satisfactory. The quantity $H + h$ presumably represents the difference in the strengths of a few hydrogen bonds between protein and water and between protein and urea. It is difficult to believe that this could be as large as 10 kcal., especially in the light of the small heat effect which Schellman[73] found in the polymerization of urea in water. Furthermore, the remarkable results of Jacobsen and Christensen[50] on the interaction of urea and β-lactoglobulin at 0° and at 20°, if they have any general significance at all, indicate that entirely different denaturation mechanisms may be involved above and below 20°, which is not consistent with our formulation of Hopkins' mechanism. The pH dependence of the rate of heat denaturation of ovalbumin (see Lewis[32]) is also somewhat different from that of the rate of urea denaturation, the range over which the rate is independent of pH being much narrower for the former. This makes somewhat questionable our identification of the constant, k_0, in our model with the rate of heat denaturation measured by Lewis.

The difference in the pressure dependence of the rates of heat and urea denaturation, while not inconsistent with our model, shows that at best water and urea probably do not break hydrogen bonds in the same way. Furthermore, we have seen that there are large and unexplained effects of electrolytes, pH, detergents, and organic substances on the rate of urea denaturation. These would seem to indicate that the rupture of hydrogen bonds is only one of several processes which may control the rate of unfolding of the ovalbumin molecule in urea.

It therefore appears that, in spite of many attractive qualitative arguments for the essential identity of the mechanisms of heat and urea denaturation, we can still not be sure that they do not occur by fundamentally different mechanisms.

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The Kinetics of Protein Denaturation. II. The Optical Rotation of Ovalbumin in Solutions of Guanidinium Salts

BY J. SCHELLMAN, R. B. SIMPSON AND W. KAUZMANN

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The change in rotation of ovalbumin in guanidinium chloride and in mixtures of urea with guanidinium chloride and nitrate has been studied. The behavior closely resembles that found in urea solutions, as shown by a similar dependence of both the rate of change and the final optical rotation on the concentration, the temperature and the pH.

The denaturing action of guanidine salts on proteins is similar to that of urea except that guanidinium ion is effective at much lower concentrations.[1] It was therefore felt desirable to see if the unusual kinetics of the denaturation of ovalbumin by urea[2] also occur with guanidinium salts.

Methods and Materials.—Guanidinium nitrate and chloride were recrystallized from methyl alcohol by the addition of ether.[3] Otherwise the procedures were the same as those described in Part I.[2]

Results and Discussion

Much lower concentrations are required with guanidinium salts than with urea in order to obtain rates of change of the optical rotation which are slow enough to be measurable. Unfortunately these low concentrations of guanidinium salts are insufficient to keep the denatured ovalbumin in solution, so that as the reaction proceeds the solutions become turbid and the rotations cannot be followed indefinitely. The use of dilute protein solutions reduces this tendency somewhat, but also makes the results less precise. Higher concentrations of guanidinium salts, which cause a very rapid increase in the optical rotation, will keep the denatured protein in solution, so that a value of the final rotation, though not of the half time, is readily determined. (There appears, however, to be a very slow decrease in the levorotation after the immediate attainment of $[\alpha_f]$ at these higher concentrations.)

Figure 1 shows the change in rotation with time for ovalbumin in various concentrations of guanidinium chloride at 30°. As with urea the shape of the rotation *vs.* time curve does not fit that of a simple first-order reaction, and at the higher concentrations primary and secondary reactions can be distinguished. The rate of change of the primary reaction depends strongly on the concentrations of guanidinium chloride. If the half time of this primary change is computed in the manner outlined in Part I, a log–log plot of the half time against the concentration of guanidinium chloride gives a straight line having a slope of 13.5 ± 1. (Figure 2; because of the overlapping of the primary and secondary reactions the two points at the lowest concentration in this plot are rather uncertain.) Activity coefficients of guanidinium chloride are not available, so the true reaction order cannot be found but it is undoubtedly close to 13. This is practically the same as the reaction order with respect to urea at this temperature. Guanidinium chloride is, however, effective at about one third the concentrations which have to be used with urea.

The change in rotation in 2.48 M guanidinium chloride also was followed at different temperatures. The results are shown in Fig. 3. These curves are rather difficult to analyse precisely be-

(1) H. Neurath, J. P. Greenstein, F. W. Putnam and J. O. Erickson, *Chem. Revs.*, **34**, 157 (1944).

(2) R. B. Simpson and W. Kauzmann, THIS JOURNAL, **75**, 5139 (1953).

(3) J. P. Greenstein and W. V. Jenrette, *J. Biol. Chem.*, **142**, 176 1942).

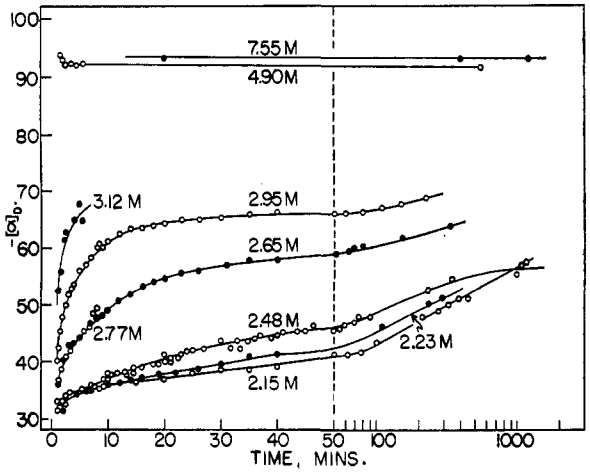

Fig. 1.—Change in optical rotation of ovalbumin in different concentrations of guanidinium chloride at 30° and *p*H 7 to 8 (0.035 *M* sodium phosphate buffer containing 9 parts dibasic salt to 1 part of monobasic salt). Protein concentration 1 to 2%. Numbers on curves indicate molarities of guanidinium chloride.

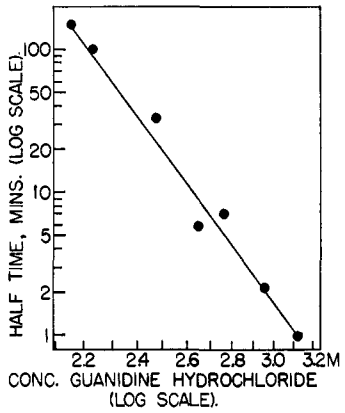

Fig. 2.—Dependence of half time for the change in optical rotation on guanidinium chloride concentration at 30°. (In obtaining these half times final rotations in 2.77 and 3.12 *M* were interpolated from values at other concentrations. Half times in 2.15 and 2.23 *M* are somewhat uncertain.)

cause of the different temperature coefficients of the primary and secondary reactions, and because turbidity makes it difficult to follow the final stages of the reaction. It is, however, apparent that the rate of the primary reaction is much smaller at 20° than at 0° and that it increases again with temperatures above 30°. If half times are computed using the (in this case) more or less arbitrary procedure described in Part I, a curve of temperature *vs.* half time is obtained which bears a striking resemblance to that found for urea (Fig. 4 of Part I[3]). The final rotation also decreases markedly with increasing temperature, as was observed with urea.

It is interesting that the final rotation is independent of the guanidinium chloride concentration above about 4 *M*, and that the rate of change of the rotation is very low below 2 *M*. Greenstein[4] found that the amount of cysteine liberated in 45 minutes from ovalbumin by guanidinium chloride was independent of the concentration above about 4 *M*, and that no cysteine was liberated below 2 *M*.

(4) J. P. Greenstein, *J. Biol. Chem.*, **130**, 519 (1939).

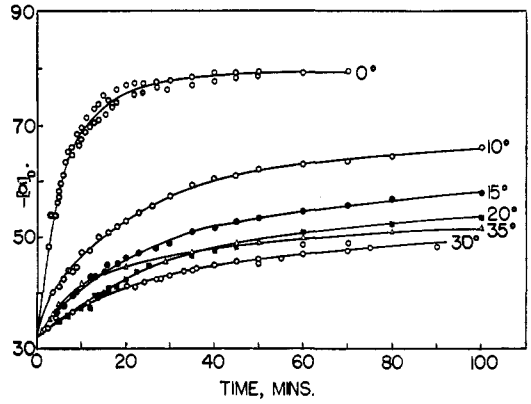

Fig. 3.—Temperature dependence of change in optical rotation in 2.48 *M* guanidinium chloride. Buffer as in Fig. 1, protein concentration 1.47%.

Experiments in unbuffered solutions at *p*H values between 6 and 9 showed that the *p*H dependence of the rate of denaturation in guanidinium chloride is similar to that in urea, there being a broad minimum in the rate in the *p*H range 6.5 to 8. The development of turbidity before the denaturation has progressed very far makes it impossible, however, to interpret the results quantitatively.

In order to study further the effects of guanidine salts, various concentrations of guanidinium chloride and nitrate were added to 6.0 *M* urea, in which the denaturation is very slow but in which the denatured protein is soluble. The effect on the half time at 30° is shown in Fig. 4.

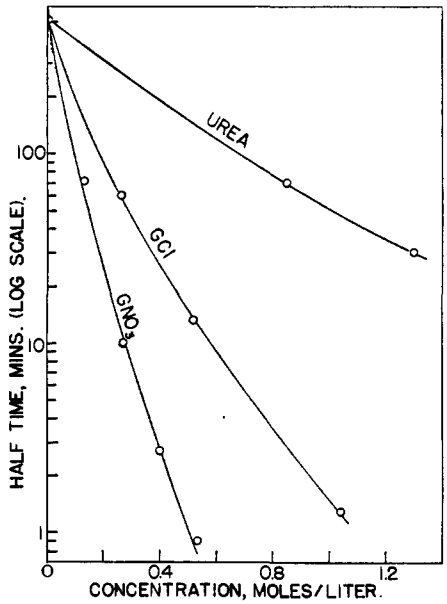

Fig. 4.—Effect of adding urea, guanidinium chloride and guanidinium nitrate on half time for change in optical rotation of ovalbumin in 6.0 *M* urea at 30°. Buffer as in Fig. 1, protein concentration, 1.92%.

The nitrate has a considerably greater effect in reducing the half time than does the chloride. This is in accordance with the similar relative effects observed in urea with sodium nitrate and sodium chlo-

ride.[3] It is interesting that in 0.3 M sodium nitrate the rate is four times greater than that in 0.3 M sodium chloride, whereas in 0.3 M guanidinium nitrate the rate is six times greater than that in 0.3 M guanidinium chloride; the quantitative effects of chloride and nitrate on the rate are apparently not very different with the two cations. The relative effects on the final rotations, however, are not comparable, $[\alpha_f]$ being considerably more negative in guanidinium nitrate than in guanidinium chloride, while neither sodium chloride nor sodium nitrate have much effect on $[\alpha_f]$.

Figure 4 shows that the addition of X moles of guanidinium chloride to a 6 M urea solution has nearly the same effect on the half time as the addition of $3X$ moles of urea to the same solution. This illustrates once again the threefold greater potency of guanidinium chloride over urea as a denaturant. It is consistent with the hypothesis that urea and guanidinium ion attack ovalbumin in the same way (though with different effectiveness), and that molecules of the two substances can replace one another at random in this attack.

It is interesting that at 30° the final specific rotation reached at the highest concentration of either urea or guanidinium chloride or in a combination of the two is not far from −95°. This indicates that the denatured protein has a similar structure in the presence of both substances.

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The Kinetics of Protein Denaturation. III. The Optical Rotations of Serum Albumin, β-Lactoglobulin and Pepsin in Urea Solutions

BY W. KAUZMANN AND R. B. SIMPSON

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The optical rotation of bovine serum albumin increases instantaneously on exposure to urea at all concentrations above two molar. After the initial increase there is no further change with time. The amount of the increase depends on the temperature and the urea concentration and is reversible with respect to changes in both temperature and urea concentration as long as the temperature is kept below 40°. Above 40° complicated changes occur, especially at lower urea concentrations. Small amounts of sodium dodecyl sulfate prevent the increase in optical rotation in urea. The optical rotation of β-lactoglobulin in urea changes in a manner intermediate between ovalbumin and serum albumin; there is an instantaneous change followed by a further change whose rate is very sensitive to the urea concentration and pH. The optical rotation of pepsin is practically unaffected by urea, even at the highest concentrations. This difference in behavior parallels the ability of these four proteins to adsorb dyes. The "configurational adaptability" proposed by Karush to account for the adsorptive power of serum albumin may therefore be a manifestation of a lack of internal rigidity which is also responsible for the ease with which the optical rotation of serum albumin is changed by urea.

The behavior of the optical rotation of ovalbumin in urea solutions has been described in detail in Part I.[1] When a similar study was attempted with other proteins rather different results were obtained; these results will be described in this paper.

Experimental

Crystalline bovine plasma albumin ("serum albumin") was obtained from Armour and Company. The concentration of the stock solution was determined from the optical rotation, assuming a specific rotation with sodium D-light of −61.4°, an average for several solutions prepared by accurately weighing out the constituents according to the procedure of Frensdorff, Watson and Kauzmann.[2] The stock solutions were stored in the refrigerator in the presence of saturated toluene vapor.

β-Lactoglobulin was prepared by the modified method of Palmer suggested by Bull and Currie.[3] It was twice recrystallized. The stock solution contained 0.5% sodium chloride and the concentration of protein was found by drying the solution to constant weight at 100°. The specific rotation was $[\alpha]_D$ −23.7°.

Crystalline porcine pepsin was obtained from Armour and Company. The protein concentration of the stock solution was determined by drying at 100°. The specific rotation of the freshly prepared solution was $[\alpha]_D$ −66°, but the rotation became more positive by nearly 10° on standing for several days.

Results

1. Bovine Serum Albumin.—The addition of urea to serum albumin results in a large change in the optical rotation, but the kinetics of this change are very different from those found with ovalbumin. At all urea concentrations and at all temperatures below 40°, the rotation appears to have reached its final value before the first reading can be made with the polarimeter (*i.e.*, within about one minute after adding the urea). The specific rotation which is attained depends on the urea concentration and on the temperature in much the same way as does the value of $[\alpha]_f$ of ovalbumin (compare Fig. 1 of this paper with Fig. 5 of Part I).

The dependence of the specific rotation in 8.0 M urea on the pH was studied between pH 3 and 11. The specific rotations are independent of the time in this entire range except for a three degree increase in levorotation overnight at pH 10. The specific rotations obtained were similar to those found by Pauli and Kölbl[4] for horse serum albumin in urea, being about 10° more levorotatory at low and high pH than in neutral solution.

To study further the effects of time of standing in urea at various temperatures, the following experiments were performed. Urea–albumin solutions were prepared at 0° and heated and cooled in 20° steps in a sequence of cycles, the rotation being measured at each temperature. On the first cycle the solution was heated to 20°, then returned to 0°. On the second cycle it was heated to 20°, then to 40°, then cooled to 20° and finally to 0°. On the next cycles 60°, 80° and in some instances 95° were

(1) R. B. Simpson and W. Kauzmann, THIS JOURNAL, **75**, 5139 (1953).

(2) H. K. Frensdorff, M. T. Watson and W. J. Kauzmann, *ibid.*, **75**, 5167 (1953).

(3) H. Bull and B. T. Currie, *ibid.*, **68**, 742 (1946).

(4) W. Pauli and W. Kölbl, *Kolloid-Beih.*, **41**, 417 (1935).