ON THE NATURE OF FORCES OPERATING IN BLOOD CLOTTING

II. The Clotting of Fibrinogen As a Two-Step Reaction

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1. INTRODUCTION

In the foregoing paper it has been shown that somewhere in the process of transformation of fibrinogen into fibrin a process of complex or autocomplex coacervation, that is an aggregation due to electrostatic attractions, takes place. Of course this result still allows for a variety of explanations of the mechanism of the clotting process. For instance, it might be supposed that the transition of fibrinogen into fibrin is essentially a denaturation, and that the denatured fibrinogen coagulates because of the liberation of ionogenic groups, which cause an autocomplex coacervation of the particles.

That the clotting of fibrinogen is a denaturation caused by thrombin is admitted rather generally; it is especially propagated by Wöhlisch (10) who considers thrombin to be a specific denaturing enzyme which he therefore calls fibrinogen denaturase. The main argument in favour of this view is a certain similarity in the appearance of fibrin and fibrinogen denatured for instance by heat. It will be clear that this is no real proof; fibrinogen is a rather labile protein, which, in accordance with its anisometric molecular shape, has a strong tendency to form fibrous gels; such gels are always formed when the solubility is decreased sufficiently to cause precipitation; this can be effected by a decrease of the salt contents of the solvent, by salting out, by denaturation, or by thrombin, and the appearance of the precipitate is determined in all these cases mainly by the properties of the fibrinogen. Further there is a synergy between denaturation and the action of thrombin; as both influences have the same effect, *viz.* making the fibrinogen clot, this synergy is quite understandable. None of the arguments of Wöhlisch or other authors give a real proof in favour of the denaturation theory.

In this paper a number of experiments are described which offer another explanation of the mechanism of the action of thrombin, and which make the assumption of a denaturation of fibrinogen by thrombin superfluous and improbable.

2. The Two Steps of the Reaction

The clotting of pure fibrinogen with purified thrombin is possible only at pH values above the isoelectric point of the fibrinogen (pH 5.3). If, however,

a reaction mixture, containing fibrinogen and thrombin, is kept at for instance pH 5.1 and than neutralised, a normal clot is formed. This shows that at this pH no irreversible damage of fibrinogen or thrombin occurs.

Now it has been discovered by Laki (unpublished data; Laki and Mommaerts, 5) that after neutralisation clotting occurs the sooner (clotting time t_c), the longer fibrinogen and thrombin are kept together at pH 5.1 (reaction time, t_r). Fig. 1 shows the result of such an experiment; it proves that the

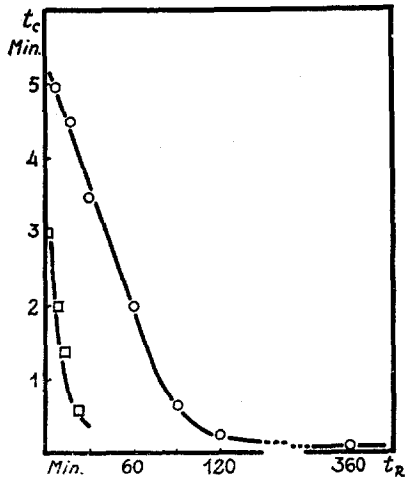


FIG. 1. Explanation in the text.

transformation of fibrinogen into fibrin takes place in two steps. The primary combination between thrombin and fibrinogen, apparently a slow reaction which limits the velocity of clotting, proceeds at pH 5.1, whereas the formation of the fibrin gel takes place only at higher pH values. Of course it is not intended to say that at acid reaction the primary process proceeds with the same speed as in neutral solution.

In this paper, the primary product will be called profibrin. The word profibrin has been used by Apitz (1, 2) to designate the still soluble products appearing in a very slowly clotting solution of fibrinogen with a small quantity of thrombin. As will be discussed elsewhere, these products are a quite heterogeneous mixture, for which no special name may be claimed.

3. The Effect of Neutral Salts

The problem arises as to which one of the two steps of the reaction is of coacervate nature. This question may be decided by the study of the effect of neutral salts on the two reactions separately.

The effect of salts on the first reaction may be investigated by making use of the fact that for most salts a certain concentration exists which prevents

clotting completely, whereas a two times lower concentration still allows clotting in a comparatively short time. Of course, the absolute value of that critical concentration depends on the nature of the ions. If now thrombin and fibrinogen are kept together for different times (t_r) in the presence of any salt in the critical concentration, and then diluted with an equal volume of distilled water, it is found that again the clotting time (t_c) decreases with longer times of reaction (two examples in Fig. 2). This means that the primary reaction proceeded in the presence of the salt, and thus that neutral salts do not inhibit

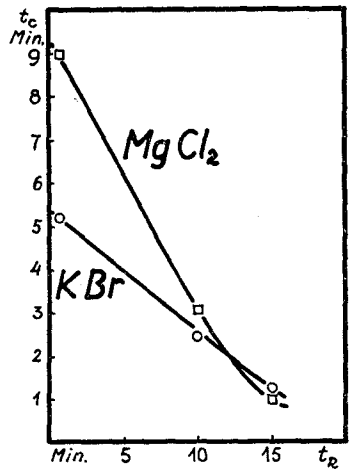


FIG. 2. Explanation in the text.

the primary reaction in a measurable degree. The primary reaction therefore is no coacervation process.

Whether neutral salts inhibit the second reaction may be tested in the following way: thrombin and fibrinogen are allowed to react at pH 5.1, and then neutralised with or without simultaneous addition of salt. An example:

2 cm.^{3} fibrinogen, 0.4 cm.^{3} thrombin, 10 units per cm.^{3}, 2 cm.^{3} M/5KH_2PO_4; after 20 minutes' neutralisation of 0.4 cm.^{3} with 0.2 cm.^{3} M/5Na_2HPO_4, with and without addition of salts, dissolved in 0.2 cm.^{3}.

Some results are shown in Fig. 3. The positions of the curves are not exactly the same as in Figs. 2 and 4 of the foregoing paper. This difference is due to the fact that in the experiments of the kind shown in Fig. 3 the solution contains a fairly high quantity of phosphate ions (~M/15) which likewise inhibit the clotting considerably. Between these and the added ions certain effects of synergy and antagonism occur, which have not yet been studied separately, so that the deviations cannot yet be explained in detail. The general trend of the curves is, however, clear.

We see therefore that the second phase of the reaction, the gelatination of the profibrin, is a coacervation process.

4. Nature of the Primary Reaction

An indication of the possible nature of the primary reaction between thrombin and fibrinogen may be obtained by the study of the inhibition of the clotting process by urea and similiar compounds.

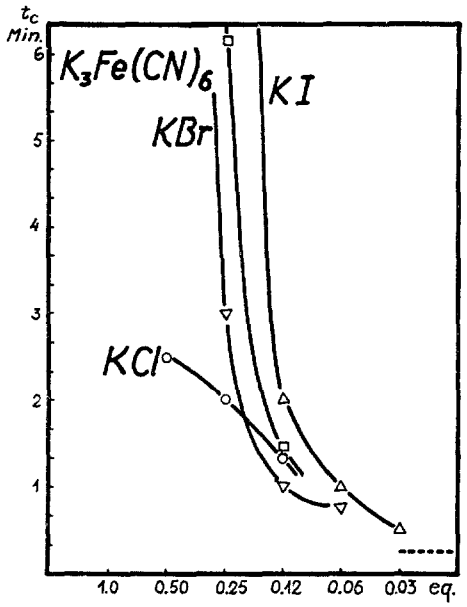


FIG. 3. Inhibition of the second reaction by some salts in different concentrations. The dotted line marks the clotting time without addition of salt.

That urea inhibits the clotting of fibrinogen with thrombin is shown in Fig. 4. Guanidine and other substances have the same effect. The question arises whether the first or the second reaction is affected.

The effect of urea on the primary reaction is investigated in the same way as in the case of salts: a mixture of thrombin and fibrinogen in the presence of urea in a concentration of 10 per cent does not clot, whereas after dilution of the system with an equal volume of distilled water gelatination occurs in a few minutes. The primary reaction can be studied by carrying out this dilution after different periods of time (Fig. 5).

Contrary to the experiments with neutral salts mentioned in section 3 the clotting time is not reduced by long periods of reaction between thrombin

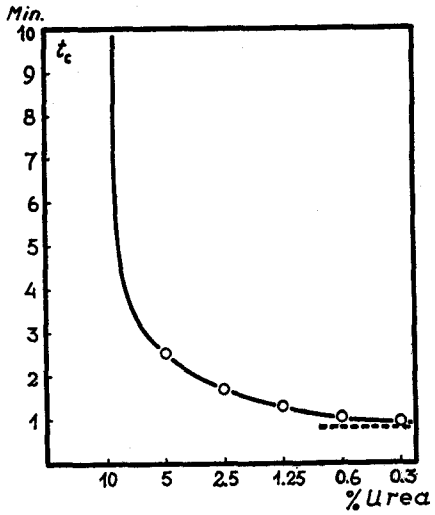


FIG. 4. Inhibition of the clotting of fibrinogen by thrombin by urea. The dotted line indicates the clotting time without addition of urea.

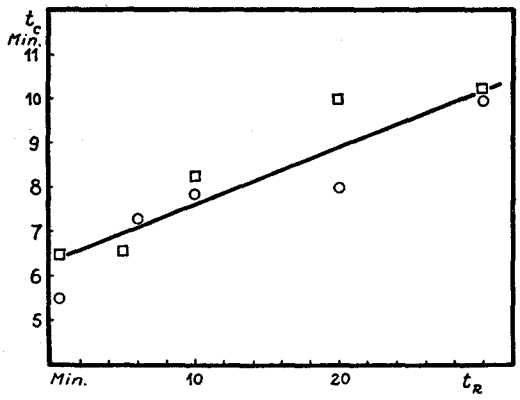


FIG. 5. Inhibition of the primary reaction by 10 per cent urea. For explanation see text.

and fibrinogen. On the contrary, in some experiments the clotting time increased somewhat. That means that in this concentration urea completely inhibited the primary combination of thrombin with fibrinogen.

Does urea affect the second reaction too? In order to get an answer on this question I studied the clotting of profibrin (after neutralisation of a reaction

mixture of thrombin and fibrinogen kept at pH 5.1) in the presence of urea. The following experiment may be cited:

1 cm.^{3} fibrinogen, 20 mg. per cm.^{3}
1 cm.^{3} M/5 KH_2PO_4
0.3 cm.^{3} thrombin, 3 units per cm.^{3}

After different times 0.4 cm.^{3} of the solution mixed with 0.2 cm.^{3} M/Na_2HPO_4 and 0.2 cm.^{3} distilled water or urea 20 per cent.

Time of reaction	Clotting time	The same with urea
30 sec.	6 min., 30 sec.	
10 min.	5 " 15 "	
20 "	4 " 30 "	>10 min.
40 "	3 " 10 "	>10 "

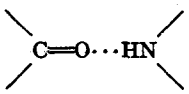
It is found therefore that the second phase is inhibited too. In other experiments, however, in which a great deal of thrombin was present, no inhibition was observed. Using a 30 times stronger thrombin solution than in the above experiment, I found that, if the mixture was neutralised after 5 minutes, clotting took place instantaneously, and that urea did not cause an observable inhibition. This could be explained by the assumption that urea inhibits the second reaction in an indirect way, by splitting the profibrin. I found that even a clotting time of 1 to 2 seconds is sufficient to allow for an inhibitive action of urea; an eventual displacement by urea of the thrombin already bound should therefore take place in a very short time. Another possible explanation, however, is that urea directly inhibits the second reaction, and that this inhibition may be neutralised to a certain degree by thrombin in higher concentration. As will be explained in section 6, this assumption is not in disagreement with the fact that the second reaction is caused by electrostatic attraction.

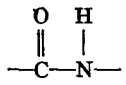
5. The Nature of Thrombin Action

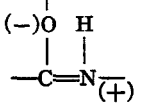
In connection with the results dealt with in section 4 some remarks must be made concerning the aggregative forces operating between two parallel peptide chains.

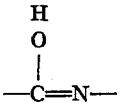
In recent years some investigators (for instance Lotmar and Picken, 6; Mirsky and Pauling, 7) expressed the view that in a protein molecule the peptide chains are kept together (or kept in a folded state) by hydrogen bridges. With that term usually two different kinds of bonds are meant: attraction between a dissociated carbonyl and a dissociated amino group (formula I), and a resonance bond between the >C=O and the >NH- groups of two carbon amide structures (formula II).

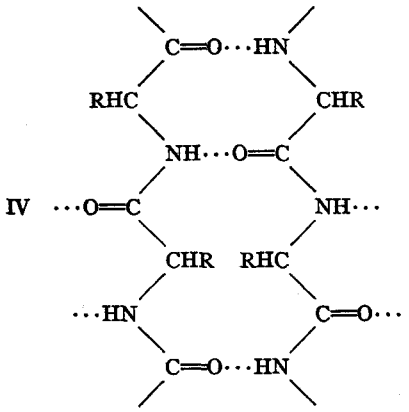
I $R\cdot COO^- + H_3N\cdot R$

II 

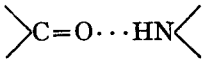
III*a* 

III*b* 

III*c* 

IV 

In this paper only the second group will be called a hydrogen bond, because the first type may better be considered as an electrostatic bond.

Recently a detailed study of the reactivity of the carbon amide structure has been made by Arndt (3). He found that substances containing this structure (formula III*a*) generally do not have the tendency to complete tautomerisation; that is, of electromery + prototropy (III*c*). Unlike the situation in the case of keto-enol tautomery, however, there is a possibility of electron displacement without prototropy (III*b*). The formation of III*b* implies the appearance of a dipole and asks therefore for additional energy, so that in isolated molecules this constellation will not occur. In the solid state, however, neighbouring molecules are linked through  bridges, the energy of which suffices to stabilise the configuration III*b*.

Between two peptide chains, or between two parts of a folded peptide chain, formation of hydrogen bonds can take place in the same way (formula IV). Urea, which disaggregates proteins, likewise possesses the  structure, and may be supposed to displace two peptide chains from each other by competition. Guanidine and other substances act in very much the same way. An investigation of other substances has been taken up already by the author.

Returning now to the process of blood clotting, the fact that urea inhibits the primary reaction between fibrinogen and thrombin suggests that in the primary reaction thrombin and fibrinogen are linked together with hydrogen bonds, although I admit that at the present state of knowledge evidence is not yet conclusive.

The nature of the second reaction is understandable by the discovery (to be described in a future paper on thrombin) that the isoelectric point of thrombin is situated far in the alkaline range. At pH values at which clotting is possible, the thrombin molecules have a strong positive charge and profibrin has a pattern of positive and negative charges; it aggregates then as an autocomplex coacervate.

The experiments described in this paper form an argument against the view that the clotting process is a denaturation of the fibrinogen. Coagulation of proteins due to denaturation is generally maximal near the isoelectric point because denaturation consists of an unfolding of the molecule, liberating the internal ionogenic groups of the molecule (*cf.* Haurowitz, 4) followed by an autocomplex coacervation of the stretched particles. Coagulation of fibrinogen with thrombin, however, does not take place near the isoelectric point of the fibrinogen, but only at higher pH values. Further, denaturation of fibrinogen is greatly increased, clotting by thrombin, however, always inhibited by NaCl.

Although the process of clotting can be explained without denaturation, and there exist strong arguments against its participation in the clotting process, I shall not yet exclude the possibility that after the formation of fibrin certain changes might occur in the fibrinogen part of the fibrin, which are similar to denaturation. However, up to now there have been no arguments in favour of such a view.

6. The Mechanism of the Clotting Process

As has been shown in the foregoing paragraphs, the first event in the clotting of fibrinogen is the formation of a compound of fibrinogen and thrombin, called profibrin. This reaction can be studied separately at pH 5.1; it does not cause any physical change of the fibrin solution which might give an indication of a beginning polymerisation, or of a change of form of the fibrinogen molecule.

At pH values at which clotting is possible fibrinogen is negatively, thrombin positively charged and the profibrin has a pattern of positive and negative charges. As in the formation of an autocomplex coacervate, these changes cause an aggregation of the profibrin by electrostatic attraction.

One must keep in mind, however, that these electrostatic forces are not the only attractions operating between two profibrin particles. The numerous

groups likewise attract each other; this attraction alone is insufficient to make the fibrinogen insoluble, but it supports the electrostatic effect. By means of salts the electrostatic effect is ruled out, clotting therefore prevented; with urea, however, the attraction of the second kind is decreased, so that likewise the attraction between the profibrin molecules is lowered.

It is therefore not astonishing that at low concentrations of thrombin urea inhibits not only the reaction between thrombin and fibrinogen, but the aggregation of the profibrin too; with much thrombin, however, the electrostatic

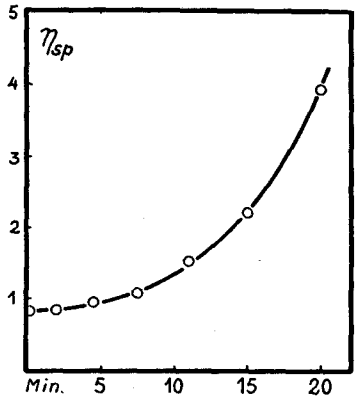


FIG. 6. Increase of the viscosity of a fibrinogen solution, which clots slowly under the action of a small amount of thrombin. 3 cm.^{3} fibrinogen $\times$ 0.2 cm.^{3} thrombin 2 units per cm.^{3}.

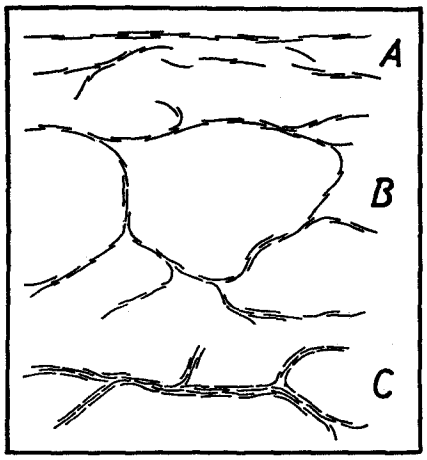


FIG. 7. Schematical representation of the polymerisation of profibrin to fibrin.

attraction dominates to such a degree that now urea hardly affects the second phase of the reaction (section 4). Such a parallel action of electrostatic and hydrogen bonds is not a special property of profibrin. It has been found by the author (9) in the case of aggregated myosin, and in unpublished investigations on the gelatin micelles, and probably the same will be found in other proteins too.

If fibrinogen is allowed to stand with a small quantity of thrombin, so that clotting takes a considerable time, it is found that from the very first moment the viscosity of the solution gradually rises (Fig. 6). Now the viscosity of the solution of an anisometric protein is determined by the proportion l/d of length and diameter of the molecules (compare the author's analysis of the viscosity of myosin, 8). Thus under the action of thrombin the asymmetry of the anisometric fibrinogen molecules increases. The aggregation of the profibrin thus takes place mainly or exclusively in longitudinal direction (Fig. 7, A).

In further stages of the process the different micelles, while still increasing in length, are more and more connected mutually, till at the end the entire solution solidifies to a gel (Fig. 7, B). But after solidification the reaction proceeds still further, under the formation of crystalline micelles (Fig. 7, C) (the existence of which is proved by Röntgen analysis) till all the thrombin and fibrinogen is exhausted.

SUMMARY

It is found that clotting of fibrinogen by thrombin does not occur on the acid side of the isoelectric point of the fibrinogen. At such pH values, however, a primary reaction between thrombin and fibrinogen takes place, leading to the formation of profibrin, a compound of thrombin and fibrinogen.

At pH values at which clotting is possible, fibrinogen is negatively, thrombin positively charged, whereas profibrin has a pattern of positive and negative charges.

The primary reaction, the formation of profibrin by combination of thrombin and fibrinogen, is inhibited by urea but not by neutral salts. The combination of thrombin with fibrinogen most probably takes place by hydrogen bonds.

The second reaction, the polymerisation of profibrin to fibrin, is inhibited by neutral salts in the same way as complex or autocomplex coacervates. It is caused therefore by electrostatic attraction between the positive and the negative charges of the profibrin.

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