**Fibrinase***

**IV. EFFECT ON FIBRIN SOLUBILITY**

 Ariel G. Loewy, Jonathan A. Gallant, and Katy Dunathan

*From the Department of Biology, Haverford College, Haverford, Pennsylvania*

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Fibrinase is a calcium-activated enzyme responsible for the transformation of the soluble fibrin into the insoluble fibrin (1, 2). Solubility has in the past been defined as dispersion in 0.01 N sodium hydroxide (3), 0.01 N hydrochloric acid (4), 5 M urea (5), 2% monochloroacetic acid (6), or 2% acetic acid (7).

Loewy and Edsall proposed that the formation of insoluble fibrin is brought about by a disulfide exchange mechanism whereby intramonomer disulfide bonds exchange to form intermonomer disulfide bonds (8). The hitherto scant lines of evidence in support of this hypothesis are as follows. (a) Fibrin is insoluble in all solvents that do not break the known covalent bonds in protein molecules. This suggests that covalent bonds are established during the formation of insoluble fibrin. (b) Thioglycolate in the presence of urea has been reported to dissolve fibrin (9). This suggests that the new covalent bonds are disulfide bonds. (c) Both fibrinogen (10) and soluble fibrin contain less than one sulfhydryl group per molecule.1 This rules out fibrin formation by sulphydryl oxidation and suggests that disulfide exchange is the mechanism by which intermonomer disulfide bonds are formed. (d) Fibrinogen and fibrinase are not inactivated by pretreatment with iodoacetate, but this reagent inhibits the formation of fibrin when present in the reaction mixture (1). This observation is consistent with the disulfide exchange mechanism which requires the liberation of new sulphydryl groups during the exchange process.

Our inability to repeat Lorand's observation (9) that insoluble fibrin can be dissolved in a mixture of thioglycolate and urea led us to a detailed investigation of the solubility of fibrin. By studying the “solubility profiles” of fibrin formed under a variety of conditions, we have been able to demonstrate the existence of an intermediate form which we have named “stabilized fibrin.” It has also been possible to obtain some information regarding the nature of the bonds cross-linking soluble, stabilized, and insoluble fibrin.

**EXPERIMENTAL PROCEDURE**

Fibrinase (Peak 4 eluate), fibrinogen low in fibrinase activity (Fraction 8), and fibrinase-rich fibrinogen (Fraction 3) were prepared by the method of Loewy, Dunathan, Kriel, and Woffinger (1).

“Solubility profiles” were established by determining whether a series of fibrin clots were dispersed in solutions of variable pH and solvent concentration. Fibrin clots were prepared by mixing in a 10-ml test tube 0.1 ml of fibrinogen (2 mg per ml), pH 7.0, dissolved in 0.15 M sodium chloride; 0.1 ml of solution at pH 7.0 containing the reagent used in a given experiment (e.g., calcium chloride, EDTA, cysteine or sodium sulfite); and 2.5 units in 0.01 ml of human thrombin (100 to 200 units per mg of protein). Incubation of the clotting mixture took place at room temperature for specified lengths of time, after which 2 ml of various solvent concentrations and 0.2 ml of buffer mixtures of differing pH values were added to the clots. These were detached from the test tubes with a fine metal spatula, and the tubes were tightly sealed with rubber stoppers and stored at room temperature. Periodic readings of the presence or absence of clots were made, usually over a span of 48 hours. As a rule, no changes were observed to occur after 24 hours of incubation with solvent. Finally, the pH values of the supernatant in each test tube were measured and the presence or absence of clots were plotted as “+” or “0” signs as shown in Fig. 1. The solubility profile represents an area formed by a line which is drawn to enclose all the + signs and exclude all the 0 signs. Thus, the solubility profile encloses a range of solvent concentrations and pH values at which the fibrin clot is not completely dispersed by the solvent. To facilitate the reading of these solubility profiles, we introduced shading on the side of the boundary that encloses the insoluble clots.

Two types of buffer mixtures were used in the course of these experiments. Buffer mixture A was prepared by mixing 1 ml solutions of Tris, sodium acetate, monobasic sodium phosphate, dibasic sodium phosphate, and glycine. These buffer mixtures were divided into 12 aliquots and adjusted to pH values of 1.0, 2.0, 3.5, 4.5, 5.5, 6.5, 7.5, 8.0, 8.5, 9.0, 10.0, and 11.0 with concentrated hydrochloric acid or 10 N sodium hydroxide. When 0.2 ml of the 1 M buffer mixtures was added to the 0.2 ml of fibrin and 2 ml of solvent, some changes in pH occurred, especially at the acid and alkaline extremes of the range. We therefore found it desirable to determine the pH for each solution at the end of the experiment.

The ratio of clot to solvent volume of 1:10 (0.2 ml of clot containing 2 mg of fibrin and 2.2 ml of solvent) was chosen after a variety of ratios were tried with soluble fibrin clots at pH 6 and 8 over a range of urea concentrations. It seemed that the use of clot to solvent volume ratios of 1:20 and 1:40 did not modify...
the results obtained with 1:10 ratios. We concluded that the transition with rising solvent concentration from insoluble to soluble clots is so abrupt that the effect is virtually unmodified by variations in solvent volume.

Because of the use of the detergent SDS, it was necessary to exclude potassium from these experiments; therefore, sodium salts were used throughout. Whenever necessary, protein preparations containing potassium chloride were dialyzed exhaustively against 0.15 M sodium chloride.

SDS and CTAB were obtained from Dr. Fred Karush.

A Beckman model G pH meter was used with a special type E glass electrode for measurements in the alkaline region.

RESULTS

Soluble and Insoluble Fibrin

Fig. 1 is a solubility profile of "soluble fibrin" in urea. It will be seen from another experiment (Fig. 3) that the fibrinogen (Fraction 8) used contained very small traces of fibrinase. To avoid the effect of this slight fibrinase contamination, the fibrinogen was clotted in the presence of 10⁻⁴ M EDTA. The solubility profile shows that soluble fibrin is a very weak gel which is dispersed in buffer at pH values below 5 and above 9. At its solubility minimum (pH 8 to 8.5), it is dispersed at the relatively low solvent concentration of 1 M urea.

Fig. 2 is a solubility profile of "insoluble fibrin" formed by clotting fibrinase-rich fibrinogen (Fraction 3) in the presence of 5 x 10⁻³ M calcium chloride. This form of fibrin, generally called "insoluble fibrin," is dispersed in urea only at high pH values.

Transition to Insoluble Fibrin

Intermediate stages of fibrin solubility can be obtained by using (a) fibrinogen preparations containing traces of fibrinase (Fraction 8) and incubating with calcium and cysteine for varying periods of time or (b) by adding to the fibrinogen (Fraction 8) various amounts of fibrinase at constant incubation time. Figs. 3 and 4 represent solubility profiles obtained by the first and second procedure, respectively. Both experiments show that the action of fibrinase has two initial effects: (a) the pH 8 to 9 peak increases in height and area without any gross pH shift in the solubility minimum, and (b) a new region of clot stability appears at low pH. It should be noted that the measurement of the solubility minimum at pH 8 (Figs. 3 and 4) permits the detection of traces of fibrinase which would escape detection if the criterion of solubility in 5 M urea alone were used. Fig. 4 shows clearly the transition between early and late stages in the formation of insoluble fibrin, the early stages being comparable to those depicted in Fig. 3 and the final form being identical with the one shown in Fig. 2.

Fig. 5 shows a solubility profile of a fibrin clotted in 0.9 M KCl. It seems that at high ionic strength, fibrin stability at low pH develops very much more rapidly than at low ionic strength. Conversely, fibrin stability in the pH 8 to 9 region develops more slowly at high ionic strength than at low ionic strength.

Stabilized Fibrin

Fig. 6 represents a study of the rate of change of the solubility profile of insoluble fibrin. We had noticed that this process was

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2 The abbreviations used are: SDS, purified sodium dodecyl sulfate; CTAB, cetyl trimethyl ammonium bromide.
a much slower phenomenon than comparable changes observed with soluble or intermediate forms of fibrin. It occurred to us that the dispersion in urea of insoluble fibrin at high pH might be brought about either by disulfide hydrolysis (12) or by disulfide exchange (13). To test either of these hypotheses, we studied the rate of movement of the boundary in the alkaline region of the solubility profile, using fibrin clotted in the presence of cysteine or sodium sulfite. We were able to show that in fibrin preparations clotted in the presence of the disulfide-cleaving reagents, the alkaline boundary of the solubility profile achieved its final position much more rapidly than their respective controls. Fig. 7 shows the results of one such experiment performed with fibrin clotted in the presence of 0.005 M cysteine which after 5 hours of equilibration with solvent achieved a boundary position comparable to the 16-hour position in Fig. 6, whereas the boundary of the control experiment, clotted in the absence of cysteine, lagged far behind.

Thus, we have observed that treatment with cysteine or sulfite causes a change in the insoluble fibrin which renders it susceptible to rapid dispersion in 2 to 6 M urea at pH values of 9.5 to 10.5 (stabilized fibrin\(^3\)). In the absence of cysteine or sulfite, insoluble fibrin is obtained which is dissolved by 2 to 6 M urea above pH 9.5, but this requires prolonged exposure to the alkaline urea solvent. We suggest that the difference between the rapidly soluble (stabilized) fibrin and the slowly soluble (insoluble) fibrin is the absence or presence, respectively, of intermonomer disulfide bonds. In the presence of cysteine or sulfite, such disulfide bonds are presumably cleaved or prevented from forming, so that this form of fibrin is rapidly soluble in urea at alkaline pH. The solubilization of insoluble fibrin, however, requires prolonged treatment at alkaline pH which presumably causes disulfide hydrolysis possibly accompanied by some disulfide exchange.

The criterion for the detection of stabilized fibrin, namely the increased rate of solution at high pH, is an inconvenient one because it involves the overlapping of two effects that occur at high pH: (a) the cleavage of intermonomer disulfide bonds by hydrolysis or exchange, and (b) the dispersion of the clot presumably by net negative charge. It would be convenient if it were possible to separate these two effects, so that "equilibrium" instead of "kinetic" criteria could be used. The anionic detergent, SDS proved to be an excellent reagent for this purpose. Binding of the anionic detergent gives the fibrin monomer, at relatively low pH, a negative charge normally encountered only at high pH. Thus, at pH 8 in the presence of the detergent, the fibrin is in a pH region where disulfide hydrolysis does not occur, yet sufficient charge is accumulated by SDS binding to disperse the stabilized fibrin clot. Fig. 8 shows that, unlike the insoluble fibrin control, stabilized fibrin is indeed dispersed by SDS at relatively low pH values. In this instance, the stabilized fibrin

\(^3\) We will call fibrin clotted in the presence of fibrinase, calcium and a disulfide-cleaving reagent “stabilized fibrin.” In 6 M urea, this fibrin is insoluble at pH 8 but rapidly soluble at pH 9.5.
was formed by clotting in the presence of sulfite. The solubility profiles obtained permit us to devise a simple test for stabilized fibrin, namely solubility in 0.05 M SDS at pH 8.0 and insolubility in 5 M urea at pH 8.0.

Stabilized fibrin can also be obtained by reversing the formation of insoluble fibrin through subsequent treatment with cysteine or sulfite. The procedure usually used was to bathe 0.2 ml of insoluble fibrin clots in 2 ml of 0.15 M NaCl containing 0.01 M cysteine or sodium sulfite at pH 7.0. Fibrin treated in this manner was also soluble in 0.05 M SDS at pH 8.0 and insoluble in 5 M urea at pH 8.0.

Upon standing, stabilized fibrin is converted into insoluble fibrin. This reaction is rapid with cysteine-treated and relatively slow with sulfite-treated fibrin. When stabilized fibrin clots are washed free of cysteine or sulfite with several changes of 0.15 M KCl, more rapid conversion to insoluble fibrin is observed. Cysteine-treated fibrin requires 3 hours and sulfite-treated fibrin requires 12 hours to be converted to insoluble fibrin. The reversal of disulfide reduction, which had been produced by thiol reagents, has been reported on numerous occasions (12). The reversal of disulfide cleaving with sodium sulfite has also been previously observed (14–16). The formation of insoluble fibrin from stabilized fibrin obtained by treatment with cysteine can be prevented by washing the fibrin with large volumes of 0.0005 M iodoacetate. We found to our surprise that stabilized fibrin formed by the action of sulfite could be prevented from forming insoluble fibrin by washing with large volumes of 0.0005 M EDTA.

This effect may be explained by the observation that cupric ion catalyses the oxidation by sulfite of thiol groups to thiosulfate (15). Since this reaction is known to be reversible, it is conceivable that the reverse reaction which occurs when sulfite is washed out of the system, is also catalyzed by cupric ion. According to this explanation, EDTA acts by removing traces of cupric ion, catalyzing the formation of sulfhydryl from thiosulfate.

The above experiments performed with stabilized fibrin formed by treating insoluble fibrin with sulfites or cysteine were repeated with stabilized fibrin formed by including these reagents in the fibrinogen-fibrinase-calcium clotting mixture. Identical results were obtained, indicating that the formation of insoluble fibrin from stabilized fibrin is reversible.

**Some Additional Solubility Profiles**

As we have seen, stabilized fibrin can be dispersed with an anionic detergent which presumably increases the negative charge on the fibrin monomers. It is therefore likely that stabilized fibrin will also be dispersed by a cationic detergent. Fig. 9 shows that this is the case, but that the solubility profile of stabilized fibrin in CTAB is of a more complex nature. There are two pH regions (7 to 10 and 2 to 4) in which the clots are soluble in 0.05 M detergent.

The extreme resistance of stabilized fibrin to dispersion in urea can be reduced by raising both temperature and urea concentration. Fig. 10 is a solubility profile in urea at 45° of stabilized fibrin obtained by clotting in the presence of sulfite. It shows that at the higher temperature, stabilized fibrin is urea-soluble at low pH as well as high pH. Insoluble fibrin on the other hand is insoluble at 45° over the entire pH range of 2.2 to 9.5 in 8.3 M urea and even in a solution consisting of 8.3 M urea and 1 M SDS at 45°. Figs. 11 and 12 represent the remaining permutations of solubility profiles which can be determined by using the three
forms of fibrin and the three types of solvent. These are solubility profiles of soluble fibrin in SDS and CTAB, respectively. It should be noted that these curves illustrate a phenomenon not encountered in the previous solubility profiles. This is the initial reduction in fibrin solubility by relatively low concentrations of detergent. Thus, it should be noted that SDS reduces fibrin solubility in the acid region and CTAB reduces fibrin solubility in the alkaline region.

**DISCUSSION**

We have established the existence of three forms of fibrin differing significantly in their solubility properties.

1. **Soluble fibrin** obtained by clotting fibrinogen in the presence of EDTA can be recognized by its solubility in buffer at pH values below 4.5 and 9 and by its solubility in 1 M urea at pH 8.

2. **Stabilized fibrin** obtained by clotting in the presence of fibronectin, calcium, and cysteine or sulfite, is insoluble in 6 M urea at pH 8 but soluble in 10^{-2} M SDS at the same pH.

3. **Insoluble fibrin** obtained by clotting in the presence of fibronectin and calcium is insoluble even in 8 M urea dissolved in 1 M SDS at 45°, at all pH values between 2.2 and 9.5.

We now consider in some detail the shape of the solubility profiles we have presented, in order to obtain some information as to the nature of the bonds involved in the stabilization of these gels.

**Soluble Fibrin**

The solubility profile of soluble fibrin in urea (Fig. 1) shows that this gel is stabilized by very weak bonds capable of being ruptured by low concentrations of urea and by rather small changes in net positive or negative charge on the fibrin framework. The shape of the solubility profile is in agreement with the view of a number of investigators (17–19), particularly Scheraga and his group (20), that soluble fibrin is cross-linked with hydrogen bonds between uncharged histidyl acceptors and uncharged tyrosyl donors. One would expect that a gel cross-linked by this type of hydrogen bond would have a solubility minimum at a pH value where the maximal number of bonds occurred, namely, at approximately pH 8.5, which is halfway between the pK values of the histidyl and tyrosyl groups. The steeper downward slope of the solubility profile on the alkaline side of the peak is explainable in terms of the additional effect of increasing negative charge.

The solubility profile of soluble fibrin in SDS (Fig. 11) illustrates nicely the effect of detergent binding and charge on fibrin dispersal. The interesting aspect of this diagram is the large area of insolubility in regions of low pH. Let us for example consider what happens in the pH 3 region. At this pH, as we have already seen, soluble fibrin is dispersed in buffer presumably because of a net positive charge. In 0.0005 M SDS, however, the positive charge is sufficiently neutralized to stabilize the fibrin. In 0.005 M SDS, it would appear that further detergent binding generates a net negative charge of sufficient magnitude to disperse the clot. Since it is very unlikely that at pH 3 there can be any tyrosyl-histidyl hydrogen bonds, the presence of undispersed clots at that pH suggest at least one other type of weak bond capable of existing in the pH 2 to 4.5 region. These bonds seem to be sufficiently weak to be ruptured in buffer by the net positive charge, which is attained below pH 4.5. The shoulder in the pH 6 to 8 region of the SDS solubility profile would appear to be due to the hydrogen bonds of the histidyl-tyrosyl interaction mentioned above.

The solubility profile of soluble fibrin in CTAB (Fig. 12) is essentially a reverse of the SDS profile. Here, binding of the cationic detergent at alkaline pH causes a stabilization of the gel. CTAB does not appear to be bound as readily as SDS, so that dispersal by charge reversal only occurs when the negative charge on the protein is reduced by lowering pH. Here again, there is a marked shoulder in the curve but its presence as far down as pH 6.5 suggests that CTAB binding in the vicinity of histidyl groups lowers the pK of these groups, thus extending the tyrosyl-histidyl hydrogen bond interaction to lower pH values.

In summary, our observations on soluble fibrin suggest that this form of fibrin is cross-linked with (a) hydrogen bonds between histidyl and tyrosyl groups which can be disrupted with urea, and (b) other bonds of unknown character which are normally ruptured by excess net negative or positive charge.

**Stabilized Fibrin**

The solubility profile of stabilized fibrin in urea (Fig. 7) shows why we were at first unable to repeat Lorand's (9) observation that insoluble fibrin dissolves in a mixture of thioglycolate and urea. It shows that stabilized fibrin is soluble in 5 M urea only at pH values above 10. Presumably, Lorand's experiments were carried out at alkaline pH, whereas our attempts to repeat these observations were carried out at neutral pH.

The solubility profile of stabilized fibrin in urea at 45° (Fig. 10) shows that the combined effect of elevated temperature and net positive charge eliminates the interaction at low pH which can be observed in Figures 3, 4, and 7. The remarkable resistance to disaggregation by urea of stabilized fibrin at neutral pH, even in 8 M urea, suggests that hydrogen bonds are not involved in this interaction.

The solubility profile of stabilized fibrin in SDS (Fig. 8) shows a startlingly rapid transition at pH 6 to 7. At pH 2.5, 1 M SDS does not appear to bind in sufficient quantities so as to reverse the charge and disperse the clot. Within 1 pH unit between pH 6 and 7, SDS is able to disperse the clot at 0.01 M. It seems to us unlikely that the loss of positive charge of the histidyl groups alone, with a consequent increase in net negative charge, could
account for such an extensive change in solubility properties. We suggest that this remarkable solubility of stabilized fibrin in SDS may be due to the rupture of hydrophobic bonds (21) established during the formation of stabilized fibrin. The presence of such bonds would explain the previously noted insolubility in 8 M urea at 15°C. Such hydrophobic bonds would be ruptured by excessive net positive and negative charge but not by urea. The region of insolubility below pH 7 in SDS (Fig. 8) could be due to a combination of factors: (a) excessive net positive charge is not developed due to SDS binding; (b) hydrogen bonds involving the previously suggested uncharged carboxyl groups are not ruptured by SDS; (c) SDS binding raises the pK of the carboxyl groups to higher pH values; (d) the involvement of the carboxyl groups in hydrogen bonding may also contribute to the raising of their pK values. Thus, the rapid transition of the solubility profile at pH 7 may be due to the combined effect of the dissociation of carboxyl groups resulting in the rupture of hydrogen bonds, the appearance of negative charges and the disappearance of positive charges due to the dissociation of histidyl groups.

The solubility profiles in urea of fibrin in the process of transition from soluble to insoluble fibrin (Figs. 3, 4, and 5) are useful because they provide information on the primary effects of fibrinase. It is clear that one effect of fibrinase is to increase considerably the stabilization in the pH 5 to 9 region. More histidyl-tyrosyl bonds may be formed but, it is also possible that new hydrogen bonds involving the interaction of histidyl and lysyl ε-amino groups might be involved (22-25). The action of fibrinase also reveals a stabilization at low pH. This could be due to hydrogen bonds involving uncharged carboxyl groups as the donors. The velocity with which the low and intermediate pH stabilizations develop is markedly dependent on the ionic strength of the clotting mixture. Low ionic strength favors the pH 5 to 9 stabilization and high ionic strength favors the pH 2 to 4 stabilization. Since it is known that high ionic strength favors end to end association during the clotting process (26-28), it is possible that the low pH stabilization involves the end to end interactions of the monomers in the fibrin clot.

The solubility profile of stabilized fibrin in CTAB (Fig. 9) is of considerable complexity. In the region of pH 9 to 11, 0.05 M CTAB stabilizes the clot, presumably by lowering net negative charge. At higher concentrations of CTAB (0.1 M) we have clot dispersal, presumably by increased binding and charge reversal. The dispersal becomes increasingly effective as the pH is lowered from 11 to 8. The massive peak between pH 8 and 3 may be in part due to the histidyl-tyrosyl hydrogen bonding with the maximum shifted to lower pH values because of the effect of CTAB binding on the pK values of histidyl groups. It may also in part be due to the above mentioned hydrophobic bonds which may be less susceptible to CTAB than to SDS. One might imagine hydrophobic regions with neighboring positive charges to account for this difference in the effect of CTAB and SDS.

Finally, the stability below pH 3 (Fig. 9) previously encountered in Figs. 3, 4, and 5 may be similarly explained by hydrogen bonds involving uncharged carboxyl groups. The pK of these groups is apparently lowered by the effect of CTAB binding in their vicinity.

In summary, the solubility profiles of stabilized fibrin can be explained by assuming the appearance of hydrophobic bonds and of hydrogen bonds involving uncharged carboxyl groups. The presence of such bonds would explain (a) the high solubility in SDS at pH 8, (b) the low solubility in 8 M urea at the same pH, and (c) the insolubility both in CTAB and SDS below pH 3.

**Insoluble Fibrin**

The solubility profiles of insoluble fibrin (Figs. 2, 4, and 6) show that the solubility of this material is restricted to the alkaline region beyond pH 9.5. Solubility in this instance is presumably brought about by the hydrolysis of disulfide bonds. Should disulfide hydrolysis not be sufficiently rapid to account for the rate of clot dispersion at pH 9.5, it is possible that the dispersion of insoluble fibrin at this pH (Figs. 2, 6) may be due to a slow hydrolysis of disulfide bonds generating some free sulfhydryl groups which then catalyze a reversal of the disulfide exchange reaction. We suggest the possibility that the reversal of such a disulfide exchange reaction could be brought about by the "swelling action" or reduction in constraints caused by the urea solvent.

The solubility profile of insoluble fibrin shown in Fig. 2 can be reproduced even in the presence of 8 M urea dissolved in 1 M SDS at 45°C. This strongly suggests that the fibrin monomers in insoluble fibrin are cross-linked with covalent bonds. The fact that insoluble fibrin can be converted to stabilized fibrin with dilute solutions of cysteine or sodium sulfite constitutes strong evidence that these covalent bonds are disulfide linkages. It is, of course, possible that these disulfide cleaving reagents solubilize the gel by opening intramonomer disulfide bonds that cross-link separate peptides in the fibrin monomeric unit. We plan to investigate this possibility by a study of the subunits of the fibrinogen molecule.

**Mechanism of Insoluble Fibrin Formation**

The spontaneous conversion of stabilized fibrin to insoluble fibrin, and the inhibition of that reaction by iodoacetate treatment of the cysteine-generated stabilized fibrin, substantiate the theory that insoluble fibrin is cross-linked with disulfide bonds. The absence of free sulfhydryl groups in the soluble fibrin monomer would seem to eliminate the possibility that the disulfide bonds generated in the formation of insoluble fibrin are the results of sulfhydryl oxidation. We therefore conclude that the disulfide bonds are created by a disulfide exchange reaction in which the intramonomer disulfides exchange to form intermonomer disulfides. It has been shown that these reactions require traces of sulfhydryl groups to catalyze disulfide exchange (13). We have observed that the inhibition of insoluble fibrin formation by sulfhydryl reagents is much more effective if the reagent is added to the clotting mixture than if the fibrinogen and fibrinase are pretreated (1). For instance, neither fibrinogen nor fibrinase is susceptible to iodoacetate before the reaction but iodoacetate inhibits insoluble fibrin formation when added to the clotting mixture. This suggests that new sulfhydryl groups appear and is consistent with a disulfide exchange hypothesis.

The fact that the transformation of insoluble fibrin into stabilized fibrin is a reversible reaction and does not require active fibrinase suggests that the role of fibrinase precedes the disulfide exchange reactions. Thus, it is likely that fibrinase modifies the fibrin monomer in such a manner as to make the disulfide exchange possible. That native proteins must be denatured before they can undergo disulfide exchange has been first shown by Huggins, Tapley, and Jensen (29). It would seem that fibrinase achieves under physiological conditions what has been artificially induced by urea denaturation, namely a sufficiently close
juxtaposition of disulfide groups to permit disulfide exchange. Our results indeed show that stabilized fibrin is much less readily dispersed by urea and detergents than is soluble fibrin. Thus, the initial action of fibrinase is to increase the degree of interaction between the fibrin monomers caused by secondary bonds.

We have, so far, no direct evidence concerning the precise mechanism by which fibrinase might bring about this closer interaction of the fibrin monomers. We would like to suggest the possibility that fibrinase is a specific protease that acts by loosening or removing regions that interfere with the close association of the fibrin monomer units, which is required for the formation of stabilized fibrin and for the disulfide exchange reaction which ensues.

The preliminary study of Middlebrook (30) on the terminal amino end groups of soluble and insoluble fibrin is in agreement with a proteolytic mechanism for the action of fibrinase. He was able to show that upon washing insoluble fibrin with urea or monoiodochloroacetic acid, one could detect the disappearance of one glycine amino end group while at the same time recovering in the supernatant a dinitrophenyl peptide with a glycyl amino end group. This important work will have to be repeated with the purified components which are now available.

**Some Physiological Considerations**

We have now the opportunity to re-evaluate the physiological role of thrombin and fibrinase in the clotting process. The elastic, resistant fibrin clot required for its physiological role in the higher organism is generated by fibrinase. The role of thrombin is to form soluble fibrin, thus initiating weak interactions which align the molecule in their required geometrical pattern, but this clot is only a tenuous array of particles without the structural properties necessary for its physiological function. An hereditary abnormality has recently been described (31) in two siblings whose blood is incapable of forming urea-insoluble fibrin even when clotted in the presence of calcium and cysteine. This clotting abnormality is correlated with "hemorrhagic diathesis associated with poor wound healing" and the authors conclude that this condition is due to a deficiency of fibrinase. It seems as if the "vulcanization" of the fibrin clot by the enzyme fibrinase is indeed of considerable physiological importance.

**Summary**

A study of the solubility properties of fibrin in different solvents and at a variety of pH values has revealed the presence of three forms of fibrin.

1. "Soluble fibrin" is formed in the absence of fibrinase or calcium. It is a very weak clot which can be dispersed in buffer below pH 4.5 and above pH 9.0. At pH 8, it is soluble in 1 M urea, 0.001 M sodium dodecyl sulfate or 0.05 M cyclohexyl ammonium bromide.

2. "Stabilized fibrin" is formed in the presence of fibrinase, calcium and 0.05 M cysteine or sulfite. It can also be prepared by treating insoluble fibrin with cysteine or sulfite. The clot is much stronger than that of soluble fibrin, since it does not disperse in 8 M urea at pH 8 but readily disperses in 0.01 M sodium dodecyl sulfate or 0.01 M cyclohexyl ammonium bromide at the same pH. Upon standing, stabilized fibrin is converted to insoluble fibrin which can again be changed to stabilized fibrin by treatment with cysteine or sulfite. Stabilized fibrin formed by treatment with cysteine will not convert to insoluble fibrin if it is subsequently treated by an excess of iodoacetate. The same is true for sulfite-generated stabilized fibrin when treated with ethylene diaminetetraacetate.

3. "Insoluble fibrin" does not disperse at pH 8 even when treated with 8 M urea and 1 M sodium dodecyl sulfate at 45°. It will dissolve after prolonged exposure to urea or sodium dodecyl sulfate above pH 9.5.

The fact that insoluble fibrin is not dispersed by 8 M urea in 1 M sodium dodecyl sulfate is interpreted to mean that it is cross-linked by covalent bonds. Since insoluble fibrin can be reversibly transformed into stabilized fibrin by reagents that cleave disulfide bonds, it is concluded that the covalent bonds are disulfide bonds. Since free sulfhydryl groups can only be detected in trace amounts on the fibrin monomer, it is concluded that the formation of the intermonomer disulfide bonds is the result of a disulfide exchange reaction.

The primary action of fibrinase is the conversion of soluble fibrin into stabilized fibrin. It is suggested that fibrinase may be a specific protease which causes structural alterations in the fibrin monomers, thus bringing them into more intimate association with each other. The stabilized fibrin thus formed then undergoes spontaneous disulfide exchange reactions causing the formation of an insoluble network cross-linked by disulfide bonds.

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