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Properties of Fibrin Dissolved in Urea Solutions

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Several investigators of the late 19th century reported the solubility of fibrin clots in urea solutions.[1,2] Wöhlisch and his co-workers, however, could not confirm this finding;[3,4]; they found the fibrin insoluble at room temperature in concentrated urea solutions, though when heated to 100° C it dissolved easily.

The problem has considerable importance, because the protein gels and coagula — heat-denatured egg albumin, tobacco mosaic virus, *etc.* —, where the particles are bound by weak secondary forces, are all soluble in urea solutions. Insolubility may thus be an indicator of stronger primary chemical bonds between the particles.

It seems that the negative results of Wöhlisch *et al.* have been accepted without further proof by most of the authors. Indeed, Ferry in his recent rewiev on protein gels[5] considered the insolubility of fibrin in urea solutions as an argument in favor of the theory of polymerization of fibrinogen through co-valent bonds to form the fibrin gel.

Loránd[6], reinvestigating the problem, found fibrin resulting from the action of thrombin upon pure fibrinogen to be readily soluble in concentrated urea solutions. On the contrary, fibrinogen clotted by thrombin in the presence of serum and calcium ions, as well as the clot obtained by the recalcination of oxalated plasma, was insoluble. It is possible that the failure to dissolve fibrin reported by the authors cited above was due to contamination of their fibrinogen preparation with the serum factor and calcium. Wöhlisch and his associates used fibrinogen prepared by salting out with NaCl. It is known that the NaCl precipitation of fibrinogen is less sharp than that by $(NH_4)_2SO_4$[7], thus favoring the precipitation of other plasma proteins with the fibrinogen.

In the experiments which will be described, the solubility of fibrin in concentrated urea solutions was definitely confirmed and some of the properties of the solutions of fibrin in urea investigated.

EXPERIMENTAL

Materials

Fibrinogen was prepared from oxalated cattle plasma by salting out with $(NH_4)_2 SO_4$ at 0.24 saturation. The details of the method have already been described [8].

The reagents employed were of analytical purity. Impure urea preparations gave unsatisfactory results, the dissolution of fibrin being very slow and incomplete when they were used.

Fibrin solutions were prepared in the following way: 50 ml of solution containing 20 mg fibrinogen per milliliter were clotted by adding 1 ml of thrombin solution. Hoffmann la Roche's commercial thrombin preparation was used in the concentration of 2 mg per milliliter. After two hours, the clot was dissolved at 0° C by adding 15 g of solid urea. The dissolution was complete in about one hour. The solution was then filled up to 100 ml with distilled water. The water must be added cautiously, under continuous stirring: otherwise, the fibrin precipitates. This stock solution was stored at 0° C; and when used, was diluted with distilled water and 60 per cent urea solution for the preparation of solutions of different composition.

Methods

The viscosity of the solution was determined with an Ostwald viscosimeter at 0° C. At this temperature the distilled water had an outflow time of 23 seconds. Owing to the thixotropy of the solutions, the outflow time decreased in successive repetitions of the determination. A nearly constant value was reached finally, sometimes after 8 to 10 determinations, and this was considered as corresponding to the viscosity of the solution. The viscosities were calculated relative to that of distilled water at the same temperature.

The double refraction of flow was investigated qualitatively with the apparatus described by Gerendás [9].

Results

A 'coarse type' gel dissolves more easily in urea solutions than a 'fine type' gel. The coarse and fine qualifications of fibrin gels are in accord with the definitions of Ferry and Morrison [10]. More rapid dissolution was observed if the urea was added immediately after gelation than if it was added later. The following experiment serves to illustrate this: Samples of 10 ml of a solution containing 10 mg fibrinogen per milliliter were clotted by adding 1 ml of thrombin solution. In about 30 seconds the solution clotted. The clot was then broken with a glass rod, 3 g of solid urea were added, and the mixture continuously stirred. If the urea was added 30 seconds after the clotting, the dissolution was complete in about 2 minutes. After 5 minutes of waiting

it took 25 to 30 minutes, while after 15 minutes it required 40 to 50 minutes. It is apparent that the gelation of the solution does not mean the completeness of the reaction. The reaction continues for a long time, as shown by the growing resistance against the dissolving action of urea. Similarly Ferry and Morrison [19] showed a continued increase of the tensile strength and opacity of fibrin clots for a long time after the moment of gelation.

A. Reconstitution of the fibrin gel after removal of the urea. When the urea from a fibrin solution is dialysed out, the cellophane tube will be completely filled out by a fibrin gel, which has all the characteristics of the original gel. The pH of the fibrin solution in urea in the experiment here reported was brought to approximately 6.5. The solution was then dialysed against a $M/10$ phosphate buffer of the same pH. The result was an opaque coarse type gel. On the contrary, when a slightly alkaline fibrin solution was dialysed against $M/10$ phosphate buffer of pH 8, a fine type gel was formed.

The quality of the resulting gel is determined only by the pH during the dialysis and not by the type of the original fibrin gel. A fine type gel can be converted in this way into a coarse one and vice versa; and this transformation can be repeated at will.

In the interpretation of these findings the question arises wheter the urea reversed completely the fibrinogen-fibrin transformation or whether it merely split the gel into modified fibrinogen particles. The thrombin which served for the clotting of fibrinogen is present in the fibrin solution and its activity can be demonstrated after the urea has been removed. If the first assumption is the correct one, the reclotting would occur as a result of the action of thrombin on the reconstituted fibrinogen molecules; while if the second is the explanation, then the preformed particles would gelify without the intervention of thrombin with removal of the urea. It is easy to verify the latter assumption. Indeed a fibrin solution in 30 per cent urea gelifies instantaneously when it is diluted with four volumes of distilled water. The rapidity of the reaction demonstrates the presence of preformed particles.

B. Effect of pH and urea concentration on the gelation of fibrin solutions. The stock fibrin solution was mixed with suitable quantities of distilled water and 60 per cent urea solution in order to have after the addition of 1/10 volume of buffer a final protein cencentration of 5 mg per milliliter and urea concentrations of, respectively, 7.5, 10, 15, 20, and 30 per cent. To cover the pH range from 5.9 to 12.0 primary-secondary phosphate, borate, and secondary-tertiary phosphate buffers were used in $M/1$ concentration. The mixtures were kept at 0° C for 24 hours, then their viscosity determined at the same temperature.

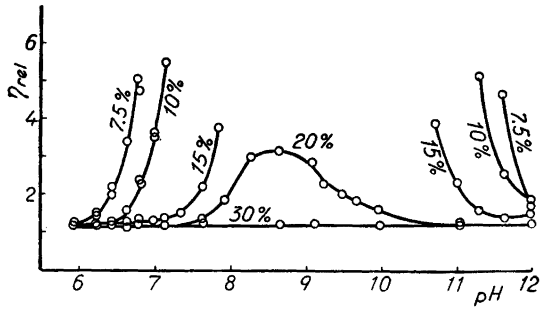

Fig. 1. The viscosity of fibrin dissolved in urea solutions at different urea concentrations and pH. 5 mg fibrin per milliliter, 0° C.

The results are summarised in Fig. 1. Over the entire pH range studied the viscosity of fibrin solutions in 30 per cent urea did not differ from that of fibrinogen solutions in similar conditions. These solutions had Newtonian viscosity and showed no double refraction of flow.

At 20 per cent urea concentration the viscosity rose at pH values more alkaline than 7.6 and reached a maximum at pH 8.6; it then decreased again to the original value. At still lower urea concentrations the increase of viscosity started at a pH the more acidic, the lower was the urea concentration. At 15 per cent urea concentration and below, the increase of viscosity finally led to a gelation of the solution. At considerably alkaline pH values, the solutions remained in the sol state. As it can be seen in Fig. 1, the pH range of gelation was widened by lowering the urea concentration.

In the regions of rising viscosity, the solutions became more and more thixotropic. The phenomenon was more accentuated in strong alkaline solutions on the redissolution side and, parallel with this, the viscosity showed a strongly anomalous character. The specific viscosity was no longer constant but rose with increase of the fibrin concentration; and, at the same time, a pronounced double refraction of flow appeared. With the highly viscous solution shaking the tube containing the solution was sufficient to obtain a strong and persistant double refraction. It is obvious that in this region what was determined was only an apparent viscosity resulting from the true viscosity and the structural rigidity of the solutions.

After the addition of buffer the viscosity did not instantaneously reach an end value. At high urea concentrations and at pH values far from the optimal value for gelation, the process requires several hours. This was the reason why the determinations were made after 24 hours, a time sufficient

to ensure stabilization of the viscosity. The increase of viscosity, however, was very rapid and gelation occured practically immediately after the addition of buffer at low urea concentrations and at the optimal pH for gelation *i. e.*, at pH 8.6.

DISCUSSION

As it has been shown, the fibrin clot resulting from pure fibrinogen readily dissolves in concentrated urea solutions. Neither the -S-S-, nor the -NH-CO-, nor any other co-valent bond immaginable between the chemical groups present in proteins can be broken by urea. This seems to justify the conclusion that by the formation of fibrin clots no co-valent bonds take part.

Urea affects the electrostatic forces between the charged groups by increasing the dielectric constant of the medium. Although this effect may have some importance, it is far from being the cause of the dissolution of fibrin. The dielectric increment of urea is not very high, dipolar ions like glycine for example, having a much greater effect on the dielectric constant. The dielectric constant of a 15 per cent urea solution is 93.5, while that of a 2 molar glycine solution is 125. A fibrin solution in 15 per cent urea, when diluted with 2 molar glycine solution, gelified exactly at the same degree of dilution as when it was diluted with distilled water, in spite of the fact that the dielectric constant of the medium was still further increased by dilution with the glycine solution.

Most probably the urea affects the hydrogen bonds of the protein molecules [11, 12]. Hydrogen bonds are formed between the —NH— and —CO— groups of adjacent polypeptid chains. The structural resemblance of urea and these groups makes possible a competition of the urea molecules for the same places as those by which the polypeptid chains are bound together. If this mechanism is really the cause of the dislocation of the protein particles, the hydrogen bonds must play a considerable role in the building of the fibrin gel. Mommaerts [13] has already arrived at a similar conclusion on the basis of the effect of urea on the clotting of fibrinogen.

Laki and Mommaerts [14] have shown that the gelation of fibrin is possible only above a certain pH. Fibrinogen incubated wirh thrombin at a pH below this value is transformed into modified particles, which gelify instantaneously if the pH is brought to a proper value. Lyons [15] showed the same phenomenon with fibrinogen incubated with thrombin at a pH more alkaline than 10. There is a pH zone where the fibrin gel can be formed. The dissolution of the gel occurs only at much lower and much higher pH values than the limits of this zone — a fact described by several authors [16, 17, 18, 19]. It is apparent from the experiments described in this paper that urea narrows

the pH stability range of the fibrin gel. At 20 per cent urea concentration, of the ability to form gels only some rise of viscosity was left; while at 30 per cent urea concentration, this capacity was completely anihilated. The role of the pH in the gelation process is still not clear; it is apparent only that the dissolving effect of urea is counterbalanced to some extent by the rise of pH in the zone more acidic than 8.6, and it is favored by the rise of pH in the zone more alkaline than 8.6.

The viscosimetric behavior of fibrin solutions in 30 per cent urea, as well as of those in lower urea concentrations in certain pH ranges, indicates that the particles, in respect to their shape and size, are identical with those of fibrinogen. The thrombin does not alter the shape and size of the fibrinogen molecules; it only modifies some of their physicochemical properties, increasing their tendency to form gels. Laki and Mommaerts [14] arrived at the same conclusion in an investigation of fibrinogen solutions which were incubated with thrombin at acidic pH.

Increase of viscosity and gelation were observed in the urea denaturation of fibrinogen. [20]. The possibility of denaturation must, therefore, be excluded in the experiments described in this paper. As reported in a previous paper [21], at 20 per cent and lower urea concentrations the urea has no perceptible denaturing action on fibrinogen and fibrin at 0° C in the pH range investigated. The viscosity changes, therefore, which were observable in fibrin solutions, could not have been caused by denaturation. The viscosity of the solutions once arrived at a constant value, did not change for a period as long as two weeks, and the viscosity of fibrinogen solutions under exactly the same conditions did not show any increase in four days. In the presence of 30 per cent urea the fibrin was more or less denatured, but this seemed to have no influence on the viscosity of the fibrin solutions. It has already been reported [20] that the denaturation of fibrinogen in 30 per cent urea also leaves unaffected the viscosity of the solution, fibrinogen and fibrin being similar in this respect.

SUMMARY

1. Fibrin clots are soluble in concentrated urea solutions.

2. When urea is dialysed out, the clot is reconstituted. The pH of the fibrin solution during the dialysis decides whether a coarse or a fine type gel will be formed.

3. The viscosity of fibrin in 30 per cent urea solution is normal and equal to that of fibrinogen in similar conditions. The pH has no influence on the viscosity. At 20 per cent urea concentration the viscosity is increased by increasing the pH up to 8.6. Further increase of pH again decreases the

viscosity. At still lower urea concentrations, the increase of pH leads to gelification; and at more alkaline pH to the redissolution of the gel.

4. The results are discussed.

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