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The Urea Denaturation of Fibrinogen

I. Kinetic Aspects

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Because of its property of increasing the solubility of proteins, urea is a reagent often employed by biochemists. It has been shown [1] that fibrin, the insoluble protein resulting from the action of thrombin on fibrinogen, is also soluble in urea solutions. Several recent investigations, [2,3] making use of this solubility, have dealt with the chemical and physicochemical differences between fibrin and its precursor.

The advantage of working in homogeneous medium is obvious; but a question which arises immediately when using urea in experiments with proteins is whether or not it has a denaturing effect on the protein in question. Meisner and Wöhlisch [4] considered fibrinogen resistant to the denaturing effect of urea, and even thought to avoid its spontaneous denaturation by storing it at 0° C in solutions of 30 per cent urea. Diebold and Jühling [5], continuing investigations along this line, found that urea denatured fibrinogen, but at 4° C, the denaturation was extremely slow. With higher temperatures the rate of denaturation was found considerably increased. Since Diebold and Jühling report qualitative, rather than quantitative data, and since the results of investigations on fibrinogen and fibrin in the presence of urea are difficult to interpret without a clear picture of its denaturing effect under different conditions, it seemed desirable to investigate the problem more thoroughly.

The urea denaturation of proteins has been subjected to much study, but very few data are presented on the kinetics of the process. Hopkins [6] showed that the urea denaturation of egg albumin has a negative differential rate-temperature coefficient. The same observation has been made recently in an investigation of the urea denaturation of β-lactoglobulin [7]. As pointed out by Clark [8], the negative temperature coefficient may be the result of two opposite actions of urea on the egg albumin molecules: (a) denaturation,

which renders the protein insoluble, and (b) splitting of the denatured molecules into soluble products. Usually the rate of denaturation is determined on the basis of the appearance of insoluble protein. If the tenperature coefficient of the second reaction is greater — both being positive — the resulting differential rate-temperature coefficient should be negative. The second effect is apparent only at fairly high urea concentrations. At lower urea concentrations than those employed by Hopkins, Clark [9] found a positive temperature coefficient of the denaturation of egg albumin.

Although the experiments along this line are not conclusive, the reaction seems to be of the first order in the case of chorion gonadotropin [10], with a positive temperature coefficient[11]. Lauffer[12], in his very extensive study on the denaturation of tobacco mosaic virus protein by urea, also found the reaction to be of the first order, but the velocity constant was a linear function of the reciprocal of the initial virus concentration. The temperature coefficient was negative below 23° C and positive above this temperature. He made the assumption that the urea denaturation consists of two or more parallel simultaneous reactions, one having a negative differential rate-temperature coefficient. As Hopkins did in the case of egg albumin denaturation, Lauffer supposed the formation of an urea-tobacco mosaic virus complex, and the subsequent denaturation of this.

There is complete lack of data on the reaction type of urea denaturation of other proteins. The investigation here reported was undertaken also with the idea of contributing to the data on the kinetics of urea denaturation of proteins.

EXPERIMENTAL

Materials

Fibrinogen was prepared from oxalated bovine plasma. Four liters of blood were mixed with 1 liter of 2 per cent sodium oxalate solution and the cells removed by centrifugation. The plasma was cooled to 0° C and the fibrinogen precipitated by adding ice-cold, saturated $(NH_4)_2\,SO_4$ solution to 0.24 saturation. The precipitate was centrifuged, and redissolved in 500 ml physiological saline. The insoluble residue, mainly red corpuscles, was centrifuged down. The clear solution was diluted with saline to 3 000 ml and the fibrinogen precipitated again by adding saturated $(NH_4)_2\,SO_4$ solution to 0.24 saturation. The resulting precipitate was centrifuged, washed with ice-cold distilled water, and dissolved in 200 ml of physiological saline solution. All the above operations were performed at a temperature as close as possible to 0° C. The fibrinogen solutions were slightly opalescent.

The method described above was followed in order to obtain quickly, through few steps, material pure enough for the kinetic study of the denaturation. This was attained by precipitating the second time from a rather big volume. The fibrinogen is very sensitive to repeated precipitations. In order to obtain a preparation as close to the native state as possible, further purification by reprecipitations and the removal of $(NH_4)_2 SO_4$ by dialysis were omitted. The fibrinogen preparations used contained, therefore, a small amount of $(NH_4)_2 SO_4$.

The concentration and purity of preparations were determined by clotting an aliquot of the fibrinogen with thrombin, and then washing, drying, and weighing the resulting clot. From another aliquot the total protein concentration was determined by means of precipitation with 20 per cent trichloracetic acid. The resulting precipitate was washed, dried, and weighed. The solutions contained 30 to 40 mg fibrinogen per milliliter and their purity ranged from 85 to 95 per cent.

All the other substances used were analytical reagents.

Methods

The denaturation is manifested by a loss of or a change in the specific properties of the protein. The most apparent of these changes is that of solubility. The denatured protein becomes insoluble in a solvent in which it was soluble in the native state. The estimation from time to time of the protein fraction which became insoluble is the easiest and most commonly used method for the determination of the denaturation rate of a protein.

Conditions under which the denatured protein is completely insoluble, while the solubility of the native protein is unaffected, must be carefully chosen, if an effective separation is to be secured. The urea denatures the protein, but in moderate and high concentrations it keeps the denatured product in solution. Therefore the urea should be removed by dialysis or its concentration lowered by dilution until it is not in sufficient amount to keep the denatured protein in solution. By either of these procedures the denatured protein precipitates, provided the pH of the solution is suitable. The precipitation is optimal at the isoelectric point and the denatured protein is more or less soluble at moderate alkaline or acidic pH.

In the preliminary investigations the precipitation of the denatured fibrinogen was effected by dialysing out the urea against 0.9 per cent NaCl solution the pH of which was brought to approximately 7 with a few drops of $N/1$ NaOH. After complete removal of the urea, the pH of the solution was adjusted to 6.3 with phosphate buffer; and the precipitate formed during the dialysis

and by the addition of the buffer solution was centrifuged down. The unaffected fibrinogen remained in the supernatant liquid, was clotted by thrombin, and its amount estimated. The fraction of the fibrinogen originally present recovered as fibrin clot gave a measure of the extent of denaturation. In these experiments the pH of the solution was not the most favorable for the separation of the denatured fibrinogen. Presumably a fraction of the denatured fibrinogen remained in solution after adjusting the pH at 6.3, and it was thereafter incorporated in the clot formed by the native fibrinogen. The extent of denaturation is, therefore, somewhat greater than determined by the above method.

The method of dialysis is not applicable to kinetic studies. Since urea also exerts its action during the dialysis the reaction time is uncertain. For this reason the method of precipitation by dilution was adopted. The buffered fibrinogen solution was kept in a constant temperature water bath, and sufficient time was allowed for reaching the temperature equilibrium; urea solution at the same temperature was then added, and the solution thoroughly mixed. During the experiment the solution was stirred continuously. At definite time intervals after mixing the fibrinogen solution with urea, samples of 5 ml were withdrawn and diluted in centrifuge tubes with 50 ml of M/10 phosphate buffer of pH 5.9. The pH of the final mixture was 6.05. The denatured fibrinogen precipitated in the early stage of the reaction as a fine fluffy precipitate, later as long filaments clumped together. To assure complete separation of the denatured material, the solutions were allowed to stay three hours at room temperature, then centrifuged. It was easy with the aid of a spatula to collect the sticky precipitate in a compact pellet. The precipitate was washed with physiological saline solution, then two times with distilled water, dried at 105° C, and weighed.

Attempts have been made to separate the denatured from the native fibrinogen by salting out. The salting out curves of the two substances were determined and are shown in Fig. 1. Denatured fibrinogen, which precipitated completely by dilution with buffer of pH 5.9, was only partially precipitated by dilution with neutral $(NH_4)_2SO_4$ solutions up to 0.15 saturation. Ammonium sulfate had, at low concentrations, a salting in effect, followed at higher concentrations by a salting out effect on the denatured fibrinogen. As Fig. 1 shows, the most favorable salt concentration for the separation of native and denatured fibrinogen is approximately 0.15 $(NH_4)_2SO_4$ saturation, but even in this case about 10 per cent of the denatured fibrinogen may remain in solution.

The above experiments made us prefer separation of denatured fibrinogen by dilution with buffer of pH 5.9, rather than by precipitating it by salting out.

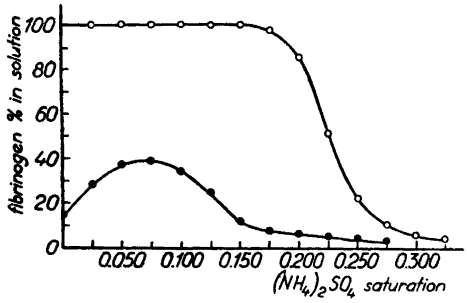

Fig. 1. Salting out curves of native and urea denatured fibrinogen. 100 per cent corresponds to 1.47 mg protein per milliliter.
○ *native fibrinogen.*
● *denat. fibrinogen.*

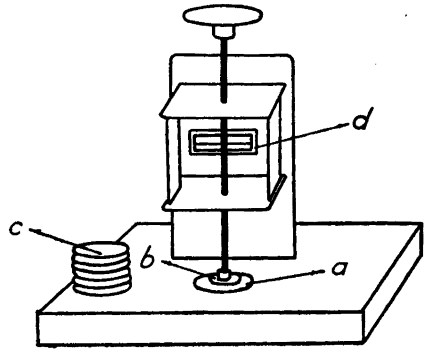

Fig. 2. Apparatus for the determination of the compressibility of fibrin gels. For explanation see text.

Difficulties were encountered when attempts were made to determine the urea denaturation of fibrin, and none of the above methods was found practicable. Fibrin dissolved in urea was denatured to various extents. When the urea was removed by dialysis or the solution diluted with buffers of different pH, all the fibrin precipitated out, denatured as well as unaffected.

When the urea was dialysed out against a buffer of pH 7.6 — a procedure which favored the formation of a 'fine type' gel [13] — the fibrin solutions which were exposed to the effect of urea at different pH, temperature, etc., yielded gels with quite different physical properties. The gels, where denaturation was expected according to the results obtained by fibrinogen, were opaque and soft, while those where presumably denaturation did not occur were transparent and elastic. The tensile strength of 'coarse type' gels, obtained by dialysing out the urea at pH 6.3, was lowered in those in which denaturation occured. It is probable from the above results that the mechanical properties of a fibrin gel are proportional to the amount of undenatured fibrin present and are not influenced by the inclusion of denatured fibrin. On the basis of this assumption, the extent of denaturation of fibrin has been determined by measuring the compressibility of the gels formed after the removal of the urea.

A small apparatus, shown in Fig. 2, was constructed to make possible the quantitative determination of the compressibility. The fibrin solutions, after undergoing the action of the urea at different conditions, were brought to about pH 7.6, poured into cellophane tubes, and then dialysed at 0° C for

48 hours against M/10 phosphate buffer of pH 7.6. After dialysis, the tubes contained cylindrical gels of 18 mm diameter. Small discs, of 10 mm height, were cut from them with the aid of a knife constructed from two shaving blades, fixed parallel at a distance of 10 mm from each other. The gel disc was cautiously transferred into the cylindrical hole of the apparatus, *a*, which had the same diameter as the gel, and so placed that the cut surface was horizontal. Compression was exerted by a round plate, *b*, of 14 mm diameter, which could be loaded with loads of different weight, *c*. The compression of the gel was followed by projecting on a centimeter scale the image of a hair fixed perpendicular to the axis in the frame *d*. With an enlargement of 1 : 20, it was possible to determine easily a deformation of 0.05 mm. The position of the image on the scale was observed, and the gel then loaded with 10 g every 30 seconds. The loading was continued at a a rate of 10 g per 30 seconds up to 50 g and then decreased in the same manner. The results were reproducible with different pieces cut off from the same gel.

Results

A. *The reaction type of the urea denaturation of fibrinogen.* Fibrinogen solutions of 15 to 40 mg per milliliter concentration were used. Twenty milliliters of fibrinogen solution were mixed with 5 ml. *M*/1 sodium phosphate buffer of pH 7 and 25 ml of 60 per cent urea solution at 30° C in the manner already described. Thus the experiments were performed in the presence of rather high salt concentrations, the final ionic strength being approximately 0.34. In such high salt concentration the small individual variations in the $(NH_4)_2\ SO_4$ content of different fibrinogen preparations had no significance. By using high buffer concentration the adjustment to the desired pH was assured and a possible shift of pH during the denaturation process avoided.

Fig. 3 shows the results obtained with three different fibrinogen concentrations : 8.0, 12.6, and 16.2 mg fibrinogen per milliliter in the final mixture. The amount of denatured fibrinogen is plotted against the time of standing in contact with 30 per cent urea. All points corresponding to the three different concentrations are apparently on the same curve. Plotting the logarithm of the concentration of unaffected fibrinogen against the reaction time, as done in Fig. 4, gave a straight line. In Fig. 3 the smooth curve corresponds to the straight line of the logarithmic graph. The experimental points do not deviate more than 1 to 3 per cent from this ideal curve. Above 90 per cent denaturation this error — common to a gravimetrical protein determination — causes pronounced scattering of the experimental points on the logarithmic graph. On the basis of the above findings the reaction must be considered

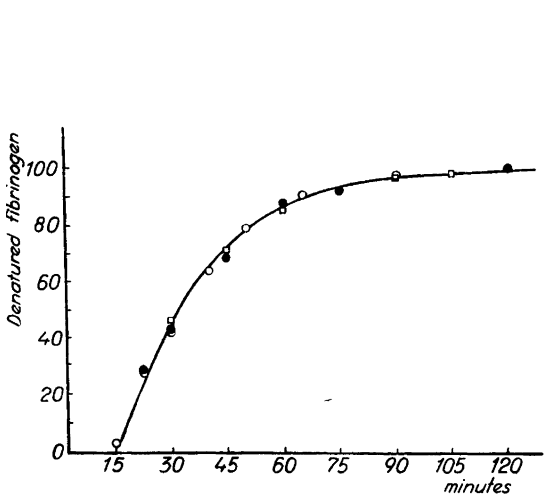

Fig. 3. Urea denaturation of fibrinogen. 30 per cent urea concentration, 30° C, pH 7.
○ *16.2 mg fibrinogen/ml*
● *12.6 mg fibrinogen/ml*
□ *8.0 mg fibrinogen/ml*

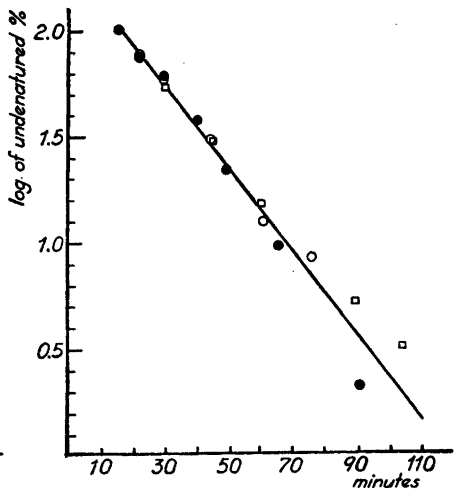

Fig. 4. Logarithm of undenatured fibrinogen plotted against time. Same experiments as in Fig. 3.

as one of the first order. In all the experiments, performed at various temperatures, different urea concentrations, and different pH, these conditions were fullfiled.

As can be seen in Figs. 3 and 4 in the first 15 minutes the amount of precipitated denatured fibrinogen was very small, if any. The data suggest an inductive period, after which the reaction started as a first order one. To explain this, it is supposed that the urea denaturation of fibrinogen consists of two distinct processes. The primary process is a definite chemical reaction of the first order; the result of this is fibrinogen molecules already denatured, but still possessing a considerable degree of hydration and, consequently, a rather high solubility. They are not precipitated at pH 6, but only after a secondary action of urea, which diminishes their hydration and renders them insoluble at the pH in question. The secondary alteration has the same velocity for all the molecules affected during the primary process, which means that the precipitation of denatured fibrinogen will follow the course of the primary change, but with a shift in time equal to the duration of the secondary changes. The change in the solubility of denatured fibrinogen is more apparent at a pH close to the isoelectric point, and becomes less pronounced at a pH distant from it. In other words, a very short secondary action of the urea

will suffice to render the molecules insoluble at a pH near the isoelectric point, while at a higher pH the secondary action will be longer the more alkaline is the pH of the diluting solution.

To test the correctness of this assumption, fibrinogen was denatured at pH 7 and 30° C in contact with 30 per cent urea. The reaction was followed as usual by diluting samples at different time intervals, but using for this purpose three buffers of different pH : 5.5, 5.9, and 6.4. Fig. 5 presents the results in logarithmic plot. As was expected, the inductive periods are increased proportionally with the pH of the diluting solution, but the straight lines in all cases have equal slopes, showing that the velocity constants are the same with only the precipitation of the denatured product suffering a retardation proportional to the pH of the diluting buffer.

Findings similar to these are reported by Hopkins [6] in connection with the urea denaturation of serum proteins. This author found that the nitroprusside test was positive very soon after the addition of urea, but the denatured serum proteins began to precipitate on dialysis or dilution only after some hours of previous standing in contact with urea. He concluded: 'It would seem as though these proteins undergo with readiness the chemical changes which in all cases are associated with denaturation, but the colloid particles of the product are more resistant than those of egg albumin to the dehydration which characterizes the change from lyophil to the lyophobe condition.'

The solubility of the denatured product would offer another possible explanation of the inductive period. In this case the precipitation would start only after the saturation of the solution with the denatured fibrinogen. It is very easy to convince one's self of the wrongness of this assumption. Indeed, the amount of finally precipitated denatured fibrinogen was always equal to or very near by 100 per cent of the fibrinogen originally present. In the case of a solubility of the denatured fibrinogen the precipitate at the end of the denaturation process would be obviously less than 100 per cent; and the reaction would apparently slown down.

Fig. 5 shows also the curve obtained by precipitating the denatured fibrinogen with 0.15 saturated $(NH_4)_2SO_4$ solution. It was already mentioned that the denatured product is slightly soluble in this solution even after a prolonged action of urea. Accordingly, it can be seen that the reaction is apparently slowed down and the amount of precipitated fibrinogen did not reach 100 per cent of the originally present.

Thus the assumption of a primary process, followed by secondary physicochemical changes in the state of denatured molecules seems to be justified. A further proof of this concept will be presented in section II of this paper.

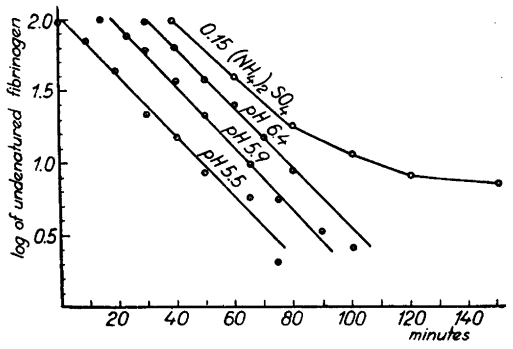

Fig. 5. The urea denaturation of fibrinogen followed by diluting samples with buffers of different pH and 0.15 saturated $(NH_4)_2SO_4$ solution. Denaturation at 30° C, pH 7, and 30 per cent urea concentration.

From the results described above it seemed probable that a buffer which has a pH equal to the isoelectric point of fibrinogen would be the most effective for precipitating denatured fibrinogen in the earliest stage of denaturation. Such a buffer can be used to dilute fibrinogen solutions which have stood in contact with 30 per cent urea, because the urea concentration after dilution is still sufficiently high to avoid the isoelectric precipitation of the native fibrinogen; but is not applicable to the dilution of solutions with lower urea concentrations where the native fibrinogen will precipitate also. This was the reason why the precipitation of denatured fibrinogen with $M/10$ phosphate buffer of pH 5.9 was utilised throughout this work.

The secondary process seems to be affected in the same way as the primary by temperature and urea concentration. The slower the denaturation process, the longer the inductive period; the quicker the denaturation, the shorter the period.

B. *Effect of temperature and urea concentration on the denaturation rate.* In order to investigate the effect of the urea concentration on the reaction rate, 20 ml fibrinogen solution of 30 mg per milliliter concentration was mixed with 5 ml $M/1$ sodium phosphate buffer of pH 7 and 25 ml of urea solution of, respectively, 20, 40, and 60 per cent concentration. Thus the final urea concentrations were 10, 20, and 30 per cent. The mixtures were kept at different temperatures and the reaction rate determined. In all the cases an inductive period was observable, the significance of which has already been discussed. The velocity constants were calculated from the slope of the straight lines obtained by plotting the logarithm of the concentration of unaffected fibrinogen against the time of standing in contact with urea. In each case at least two independent experiments were made, and the given values correspond to the

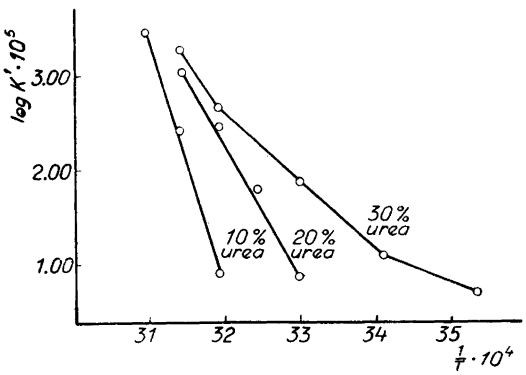

Fig. 6. The Arrhenius plot of the velocity constants of the urea denaturation of fibrinogen.

straight lines which best fitted all the experimental points. The results are summarised in Table 1.

It is evident that an increase in temperature brought about an increase of the reaction rate in all instances. The differential rate-temperature coefficient of the denaturation process is thus always positive — a result in contrast with the findings on egg albumin, β-lactoglobulin, and tobacco mosaic virus protein. Fig. 6 shows the Arrhenius plot of the results; *i. e.*, the logarithm of the velocity constants plotted against the reciprocal of the absolute temperature. It is apparent that in the presence of 10 and 20 per cent urea in the investigated temperature range the Arrhenius law is valid, the logarithm of

Table 1. Velocity constants and half reaction times of the urea denaturation of fibrinogen at different temperatures and urea concentrations. pH 7.

t (°C)	Urea conc.	k'	Half reaction seconds
40	10 %	7.85×10^{-5}	8 833
45	10 %	3.15×10^{-3}	220
50	10 %	1.35×10^{-2}	51
30	20 %	7.20×10^{-5}	9 625
35	20 %	6.19×10^{-4}	1 119
40	20 %	2.86×10^{-3}	242
45	20 %	1.06×10^{-2}	65
10	30 %	5.03×10^{-5}	13 750
20	30 %	1.26×10^{-4}	5 500
30	30 %	7.50×10^{-4}	924
40	30 %	4.56×10^{-3}	152
45	30 %	1.83×10^{-2}	37

the velocity constant being a linear function of the reciprocal of the absolute temperature. The considerable temperature coefficient of the reaction rendered difficult the covering of a larger temperature range than 10 to 15° C. At 30 per cent urea concentration, it was possible to investigate the reaction rate over a wider range. Between 20 and 40° C, the Arrhenius plot gave a straight line, but above and below this temperature there was a marked deviation from this line. It is possible that a similar departure would also be detectable in the presence of 10 and 20 per cent urea, if the determinations could have been extended over a larger temperature range.

The heat, the free energy and the entropy change of activation were calculated using the equations derived by Eyring and Stearn [14, 15] from the theory of absolute reaction rates:

$$\Delta H^{\ddagger} = RT^2 \frac{\mathrm{d} \ln k'}{\mathrm{d}\, T} - RT \tag{1}$$

$$\Delta F^{\ddagger} = -RT \ln k' + RT \ln \frac{kT}{h} \tag{2}$$

$$\Delta S^{\ddagger} = \frac{\Delta H^{\ddagger} - \Delta F^{\ddagger}}{T} \tag{3}$$

It can be seen that the heat of activation has approximately the same value as the classical energy of activation given by the well known Arrhenius relation:

$$E = RT^2 \frac{\mathrm{d} \ln k'}{\mathrm{d}\, T} \tag{4}$$

From equations (1) and (4),

$$\Delta H^{\ddagger} = E - RT \tag{5}$$

The energies of activation were calculated from the slopes of the Arrhenius plot and then substituted in equation (5) to obtain the heats of activation.

The values calculated are listed in Table 2. It is apparent that the heat of activation of the denaturation process is lowered to a great extent by urea. Considering only the segment between 20, and 40° C in the presence of 30 per cent urea, the heats of activation are, respectively, 120 000, 63 000, and 32 000 calories for 10, 20 and 30 per cent urea concentration. Thus, as while the urea concentration is increased arithmetically, the heat of activation decreases geometrically. At constant temperature the entropy of activation

Table 2. The heat, the free energy, and the entropy change of activation by the urea denaturation of fibrinogen at different temperatures and urea concentrations. pH 7.

t (°C)	Urea conc.	$\Delta H^{\ddagger}$	$\Delta F^{\ddagger}$	$\Delta S^{\ddagger}$
40	10 %	120 200	24 200	306.7
40	20 %	63 000	22 000	130.9
40	30 %	31 900	20 700	35.7
10	30 %	14 600	22 100	−26.5
30	30 %	31 900	22 075	32.4
45	30 %	54 300	21 100	104.4

is also considerably lowered by the increase of the urea concentration, whereas at constant urea concentration the rise of the temperature increases the entropy of activation.

The results may be explained in terms of the protein denaturation theory of Mirsky and Pauling [16]. These authors suppose that the activation process involves the breaking of hydrogen bonds. The great increase in entropy is the result of the increase in freedom of the groups liberated by this process. The liberated groups are unable to form new hydrogen bonds with water, but urea has a much greater tendency than water to from such bonds. It is probable that this tendency even exceeds that of the groups linked in the protein molecules; urea being thus able to break the hydrogen groups between these groups. The binding of urea molecules is an exothermic reaction, the heat liberated compensating in part the heat expenditure of the activation process. At the same time the freedom of the groups which bound urea is decreased, the result being a drop in the entropy change of the activation process. At high urea concentrations and low temperature the drop of the entropy caused by the binding of urea molecules, may exceed the entropy increase of the activation process, the result being a negative entropy of activation.

The free energy change of the activation was only moderately decreased by the presence of urea, because the heat and the entropy of activation were lowered in such a way as to compensate each other.

The urea-protein complex formation is exotherm; the number of bound urea molecules is, therefore, decreased when the temperature rises. A consequence of the decrease of the number of bound urea molecules with increasing temperature is the increase of the heat and of the entropy of activation. This can be actually observed on the data obtained in the presence of 30 per cent

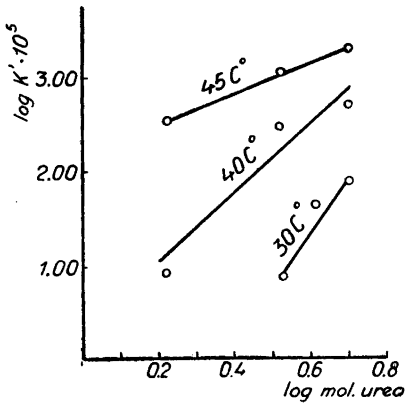

Fig. 7. The logarithm of the velocity constants plotted against the logarithm of the urea concentration.

urea (see Table 2). At high temperature it is evident that the number of bound urea molecules will be equal to zero, the heat of activation approaching thus a value equal with that found in the absence of urea. Fig. 6 clearly shows this tendency.

At constant temperature the increase of urea concentration brings about an increase in the velocity constant. This is easily explained by an increase in the number of bound urea molecules. According to Lauffer's derivations [12], the velocity constant at constant temperature is proportional to a power of the urea concentration. Thus, when log k' is plotted against log urea concentration, a straight line should result. The lines corresponding to different temperatures — according to the theory — are the steeper, the lower is the temperature. This straight line relation has been verified by Lauffer on tobacco mosaic virus and the change in the slope at different temperatures was also observed.

Fig. 7 shows a similar plot of the data obtained on fibrinogen. At 45° C, a straight line was obtained, but a 40° C, the data fitted less satisfactorily with a straight line relation. In spite of the incompleteness of data, it can be seen that the lines become much steeper when the temperature decreases. The slopes are equal with the average number of urea molecules bound, which are respectively 4.0, 3.2, and 1.4 at 30, 40, and 45° C. In the case of tobacco mosaic virus, Lauffer found 8.1 at 0° and 5.7 at 45° C. Comparing the results obtained with the two proteins, it is apparent that at the same temperature (45° C) fibrinogen bound less urea than the virus and that the rise of temperature dissociates the fibrinogen complex more rapidly.

C. *The effect of pH on the denaturation rate.* The determination of the reaction rate was conducted as in the preceding experiments. Twenty milliliters of fibrinogen solution containing 30 mg per milliliter, was mixed with 5 ml of buffer of different pH. Molal acetate buffers of pH 4.6 and 5.3, $M/1$ phosphate buffers of pH 5.9, 6.5, 7.0, and 7.6, and a borate buffer of pH 8.6 — equal volumes of $M/1$ boric acid and $M/4$ borax — were used. The solutions have the same molal concentration, but very different ionic strength. In spite of this, the results seem coherent, which shows that at such high values the variation in ionic strength has little effect. The buffered fibrinogen was mixed with 25 ml of 60 per cent urea solution. The pH of the solution was not altered significantly by the addition of urea. The mixture was kept at 20° C, then samples of 5 ml were diluted with 50 ml of buffer solution which composition was calculated in each case to give, after mixing, a final pH of 6.05. The amount of precipitate was determined in the described manner. In Table 3 are summarised the velocity constants obtained. The reaction rate was nearly constant between pH 7 and 8.6, whereas lowering the pH below 7 increased considerably the velocity constant. Below pH 7 the reaction rate was proportional with the 0.4 power of the hydrogen ion concentration. When, in this range, log k' was plotted against pH, a straight line was obtained, with the slope —0.4 (see Fig. 8) According to Steinhardt[17], this demonstrates that the activation of the molecules is accompanied by the binding of 0.4 proton per mol. of fibrinogen. It is hard to believe, however, that the above figure has this significance, since it is too small to have such a pronounced effect on the state of the molecule. Most probably the hydrogen ions have a catalytic effect.

In early orienting experiments, the effect of pH on the denaturation of fibrinogen by urea was studied by a method of dialysis. Four milliliters of

Table 3. Velocity constants and half reaction times of the urea denaturation of fibrinogen at different pH.

Urea conc.	t (°C)	pH	k'	half reaction (seconds)
30 %	20	4.6	1.10×10^{-3}	630
30 %	20	5.3	6.37×10^{-4}	1 087
30 %	20	5.9	3.88×10^{-4}	1 786
30 %	20	6.5	2.18×10^{-4}	3 178
30 %	20	7.0	1.26×10^{-4}	5 500
30 %	20	7.6	1.38×10^{-4}	5 021
30 %	20	8.6	1.39×10^{-4}	4 941

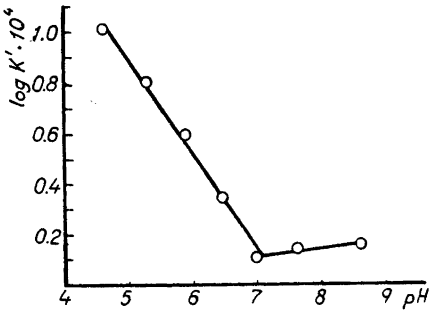

Fig. 8. The logarithm of the velocity constants plotted against pH. 30 per cent urea concentration, 20° C.

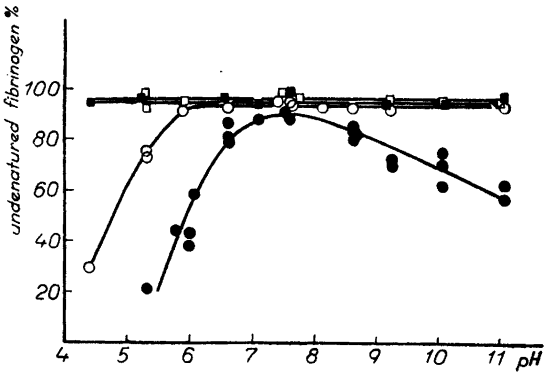

Fig. 9. The urea denaturation of fibrinogen at different urea concentrations and pH. Time of standing in contact with urea: 24 hours. 0° C.

□ *0* % *urea*
■ *10* » »
○ *20* » »
● *30* » »

fibrinogen solution of 25 mg per milliliter concentration were mixed with 1 ml *M*/1 buffers of different pH, and 5 ml of urea solution at 0° C. Urea solutions of 20, 40, and 60 per cent were used. The mixtures stood 24 hours at 0° C; their pH was then brought to approximately 7 with a few drops of *M*/1 NaOH or *M*/1 HCl; the solutions were then dialysed, the precipitated fibrinogen centrifuged down, and the unaeffected fibrinogen clotted by thrombin.

The results are shown in Fig. 9. It can be seen that at 10 per cent urea concentration the amount of fibrinogen recovered as fibrin clot is equal, over the whole pH range studied, to the amount obtained in controls containing physiological saline solution instead of urea. The same is true for 20 per cent urea concentration above pH 6.0. Below this pH, the amount of denatured fibrinogen increases rapidly with the lowering of pH. At 30 per cent urea, the maximal stability is approximately at pH 7.6 and the amount of unaffected fibrinogen falls on either side of this point. According to Wu and Yang [17] the pH of maximal stability of egg albumin against urea denaturation is 7, the two proteins being similar in this respect.

The results obtained by the study of the influence of pH on the velocity constants of denaturation are in accord with the above findings. The pronounced accelerating effect of the acidic reaction and the relatively small change

brought about by the increase of pH from 7 to 8.6 in the presence of 30 per cent urea can be seen here also.

D. *The urea denaturation of fibrin.* Fifty milliliters of fibrinogen of 40 mg per milliliter concentration were clotted by adding 1 ml of thrombin solution. The clot was allowed to stay at room temperature for three hours, then cooled to 0° C, and dissolved by adding 15 g of solid urea. The fibrin solution obtained was divided in three equal portions and calculated amounts of distilled water and 60 per cent urea solution added in order to have after addition of buffer a final protein concentration of 13.3 mg per milliliter, and a final urea concentration of, respectively, 10, 20, and 30 per cent. Three sets of tubes were prepared with the solutions of different urea concentrations, each tube containing 9 ml fibrin solution and 1 ml of $M/1$ buffer of different pH. The mixtures were kept at 0° C for 24 hours, then neutralised, and dialysed against $M/10$ phosphate buffer of pH 7.6, as described in the section on methods.

Fig. 10 shows the maximal compression obtained with load of 50 g by the gels which were obtained from fibrin solutions treated with 10, 20, and 30 per cent urea at different pH. The compressibility of the gels obtained from the fibrin solutions with 10 per cent urea was constant over the whole pH range studied. With 20 per cent urea constant compressibility was observed above pH 6.0, but below this pH the gels softened proportionally with the increase in acidity. Thirty per cent urea concentration had a marked effect below pH 7 and above pH 9; between pH 7 and 9, the mechanical properties of the fibrin gels were very alike.

A comparison of the curves with those in Fig. 9, which represent the denaturation of fibrinogen in similar conditions, reveals a great similarity between the resistance of the two proteins against urea denaturation. The above findings suggest that the urea denaturation of fibrin does not differ from that of fibrinogen.

SUMMARY

1. The urea denaturation of fibrinogen is a reaction of the first order. The primary reaction is followed by secondary changes characterized by a gradual decrease of the solubility of the denatured product.

2. The denaturation process has a positive differential rate-temperature coefficient. The Arrhenius law is valid over small temperature intervals only.

3. Increase of urea concentration increases the reaction rate and decreases the activation energy and entropy of the process. The free energy change of the activation process is lowered only moderately by urea.

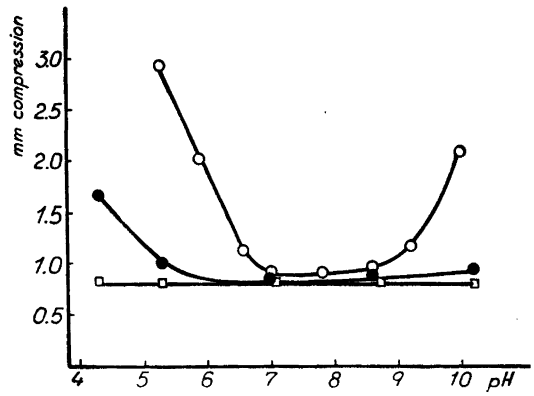

Fig. 10. Compression of fibrin gels obtained with load of 50 gm.
○ *30 % urea*
● *20 » »*
□ *10 » »*

4. The reaction rate is increased by lowering the pH below 7, in the presence of 30 per cent urea. Between pH 7 and 8.6, the reaction rate is nearly constant.

5. The urea denaturation of fibrin does not differ from that of fibrinogen.

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