THE CONVERSION OF FIBRINOGEN TO FIBRIN

XIII. DISSOLUTION OF FIBRIN AND INHIBITION OF CLOTTING BY VARIOUS NEUTRAL SALTS*

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INTRODUCTION

Fibrin, when formed from purified fibrinogen and thrombin, with the exclusion of either calcium or an unidentified serum factor (or both), can be dissolved in several rather mild reagents. Dissolution has been reported in dilute acid (1) and in moderately concentrated solutions of urea (2-5), guanidine hydrochloride (2-5), and histamine dihydrochloride (5). In urea, the dissolved particles have the same gross size and shape as the original fibrinogen (4, 5), and have been postulated to be identical with the initial reaction product of fibrinogen and thrombin, “activated” fibrinogen; with a decrease in urea concentration, they repolymerize very much as activated fibrinogen does (5). At lower concentrations, urea, guanidine, and histamine act as inhibitors which prevent the clotting of fibrinogen by thrombin, although (in the case of urea and guanidine at least) they permit a partial polymerization.

It has now been found that there is a considerably wider variety of substances which can act as solvents for fibrin and, at lower concentrations, as inhibitors of the clotting process. The present paper reports the use of several simple neutral salts for this purpose—lithium chloride and bromide, and sodium bromide and iodide; and it gives some additional information on guanidine hydrochloride.

Materials and Methods

The fibrinogen used in these experiments was refractionated from Armour bovine fraction I, preparation 128–163, by ammonium sulfate, to give the fraction designated

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as I-L in Paper X of this series (6). The clottabilities of different preparations ranged from 88 to 97 per cent. In spite of the high clottability, all these preparations contained the serum factor of Laká and Lóránd which, in the presence of 0.001 m calcium, renders fibrin insoluble in concentrated urea solutions; the fractionation procedure did not include a precipitation near pH 5, which has been shown by others to remove or destroy the serum factor (3, 7). In most of the experiments, the thrombin was a highly purified bovine preparation which was kindly given us by Dr. W. H. Seegers of Wayne University. It was dissolved in the presence of bovine plasma albumin as described elsewhere (8). In a few experiments, the thrombin was a bovine preparation (No. 51341) containing 32 units/mg., kindly given us by Dr. E. C. Loomis, Parke, Davis and Company. The salts employed as dissolution reagents were commercial products of reagent grade.

Fibrinogen solutions were prepared and filtered as described previously (8). Clots were formed at 4 gm. fibrinogen/liter, 1 unit thrombin/milliliter, pH 6.2, ionic strength 0.45 of which 0.40 was contributed by sodium chloride and 0.05 by phosphate buffer. Under these conditions, the clotting time is about 10 minutes; the clots were allowed to stand for about 20 hours before dissolution tests, to insure complete conversion of the fibrinogen to fibrin (9). In some preliminary experiments to test dissolution, 4.5 cc. of the dissolving reagent was poured on a 0.5 cc. clot. In most experiments, in which the dissolved protein was to be subjected to physical measurements, the clots were formed in cellophane tubes; and dissolution was carried out by dialysis against a large volume of the reagent, as in earlier experiments with urea (5). The slight change in protein concentration during dialysis was determined, when necessary, by weighing the tube contents. The pH of the dissolved fibrin was usually somewhat lower than that of the original fibrinogen solution, owing to the effect of the concentrated salt on the dissociation constants of buffer and protein; in some experiments, this pH change was decreased or eliminated by adding small amounts of sodium hydroxide to the reagent before the dialysis. Sedimentation measurements on the dissolved fibrin were made in the Svedberg oil turbine ultracentrifuge (through the kindness of Professor J. W. Williams). Viscosity measurements were made in Ostwald viscosimeters in which the average velocity gradient was of the order of 1000 sec.⁻¹. The viscosities and densities of the various solvents were also determined, for reducing sedimentation constants to the usual standard solvent of water at 20°C. The sedimentation constants were corrected for the temperature of the rotor cell based on calibration measurements of the melting point of diphenyl ether (10).

In control experiments to test the effect of the dissolving reagents on fibrinogen, solutions of fibrinogen contained in cellophane tubes were dialyzed first against the dissolving reagents, each for the time which had been used for fibrin dissolution, and then against the original solvent of 0.45 m sodium chloride–phosphate. In no case did precipitation occur. Sedimentation measurements were made on the final solutions. Inhibited clotting mixtures in the various reagents were made by mixing stock

1 In one experiment the phosphate was omitted and the entire ionic strength was contributed by sodium chloride. The resulting clots dissolved in urea, lithium bromide, sodium bromide, and guanidine hydrochloride under the same conditions as clots containing the usual phosphate.
solutions as in previous studies of inhibition by hexamethylene glycol (11) and urea (5). The mixtures were allowed to stand at room temperature (26 ± 1°C.), and aliquots were withdrawn at intervals for sedimentation measurements.

RESULTS

Dissolution Experiments

The minimum concentrations of lithium chloride and bromide, sodium bromide and iodide, and guanidine hydrochloride required for dissolution of fibrin under the conditions of these experiments are indicated in Table I. Sedimentation constants measured at the higher of the two reagent concentrations given in column 2, and at the pH and fibrin concentrations given in columns 3 and 4, are listed in column 5. The sedimentation constant of fibrin-exposed to each reagent for the period necessary for fibrin dissolution is also shown.

The concentrations required for dissolution appear to decrease somewhat with increasing pH, and do not differ greatly for the alkali halides listed in the table. In other qualitative experiments, however, dissolution was found not to occur in 4 M sodium chloride, 4 M lithium acetate, or 2 M lithium sulfate.

TABLE I

Minimum Dissolution Concentrations and Sedimentation Constants

<table>
<thead>
<tr>
<th>Reagent</th>
<th>Minimum dissolution concentration</th>
<th>pH</th>
<th>Fibrin concentration</th>
<th>$s_{19W}$</th>
<th>Time of treatment</th>
<th>$s_{19W}$</th>
</tr>
</thead>
<tbody>
<tr>
<td>Lithium chloride</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>2-3</td>
<td>5.5</td>
<td></td>
<td></td>
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<td></td>
<td></td>
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<tr>
<td>1.5-2</td>
<td>6.0</td>
<td></td>
<td></td>
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<td></td>
</tr>
<tr>
<td>Lithium bromide</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>1-2</td>
<td>5.7</td>
<td>4.3</td>
<td>7.6</td>
<td>2.5</td>
<td>8.3</td>
<td></td>
</tr>
<tr>
<td>&lt;2</td>
<td>5.9</td>
<td>4.8</td>
<td></td>
<td></td>
<td></td>
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<td>0.5-1</td>
<td>6.2</td>
<td>5.0</td>
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<td></td>
</tr>
<tr>
<td>Sodium bromide</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>1-2</td>
<td>5.9</td>
<td>3.4</td>
<td>6.6</td>
<td>6</td>
<td>8.6</td>
<td></td>
</tr>
<tr>
<td>0.5-1</td>
<td>6.3</td>
<td>5.0</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Sodium iodide</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>0.5-1</td>
<td>6.2</td>
<td>4.3</td>
<td>8.3</td>
<td>4</td>
<td>8.2</td>
<td></td>
</tr>
<tr>
<td>Guanidine hydrochloride</td>
<td>&lt;0.5</td>
<td>6.0</td>
<td>4.3</td>
<td>8.2†</td>
<td>6</td>
<td>8.6†</td>
</tr>
<tr>
<td>&lt;0.5</td>
<td>6.1</td>
<td>4.8</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

* Fibrinogen dialyzed against the higher concentration of reagent given in column 2, and then dialyzed against the original solvent of 0.45 M sodium chloride-phosphate.
† In 1 M guanidine hydrochloride.
All the sedimentation diagrams from which the data of Table I were derived showed single sharp peaks, indicating homogeneity. The sedimentation constants are close to the value of 8.0 (extrapolated to infinite dilution) for native fibrinogen, which has been obtained from both oil turbine and spino centrifuges when proper temperature corrections are applied (10). The similarity of the sedimentation constants indicates that the fibrin dissolution fragments have the same size and shape as fibrinogen, just as fibrin dissolved in urea has not only a sedimentation constant but also an intrinsic viscosity and a molecular weight from light scattering close to those of fibrinogen (4, 5).

The sharpness of the sedimentation peaks, together with the observation that no precipitation occurs when the reagents are brought into contact with fibrinogen and subsequently removed by dialysis, indicates that no profound intramolecular changes occur in either fibrinogen or fibrin under these conditions. One light scattering experiment was performed to test this conclusion. Fibrinogen at a concentration of 25 gm./liter was dialyzed against 2 M sodium bromide for 3 hours and then against the original sodium chloride–phosphate buffer; the intensity of scattered light at 90° was measured at various fibrinogen concentrations from 0.7 to 1.8 gm./liter on both the original and the sodium bromide–treated material, using the apparatus and procedures previously described (6). The values of $Kc/R_{900}$ (in which the symbols have their conventional significance (6)) were 2.70 and $2.44 \times 10^{-4}$, respectively, and the dissymmetries, $R_{45}/R_{90}$, were 1.10 and 1.09. The clottabilities were 94 and 90 per cent, respectively, before and after treatment. The fact that the scattering intensity (which corresponds to a slightly higher apparent molecular weight than previously reported (6), because the clarification procedures were not quite so rigorous) and clottability are only slightly affected, and the dissymmetry unchanged within 1 per cent, supports the conclusion that the concentrated salt does not disrupt the fibrinogen molecule. Further evidence for absence of changes which might be called denaturation is provided by the fact that a solution of fibrin in any of the reagents of Table I can be gelled again by dialysis against the original solvent of sodium chloride–phosphate, yielding a product closely resembling the original clot both in macroscopic appearance and under the electron microscope.  

It should be remarked, however, that more severe treatment of fibrin yields solutions in which denaturative changes have occurred, as indicated by abnormal, very broad sedimentation diagrams; correspondingly, fibrinogen under similar treatment precipitates after removal of the reagent by dialysis. Such behavior has been found for urea at concentrations above 4 M or for prolonged times of exposure (5, 12), and in the present study for 1.75 M guanidine hydrochloride, as well as for 1 M sodium thiocyanate.

3 Electron microscope observations were made by Dr. P. Kaesberg and will be reported elsewhere.
Fibrin clots formed in the presence of 0.001 M calcium chloride were not dissolved by any of the reagents at the concentrations given in Table I. Since the fibrinogen contained the serum factor of Laki and Lórán, as shown by dissolution tests in urea, it is evident that the stronger bonds formed in the presence of calcium and serum factor (as demonstrated by stress relaxation measurements (8)) affect solubility in the above neutral salts in the same way as solubility in urea.

**Inhibition Experiments**

The minimum inhibiting concentrations for lithium chloride and bromide and sodium bromide and iodide were determined by the procedure described in Paper IV of this series (13); the choice of stock solutions was such that the total ionic strength was slightly higher than the value of 0.45 used in most previous work. The data are given in Table II, together with a few earlier results (13) for comparison. All these salts are quite effective inhibitors and of roughly equal effectiveness. Sodium chloride, by contrast, does not inhibit at a concentration of 2 M, above which salting out of fibrinogen occurs.

<table>
<thead>
<tr>
<th>Reagent</th>
<th>pH</th>
<th>Total ionic strength</th>
<th>Minimum inhibiting concentration M</th>
</tr>
</thead>
<tbody>
<tr>
<td>Lithium chloride</td>
<td>5.9</td>
<td>0.60-0.72</td>
<td>0.20-0.35</td>
</tr>
<tr>
<td>Lithium bromide</td>
<td>6.0</td>
<td>0.56-0.62</td>
<td>0.22-0.34</td>
</tr>
<tr>
<td>Sodium bromide</td>
<td>6.0</td>
<td>0.62-0.68</td>
<td>0.34-0.45</td>
</tr>
<tr>
<td>Sodium iodide</td>
<td>6.1</td>
<td>0.56-0.62</td>
<td>0.22-0.34</td>
</tr>
<tr>
<td>Guanidine hydrochloride*</td>
<td>6.2</td>
<td>0.45</td>
<td>0.24-0.35</td>
</tr>
<tr>
<td>Sodium thiocyanate</td>
<td>6.2</td>
<td>0.45</td>
<td>0.36-0.42</td>
</tr>
<tr>
<td>Urea*</td>
<td>6.2</td>
<td>0.45</td>
<td></td>
</tr>
</tbody>
</table>

* From reference 13.

Inhibited clotting systems were made up in lithium bromide at several concentrations somewhat higher than the minimum inhibiting value, the initial concentration of fibrinogen being in all cases 4.0 gm./liter. Sedimentation studies on aliquots withdrawn at intervals revealed the same behavior as previously found in clotting systems inhibited by hexamethylene glycol (11) and urea (5). The sedimentation diagram showed at first one sharp peak with the sedimentation constant of fibrinogen; after a longer reaction time, a faster peak appeared; with increasing time of reaction, the area of the second peak increased at the expense of the first, while the two sedimentation constants
remained unchanged. In some cases aliquots were diluted with a solvent of the same pH, ionic strength, and inhibitor concentration as the original system, sedimentation measurements being made within an hour after dilution.

The sedimentation constants are given in Table III. It is clear that the two components are very similar to those observed in hexamethylene glycol and urea; the sedimentation constants extrapolated to infinite dilution for the fast and slow components, respectively, are 8.0 and 23 in the glycol and 7.8 and 23 in urea. It may be concluded that the course of arrested polymerization is much the same in lithium bromide as in the other two inhibitors. The fast peak appears sooner in 0.40 M than in 0.56 M lithium bromide.

An isolated determination on an inhibited clotting system in 0.34 M sodium iodide, 4.0 gm. fibrinogen/liter, pH 6.05, ionic strength 0.45, reaction time 41 hours, showed the same two components with sedimentation constants of 8.0 and 19.8 respectively.

* The earlier values of 8.5 and 24.5 for the hexamethylene glycol system have been revised by applying a temperature correction for the oil turbine ultracentrifuge, using the observed ratio of extrapolated sedimentation constants and assuming that the true value for the slow component is equal to that of fibrinogen (10).
DISCUSSION

The following steps have been identified in the conversion of fibrinogen to fibrin (5, 14):

$$\begin{align*}
F & \xrightarrow{T} f \xleftarrow{2} f_{n} \xrightarrow{3} \text{fibrin}
\end{align*}$$

in which $F$ is fibrinogen, $T$ is thrombin, and $f$ is activated fibrinogen; $f_{n}$ represents a series of intermediate polymers which appear as the fast peak in the ultracentrifuge in inhibited systems (5, 11) and under various other conditions before the moment of clotting (15). The salts described in this paper share with urea and hexamethylene glycol the faculty of inhibiting step 3. These different inhibitors would be expected to interact with the protein in different ways; the glycol blocking largely non-polar groups (13, 16) and the urea largely polar groups, while the ions of lithium, bromide, etc., might exert their dispersive effect by adsorption with a consequent change in the pattern of electric charges (17). This suggests that the forces leading to association of the $f_{n}$ units involve all three types of groups, although the non-polar groups are probably of particular importance (15).

The salts, like urea, dissolve fibrin to form fragments which resemble fibrinogen in size and shape and are probably identical with $f$. It has not yet been possible to choose conditions which will dissolve fibrin to form $f_{n}$ directly; thus, step 3 does not appear to be easily reversible. In other words, the dissolution agents act by detaching $f$ units from the clot singly. Indeed, it might be kinetically a very slow process to detach a unit of such huge size as $f_{n}$ (cf. the comments of Spurlin (18) on the solubility and rate of solution of high polymer molecules). It has been postulated (14, 15) that the forces holding the $f$ units together involve primarily electrostatic and polar groups, so it is reasonable that urea and the salts described here should be effective in disrupting these, whereas dissolution has not been achieved in hexamethylene glycol.

It may be added that salts such as those described here also depolymerize actomyosin (19) and actin (20), and at higher concentrations disrupt the configuration of corpuscular proteins (21). On fibrin as on the other proteins their order of effectiveness follows the familiar Hofmeister or lyotropic series. While it was recently believed that the polymerization of fibrinogen involved primary chemical bonds (22), and even the solubility of fibrin in urea did not preclude such a possibility (8), it now seems very likely that only electrostatic forces and secondary bonds are involved.

We are indebted to Mr. Edwin M. Hanson for performing the sedimentation experiments, and to Miss Carol Braatz for technical assistance.
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SUMMARY

1. Fibrin clots prepared in the absence of calcium can be dissolved in solutions of lithium chloride and bromide and sodium bromide and iodide, as well as of guanidine hydrochloride and urea. These salts do not denature fibrinogen under the same conditions of concentration, temperature, and time. Sedimentation experiments on the fibrin solutions show in each case a single sharp peak with a sedimentation constant close to that of fibrinogen.

2. At lower concentrations, these salts inhibit the clotting of fibrinogen by thrombin, but in the case of lithium bromide and sodium iodide, at least, allow an intermediate polymer to accumulate whose sedimentation constant is close to that of the polymer observed in systems inhibited by hexamethylene glycol or urea.

BIBLIOGRAPHY