autolysate showed no stronger precipitin reactions against the crystalline serum albumin than did the serum from the control animals. The intraperitoneal injection of 0.02 grams of the crystalline serum albumin into each of these sensitized guinea pigs caused no apparent shock. These results indicate that no undenatured serum albumin

was present in the fibrin autolysate used.

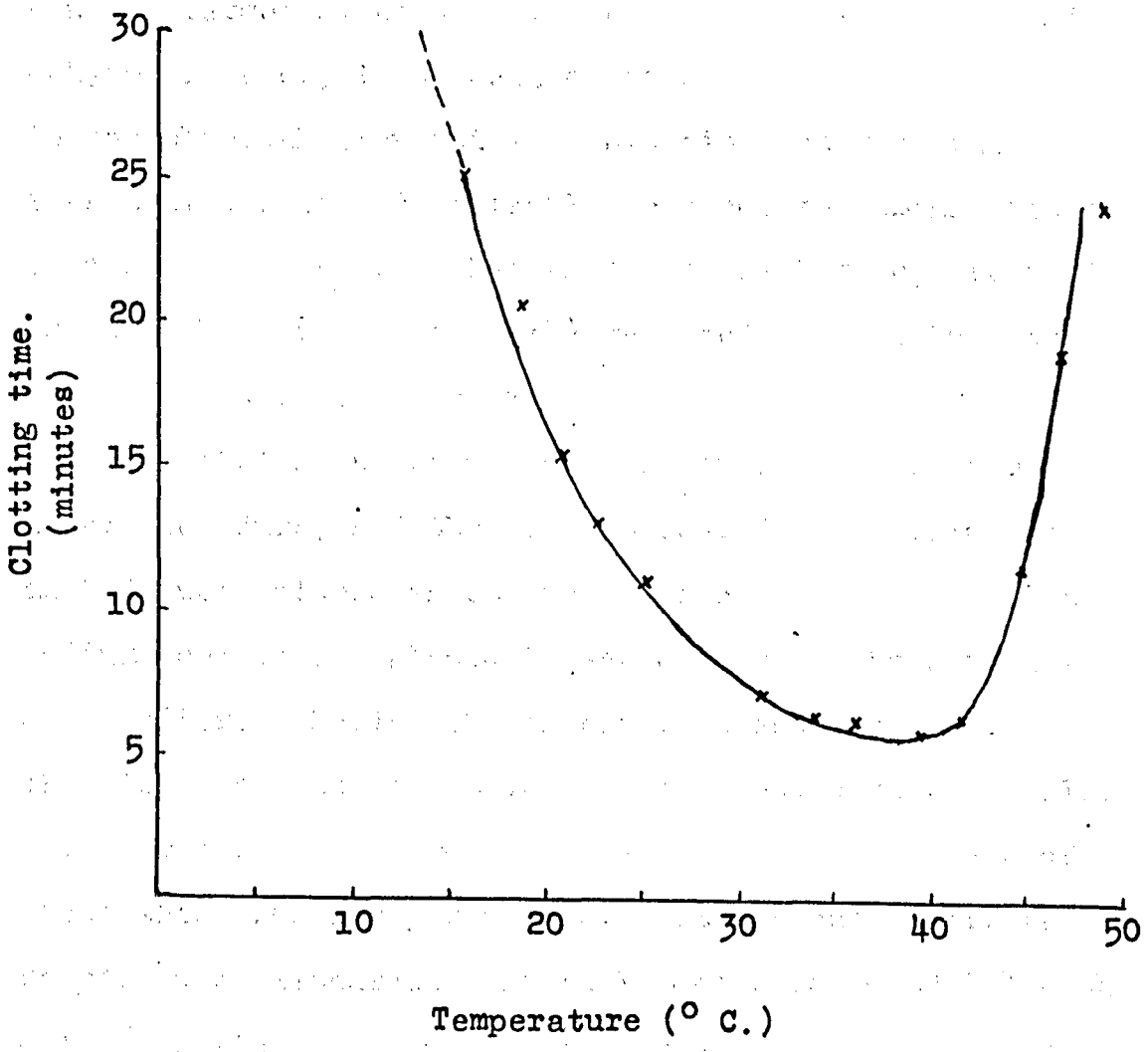
Effect of Temperature on the Velocity of Clotting.

The earlier discussion of the findings in regard to the relation of temperature to clotting time showed that, with the possible exception of the work of Mellanby (22), theeffect of the calcium ion concentration had been neglected. For this reason a study of this relationship was made in which optimum calcium concentrations were used. These optima were established for a sample of citrated plasma at each of a number of different temperatures by adding various quantities of calcium chloride to equal volumes of plasma at a given temperature and determining the clotting times. The additions were such that the calcium concentrations increased in 0.005 molar steps. The plasma volumes were kept equal by the addition of the necessary quantity of isotonic salt before the calcium was added. It was noticed that as the temperature increased, the optimum quantity of calcium decreased slightly. Having determined the optimum calcium concentrations for a given sample, clotting time determinations were made on the same plasma at the various temperatures and using the optimum amounts of calcium. The determinations were made simultaneously to avoid slight but

appreciable errors due to the slow deterioration of the plasma. The results are expressed in the graph on the following page and indicate that the reaction is enzymic with an optimum temperature of 39° C. The destruction of thrombin at a little above 50° C. is evident. That the decreased speed of clotting at temperatures above the optimum was not entirely due to the destruction of thrombin is shown by the following experiment. Two samples of plasma were clotted, the one at 39° C. and the other at 46° C. The ratio of coagulation times was 5.5:19.0 or 1:3.45. The serums from each of these samples were collected by centrifuging and 0.2 cc. of each serum was added to different 1.0 cc. samples of plasma. The clotting times of these samples were 0.5 minutes and 0.75 minutes, or in a ratio of 1 : 1.5 . There was nearly as much thrombin in the serum from 46° C. as in that from 39° . As Mellanby has shown that the time of clotting is inversely proportional to the amount of thrombin present, it is seen that not all of the increased time of coagulation at 45° C. is due to the destruction of thrombin.

(Insert Figure.)

Effect of temperature on clotting time.



Thrombin Action and Chemical Constitution.

The work of Collingwood (52) and of Dunn (54) showed that the treatment of fibrinogen with even small amounts of formaldehyde destroyed its activity. Small amounts of formaldehyde inhibited the action of thrombin and larger amounts destroyed it completely. Formaldehyde, however, is capable of causing drastic changes in the physical properties of proteins, and for this reason it was deemed advisable to block the amino groups by some other means. For this purpose ketene, prepared as described by Herriott (73) was used to acetylate the amino and possibly other groups.

Fibrinogen, prepared from horse blood by the usual procedure, and thrombin, prepared by Eagle's method, were placed in separate collodion bags. 2.0 M sodium acetate was placed in and around the bags to act as a buffer. During the preparation and treatment of the fibrinogen and thrombin the solutions were kept cold. Ketene was bubbled through the solutions at the rate of about three bubbles per second. At various intervals samples were withdrawn, carefully adjusted to pH 7.4 with dilute acetic acid, and tested for activity by addition to a portion of the other reactant which had not been so treated. The results given in Table IX confirm the

findings of Dunn (54). It is seen that the ability of fibrinogen to clot upon the addition of thrombin is completely destroyed by ten minutes' exposure of the fibrinogen to ketene. The activity of thrombin is also affected but some activity remains to a detectable extent for forty minutes.

(Insert Table IX.)

Within the period of time employed it is probable, but not absolutely certain, that only amino groups were attacked by the ketene. The loss of activity of thrombin upon acetylation is quite possibly due to the destruction of cephalin as rapidly as it was liberated by the dissociation of thrombin although this was not determined. In any event, the relative stability of thrombin and the rapid inactivation of fibrinogen by ketene indicate that thrombin is an amino proteinase as Dunn believed.

Table IX.

Effect of ketene on thrombin and fibrinogen.

Time of clotting (in minutes) after addition of 2 cc. of thrombin to 5 cc. of fibrinogen, one component having been treated with ketene.

Total dilution 9 cc. Temperature 25° C.

Substance exposed to ketene	Time of exposure in minutes									
	0	5	10	15	20	25	30	35	40	45
Fibrinogen	0.25	3	-	-	-	-	-	-	-	-
Thrombin	0.25	1.25	2.5	4	6	12	20	32	45	-

The Effect of Cyanide on Clotting.

Since certain proteolytic enzymes are activated by cyanides, Professor Mathews suggested that cyanides might have some effect upon the activity of thrombin. This possibility was strengthened by the fact that it has long been known that oxygen plays a part in the clotting of blood, and that cyanides have the power of disturbing oxydation-reduction mechanisms.

Dilute solutions of potassium cyanide were titrated with dilute hydrochloric acid to as near pH 7.36 as possible, using bromthymol blue as an external indicator. The solutions were then made up to known values with a boric acid-sodium borate buffer of pH 7.36. Equivalent solutions of potassium chloride in the same buffer were then prepared. To each of a series of test tubes was added the same amount of citrated plasma and a varying amount of the cyanide solution. The potassium content was made the same in each tube by the addition of the necessary amount of the potassium chloride solution. Any further dilutions were made with the borate buffer. After definite periods of incubation identical amounts of calcium chloride were added to each tube and the clotting times recorded. The period of incubation with the cyanide was found to have no effect. Marked prolongation of the clotting times was found to take

place.

The degree of inhibition of clotting by a given amount of cyanide varied with the sample of plasma. That this might be due to the union of some of the cyanide with factors not involved in clotting is possible, and the work should be repeated with pure fibrinogen and thrombin. Table X shows the degree of inhibition.

(Insert Table X.)

If, in the above experiment, the inhibition by cyanide were due to its action on sulfhydride groups, the addition of a sulfhydride compound should lessen the inhibiting effect on clotting. Similarly, if the inhibition were due to an interference with oxidation elsewhere in the molecule, oxygenation should decrease the inhibition. On the basis of these considerations an experiment was performed similar to the preceding one, but in which the cyanide concentration was held constant. In this experiment oxygen was bubbled through some of the tubes for three minutes, after the cyanide had been added; hydrogen sulfide was bubbled through other tubes under the same conditions. Table XI gives the results of one of these experiments. In the instance tabulated the pH of the tubes through which hydrogen sulfide was passed changed from 7.36 to 7.2 but such a change would have no

Table X.

Inhibition by cyanide.

5 cc. of plasma used in each case.

Total volume 1.65 cc.

Sample	Concentration of HCN	Clotting time
1	0.0000 %	10 minutes
2	0.0673 %	30 minutes
3	0.101 %	50 minutes
4	0.135 %	24 hours
5	0.168 %	None in 72 hrs.

appreciable effect on normal clotting.

(Insert Table XI.)

The decrease of inhibition by oxygen and the increase by hydrogen sulfide are strongly indicative of the dependence of thrombin action upon an oxidation or reduction mechanism. In this connection it is significant that Kuhnan and Morgenstein (74) have shown sulfhydride compounds to inhibit clotting, and Hirose (36) has shown the re-solution of fibrin to take place most rapidly when large amounts of oxygen were present. Cyanide is known to reduce cystine to cysteine and it may be acting on glutathione. The relatively large amounts of cyanide necessary indicate some such action rather than its combining with minute amounts of iron or copper catalysts.

Table XI.

Effect of oxygen and of hydrogen sulfide upon the inhibition of clotting by cyanide.

0.5 cc. of plasma used in each case.

Total dilution 1.65 cc.

Sample	Concentration of HCN	O_2	H_2S	Clotting time
1	0.00 %	-	-	3.0 minutes
2	0.00 %	-	-	3.0 minutes
3	0.12 %	-	-	7.5 minutes
4	0.12 %	-	-	7.75 minutes
5	0.12 %	-	3 min.	18 hours
6	0.12 %	-	3 min.	19 hours
7	0.12 %	3 min.	-	4.75 minutes
8	0.12 %	3 min.	-	5.0 minutes

Summary and Conclusions.

1. When a solution of purified fibrinogen is clotted by thrombin about 9.5% of its nitrogen remains in the solution. The fibrin contains only 91% of the nitrogen in fibrinogen.
2. There is an increase of about 5% in free amino groups as determined by the Sorensen method when purified fibrinogen clots to fibrin.
3. Both fibrinogen and fibrin autolyse in sterile dilute salt solution, but fibrin more rapidly.
4. Purified fibrinogen treated in solution with precipitated calcium phosphate and charcoal is much more stable and autolyses very slowly. Presumably prothrombin or some other proteolytic enzyme has been removed from it.
5. In the process of autolysis of fibrinogen and fibrin globulins are formed which have the coagulation temperatures of and appear to be euglobulin and pseudoglobulin. No serum albumin could be found, but possibly longer digestion might have yielded it. Proteases and lower peptides (non-protein nitrogen) are also set free.
6. The clotting of fibrinogen by thrombin and of its autolysis is blocked by acetylation or by formalinization of the fibrinogen.

7. Acetylation also inactivates thrombin but at a very much slower rate. How this is done is undetermined. Nor were attempts made to regenerate the thrombin.
8. The marked slowing of clotting occurring at temperatures of about 45° C. is not due chiefly to the destruction of thrombin.
9. Clotting is prolonged by HCN and by H_2S. Bubbling oxygen through the HCN plasma before recalcification removes the inhibition of the HCN; bubbling H_2S greatly increases the inhibition due to HCN.
10. The general conclusion is that thrombin is a proteolytic enzyme; that fibrin is a first proteolytic cleavage product of fibrinogen; that when fibrin is formed some fibrinoglobulin and other forms of protein nitrogen are set free; that on further action of thrombin other globulins similar to and possibly identical with euglobulin and pseudoglobulin appear, as well as proteases and simpler hydrolytic splitting products.
11. Thrombin is so far as known specific for fibrinogen, and is an amino protease. An oxidation-reduction system possibly involving glutathione seems to be involved in its activity.
12. Serum globulin possibly originates from the hydrolysis of fibrinogen of the blood by the action of some

proteolytic enzyme. No light has been shed by these experiments as to the origin of serum albumin.

Acknowledgement

In closing I wish to thank the William S. Merrell Company for their generous support of this work for the first two years; and Professor S. Tashiro who placed at my disposal part of the Hepatic Research fund in the last year. This financial support has made the work possible. I also wish to thank Professor Mathews and Professor Tashiro for their constant helpful criticisms and advice.

Bibliography

1. Buchanan: Lond. Med. Gaz., 18, 50, 1835.*
2. Buchanan: ibid. (new series), 1, 617, 1845.*
3. Chaptal: Ann. de Chemie, 21, 290, 1795.
4. Denis, P. S.: Memoire sur le sang (Paris), 1859.*
5. Schmidt, A.: Archiv. f. Anat. u. Physiol., 545, 1861.
6. Schmidt, A.: ibid. 428, 533, 1862.
7. Hammarsten, O.: Pfluger's Archiv. 16, 211, 1877.
8. Schmidt, A.: Zur Blutlehre (Leipzig), 1892.*
9. Kugelmass, I. N.: Archiv. intern. de Physiol., 21, 139, 1923.
10. Stuber, B. and Sano, M.: Biochem. Z., 134, 239, 260, 1922.
11. Hekma, E.: The Blood Plasma in Health and Disease, p. 73, 1927.
12. Wadsowrth, A., Maltaner, F. and Maltaner, E.: Am. J. Physiol. 97, 74, 1931.
13. Leivi, F.: Acta. Med. Scand. 82, 268, 1934.
14. Mellanby, J.: J. Physiol. 38, 28[illegible], 441, 1909.
15. Stuber, B. and Funck, A.: Biochem. Z. 126, 142, 1921.
16. Klinke, K. and Ballowitz, K.: Z. ges. exptl. Med. 84, 224, 1932.
17. Abderhalden, E. and Buadze, S.: Fermentforschung, 14, 215, 1934.

18. Mills, C. A. and Mathews, A. P.: Arch. intern. de Physiol. 24, 73, 1924.
19. Sumner, J. B.: Compt. rendu Soc. Biol. 87, 388, 1922.
20. Rettger, L. J.: Am. J. Physiol. 24, 406, 1909.
21. Mills, C.A.: Proc. Soc. Exp. Biol. and Med., 25, 849, 1928.
22. Mellanby, J.: Proc. Roy. Soc. B., 113, 93, 1933.
23. Eagle, H.: J. Gen. Physiol., 18, 547, 1935.
24. Nausenbach: Thesis, Dorpat, 1882.*
25. Grohmann; Thesis, Dorpat, 1884.*
26. Loeb, L.: Montreal Med. News, 32, 507, 1903.*
27. Gratia, A.: Compt. rendu Soc. Biol., 82, 1247, 1919.
28. Gratia, A.: ibid., 83, 649, 1919.
29. Gratia, A.: Arch. intern. de Physiol., 18, 355, 1921.
30. Cekada, E. B.: Am. J. Physiol., 78, 512, 1926.
31. Lamb: Scien. Mem. Med. and San. Dept. of India, 1903.*
32. Martin, C.J.: J., Physiol., 32, 207, 1905.
33. Barratt, J. O. W.: Biochem. J., 14, 189, 1920.
34. Collins, W.: Thèse de Lausanne, 1-31, 1927.*
35. Cekada,: Am. J. Physiol., 78, 512, 1926.
36. Hirose, R.: Thesis, University of Cincinnati, 1932.
37. Morawitz, P.: Ergebn. d. physiol., 4, 307, 1904.*
38. Mellanby, J.: J., Physiol., 38, 28, 1909.
39. Burker: Pfluger's Archiv. 102, 65, 1904.
40. Addis: Quart. J.,Exp. Physiol., 1, 305, 1908.

41. Kuwashima, K.: J. Biochem. (Japan), 3, 91, 1924.

42. Hsü, H. and Wu, H.: Chinese J., Physiol., 7, 117, 1933.

43. Fuchs, H. and Zahrzewski, Z.: Klin. Wochschr. 13, 1511, 1934.

44. Gengou, O.: Ann. inst. Pasteur, 51, 1431, 1933.

45. Haugardy, A.: Arch. intern. de Physiol., 36, 92, 1933.

46. Mills, C. A.: Proc. Soc. Exp. Biol. and Med., 25, 849, 1928.

47. Hirose, R.: Am. J., Physiol., 107, 693, 1934.

48. Welker, W., Gilman, G. and Hektoen, L.: Am. J., Physiol., 106, 475, 1933.

49. Waldschmidt, Leitz, E., Stadler, P., and Steigerwald, F.: Z. f. physiol. Chemie, 183, 39, 1929.

50. Strughold, H. and Wöhlisch, E.: Z. physiol. Chem., 223, 267, 1934.

51. Northrop, J. and Kunitz, M.: J. Gen. Physiol., 16, 295, 1932.

52. Collingwood, B. J.: J., Physiol., 27, 58, 1924.

53. Hugounenq, H. and Loisleur, J.: Bull. Soc. Chemie Biologique, 7, 955, 1925.

54. Dunn, E. F.: Thesis, University of Cincinnati, 1930.

55. Mills, C. A. and Mathews, A. P.: Arch. intern. Physiol., 24, 73, 1924.

56. Mills, C. A.: Am. J., Physiol., 57, 395, 1921.

57. Nakamura, K.: Med. Bull., University of Cincinnati, 6, 184, 1931.

58. Eagle, H.: J., Gen. Physiol., 18, 531, 1935.

59. Van Slyke, D. D.: J., Biol. Chem., 71, 235, 1927

60. Schmiedeberg: Arch. exp. Path. 31, 8.

61. Van Slyke, D. D.; J., Biol. Chem., 83, 425, 1929.

62. Denis, P. S.: Memoire sur le sang (Paris), 51, 376, 1838.*

63. Dastre, A.: Archiv. de Physiol., 5, 661, 1893.

64. Wasteneys, H. and Borsook, H.: J., Biol. Chem., 62, 1, 1924.

65. Kylin, E. and Kristenson, H.: Acta. Med. Scand. 83, 273, 1934.

66. Halliburton, W. D.: J., Physiol., 5, 152, 1884-5.

67. Halliburton, W. D.: ibid., 7, 319, 1886.

68. Halliburton, W. D.: A Text Book of Chemical Physiol., (London), 1891.

69. Howe, P. E.: J., Biol. Chem., 49, 109, 1921.

70. Howe, P. E.: ibid. 57, 235, 1923.

71. Young, E. G.: Proc. Roy. Soc., B, 93, 15, 1922.

72. Adair, G. and Robinson, M.: Biochem. J., 24, 993, 1930.

73. Herriott, R. M.: J., Gen. Physiol., 18, 69, 1934.

74. Kuhnau, J. and Morganstein, V.: Z. phys. Chem., 227, 145, 1934.

* Quoted from Pickering, "The Blood Plasma in Health and Disease", MacMillan, 1928.