ACTA CHEMICA SCANDINAVICA 4 (1950) 351 – 358

Electrophoretic Investigation of Fibrin and Fibrinogen Dissolved in Urea Solutions

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The solubility of fibrin in urea solutions makes it possible to investigate the electrophoretic mobility of this protein. Such fibrin solutions contain modified fibrinogen molecules, which, after removal of the urea reform the original fibrin gel [1,2]. Laki and Mommaerts have shown [3] that the clotting of fibrinogen occurs in two steps. The first one is the chemical change in the fibrinogen molecule brought about by the thrombin and the second, the transformation of the system into a gel. In urea solutions this second physicochemical process is reversed and it is very probable that the particles in solution are identical with those formed in the first phase of clotting.

If the action of thrombin involves some of the ionizing groups of fibrinogen, a study of the differences in electrophoretic mobilities between fibrinogen and fibrin may give some information about the nature of the process of clotting.

EXPERIMENTAL

Materials

Fibrinogen was prepared from oxalated bovine plasma as described in a previous paper [4]. The solutions contained 25 to 35 mg fibrinogen per milliliter and their purity ranged from 90 to 95 per cent.

The commercial thrombin preparation of Hoffmann-La Roche, Basel was used.

All the other substances used were reagents of analytical purity.

Methods

The fibrinogen solutions were diluted to contain 25 mg of fibrinogen per milliliter. Ten milliliters of fibrinogen solution were clotted by adding 1 ml of thrombin. The concentration of the thrombin solution was 1 mg per milliliter. Clotting occured in about one minute. The clot was allowed to stand at

room temperature for an hour for the completion of the thrombin action; was then broken with a glass rod, and dissolved at 0° C by adding 3 g of urea. The dissolution was complete in about an hour, after which 7 ml of urea-buffer solution were added. The composition of urea-buffer solution varied from experiment to experiment, as indicated later in text.

The fibrinogen solutions were prepared in exactly the same manner, with the exception that instead of thrombin 1 ml of physiological saline solution was added. Both solutions were dialysed for 24 hours against 2000 ml urea-buffer solution. The tube containing the bags and the buffer was rocked at + 4° C. After dialysis the protein solutions were diluted to 20 ml with the urea-buffer solution.

Electrophoretic mobility determinations were made with the Tiselius apparatus. The potential gradient varied in the different experiments between 5 and 10 volts/cm (voltage between 200 and 350 volts). In general the migration was followed for four hours, and readings were made every half hour. The migration per hour was determined graphically, and the arithmetical mean of the migrations of the ascending and descending boundaries was used for the calculation of the mobility.

The difference between the mobilities of fibrinogen and fibrin is small. To exclude the possibility that the observed differences were due to experimental error, a control run was made with a mixture of fibrinogen and fibrin at each pH studied. The solutions of the two proteins were prepared as described above. In order to minimize the possible action of thrombin on fibrinogen during the dialysis, the two solutions were mixed just before filling the electrophoresis apparatus, although the high urea concentration employed certainly inhibits completely the action of thrombin [5]. In these experiments the duration of the electrophoresis was 10 to 12 hours, and a lower potential gradient was used, about 5 volts/cm. The descending boundary was mostly blurred; in these experiments, therefore, only the migration of the ascending boundary was followed. The observed components were identified by means of the mobilities determined by runs with a single component.

The conductivity of the solutions was determined in the water bath of the electrophoresis assembly to an accuracy of $\pm$ 1 ohm. The temperature of the water bath was + 0.1° C. For the calculation of the potential gradient, the mean value of the conductivity of the protein and of the urea-buffer solution was used. When only the migration of the ascending boundary was followed, the conductivity of the urea-buffer solution served for the calculation of the potential gradient, as recommended by Svensson [6]. The pH of the solutions was controlled with the glass electrode.

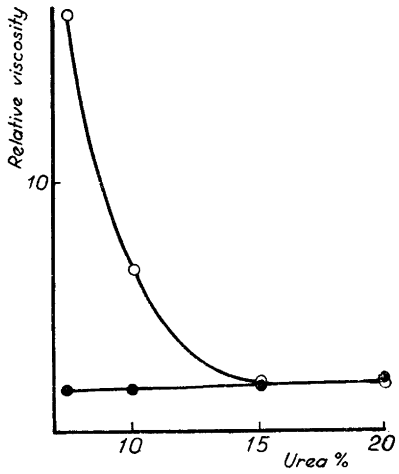

Fig. 1. The relative viscosity of fibrinogen and fibrin in urea solutions of different concentrations. 10 mg, protein per milliliter, 0.4 ionic strength, pH 6.8.

○ *fibrinogen*
● *fibrin*

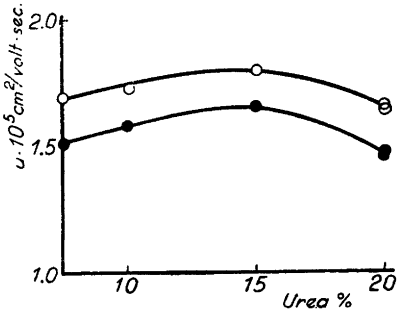

Fig. 2. The electrophoretic mobility of fibrinogen and fibrin in urea solutions of different concentrations. 10 mg. protein per milliliter, 0.4 ionic strength, pH 6.8.

○ *fibrinogen*
● *fibrin*

An Ostwald viscosimeter served for the viscosity determinations at 0° C. The reported viscosities are relative to the viscosity of distilled water at 0° C.

Results

In previous investigations it was found that between pH 5.9 and 11.0 urea solutions of 30 per cent concentration have a denaturing action on fibrinogen, while 20 per cent urea has not [7]. Therefore, in this pH range, the experiments were performed in 20 per cent urea solutions. Below pH 5.9 urea causes denaturation also in 20 per cent concentration. At pH 5.3 and 4.4, therefore, solutions of 10 per cent urea were used, which do not denature fibrinogen even at this acidic pH.

In 20 per cent urea solutions in the pH range from 7.6 to 11.0 the viscosities of fibrin solutions are higher than those of fibrinogen solutions of the same concentration. The difference is maximal at pH 8.6, where, at the protein and salt concentration used, the fibrin solution has a relative viscosity of 7.13; the fibrinogen, only 1.46. In spite of the fact that in 30 per cent urea solution these viscosity differences are absent, for the reason mentioned above, higher urea concentrations than 20 per cent were not used.

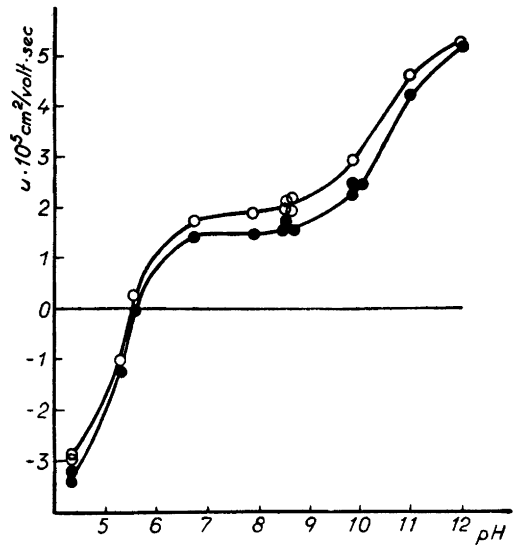

Fig. 3. The electrophoretic mobility of fibrinogen and fibrin plotted against pH. 10 mg protein per milliliter, 0.1 ionic strength, 10 respectively, 20 per cent urea.
○ *fibrinogen.*
● *fibrin.*

A. Influence of the urea concentration on the mobility of fibrinogen and fibrin. To determine to what extent the viscosity influences the electrophoretic mobilities, experiments were performed in which, at the same pH, ionic strength, and protein concentration, the concentration of urea was varied. With urea concentrations above 15 per cent at pH 6.8 in phosphate buffer of 0.4 ionic strength, the viscosities of fibrin and fibrinogen are equal. Lowering of the urea concentration from 15 to 7.5 per cent increases to a considerable degree the viscosity of fibrin solutions, whereas the viscosity of fibrinogen solutions is not affected by this change in the urea concentration.

The viscosity of the two proteins is plotted against the urea concentration in Fig. 1 and the electrophoretic mobilities of the same solutions are shown in Fig. 2. The mobility curves of fibrin and fibrinogen are parallel, in spite of the fact that the viscosity curves are strongly divergent. The mobility of fibrin in 7.5 per cent urea is the same as in 20 per cent urea, whereas the viscosity is ten times higher in the first case than in the second — 16.50 and 1.62. Both of the proteins possess a maximum mobility in 15 per cent urea solution.

B. Influence of ionic strength on the mobility of fibrinogen and fibrin in urea solutions. In the presence of 20 per cent urea, the ionic strength seems to have but little influence on the electrophoretic mobility of the two proteins. At pH 6.8 the ionic strength was increased from 0.1 to 0.4 by increasing the concentration of the phosphate buffer. The mobilities were the same over this

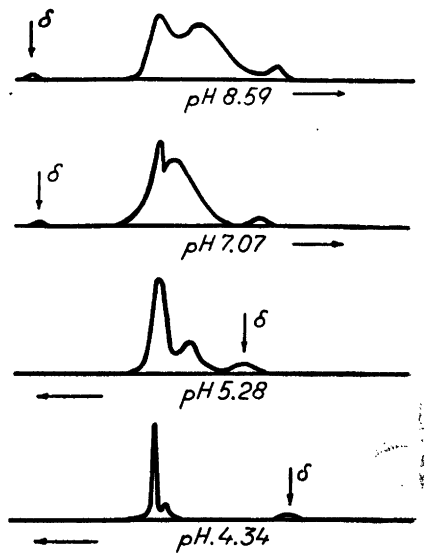

Fig. 4. Electrophoretic patterns of mixtures of fibrinogen and fibrin, (1 : 1), 10 mg. protein per milliliter, 0.1 ionic strength, 20, respectively 10 per cent urea, pH 8.59, 7.07, 5.28, 4.34; potential gradient 5 volts/ cm, duration of the experiment 12 hours.

ionic strength range. Constant mobilities were obtained also when, at pH 8.6 and 20 per cent urea concentration, the ionic strength was increased from 0.05 to 0.2 by increasing the concentration of the borate buffer, and even when it was increased up to 0.55 by adding NaCl to the buffer.

C. *Influence of pH on the mobility of fibrinogen and fibrin.* In all the determinations performed in order to determine the pH dependence of mobility the ionic strength of the buffers was 0.1. Acetate buffers were used from pH 4.4 to 5.3, phosphate buffers from 5.6 to 7.9, borate buffers from 8.6 to 10.0, and secondary-tertiary phosphate buffers from 11.0 to 12.0. The mobility of fibrinogen and fibrin as a function of pH is plotted in Fig. 3. It can be seen that the mobility curve of fibrin is always below that of fibrinogen; *i. e.*, in the region alkaline to the isoelectric point the fibrinogen is the faster, whereas in the region acidic to the isoelectric point the fibrin is the more rapid component. Between pH 5.3 and 6.8 the two curves are very close together, but at a more basic pH they separate, rejoining again at very alkaline values.

The mobilities determined in mixtures of fibrinogen and fibrin were the same as those determined in runs with a single component. The two components separate slowly, owing to the small difference in their mobility. In Fig. 4 can be seen the electrophoretic pattern of an 1 : 1 mixture of fibrinogen and fibrin at four different pH. At pH 4.4 and 5.3, the faster component is fibrin, whereas at pH 7.8 and 8.6 the fibrinogen is the faster component. In both upper patterns there can be seen a very small component faster than

fibrinogen and fibrin — a component which probably corresponds to the globulins co-precipitated with the fibrinogen.

In all the runs performed the fibrin has shown a much sharper boundary than the fibrinogen — a finding pictured in the patterns of Fig. 4, where the steeper, higher peaks correspond to fibrin and the broader to fibrinogen.

The isoelectric points of the two proteins in 20 per cent urea are very close together. Judging from the mobility curves, the fibrinogen has an isoelectric point of 5.5, while that of fibrin is somewhat more alkaline, at pH 5.6.

DISCUSSION

The difference in the electrophoretic mobility of fibrinogen and fibrin may be due either to a difference in their net charge, or to differences in the shape and size of the particles, or to both of these factors. Over a distinct pH range the high viscosity of fibrin in urea solutions, as compared with that of fibrinogen in similar conditions, clearly indicates a structural difference between these two protein solutions. The high viscosity of fibrin solutions in urea may be accounted for by (a) the structural rigidity of the solution, caused by large forces between the particles, and by (b) the polymerisation of the molecules in long rod-shaped particles.

In interpreting the electrophoretic results, the question should be answered whether or not the structure of the solution or the shape and size of the particles has an effect upon the electrophoretic mobility.

The structural rigidity of the medium seems to have little or no influence in the cases reported in the literature. It has been shown that the mobility of zinc particles and air bubles remained constant in a 1 per cent gelatin solution during the sol-gel transformation [8]. The movement of the zinc particles is only an indicator of the movement of the gelatin micelles themselves; thus, the setting of the sol seems to have no influence on the mobility of the gelatin micelles. The same is true for the electrophoretic migration of red blood corpuscles in a gelatin sol or gel. There are evidences that this unexpected result is caused by the thixotropy of gels. The gel structure is destroyed before the particle, and then afterwards reconstituted. With more concentrated or non-thixotropic gels this transformation cannot occur, and the particles are immobile [9].

The shape and size of microscopic particles has no influence on the electrophoretic mobility. Abramson and Michaelis [10] demonstrated this with particles coated with gelatin — particles varying widely in size, shape, and orientation. It seems that the only determining factor is the surface charge density of the particles, and the same seems to be true also in molecular dimensions. Astrup

and Brodersen [11] found that cellulose polysulfonic acids have the same mobility, irrespective of their degree of polymerisation. The aggregation of heat-denatured serum albumin particles had also no influence on their electorphoretic mobility. The material was polydisperse in the ultracentrifuge, as shown by Pedersen [12], but was perfectly homogeneous in electrophoretic experiments. It is apparent that the degree of polymerisation is irrelevant if the charge per unit of the polymer is the same.

Stenhagen and Teorell [13] investigated the concentration-mobility relation of thymonucleic acid. By increasing concentration the electrophoretic mobility was found constant, in spite of the remarkable increase of viscosity caused by the tendency of the long rod-shaped thymonucleic acid molecules to give thixotropic gels.

In the experiments described in section A. of this paper, the viscosity of fibrin solutions was enormously increased by decreasing the urea concentration; in spite of this, the electrophoretic mobility changed but little. The small change may be explained by the increase of the true viscosity of the solvent and by the change of the dielectric constant. That the changes are due to the difference in the composition of the solvent, and not to the increase of viscosity, is shown by the fact that the mobility of fibrinogen, whose viscosity is not altered by the lowering of the urea eoncentration, is perfectly parallel with that of fibrin.

There are no viscosity differences between fibrinogen and fibrin below pH 7.5 in 20 per cent urea solutions; and, in spite of this, the mobility differences are about the same as at pH 8.6, where the viscosity of fibrin is approximately five times higher than that of fibrinogen.

The conclusion seems to be justified that the electrophoretic mobility differences found between fibrinogen and fibrin in 20 per cent urea solutions are not due to morphological differences, but to differences in the net charge of the two proteins. The net charge of the fibrin particles is smaller above the isoelectric point and larger below this point than that of fibrinogen.

The isoelectric point of fibrinogen as determined in the experiments reported is in good agreement with the values found by other authors in the absence of urea. Stenhagen [14] found 5.4 as the isoelectric point of fibrinogen by the electrophoresis of human plasma. Seegers *et al.* [15] obtained 5.5 by the electrophoresis of isolated pure fibrinogen. It seems that the urea does not shift the isoelectric point.

The mobility of fibrinogen seems also to be unaffected by the presence of 20 per cent urea. Armstrong, Budka, and Morrison [16] gave $1.78—2.3 \times 10^{5}$ as the mobility of fibrinogen isolated by the alcohol fractionation method, when investigated at pH 8.6 in diethylbarbiturate buffer of 0.1 ionic strength.

Avery and Munro [17] found, in the same conditions, 2.18—2.37 $\times$ 10^5 with the fibrinogen prepared by salting out. Dole [18], using the same buffer, reported 2.14 $\pm$ 0.25 $\times$ 10^5 as the mobility of fibrinogen in unfractionated human plasma. The value of 1.95—2.08 $\times$ 10^5 determined in our experiments in borate buffer of pH 8.6 and 0.1 ionic strength is only slightly lower than the above values, and demonstrates that at least at this pH value the urea does not affect appreciably the electrophoretic mobility of fibrinogen.

SUMMARY

1. The viscosity differences between fibrin and fibrinogen in 20 per cent urea solutions are not the cause of the electrophoretic mobility differences.

2. The ionic strength has little influence on the electrophoretic mobility of fibrinogen and fibrin in the presence of 20 per cent urea.

3. Fibrin has a lower mobility above and a higher below its isoelectric point than fibrinogen.

4. The isoelectric point of fibrinogen in solutions of 20 per cent urea lies at pH 5.5; that of fibrin, at pH 5.6.

The author wishes to acknowledge his indebtedness to Professor Hugo Theorell for inviting him to work in his laboratories, and for his very kind interest throughout the work.

He also wishes to express his thanks for a grant from The Institute for Muscle Research, New York.

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Received September 9, 1949.