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# The Urea Denaturation of Fibrinogen

## II. Physicochemical Changes

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The physicochemical properties of a number of proteins are considerably altered by urea and other related substances. Fibrinogen exposed to urea undergoes remarkable changes. In the presence of 30 per cent urea the non-Newtonian character of the viscous flow of fibrinogen solutions is greatly reduced [1] and the double refraction of flow disappears [2]. Meissner and Wöhlisch [3] showed that the opacity and the ability of fibrinogen solutions to form threads (Spinnbarkeit) is greatly diminished by urea. These effects follow almost immediately the addition of urea, and are readily interpreted by supposing a fragmentation of the rod-shaped fibrinogen molecules. The dissociating effect of urea is well known also in the case of other proteins, such as hemoglobin [4,5], myosin [6,7], tobacco mosaic virus [8,9], *etc.* In some of the cases this dissociation is reversible.

Prolonged action of concentrated urea solutions brings about changes connected with the denaturation of the molecules. Hopkins [10] found that the viscosity of egg albumin and serum protein solutions was increased and that finally gelation occured in the presence of approximately 60 per cent urea. Neurath and his co-workers [11,13] investigated in more details the viscosity and diffusibility changes of egg albumin and of serum proteins in urea solutions. They concluded that the increase of viscosity and the decrease of diffusibility may be explained by an unfolding of the protein molecules during the process of denaturation. An increase of the viscosity and opacity of fibrinogen solutions in urea was found earlier by Diebold and Jühling [14]. They correctly interpreted these findings as signs of denaturation.

In the course of investigations on the kinetics of the urea denaturation of fibrinogen, reported in section I of this paper, similar observations were made. The main object of the experiments to be described in the present section is

the investigation of these physicochemical changes and their correlation with the denaturation process.

## EXPERIMENTAL

### Materials

Fibrinogen was prepared by the method described in the first section of this paper. Throughout this work solutions containing 30 mg fibrinogen per milliliter were used.

All the other reagents used were of analytical purity.

### Methods

The amount of denatured fibrinogen was estimated by precipitation, the urea-fibrinogen mixture being diluted with 10 volumes of $M/10$ phosphate buffer of pH 5.9. The details of the method have already been given in the preceding paper.

The change in opacity of urea-fibrinogen solutions was determined in a Beckman spectrophotometer at two wave length: 350 and 600 m$\mu$. Twenty milliliters of fibrinogen solution were mixed with 5 ml of $M/1$ phosphate buffer of pH 7; were kept in a water bath until the desired temperature was reached; and were then mixed with 25 ml urea solution at the same temperature. The mixture stood in the constant temperature water bath. From time to time an aliquot was withdrawn, and its transmission coefficient determined immediately. When it became difficult to handle the solution owing to its high viscosity, it was left in the cell of the spectrophotometer, and placed with this in the water bath.

Viscosity determinations were made with an Ostwald viscosimeter, which had an outflow time of 32.9 seconds with distilled water at 35° C. The viscosimeter was placed in a constant temperature water bath. Four milliliters of fibrinogen solution mixed with 1 ml of $M/1$ phosphate buffer of pH 7 were placed in the water bath and, after the temperature equilibration, 5 ml of urea solution of the same temperature were added. At the moment of mixing, a stopwatch was started and 7 ml of the solution were withdrawn and transferred into the viscosimeter. The viscosity of the solution was determined from time to time. The increase of viscosity was rather quick in comparison with the outflow time of the viscosimeter. With the assumption of a linear increase of viscosity during single determinations, the viscosity value corresponds to the viscosity of the solution at the middle time of determination. The viscosity data were plotted in consequence against the time obtained by adding half of the outflow time to the moment of start of the determination.

Electrophoretic determinations were made with the Tiselius apparatus. Ten milliliters of fibrinogen solution were mixed with 1 ml of $M/1$ buffer of pH 7, and 3.3 g of solid urea were added. The mixture was kept at 35° C for 20 minutes, which treatment sufficed to denature almost completely the fibrinogen. Ten milliliters of an urea-buffer mixture were added, and the solution dialysed at 4° C for 16 hours against 2 000 ml of the same urea-buffer mixture. Borate buffer of pH 8.6 and phosphate buffer of pH 7 were used, each of 0.1 ionic strength and containing 20 per cent urea. After dialysis the solution was filled up to 25 ml with the urea-buffer mixture, and then subjected to electrophoresis.

The electrophoretic behavior of denatured fibrinogen was compared with that of the native. Ten milliliters of the fibrinogen solution were mixed with 10 ml of urea-buffer mixture and dialysed under the same conditions as already described. Experiments were also performed with mixtures of native and denatured fibrinogen in urea-buffer solutions.

Before and after electrophoresis the fibrinogen solutions were tested by dilution with 5.9 phosphate buffer. There was no precipitation or turbidity by the native fibrinogen, showing that the fibrinogen had not suffered any alteration during the experiment, while the denatured was completely precipitated.

The mobilities were determined by runs of four hours, with a potential gradient of approximately 8 volts/cm. The mixtures of native and denatured fibrinogen were subjected to electrophoresis for 12 hours, with a potential gradient of 5 volts/cm.

## Results

*A. Increase of turbidity.* In the presence of 10 and 20 per cent urea the increase of turbidity of the solution followed the denaturation process, but with an inductive period even longer than that occuring when the denatured fibrinogen was precipitated by dilution with a buffer of pH 5.9 (see first section of paper). The effect was studied in the temperature range of 35 to 50° C. The final turbidity was higher in the presence of 10 per cent urea than in the presence of 20 per cent. The velocity of the increase of turbidity was influenced by the temperature in the same fashion as the denaturation itself. It was greater if the temperature was higher, and at the same temperature was greater in 20 than in 10 per cent urea.

At 30 per cent urea concentration and 40° C, there was no change in the light transmission of fibrinogen solutions over a period of 90 minutes, although 15 minutes under these conditions are sufficient to denature the fibrinogen completely.

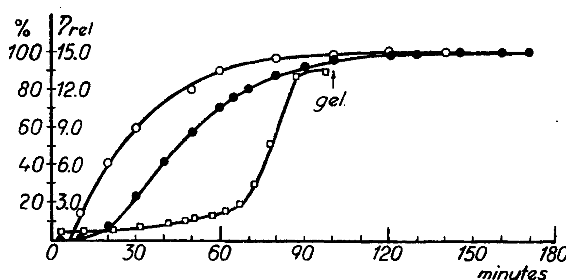


*Fig. 1. The amount of denatured fibrinogen, the opacity, and the viscosity of the solution plotted against time. 12 mg fibrinogen per milliliter, 20 per cent urea, 35° C, pH 7.*

○ % *denatured fibrinogen*
● » *increase of opacity*
□ *relative viscosity*

If the solution has no true light absorption, as in our case at 350 and 600 m$\mu$, the intensity of the transmitted light ($I$) is given by the relation: $I = I_0 - I_{sc} - I_r$, where $I_0$ is the intensity of the primary beam, while $I_{sc}$ and $I_r$ are, respectively, the light scattered in all directions except that of the primary beam, and the light reflected on the surfaces of the traversed media. Since in the Beckman spectrophotometer a blank cell is used, $I_r$ is eliminated automatically and the transmission coefficient, $\frac{I}{I_0}$, determined enables the calculation of the relative intensity of scattered light by the relation: $\frac{I_{sc}}{I_0} = 1 - \frac{I}{I_0}$ (see Nanninga *l. c.*).

From the figures calculated with this relation, the initial scattering of the solution was substracted and the differences expressed as the percentage of the finally reached constant value of the scattering.

In Fig. 1 is plotted the increase of the scattering of a fibrinogen solution in 20 per cent urea at 35° C calculated from the transmission coefficients at 350 m$\mu$ in the above described manner.

According to Rayleigh's relation — when the other factors involved are constant — the relative intensity of the scattered light, $\frac{I_{sc}}{I_0}$, is proportional to the number of scattering particles. The determination of $\frac{I_{sc}}{I_0}$ thus makes it possible to estimate the concentration of the scattering substance. Several authors have indeed attempted a quantitative protein estimation on the basis

of the turbidity obtained by various precipitating agents [15, 16]. Korányi and Hatz [17] found the protein concentration to be proportional to the relative intensity of the scattered light: while Looney and Walsh [16], and Nanninga [18] demonstrated the proportionality between protein concentration and relative light absorption $(\frac{I_0 - I}{I_0})$. These findings are in complete accord with the above derivation.

Thus the values of light scattering presented in Fig. 1 may be considered a measure of the concentration of fibrinogen molecules at a certain stage of denaturation, *viz.*, at a stage which is characterized by an increased light scattering power. The logarithmic plot of the concentration of fibrinogen molecules which had not attained this stage, calculated from the scattering, $\log (100-100 \frac{I_{sc}}{I_0})$, is shown in Fig. 2. The calculated points fall close to a straight line and the velocity constant obtained from the slope of the line is $6.4 \times 10^{-4}$ — a finding in good agreement with the $6.2 \times 10^{-4}$ calculated from the data obtained by the precipitation method (see first section of paper).

These findings seem to support the conclusion arrived at in the first part of these investigations, *i. e.*, the existence of a primary process of the denaturation, followed by secondary physicochemical alterations. Most probably the increase of the light scattering is caused by a dehydratation of the particles, which follows the primary denaturation process. The result of this is a sharpening of the refractive index gradient on the surface of the particles, which increases the light scattering.

Further analysis of the opacity can still give information about the physicochemical changes involved in the denaturation process. Heller and Vassy [19] showed that the Tyndal spectra, *i. e.*, the variation of the intensity of scattered light with wave length, enables the determination of the size of the scattering darticles. The wave length exponent in the equation: $k = c \cdot \lambda^{-n}$, where $k$ is the absorption coefficient, $c$ a constant, and $\lambda$ the wave length, is a function of the particle size. If the largest diameter of the scattering particles is smaller than 1/10 of $\lambda$, $n$ is equal to 4, but decreases very much if the particle size grows over this value. Ferry and Morrison [20] applied this principle to the analysis of the structure of fibrin gels, but they determined, instead of the wave length exponent, the ratio of the absorption coefficients at two different wave lengths. The ratio $\frac{k_{350\ m\mu}}{k_{600\ m\mu}}$ is equal to 8.6, if the wave length exponent is 4 and decreases if $n$ is less than 4. Fig. 3 shows this ratio plotted against time, the fibrinogen solution used being kept at 35° C in the presence of 20

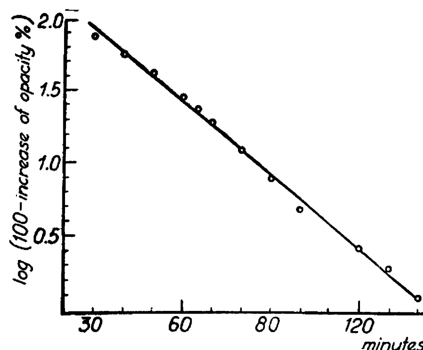


*Fig. 2. Log (100 — % increase of opacity) plotted against time. Same experiment as in Fig. 1.*

per cent urea. It can be seen that the ratio first increases, showing a decrease of the particle size — an effect which may be connected with the disintegration of fibrinogen by urea. Later the decrease of the ratio indicates a coarsening of the system, possibly brought about by the unfolding of the molecules and their association in bundles.

*B. Increase of viscosity.* The viscosity of fibrinogen in 10 and 20 per cent urea solutions increases very much and, if the protein concentration is high enough, the solution gelifies. The effect was investigated in the temperature range of 35 to 50° C. The increase of viscosity is enhanced by increase of temperature, and at the same temperature is quicker in 20 than in 10 per cent urea solution. Fig. 1 shows also the increase in viscosity of a fibrinogen solution in 20 per cent urea at 35° C. The increase of viscosity is characterised by a period of slight increase, after which the curve of increase rises sharply.

In the presence of 30 per cent urea at 40° C there is only an insignificant rise of the viscosity of fibrinogen solutions.

The changes in viscosity and opacity seem to be correlated. In the presence of 30 per cent urea both are absent, whereas at lower urea concentrations the increase of opacity is always followed by an increase of the viscosity.

The gels formed by the denatured fibrinogen in the presence of urea are very loose; if shaken they are easily fragmented. If the urea is washed out, they become elastic and very opaque; if concentrated urea solution or solid urea are added to a gel formed in the presence of 10 or 20 per cent urea, the gel dissolves. The resulting solution has a low viscosity, but the opacity does not disappear completely. If fibrinogen in 30 per cent urea solution is completely denatured and then diluted with distilled water to 20 per cent urea concentration or below, in the first moment after dilution it does not show any change, but after a few minutes becomes turbid, and finally gelifies.

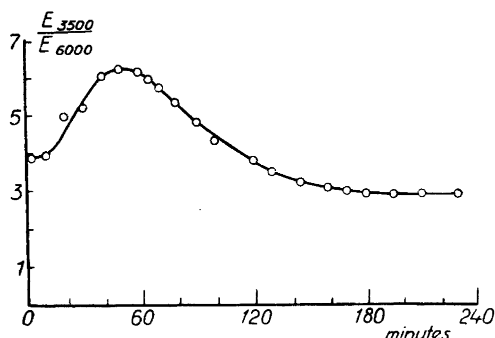


*Fig. 3. The ratio of the absorption coefficients at 350 and 600 mμ plotted against time. 12 mg. fibrinogen per milliliter, 20 per cent urea, pH 7, 35° C.*

Fibrinogen thus behaves differently from egg albumin, serum globulin, and serum albumin, which gelify even in urea solutions as concentrated as 60 per cent [10]. The denatured fibrinogen is soluble at urea concentrations higher than 30 per cent.

*C. Electrophoretic investigations.* The urea denatured fibrinogen in 20 per cent urea solutions moved as a single component with a sharp boundary. At pH 7 and 8.6 the mobility of denatured fibrinogen did not differ from that of the native, both being in 20 per cent urea solution. In order to detect small mobility differences, mixtures of native and denatured fibrinogen were subjected to electrophoresis. Only a single boundary was observed, showing that the mobilities at these pH-s were the same for both the native and the denatured fibrinogen. The viscosity of denatured fibrinogen was approximately double that of the native. The results indicate that the denaturation does not affect the net charge of fibrinogen in 20 per cent urea, and that the increase of viscosity by denaturation is without effect on the mobility of the protein.

Several authors also found denatured proteins to be homogeneous in electrophoretic experiments; but the mobilities differed from that of the native proteins. Pedersen [21] investigated the electrophoretic mobility of heat denatured serum albumin. In spite of the very accentuated heterogenity in the ultracentrifuge, the material was electrophoretically homogeneous. The pH-mobility curves of the native and denatured protein were almost parallel, but the denatured serum albumin had an isoelectric point 0.2—0.5 more alkaline than the native. Sharp, Cooper, and Neurath [22] also found urea-denatured horse pseudoglobulin, after reversal of the denaturation, to be electrophoretically homogeneous, with a higher mobility above and a lower below the isoelectric point than that of the native protein.

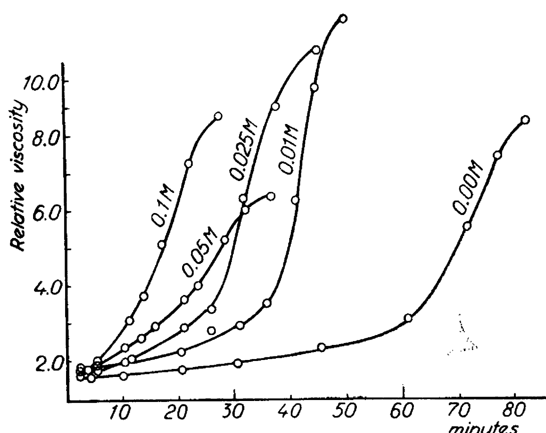


*Fig. 4. The rise of viscosity of fibrinogen solutions in 20 per cent urea, in the presence of different concentrations of sodium caprylate. 12 mg fibrinogen per milliliter, 35° C, pH 7.6.*

*D. Effect of sodium caprylate on the urea denaturation of fibrinogen.* Luck and his co-workers [23,26] investigated in great detail the preventive action of large organic anions against urea and heat denaturation of proteins. Duggan and Luck [23] tested the protective effect of a large number of organic anions against the viscosity rise of serum albumin solutions in concentrated urea. One of the most powerful stabilizers found was the caprylate anion. These authors are inclined to believe that the investigated substances protected the serum albumin molecules against the denaturation itself, and not merely prevented their aggregation.

It seemed interesting to try whether or not the caprylate anion has also a protective effect against the urea denaturation of fibrinogen. Dialysing experiments were performed, as described in section C of preceding paper. The fibrinogen was kept for 24 hours at 0° C in the presence of 30 per cent urea and 0.04 molar sodium caprylate; then the urea dialysed out. The pH of the solutions varied from 6.6 to 11.0. The above caprylate concentration proved to be very effective in preventing the urea denaturation of serum albumin [23,26]. No protective effect, however, was found in experiments with fibrinogen; on the contrary the caprylate accelerated the denaturation. The amount of unaffected fibrinogen was 45 to 80 per cent less in the set with caprylate, than in a control which did not contain it. The differences were the more accentuated, the more alkaline was the pH.

The effect of caprylate was tested also in kinetic experiments. The experimental procedure was the same as already described in section I of this paper. The denaturation in the presence of 0.01 molar sodium caprylate and 30 per

cent urea at pH 7 and 30° C proved to be strictly of the first order, with $k = 1.3 \times 10^{-3}$, whereas in the same conditions in the absence of caprylate $k = 7.5 \times 10^{-4}$. Thus the caprylate brought about a 1.73 -fold increase of the reaction velocity.

It may be that caprylate is not able to prevent the urea denaturation of fibrinogen, but, owing to its hydrotropic character, it may prevent the aggregation of the denatured particles and thus the increase of viscosity. In order to test this assumption, viscosity determinations were made in the manner described, in the presence of different concentrations of caprylate and 20 per cent urea at pH 7.6 and 35° C. A higher pH was used to prevent the precipitation of caprylate. As it can be seen from the results presented in Fig. 4, the caprylate is ineffective as a protective agent; even more, the increase of viscosity is the quicker, the higher is the caprylate concentration. Also, after a lapse of time, the solutions gelified in the presence of caprylate.

The caprylate has, thus, an effect on the urea denaturation of fibrinogen opposite from its effect on serum albumin. An intermediate position is occupied by egg albumin, caprylate having little or no effect on its urea denaturation [24].

The results clearly indicate that even where an agent has apparently the same effect — the increase of the viscosity of a protein solution — the mechanism which leads to this result may be quite different for different proteins. The efficiency of an inhibitor in one case and the inefficiency or even the opposite accelerator effect in other cases may be explained in this way.

## SUMMARY

1. The urea denaturation of fibrinogen is accompanied by an increase of the opacity and viscosity of the solutions, if the urea concentration is below 20 per cent. At 30 per cent urea concentration, neither the opacity, nor the viscosity change.

2. In 20 per cent urea solution the urea-denatured fibrinogen is electrophoretically homogeneous and its mobility does not differ from that of native fibrinogen.

3. Sodium caprylate has no preventive action against the urea denaturation of fibrinogen; on the contrary, it accelerates the denaturation.

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