The Kinetics of the Urea Denaturation of Hemoglobin. I. Beef Oxyhemoglobin*

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The kinetics of the denaturation of beef oxyhemoglobin by urea has been studied by observing changes with time of the spectrum, viscosity, and solubility. The effects of urea and protein concentrations, *p*H, buffer concentration, and temperature have been investigated. The three methods of assaying the reaction show nearly the same kinetic behavior. At urea concentrations between 6 M and 10 M the reaction is of the fifth order with respect to urea. The rate has a minimum near *p*H 6.8 and between 14° and 30° the half time for denaturation decreases linearly with increasing temperature. The protein concentration has a large effect on both the rate and the general character of the denaturation reaction: at high protein concentrations there is an induction period, whereas at low protein concentrations the reaction is more rapid and no induction period is evident. Several possible interpretations of the effect of protein concentration on the kinetics are discussed. The most plausible interpretation assumes a rapid equilibrium between half and quarter hemoglobin molecules, the half molecules denaturing through an intermediate whose properties are nearly identical with those of the native molecule. The denaturation of methemoglobin is first order with respect to the protein, and the rate is independent of the protein concentration. A few measurements were also performed on cyanomethemoglobin and carboxyhemoglobin. The viscosity of denatured hemoglobin in 8 M urea is compared with that of other proteins in urea.

Chick and Martin (1911) showed that the thermal denaturation of horse hemoglobin was a first-order reaction, their criterion of denaturation being the production of material insoluble at the isoelectric point. This study was one of the earliest examples of the application of chemical kinetics to protein denaturation. Further investigations of the heat, acid, and alcohol denaturation of horse and beef hemoglobins were made by Lewis (1926, 1927), Cubin (1929), and Booth (1930). Acid denaturation and its reversal were studied by Anson and Mirsky (1929b, 1930, 1931) and by Hill and Holden (1926) and Holden (1936, 1947), who characterized various intermediates and products spectroscopically. More recently Steinhardt, Zaiser, Ona, and Beychok have reinvestigated the acid denaturation of horse carbonylhemoglobin and methemoglobin, particularly with regard to the appearance of titratable groups at *p*H values below 4 (Steinhardt and Zaiser, 1955; Beychok and Steinhardt, 1960; Steinhardt *et al.*, 1962). Denaturation of hemoglobin by urea, salicylate, and simple amides was also studied by Anson and Mirsky (1929a, 1930), who showed that the production of material insoluble in water at the isoelectric point is accompanied by an increase in the viscosity of the protein solution in urea. More recent investigations of the effect of urea on hemoglobin and some of its modifications are those of Chalopin and Colson-Guatalla (1949) and White and Kerr (1957). The dissociation of the hemoglobin molecule by urea was demonstrated by Wu and his collaborators (Wu and Huang, 1930; Wu and Yang, 1932) and by Steinhardt (1938). The dissociation is not accompanied by denaturation if the urea concentration is below 4 M. The alkali denaturation of hemoglobins of different types has been extensively studied by Kruger (1925), Drabkin (1939), Betke (1953), Haurowitz *et al.* (1954), and many others (see Haurowitz and Hardin, 1954, for further references).

Despite this large amount of work on hemoglobin denaturation, no detailed study of the kinetics of unfolding in concentrated urea solutions has been reported. It was decided to undertake such a study using several physical properties for following the unfolding process, namely, changes in the absorption spectrum, changes in the solution viscosity, and changes in the solubility at the isoelectric point.

EXPERIMENTAL

Beef hemoglobin was prepared from freshly drawn blood, which was defibrinated by shaking with glass rings. The red cells were washed repeatedly (five to six times) with 0.9% sodium chloride in a centrifuge, then laked by dialysis against distilled water. The stroma were removed by shaking with toluene (control experiments showed that the treatment with toluene had no effect on the results). Hemoglobin concentrations were determined by weighing known volumes of solution after drying at 105°, and also, later in the work, by measuring the absorbance at 542 mμ. All hemoglobin solutions were stored at 4–6° for no more than eight days. (It

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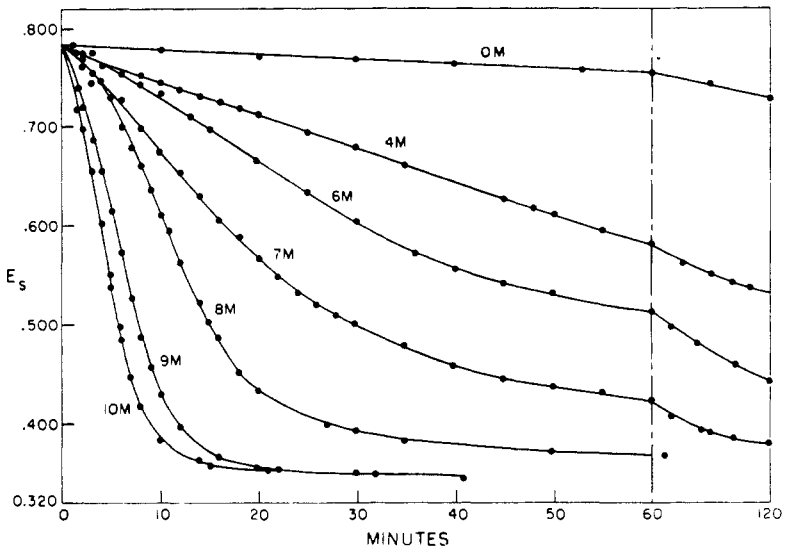

FIG. 1.—Effect of urea concentration on the rate of change of the spectral absorption at 542 mμ. Protein concentration 0.1%, 0.05 M phosphate buffer, *p*H 6.9, 30°. Urea concentrations as indicated for each curve (0 M, 4 M, etc.). Time scale compressed after 60 minutes.

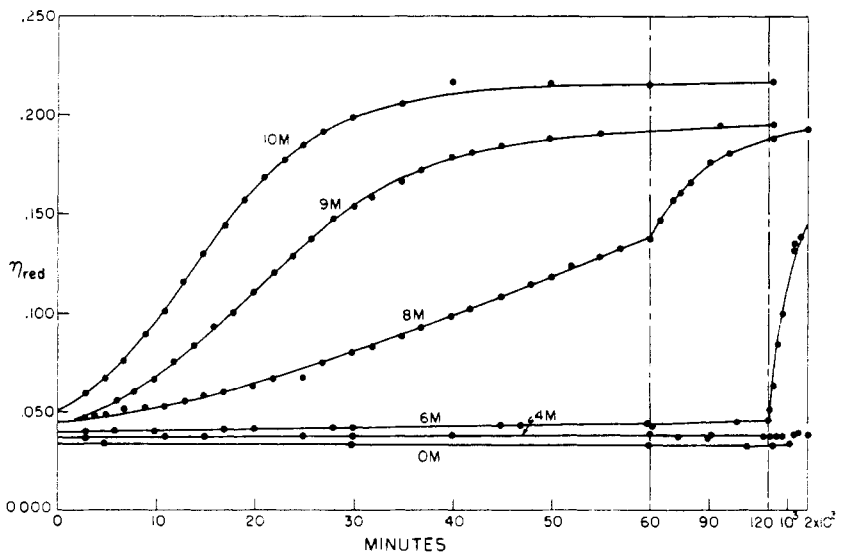

FIG. 2.—Effect of urea concentration on the rate of change of the reduced viscosity of oxyhemoglobin. Protein concentration 1.0%, other conditions the same as in Figure 1. Time scale compressed after 60 minutes and 120 minutes.

was shown that the features of the denaturation reaction to be described in this paper begin to change after this period of storage.) Spectrophotometric measurements were made with a Beckman DU spectrophotometer equipped with thermostatted cells. The spectrophotometric results are reported in terms of the specific extinction, E_s, defined as $D/10\ cl$, where D is the optical density, c is the protein concentration in g per 100 ml of solution, and l is the thickness of the absorbing layer in cm. The viscometers were of the Ostwald type, described in earlier publications from this laboratory (Frensdorff *et al.*, 1953). The *p*H values were determined with a Beckman model G *p*H meter with ordinary electrodes. All chemicals were of reagent grade. The urea was purchased from J. T. Baker Company and in most of the work was not recrystallized; effects of using recrystallized urea and other brands of urea will be mentioned below.

Beef methemoglobin was prepared by oxidizing pure oxyhemoglobin with potassium ferricyanide in 3-fold excess (3:1 $K_3Fe(CN)_6$–Fe in protein) as outlined by Drabkin and Austin (1935a). The protein was then dialyzed in distilled water in the refrigerator until free of the yellow colora-

tion of the potassium salt. Cyanide methemoglobin was prepared by the method of Drabkin and Austin (1935b).

Production of insoluble protein was followed by precipitation of denatured material in isoelectric 0.1 M phosphate buffer. Two ml samples were withdrawn from the urea-hemoglobin mixture at intervals and pipetted into 40 ml of 0.1 M 1:1 sodium phosphate buffer. The final *p*H reached was approximately 6.8, very close to the isoelectric value for mammalian blood quoted in the literature (Haurowitz and Hardin, 1954). Precipitated samples were left to stand 4 to 6 hours before filtration to allow complete flocculation. Samples were then filtered through tared medium-fine sintered glass filters, washed with 10 to 15 ml of water, and dried to constant weight at 104° to 106°. Preliminary experiments showed that the 21:1 dilution of the urea was sufficient to reduce the urea concentration well below the point at which it might solubilize the denatured protein. It was also shown that the time allowed for the precipitate to stand before filtration, the amount of water used in washing the precipitate, and the buffer concentration had no effect on the amount of protein precipitated in a run. The effect of *p*H was also studied, and it was confirmed that the maximum amount of precipitate was formed if the precipitating solution had a *p*H in the range 6.7 to 7.0.

Except when otherwise stated the temperature of all runs was 30°.

Results

The spectrum of native oxyhemoglobin has absorption peaks at 542 and 575 mμ. On standing of the oxyhemoglobin in strong urea solutions these peaks disappear and are replaced by a broad, almost featureless absorption extending over the entire visible region of the spectrum.[1] The disappearance of the peaks is a slow process which is easily followed in a spectrophotometer and thus offers a convenient method of following the denaturation process. The rates of disappearance of the absorption maxima at 542 and 575 mμ proved to be the same. The former wave length was used exclusively in the work reported here because the spectrophotometer was more sensitive at this wave length. The spectral changes are accompanied by an increase in the viscosity of the urea solution and by loss in solubility of the protein in water at its isoelectric point, as will be shown below. Therefore it can be assumed that the spectral changes are a reflection of the unfolding of the molecule by urea.

Effect of Urea Concentration.—Figure 1 shows the variation in rate of disappearance of the 542 mμ absorption peak with changes in urea concentration. These measurements were all made at a hemoglobin concentration of 0.10 g per 100 ml of solution, the *p*H being that corresponding to a minimum rate, *i.e.* between 6.8 and 6.9. All curves start with a specific extinction (E_s) of 0.784, changing to approximately 0.370 when denaturation is complete. The final value is about the same for all concentrations of urea, with a slightly greater change (lower final E_s) at higher urea concentrations. At urea concentrations greater than 6 M the curves have a slightly sigmoid shape, which will be discussed in more detail below. Once the specific extinction levels off at 0.370 the reaction is apparently complete and no further slow time-dependent changes occur.

Viscometric measurements were made under conditions similar to those just described for the spectrophotometric study, except that the protein concentration was 1% rather than 0.1%. Figure 2 shows the change in the reduced viscosity $\eta_{red} = [(\eta/\eta_0) - 1]/c$, as a function of time at various urea concentrations. All plots begin at small values of η_{red} and subsequently increase by a factor of 3 or 4. The initial values are slightly greater at the higher urea concentrations (0.051 dl/g in 10 M urea as compared with 0.036 dl/g in water). This small initial increase in η_{red} for the protein in urea solution must be caused by instantaneous changes in the native molecule when it is added to urea—possibly the splitting of the molecule into halves.

At urea concentrations up to 4 M no change in η_{red} occurs in periods of time exceeding 3000 minutes, indicating that no appreciable unfolding of the molecule accompanies the spectral change that is observed under these conditions. In 6 M urea an unfolding of the molecule occurs over periods of time of the order of 2000 minutes, and at higher urea concentrations the changes are much more rapid. The sigmoid shape of the curves is somewhat more pronounced than in Figure 1.

Effect of Protein Concentration.—An unusual characteristic of the urea denaturation of hemoglobin is the strong dependence of the rate on the protein concentration. This is observed in both the spectral measurements and the viscometric measurements (Fig. 3 and 4). Both properties change more rapidly as the protein concentration is reduced. Furthermore, closer inspection of the spectrophotometric curves shows that the sigmoid character becomes more pronounced at the higher protein concentrations. This can be seen from Figure 5, where the spectrophotometric data are plotted on a reduced scale showing the relative change in the absorption at 542 mμ *vs.* the ratio of the time of exposure to urea to the half time for complete reaction.

[1] Solutions of native oxyhemoglobin transmit light at wave lengths greater than about 600 mμ, but unfortunately denaturation increases the absorption at these wave lengths, so that it is difficult to use optical rotatory power in order to follow the change. The spectrum of the urea-denatured protein shows a broad maximum at 530 mμ and a shallow minimum at 500 mμ. The Soret band is shifted to shorter wave lengths by 20 to 30 mμ.

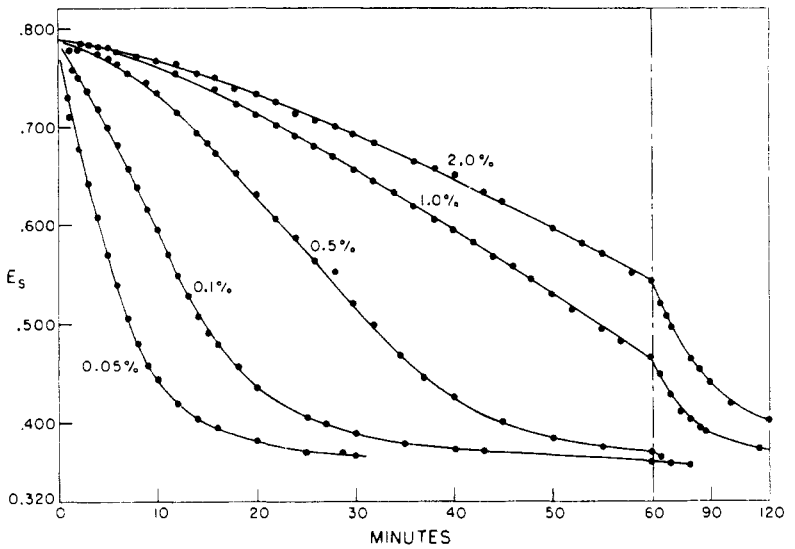

FIG. 3.—Effect of protein concentration on the rate of change of the spectral absorption at 542 mμ. Urea concentration 8 M, other conditions as in Figure 1. Protein concentrations are indicated by the figures beside each curve (0.05%, 0.1%, etc.). Time scale compressed after 60 minutes.

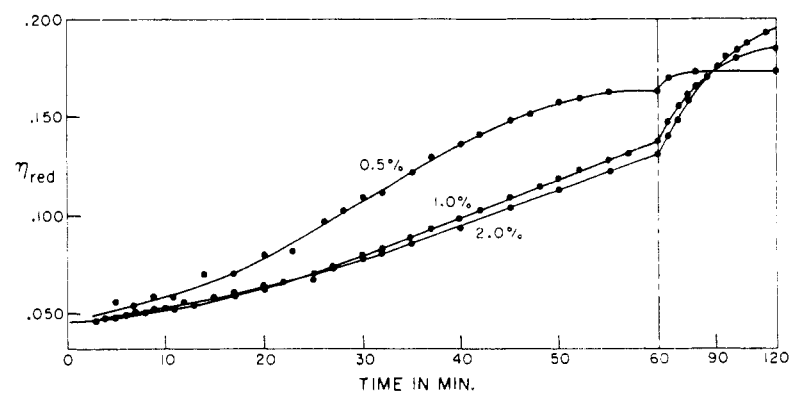

FIG. 4.—Effect of protein concentration on the rate of change of the reduced viscosity. Conditions are the same as in Figure 3. Time scale compressed after 60 minutes.

At a given protein concentration the half time for the change in absorption at 542 mμ is similar to, though not quite identical with, the half time for the viscosity change. Thus in 8 M urea and at a protein concentration of 2% the half time for the viscosity change is 56 minutes and that for the spectral change is 55 minutes; in 1% protein the respective half times are 49 and 43 minutes; and in 0.5% protein they are 31 and 25 minutes. The two properties appear to reflect essentially the same molecular change.

The measurements of the rate of the spectral change in 8 M urea cover a 40-fold range of protein concentration. A plot of the half time against the protein concentration gives, at low protein concentrations, a direct proportionality between protein concentration and half time (Fig. 6). At higher protein concentrations the half time increases less rapidly with concentration, and there is a possibility that at very high protein concentrations the half time may approach a finite limit. It is interesting, and perhaps significant, that in a plot of $c/t_{\frac{1}{2}}$ *vs.* c (Fig. 6) the points fall reasonably well along a straight line, showing that the results fit approximately the relationship

$$t_{\frac{1}{2}} = \frac{ac}{b + c} \qquad (1)$$

where $t_{\frac{1}{2}}$ is the half time at protein concentration c, and a and b are constants. A plot of the half time against the square root of the protein concentration (Fig. 6) also gives roughly, but not precisely, a straight line with an intercept of −3.7 minutes at zero concentration; this plot has dubious physical significance, however, because of the negative time intercept.

A similar effect of protein concentration on the rate was also observed in 10 M urea. The curves

for E_s *vs.* time in 10 M urea were similar in form to those shown in Figure 3 except that the change proceeds much more rapidly. The ratio of the half time at 1% hemoglobin to the half time at 0.1% is 3.4 in 10 M urea as compared with 3.7 in 8 M urea. In 10 M urea, as in 8 M urea, the sigmoid shape is more pronounced at the higher protein concentration.

Effect of pH.[2]—Oxyhemoglobin was denatured in both 8 M urea and 10 M urea over a *p*H range from 5.8 to 8.2 in order to determine the *p*H at which the rate of disappearance of the 542 mμ band was a minimum. (The experiments were performed in sodium phosphate buffers with $HPO_4^=$:$H_2PO_4^-$ ratios that varied from 0:10 to 9:1; the protein concentration was 0.1%.) At both urea concentrations there was a maximum in the half time between *p*H 6.7 and 6.9. Within the experimental error, this is the same as the isoelectric point, *p*H 6.8 in water, and it is also the same as the *p*H at which Lewis (1926, 1927) found the rate of thermal denaturation of beef oxyhemoglobin to pass through a minimum. In both 8 M and 10 M urea the rate approximately doubles when the *p*H is raised or lowered by one unit from *p*H 6.8.

Effect of Buffer Concentration.—The effect of altering the buffer concentration in 8 M urea was examined by observing the rate of the spectral change when the protein concentration was 0.1% and the buffer contained equal parts of $HPO_4^=$ and $H_2PO_4^-$. The half time was found to change from 6.4 minutes when the phosphate concentration was 0.025 M to 4.7 minutes when the phosphate concentration was 0.10 M. The variation is not great, and the buffer concentration was kept at 0.05 M in all other experiments.

Effect of Temperature.—The effect of temperature on the rate of the spectral change was studied between 14° and 30° in 8 M urea at *p*H 7.3 and a protein concentration of 0.1%. The half time decreased linearly with increasing temperature over the entire temperature range, from a value of 16.4 minutes at 15° to 5.8 minutes at 30°. There is no maximum in the half time at a temperature of about 20°, as is characteristic of the urea denaturation of ovalbumin (Simpson and Kauzmann, 1953) and tobacco mosaic virus (Lauffer, 1943).

Rate of Production of Insoluble Protein.—Figure 7 shows the production of protein insoluble in dilute buffer at *p*H 6.8 as a function of time of exposure to 8 M and 10 M urea. Weighable amounts of insoluble material are not produced during the first 20 minutes in 8 M urea and during the first 10 minutes in 10 M urea. (Some turbidity could, however, be seen on dilution of the urea solutions with buffer during the initial 10–20 minutes.) The initial "induction period" is followed by a rapid increase in the amount of precipitable protein, which then gradually levels off. After 70 minutes in 10 M urea and after 100 minutes in 8 M urea little further change is observed. When the changes are completed only about 50% of the original material can be precipitated on dilution. The rate of denaturation as measured by this technique increases with the urea concentration and decreases with increasing protein concentration, thus paralleling the viscometric and spectrophotometric results. Values of the half time for the solubility change brought about by 8 M urea agree well with those found in the spectral and viscometric studies: in 1% hemoglobin the half time for the solubility change was 46 minutes, as compared with 43 minutes for the spectral change and 49 minutes for the viscosity change. In 2% hemoglobin the respective half times were 58 minutes, 55 minutes, and 56 minutes. In 10 M urea, on the other hand, the half time for the solubility change, 25 minutes at a protein concentration of 1%, was somewhat greater than the half times observed for the spectral change (14 minutes) and the viscometric change (16 minutes) at the same protein concentration.

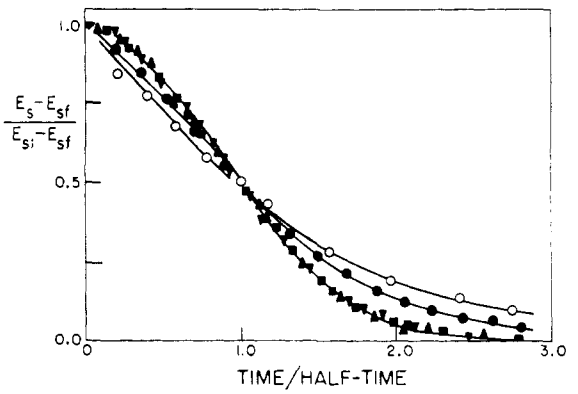

FIG. 5.—Effect of protein concentration on the shapes of the curves of spectral absorption at 542 mμ *vs.* time. Relative change in absorption is plotted against the ratio of the elapsed time to the half-time. Data from Figure 3. Key to points: Protein concentration 0.05%, O; 0.1%, ●; 0.5%, ▲; 1.0%, ▼; 2.0%, ■.

The fact that only about half of the protein would precipitate after long exposure to urea is very interesting. Possibly the denaturation reaction is reversible under the conditions of our experiment. (The reversibility of hemoglobin denaturation was long ago demonstrated by Mirsky and Anson.) It is also possible that our assay procedure precipitated only one of the two types of chains (α and β) present in hemoglobin.

Effects of Chemical Changes in the Hemoglobin.—Beef methemoglobin was denatured in urea under the same conditions as those described for oxyhemoglobin. The reaction was followed by both viscometric and spectrophotometric methods. The spectra of native methemoglobin and of

[2] In making *p*H measurements no effort was made to correct for junction potentials and other effects introduced by urea. These effects are unimportant for our purposes, however, because we wish only to show that our denaturation studies were made under conditions of relative insensitivity to *p*H changes.

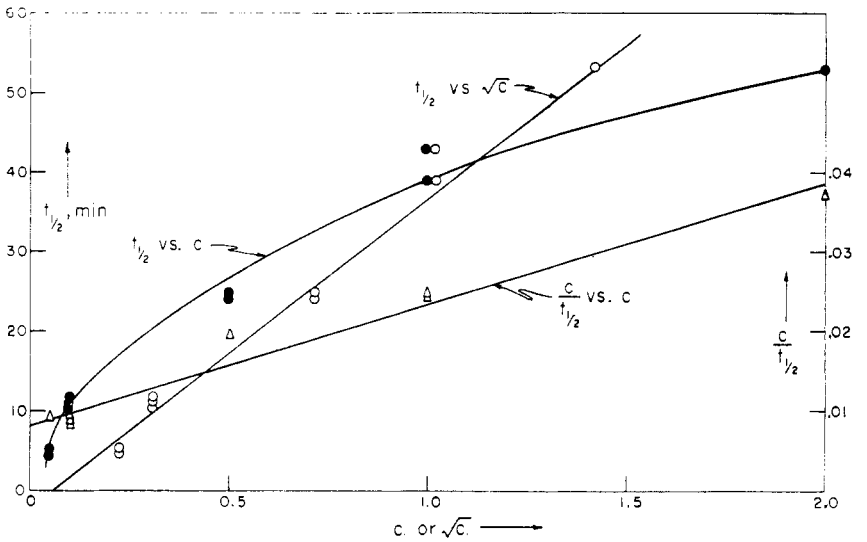

FIG. 6.—Dependence of the half-time of reaction on the protein concentration. Data from Figure 3. Three different types of plot are given: (1) $t_{1/2}$ *vs.* c, (2) $t_{1/2}$ *vs.* $\sqrt{c}$, and (3) $c/t_{1/2}$ *vs* c where c is the protein concentration in per cent.

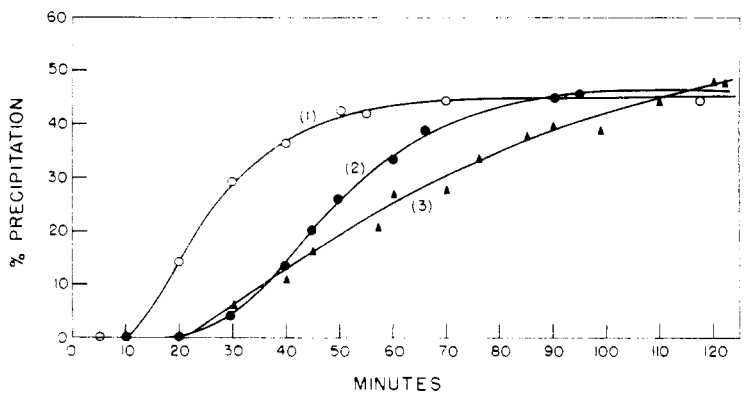

FIG. 7.—Rate of formation of hemoglobin insoluble in dilute buffer at the isoelectric point. Curve (1), urea concentration 10 M, protein concentration 1%. Curve (2), urea concentration 8 M, protein concentration 1%. Curve (3), urea concentration 8 M, protein concentration 2%. Buffer, 0.05 M phosphate, *p*H 6.9, 30°, during exposure to urea.

methemoglobin denatured in 8 M urea differ appreciably between 500 and 600 mμ, E_s for the native protein being greater than that for the denatured protein below 540 mμ, whereas the reverse is true above 540 mμ. The change in E_s with time was observed at 500 mμ, where the difference was greatest (E_s = 0.55 for the native protein and 0.39 for the denatured protein).

The kinetics of the urea denaturation of methemoglobin differs from that of oxyhemoglobin in two respects: (1) There seems to be no effect of the protein concentration on the rate, the curve for E_s *vs.* time in 1% protein being identical with that for 0.1% protein. The denaturation of methemoglobin in 8 M urea at *p*H 6.9 has a half time of about 5 minutes, as measured both spectrally and viscometrically. (2) The curves of E_s or η_{red} *vs.* time gave no indication of a sigmoid shape, and a plot of $\log[E_s(t) - E_s(\infty)]$ against the time gave a straight line, $E_s(t)$ and $E_s(\infty)$ being the values of the specific extinction at time t and at the end of the reaction respectively. Thus the urea denaturation of methemoglobin appears to be of the first order with respect to the protein. This was also found by Chalopin and Colson-Guatalla (1949) in 4 M urea.

The final value of η_{red} for 1% methemoglobin in 8 M urea is the same as that observed for oxyhemoglobin under the same conditions.

The rate of denaturation of cyanomethemoglobin in urea was studied viscometrically. The rate of change of the viscosity was very much slower than that observed for oxyhemoglobin, the half time being 660 minutes in 8 M urea at *p*H 7.2. No sigmoid shape was observed, and the half time was the same at 1% protein as at 0.5%.

The rate of change of the viscosity of a 1%

solution of carboxyhemoglobin was measured in 8 M urea at *p*H 7.2. A sigmoid shape was not observed; there was an initial rapid increase in viscosity with time, followed by a much slower further increase. The initial rapid phase amounted to about 25% of the total change, and its half time was about 40 minutes. The remaining 75% of the change had a half time of 1000 to 1500 minutes. The initial and final reduced viscosities were nearly the same as for oxyhemoglobin at the same urea and protein concentrations.

Oxidation State of the Iron in the Denatured Protein.—A standard method of testing the oxidation state of the iron in denatured hemoglobin is by observing the spectrum in slightly alkaline solution (*p*H 8–9). If the heme iron is in the plus three state parahematin is formed. It is characterized by the absence of sharp absorption bands in the spectral region between 500 and 600 mμ. Heme iron in the plus two state forms globin hemochromogen, which has a characteristic sharp absorption band at 550 mμ (Haurowitz and Hardin, 1954; Drabkin and Austin, 1935b; Keilin, 1926), and a characteristic color. Parahematin is easily converted to hemochromogen with mild reducing agents, such as sodium dithionite.

Urea-denatured oxyhemoglobin treated with sodium dithionite in slightly alkaline solution gave the typical hemochromogen spectrum, whereas if treatment with dithionite was omitted, the denatured protein gave the parahematin spectrum. This shows that the iron is oxidized from the ferrous to the ferric state during denaturation by urea. It is significant that the spectral absorption curves of denatured oxyhemoglobin and denatured methemoglobin are practically identical.

Discussion

Apparent Reaction Order with Respect to Urea.—A log-log plot of the urea concentration against the half time for the spectral change of a 0.1% hemoglobin solution gives a straight line of slope 5 for urea concentrations between 6 M and 10 M (see Fig. 8). A similar plot for the half times in 1% hemoglobin solutions gives a straight line between 8 M and 10 M urea, parallel to the line obtained at 0.1% hemoglobin. At urea concentrations below 6 M the spectral change is more rapid than would be expected from extrapolation of the linear log-log plot obtained between 6 M and 10 M urea. Thus in 4 M urea the half time for the spectral change of a 0.1% hemoglobin solution is about 60 minutes, whereas the extrapolation of the log-log plot to 4 M gives a half time of 200 minutes. This discrepancy probably results from the conversion of hemoglobin into methemoglobin, a process that causes a similar spectral change in E_s at 542 mμ and may be accelerated by lower concentrations of urea without significant unfolding. This conclusion is supported by the viscometric observations, which show that in a 1% hemoglobin solution in 4 M urea there is no time-dependent unfolding at all in 3000 minutes.

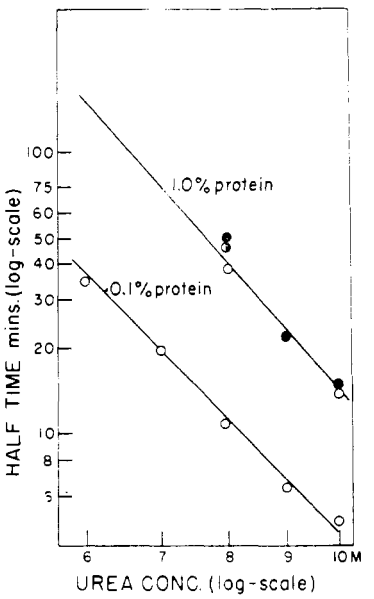

FIG. 8.—Effect of urea concentration on the rate of reaction. Conditions as in Figures 1 and 2. Half-times from spectral data, ○; from viscometric data, ●; from precipitation data, ◐.

Since the log-log plot of half time *vs.* urea concentration for 1% hemoglobin solutions leads to an expected half time in 4 M urea of about 1000 minutes, the unfolding reaction itself does not give a linear log-log plot below 6 M urea.

Mechanism of the Denaturation Reaction. 1. General Considerations on the Mechanism.—A satisfactory reaction mechanism must account for two unusual features of the denaturation process in oxyhemoglobin: (a) the induction period at higher protein concentrations, and (b) the decrease in rate with increasing protein concentration. An induction period in a denaturation reaction has, to our knowledge, not yet been reported,[3] but several examples of complex kinetics in denaturation processes are known (Wright and Shomaker, 1948; Chase, 1950), including several in which the rate decreases with increasing protein concentration (Lauffer, 1943).

These two observations imply a mechanism which has the following characteristics: (a) two reaction paths along which denaturation can occur, a slower one favored at higher protein concentration and a faster one favored at lower protein concentration, and (b) several reaction intermediates.[4]

[3] A referee has pointed out that an induction period in the acid denaturation of ferrihemoglobin azide has been reported by Steinhardt and Beychok (1960).

[4] It is also possible to account for the induction period by an autocatalytic effect in which the denatured protein accelerates the unfolding of the native molecule. This possibility was tested by adding denatured protein to the reaction mixture at the same time as the native protein was added to the urea solution. No catalytic effect by the denatured protein was observed.

A generalized reaction scheme which satisfies these requirements can be written as follows:

$$\begin{array}{ccc} P & \rightleftharpoons & P' \\ \text{(Path A)}\downarrow & & \downarrow\text{(Path B)} \\ Q & \longrightarrow & D \end{array} \qquad \text{(I)}$$

where P represents the native form of the protein molecule, Q and P' are intermediates, and D represents the denatured form. The forms P and P' denature *via* different paths, P denaturing along path A through the intermediate, Q, to give the denatured form D, and P' denaturing along a separate, more direct route (path B) to yield the denatured form D.

It is assumed that path A is followed at higher protein concentrations whereas at lower protein concentrations path B is preferred. The reaction sequence in path A is necessary in order to account for the induction period at the higher protein concentrations. The intermediate Q in path A will lead to an induction period if the reactions $P \rightarrow Q$ and $Q \rightarrow D$ proceed at about the same rate and if the properties of Q resemble those of the reactant P more nearly than they do those of the final product D. The properties of the intermediates Q and P' can be surmised to some extent from the experimental data and will be considered below.

The effect of protein concentration on the reaction can be accounted for in either of the following ways: (a) the reaction $P' \rightarrow D$ may be of a smaller reaction order with respect to the protein than the reactions $P \rightarrow Q$ and $Q \rightarrow D$, or (b) the step $P \rightleftharpoons P'$ may involve a bimolecular process such as dissociation of the native molecule. In each of these cases the equilibrium $P \rightleftharpoons P'$ must be assumed to be rapidly established as compared with the rate of the step $P' \rightarrow D$.

Perhaps the simplest way in which case (b) may be realized is by means of a reversible dissociation of the hemoglobin molecule, in which an undissociated form denatures slowly via path A and a dissociated form denatures more rapidly via path B. Since hemoglobin is known to undergo reversible dissociations of various kinds it is well to digress at this point and discuss the known dissociation behavior before proceeding with further detailed consideration of possible mechanisms.

2. Dissociation of the Hemoglobin Molecule.—Hemoglobin from certain animal species is known to dissociate reversibly in urea solutions into two parts having molecular weights of 34,000. Steinhardt (1938) has shown by ultracentrifugal measurements that horse carbonyl hemoglobin dissociates in 2 to 6 M urea and that the dissociated fragment exhibits the same spectrum as the undissociated molecule. The reaction is reversible, and the reconstituted product is the same as the native molecule except for slight differences in oxygen-binding properties. Steinhardt also noted that after long periods of time in urea solutions a small amount of insoluble material was produced, which was attributed to slow denaturation of the dissociated form. Wu and Yang (1932) have differentiated by osmotic methods between the hemoglobins of beef and horse, which dissociate in 2 to 6 M urea solutions, and those of sheep and dog, which do not.

Steinhardt (1938) has shown that in 2 to 6 M urea solutions the equilibrium $H_4 \rightleftharpoons 2H_2$ lies in favor of the dissociated form, which under the reaction conditions used in our experimental work should be the predominant species during initial stages of the reaction. (In the above equilibrium H refers to a quarter of a hemoglobin molecule, associated with a single heme group.) The dissociated form of the molecule goes on to denature, and, to quote Steinhardt, the molecule in the state H_2 has gone "one step toward denaturation."

In view of these facts there can be little doubt that the beef oxyhemoglobin molecule is split into at least two parts under the conditions used in our work. The reaction mechanism previously outlined must therefore involve the half-molecular form. No limitation in the interpretation of the data arises from the dissociation step since the half-hemoglobin molecule shows the same spectral properties, as well as almost the same oxygen-binding characteristics. The viscosimetric properties of the half-molecule may, however, differ slightly from those of the native form; the initial instantaneous increase in η_{red} on placing the protein in urea may be attributed to this splitting.

Reichmann and Colvin (1956) have shown by ultracentrifugal and osmotic methods that horse hemoglobin splits reversibly into four parts in solutions whose *p*H is less than 4, the quarter units having molecular weights of about 17,000. (Gutter *et al.*, 1956, 1957, have also found evidence for splitting into quarter units in the case of horse and human carbonyl hemoglobins in urea solutions in the presence of mercaptoethanol.) The splitting into quarters in acid does not occur as rapidly or to as great an extent as the dissociation into half-molecules. Some insoluble material is reported to be present after treatment with acid, but this could be the result of denaturation reactions that follow the splitting and does not necessarily mean that the dissociation into quarter-molecules is inherently irreversible. Hasserodt and Vinograd (1959) find that dissociation of hemoglobin also takes place at *p*H values greater than 11. Exposure of hemoglobin to low (Itano and Singer, 1958; Vinograd *et al.*, 1959) or high (Charlwood *et al.*, 1960) *p*H is used to induce dissociation preliminary to hybridization, and a great deal is being learned in this way about the nature of the dissociation reaction, which does not seem to be a simple one (Itano and Singer, 1958).

3. Possible Detailed Mechanisms. a. Mechanism Involving Dissociation of the Hemoglobin Molecule.—One mechanism which is at least

qualitatively consistent with the data involves dissociation of the half-molecule, H_2, into two fragments, and can be written as:

$$H_2 \underset{\text{rapid}}{\rightleftharpoons} 2H$$

$$\text{(Path A) } k_3 \downarrow \qquad \downarrow k_2 \text{ (Path B)} \qquad \text{(II)}$$

$$Q \xrightarrow[k_4]{} D$$

To make the mechanism consistent with the observations the equilibrium $H_2 \rightleftharpoons 2H$ must be rapidly established and must be in favor of the associated (half-hemoglobin) form of the molecule H_2, rather than the dissociated (quarter-hemoglobin) form H, at all urea and protein concentrations used in this study. In view of the work of Reichmann and Colvin and of Gutter *et al.* (1956, 1957) concerning the possibility of splitting hemoglobin into quarter-molecules, this would not appear to be unlikely.

Reaction mechanism II, involving the half-molecular form, is seen to be analogous to the general mechanism I, with the forms H_2 and H replacing P and P' respectively. The half-molecular form H_2 denatures *via* path A, characterized by the rate constants k_3 and k_4, through the intermediate Q to the denatured form D. The quarter-molecular form H denatures *via* the independent path B, with rate constant k_2, to yield the denatured form D. The slow reaction steps in this case are assumed to be first-order with respect to the protein, and the magnitudes of the rate constants lie in the order $k_2 > k_3 \approx k_4$.

From the equilibrium reaction $H_2 \rightleftharpoons 2H$ it can be seen that as the protein concentration is increased an increased fraction of the material would be present in the associated form H_2. As the protein concentration increases an increasing fraction therefore denatures by way of the slower reaction path A, the intermediate Q in reaction path A accounting for the induction period noted at higher protein concentrations.

If this mechanism is correct, and if appreciable amounts of H are present in the most dilute protein solutions studied, then η_{red} and E_s for H and H_2 must be practically identical because the observed initial values of η_{red} and E_s do not change with protein concentration over the range studied. As the protein concentration is increased the slope of the curve of E_s *vs.* time during the initial period of the reaction is seen to decrease (Fig. 3 and Table I); according to the above mechanism, this indicates that the spectral properties of Q must also be very similar to those of H_2. The slopes of the η_{red} *vs.* time curves during the initial period are possibly somewhat larger (Fig. 4), indicating that the hydrodynamic properties of Q may differ slightly from those of H_2.

Unfortunately the equations for the above mechanism cannot be solved analytically because the bimolecular step $H_2 \rightleftharpoons 2H$ introduces a non-linear differential equation.

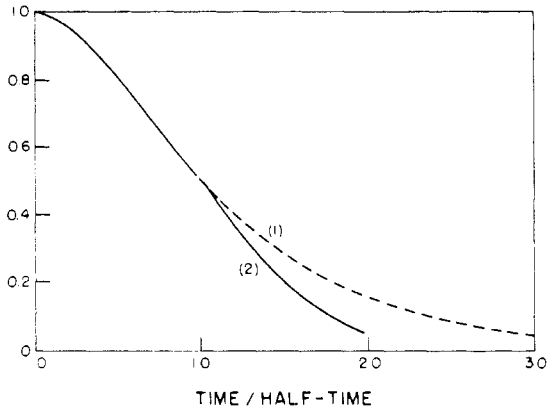

FIG. 9.—Comparison of observed change of spectral absorption with calculated change for a reaction sequence $A \overset{k_1}{\rightarrow} B \overset{k_2}{\rightarrow} C$. Solid curve from the data of Figure 5 at protein concentrations of 0.5%, 1%, and 2%. Dotted curve calculated assuming $k_1 = k_2$.

TABLE I

DEPENDENCE OF INITIAL SLOPE IN 8 M UREA ON PROTEIN CONCENTRATION

(Data from Experiments Similar to Those in Figure 3)

c = Protein Concentration (%)	Initial $\Delta E_s/\Delta t$	$c(\Delta E_s/\Delta t)$
2.0	0.015	0.03
1.0	0.020	0.02
0.5	0.035	0.018
0.1	0.16	0.016
0.05	0.43	0.022

Figure 5 shows the form that the spectrophotometric curves assume at different protein concentrations when they are plotted so that all curves pass through the same point at 50% reaction. It is seen from Figure 5 that the results for 0.5, 1.0, and 2.0% protein are almost identical when plotted in this way. It is easily shown that the reaction sequence in path A will give a curve of the same type if the absorbance of Q is identical with that of H_2 and if the rate constants, k_3 and k_4, are similar in magnitude. Figure 9 is a plot of the fractional change in spectral properties as a function of reduced time for a reaction scheme $H_2 \rightarrow Q \rightarrow D$ assuming that H_2 and Q have the same value of E_s and that the values of k_3 and k_4 are equal. For comparison, the actual data from Figure 5 for the higher protein concentrations are included. It is seen that the observed points fall close to the theroetical curve up to $t/t_{1/2} = 1.0$. At longer times, however, the observed rate of change is greater than that expected from the theoretical curve. Such a deviation would be expected because the equilibrium $H_2 \rightleftharpoons 2H$ should shift to the right as the native protein disappears; in the later stages of the reaction a larger fraction of the reaction will therefore tend

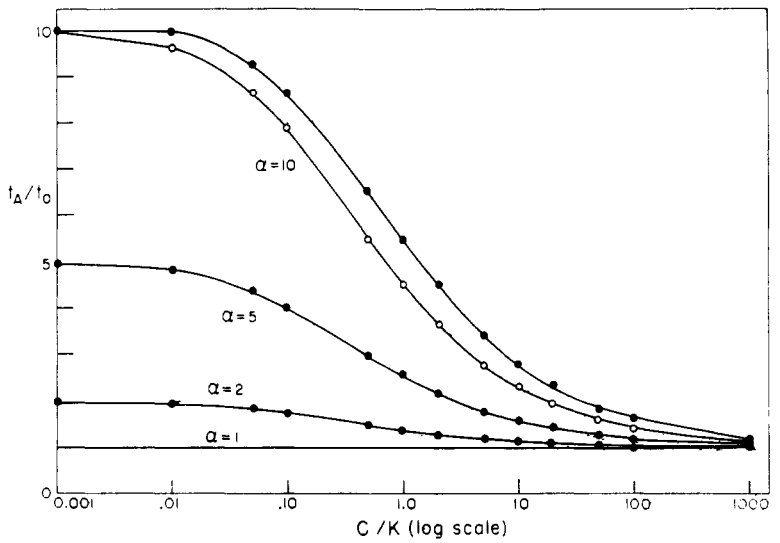

FIG. 10.—Variation of half-time for mechanism II with protein concentration. t_A and t_B are the half-times for paths A and B of mechanism II, t_0 is the over-all half-time assuming the equilibrium to be frozen as mentioned, K is the equilibrium constant for the reaction $H_2 \rightleftharpoons 2H$, c_0 is the protein concentration, and $\alpha = t_A/t_B$. Curves with solid points give t_A/t_0 assuming that the equilibrium $H_2 \rightleftharpoons 2H$ is frozen at the point corresponding to the start of the reaction. Curve with open points calculated for $\alpha = 10$ assuming this equilibrium to be frozen at the point corresponding to half reaction. The correct curve for $\alpha = 10$ lies between the two curves with open and closed points.

to go by way of path B, which is more rapid than path A.

According to reaction scheme II, the half-time of reaction should approach constant values at very high and very low protein concentrations, the value at high concentrations being that of path A, and the value at low concentrations being that of path B. Inspection of the data (Fig. 6) reveals that neither limit is reached over the range of protein concentrations employed by us.

It is somewhat surprising that a change in protein concentration by a factor as large as 40-fold is not sufficient to bring the half-time closer than it does to one or the other of the two limiting values. It can be shown, however, that this failure to attain a limiting value of the half-time is consistent with the mechanism that is under discussion. In Figure 10 the ordinate is the ratio t_A/t_0, t_A being the half-time for path A and t_0 being the observed half-time for the over-all mechanism at a protein concentration c. The abscissa in Figure 10 is $\log_{10}(c/K)$, where K is the equilibrium constant for the dissociation reaction, $H_2 \rightleftharpoons 2H$. The curves have been plotted assuming different values for the relative rates of reaction along paths A and B (*i.e.*, assuming different values of $\alpha = t_A/t_B$, where t_A and t_B are the half-times for the two paths). The calculations assume that the ratio of the concentrations of H_2 and H is constant throughout the first half of the reaction, and for all but one of the curves it is assumed that this ratio has the value corresponding to the initial protein concentration. This assumption is not strictly correct, but it is sufficiently accurate to show that t_A/t_0 may change relatively slowly with protein concentration, as is observed.

From Figure 10 it is seen that $\log_{10}(c/K)$ must change by three to four cycles (*i.e.*, c must change by 1000 to 10,000 fold) if t_0 is to change from a value near t_A to a value near t_B. The experimental data in Figure 7 cover only a 40-fold range of protein concentrations, so the fact that neither of the limiting values, t_A or t_b, of the half-time is reached in these experiments is not at variance with mechanism II.

Little of a quantitative nature can be stated about the behavior of the initial slopes of the reaction curves when the protein concentration is changed (Table I). Data during very early stages of reaction are difficult to obtain with existing techniques, and special mixing methods allowing measurement at earlier stages of reaction would be necessary in order to obtain more reliable initial slopes. If the properties of Q are the same as those of H_2, then the initial slopes are due to the small amount of denatured material produced by reaction along path B, and in the limit of high concentration they must approach zero. Furthermore, the initial slopes should vary in proportion to the reciprocal of the square root of the initial protein concentration. This does not agree with the observations, which tend to show that the initial slopes vary more nearly in proportion to the reciprocal of the protein concentration (right hand column of Table I). Better data during the early stages of reaction are necessary to be sure of this point, however.

In general, we feel that the mechanism based on dissociation of the hemoglobin molecule into quarters is satisfactory, at least qualitatively, in explaining the experimental results.

b. Mechanism Involving an Impurity in the Protein Solution.—A second possible interpretation of the results assumes that there is a protective impurity in the hemoglobin stock solutions. This mechanism is similar to one put forth by Lauffer (1943), who sought to explain a similar decrease in denaturation rate with increasing protein concentration found in the urea denaturation of tobacco mosaic virus. It invokes an equilibrium reaction between the impurity, I, and the native form of the molecule, H_2, to form a complex, H_2I, which denatures less rapidly than the uncomplexed protein H_2.

The mechanism may be written as:

$$\begin{array}{ccc} & \overset{\text{Rapid}}{\rightleftharpoons} & \\ H_2I & & H_2 + I \\ \text{(Path A) } k_3 \downarrow & & \downarrow k_2 \text{ (Path B)} \\ Q & \xrightarrow[k_4]{} & D + I \end{array} \qquad \text{(III)}$$

To make the scheme fit the observations it must be assumed that comparable numbers of impurity molecules and hemoglobin molecules are present and that $k_2 > k_3 \approx k_4$. Then as the protein concentration is decreased an increasing fraction of material is present in the uncomplexed form, and consequently path B is favored. As the protein concentration increases, path A becomes more prominent, with a slower over-all rate of unfolding and the appearance of an induction period. Furthermore, arguments similar to those presented by Lauffer (1943) lead one to expect a relationship of the general form of equation (1) between the half-time for the over-all reaction and the protein concentration.

The impurity I must possess the following characteristics: (a) it must be non-dialyzable, and therefore of a high molecular weight, and (b) it must be present in a molar concentration at least equal to the hemoglobin concentration, and therefore in a weight concentration comparable with that of hemoglobin. Ponder (1959) has shown that about 93% of the protein inside the red cell is hemoglobin. Since the hypothecated inhibitor would presumably constitute only a portion of the remaining 7% it is difficult to believe that our observations can arise from this source. Nevertheless, the mechanism cannot be entirely ruled out.

Steinhardt and Zaiser (1951) report a decrease in the rate of the acid denaturation of horse carbonyl hemoglobin with increasing protein concentration. This proved to be due to small quantities of oxygen in the system oxidizing the protein to methemoglobin. It is difficult to see how oxygen could be the hypothetical substance I in scheme (III), because the activity (or partial pressure) of oxygen did not vary when the hemoglobin concentration was varied, all solutions being exposed to air in these experiments.

c. Mechanism Involving an Impurity in the Urea Solution.—A third interpretation consistent with the data assumes the presence of an impurity in the urea solution. The impurity must be present in small amounts and must act either to denature the protein itself or to render it more susceptible to urea denaturation.

This mechanism resembles mechanisms II and III:

$$\begin{array}{ccc} & \overset{\text{Rapid}}{\rightleftharpoons} & \\ I + H_2 & & H_2I \\ \text{(Path A) } k_3 \downarrow & & \downarrow k_2 \text{ (Path B)} \\ Q & \xrightarrow[k_4]{} & D + I \end{array} \qquad \text{(IV)}$$

The impurity I combines rapidly and reversibly with the half-molecule, H_2, to give the complex, H_2I, in accordance with the relationship

$$K = \frac{(H_2I)}{(H_2)(I)}$$

If I_0 is the total concentration of the impurity present in the urea (I_0 is, of course, independent of the protein concentration), then

$$(H_2I) = \frac{K(H_2)I_0}{1 + K(H_2)}$$

If the impurity is very firmly bound by H_2 then except at very low protein concentrations (which are presumed not to have been reached in the present experiments) $(H_2I) \approx (I_0)$. Under these conditions denaturation by way of path B would be of zero order with respect to the protein and denaturation by way of path A would be of first order in the protein. If path A gives a sigmoid reaction curve with zero slope in the initial stages of the reaction, then initially only the spectral changes caused by path B would be observed. The initial slopes of the E *vs.* time curves would then vary inversely with the protein concentration. The data shown in Table I indicate that this is the case.

A number of experiments were performed with urea from different sources and also with urea that had been recrystallized by Steinhardt's method. The urea from different sources gave rates which differed by as much as a factor of two. It was found that a single recrystallization of Baker and Adamson urea by Steinhardt's method decreased the denaturation rate by as much as a factor of two, but repeated recrystallization had no further effect on the rate. Furthermore, the reaction showed the same dependence on urea and protein concentration in recrystallized and in unrecrystallized urea, so that the over-all interpretation of the results is not altered by this evidence. It is possible that elaborate purification techniques with other solvents might reveal a trace impurity not removed completely by recrystallization from alcohol.

Urea is known to be slowly converted into ammonium cyanate in aqueous solutions at room temperature. Since our urea solutions were always prepared from solid urea immediately before use, however, it is hardly likely that appreciable amounts of cyanate were present. Therefore the carbamylation reactions of cyanate described by Stark *et al.* (1960) are probably not a factor in our experiments. It is interesting to note that Steinhardt and Beychok (1960) have observed that cyanate ion stabilizes methemoglobin against denaturation by acid.

Our studies show that methemoglobin denatures more rapidly than does oxyhemoglobin. It might therefore seem possible that a trace impurity is present in the urea which instantaneously oxidizes a fixed amount of oxyhemoglobin and that path B represents the denaturation of the methemoglobin produced in this way. The half-time for the spectral change of methemoglobin (about 5 minutes in 8 M urea) is, however, too large to be able to account for the results at the lowest protein concentrations. (The half time for the spectral change of 0.05% oxyhemoglobin in 8 M urea is 5.2 minutes; since the half-time at this protein concentration varies in proportion to the protein concentration, the half-time for the denaturation of the hypothetical H_2I via path B would have to be much less than 5 minutes.)

d. Evaluation of the Proposed Mechanisms.—It is not possible to decide which of the three mechanisms described above is the correct one, since all agree at least qualitatively with the observations. Mechanism II, involving the dissociation of hemoglobin into quarter molecules, seems, however, to be the most plausible. If it is indeed responsible for the observed behavior this would point up the fact that association enhances the stability of proteins. This might be expected because association should reduce the molecular surface that is exposed to the solvent, and especially that portion of it having the least stability. Such an effect would also be consistent with the general experience of protein chemists, who have found that in the isolation and storage of proteins stability increases as the protein concentration is raised (see Taylor, 1953; Putnam, 1953).

It is worth noting that tobacco mosaic virus, like hemoglobin, is an aggregate of subunits held together by secondary forces. It also shows a marked decrease in its denaturation rate in urea as the protein concentration is raised, suggesting that this feature of denaturation kinetics might be a general one for proteins which show a strong tendency to aggregate.

Viscosity Properties of Denatured Hemoglobin in 8 M Urea.—After standing for a long time in 8 M urea, hemoglobin attains a constant reduced viscosity whose dependence on the protein concentration is given (in units of dl/g) by

$$\eta_{red} = 0.163 + 0.023c$$

If this result is expressed in the form of Huggins' equation,

$$\eta_{red} = [\eta] + k[\eta]^2c$$

we find a value of 0.163 for the intrinsic viscosity, $[\eta]$, and 0.89 for the Huggins constant, k.

The observed intrinsic viscosity is somewhat smaller than values found for other proteins in strong urea solutions. For bovine plasma albumin in 8 M urea at *p*H 10 the intrinsic viscosity is 0.22 when the disulfide cross-linkages are intact, and 0.53 when the cross-linkages have been ruptured by treatment with cysteine (Frensdorff *et al.*, 1953). For ovalbumin intrinsic viscosities of 0.24, 0.33 and 0.37 have been observed, respectively, in 7.5 M urea at *p*H 7.3, 10 M urea at *p*H 7.6, and 10 M urea at *p*H 10.2 (Frensdorff *et al.*, 1953). Part of this difference may arise from the fact that the hemoglobin viscosity was obtained at the isoelectric point, whereas the ovalbumin and plasma albumin values were obtained under conditions in which the molecules were charged. Furthermore, it is quite possible that at infinite dilution in 8 M urea the denatured hemoglobin molecule is split into quarters, giving a molecular weight considerably smaller than that of ovalbumin or plasma albumin under the same conditions. Both the absence of a net charge and the lower molecular weight tend to decrease the intrinsic viscosity of a randomly coiled polypeptide chain.

The value of 0.89 for the Huggins constant of denatured hemoglobin is rather large for a random coil. (Ordinarily high polymers give Huggins constants in the range 0.2 to 0.4.) This result indicates that there is some tendency for the unfolded hemoglobin molecules to aggregate in 8 M urea. This tendency is, however, much less than that shown by ovalbumin, whose Huggins constant is 2.0.

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Thermal Denaturation of the Heart Muscle Preparation with Respect to Its Capacity for DPNH Oxidation*

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The effects of mild thermal treatment of the Keilin-Hartree heart muscle preparation on its catalytic activity toward DPNH oxidation by oxygen, cytochrome *c*, 2,6-dichlorophenolindophenol, and ferricyanide have been studied in the presence and absence of Amytal and antimycin A. DPNH → oxygen and DPNH → cytochrome *c* activities were most sensitive to the thermal inactivation, whereas cytochrome oxidase activity was almost unaffected. Both antimycin and Amytal failed to inhibit completely any of the activities studied. The inhibitor-insensitive activities for various acceptors exhibited markedly different thermal susceptibilities when compared with the corresponding inhibitor-sensitive activities. The results suggested that, on the respiratory chain of the non-phosphorylating system, essentially only one site is available for ferricyanide and this site is insensitive to Amytal and antimycin. There are two sites for cytochrome *c:* one is inhibited by Amytal and antimycin, whereas the other is not. Dichlorophenolindophenol may interact at three sites.

Information about the effect of aging on the oxidative capabilities of particulate preparations from heart muscle is, in general, very meager; this is especially true for the oxidation of DPNH. Slater (1949b) has reported that the succinate oxidase activity of the Keilin-Hartree preparation increases upon storage at 4° for a short period but declines after several days. Recently, Redfearn and Dixon (1961) have shown that the succinate oxidase and succinate phenazine reductase activities of the heart muscle particles prepared with a Waring blendor are unstable even when the preparation is stored at −20°.

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