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

RECEIVED MARCH 24, 1953

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).

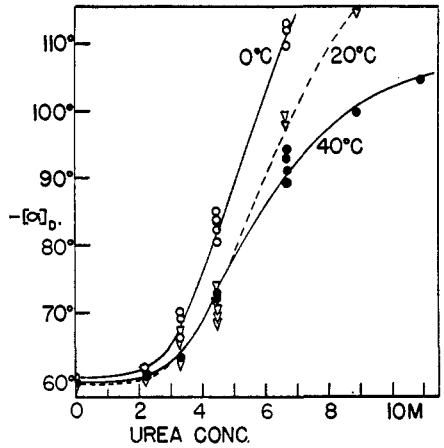

Fig. 1.—Dependence of the optical rotation of bovine serum albumin on urea concentration at 0, 20 and 40°, *p*H 7–8, no buffer.

reached in this way. When the temperature is low and the urea concentration is high the solutions rapidly become turbid.[5] Therefore the measurements at 8.88 and 10.90 *M* urea extend only to 20 and 30°, respectively. In order to make readings at even these temperatures, preliminary heating to higher temperatures is required to eliminate the turbidity.

Typical results of these measurements are shown in Fig. 2. Below 40° the rotation changes reversibly with the temperature, but on heating above 40°, especially at the lower urea concentrations, the rotations on subsequent cooling are increased above their original values. Irreversible changes clearly take place at the higher temperatures. Although the rotation in 2.22 *M* urea is almost the same below 40° as that for the native protein, the behavior on heating above 40° is quite different, showing that even low concentrations of urea accelerate the irreversible reactions at higher temperatures.

The rates of the irreversible changes must have a rather peculiar temperature dependence. For instance, heating in 4.44 *M* urea for three minutes at 40° resulted in practically no change in the rotation at lower temperatures, while heating for two hours at this temperature results in a small increase in levorotation on cooling. On the other hand, subsequent heating to 60° for only two minutes resulted in a somewhat greater increase in the low temperature rotation than did heating for two hours at 40°. Similarly, further heating at 60° for two hours had about the same effect as did two minutes heating at 80°. Thus a 20° increase in temperature seems to increase the rate by about 60-fold. The changes which occur do not, however, seem to follow a first-order law even approximately, since, for instance, the irreversible change during the first two minutes at 60° in 4.44 *M* urea was about the same in extent as that which occurred slowly during the subsequent two hours. It is as if a change which can occur slowly if at all at 40° can occur very rapidly at 60°

(5) The crystallizing agent, decanol, added in the preparation of bovine serum albumin[6] forms a crystalline adduct with urea[7] under these conditions. This adduct is responsible for the turbidity.

(6) E. J. Cohn, W. L. Hughes, Jr., and J. H. Weare, This Journal, **69**, 1753 (1947).

(7) W. Schlenk, Jr., *Ann.*, **565**, 204 (1949).

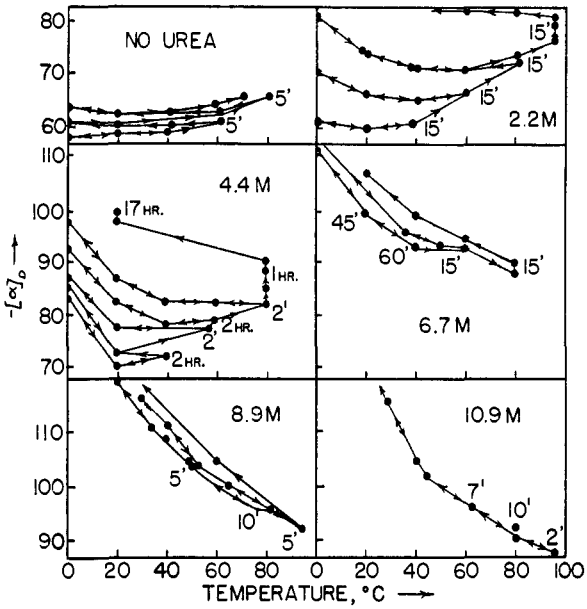

Fig. 2.—Effect of higher temperatures on optical rotation of bovine serum albumin at various urea concentrations. Solutions heated and cooled in cycles as indicated, starting at the lowest temperature, *p*H 7–8 , no buffer. Numbers along curves indicate length of time solutions were held at given temperature before cooling.

while some further change which occurs only slowly at 60° proceeds very rapidly at 80°. A complex series of equilibria or rate processes or both, having large temperature coefficients, seems to be involved here. Further and more complete experiments are necessary to enable us to say anything further, however.

Some experiments were also performed to test the reversibility of the change in the optical rotation with respect to dilution of the urea. A solution of serum albumin in 6.67 *M* urea was allowed to stand for various times and at various temperatures and then diluted to 3.33 *M*. In Table I the resulting specific rotations of the undiluted and diluted solutions are compared with those of a 3.33 *M* urea solution which had been exposed to the same temperatures.

Table I

Behavior of the Optical Rotation of Serum Albumin in Urea

	After 1 min. at 0°	After 1 hr. at 20°	After 130 min. at 40°	After 10 min. at 60°	After 120 min. at 60°
$-[\alpha]^{20}D$ in 6.67 *M* urea before dilution	101°	101°	100°	100°	105°
$-[\alpha]^{20}D$ in 3.33 *M* urea after dilution from 6.67 *M*	66°	69°	79°	80°	86°
$-[\alpha]^{20}D$ in 3.33 *M* urea	62°	63°	63°	68°	74°

In agreement with the findings of Aten, *et al.*,[8] the optical rotation change following exposure to high urea concentration is almost completely reversible on dilution of the urea, provided, however, that the solution in concentrated urea is not exposed to high temperatures. The higher the tem-

(8) H. W. Aten, Jr., C. J. Dippel, K. J. Kenning and J. van Dreven, *J. Colloid Sci.*, **3**, 65 (1948).

perature to which the original 6.67 *M* urea solution of the albumin has been heated and the longer it has remained at the high temperature, the greater is the resulting levorotation at 20° on dilution. This occurs in spite of the relatively small change in the rotation at 20° of the 6.67 *M* urea solution itself following this treatment. The increase is also greater than that which occurs in 3.33 *M* urea after heating to the same temperatures. As was observed in the denaturation of ovalbumin,[1] the reversibility is reduced to a considerable extent by merely allowing the protein to stand in concentrated urea, even though the optical rotation in the concentrated urea is hardly affected.

Duggan and Luck[9] have found that by adding 0.033 g. of sodium dodecyl sulfate (SDS) per gram of serum albumin in the solution, one can prevent the viscosity increase ordinarily obtained when serum albumin is treated with 6.0 *M* urea. Higher detergent/protein ratios do not prevent this increase.

In order to determine the effectiveness of the detergent in preventing the increase in the optical rotation of serum albumin in urea, a solution was prepared containing 0.038% SDS, 1.12% serum albumin, 6.0 *M* urea, and 0.035 *M* sodium phosphate buffer (*p*H 7.6). The urea–SDS solution was prepared first and added to the albumin. The specific rotation was −60 at 30° and remained unchanged for 24 hours. This is nearly the same as the rotation of native serum albumin and much smaller than the rotation of −93° obtained in the absence of the detergent. This result supports Duggan and Luck's theory that small concentrations of SDS prevent the denaturation of serum albumin.

The effect of a much higher detergent/protein ratio was tested by repeating the above experiment with 0.92% SDS (0.82 g. SDS/g. protein). A specific rotation of −78.5° was obtained, decreasing to −77° overnight and remaining constant for two days thereafter. This, while not so large as the rotation in the presence of 6.0 *M* urea alone, is much greater than that of native albumin.

2. **β-Lactoglobulin.**—The changes with time in the optical rotation of β-lactoglobulin at various concentrations of urea are shown in Fig. 3 (30°, 0.05 *M* sodium phosphate buffer (*p*H 6.9) 1.5% protein). The behavior is interesting in that it appears to be intermediate between those of serum albumin and ovalbumin. There is an instantaneous change in rotation as was found with serum albumin, but in 4 and 5 *M* urea this is followed by a time-dependent change whose rate appears to depend strongly on the urea concentration, as was found with ovalbumin. The rate of the latter reaction also appears to depend upon the *p*H, being much more rapid in 5 *M* urea at *p*H 8.7 than at *p*H 6.8.

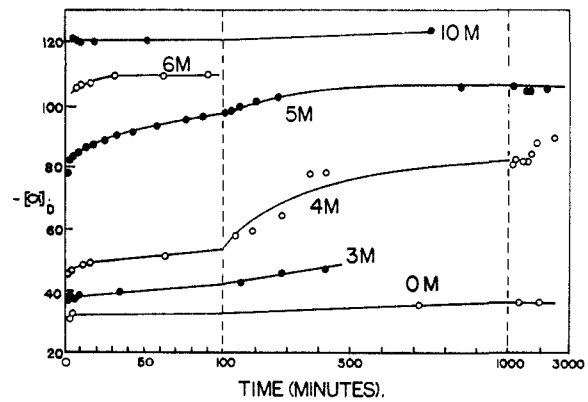

Fig. 3.—Effect of urea on optical rotation of β-lactoglobulin at 30°, *p*H 6.7–7.2 (buffer 0.05 *M* sodium phosphate containing 9 parts dibasic salt to 1 part monobasic salt).

(9) E. L. Duggan and J. M. Luck, *J. Biol. Chem.*, **172**, 205 (1948).

The composite nature of the changes is much more evident here than with either ovalbumin or serum albumin, it being, however, impossible to say at this time whether this is caused by the inhomogeneity of the protein or by the occurrence of a sequence of reactions in the denaturation. Further studies with this interesting protein are clearly called for.

3. **Pepsin.**—Pepsin is entirely different from the proteins previously studied in that its optical rotation is practically unaffected by urea. Even in 8 and 10 *M* urea at *p*H 4.3 and 30° the rotation is nearly the same as in water. This inertness toward urea accords with Steinhardt's finding[10] that pepsin is able to digest proteins in the presence of 4 *M* urea.

Pepsin is, however, known to lose its enzymatic activity very rapidly at *p*H values more alkaline than about 6.[11] It is found that in 0.1 *M* sodium phosphate buffers of *p*H 6.5 to 7.0 the specific rotation becomes more negative by 23°, the rate of the change being very strongly dependent on the *p*H. Urea has a very small effect on the rotation even at these alkaline *p*H values, however, the specific rotation in 10 *M* urea at *p*H 7.0 being only 4° more negative than that in the same buffer in the absence of urea.

Discussion

Ovalbumin, pepsin and serum albumin differ greatly in the ease with which their optical rotations are changed by urea. This dissimilarity must be a reflection of a structural difference between the three proteins, but it is not possible to say very much about the nature of this difference at the present time. It may be a consequence of radically different basic patterns of folding in these proteins, or it may result from relatively minor variations in such details as the number of free polypeptide chain ends or in the number or kind of intramolecular cross-linkages. Nevertheless, similar structural differences, be they major or minor, may well be responsible for much of the diversity in the biological functions of the proteins, and it is interesting to ask if we can correlate our findings for these proteins with any of their other properties.

There is, in fact, a very suggestive similarity between the ease with which the optical rotations of these proteins are changed by urea and their ability to adsorb certain dyes. Klotz and Urquhart[12]

(10) J. Steinhardt, *ibid.*, **123**, 543 (1938).

(11) J. Steinhardt, *Kgl. Danske Videnskab. Selskab. Math.-fys. Medd.*, **14**, No. 11 (1937).

(12) I. M. Klotz and J. M. Urquhart, THIS JOURNAL, **71**, 1597 (1949).

have found that at a given concentration of methyl orange, serum albumin adsorbs about three times as much dye as the same weight of β-lactoglobulin, and that ovalbumin and pepsin show no detectable adsorption of methyl orange. We have found that serum albumin is very easily unfolded by urea while pepsin and ovalbumin are unfolded with much more difficulty and the behavior of β-lactoglobulin is intermediate between those of ovalbumin and serum albumin.

This similarity is interesting in the light of the recent suggestion of Karush[13] that serum albumin is able to change the conformation of its adsorption sites to fit the contours of whatever molecules are available for adsorption. He calls this behavior "configurational adaptability." It is very possible that the ease with which urea unfolds serum albumin is a manifestation of a lack of rigidity in the serum albumin molecule which would also make possible configurational adaptability.

(13) F. Karush, THIS JOURNAL, **72**, 2705 (1950).

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The Kinetics of Protein Denaturation. IV. The Viscosity and Gelation of Urea Solutions of Ovalbumin[1]

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The viscosities of ovalbumin solutions in urea increase very considerably with time. The first stage of this change is caused by the unfolding process which is responsible for the change in optical rotation, but the viscosity continues to increase after the optical rotation has reached a constant value. This difference in behavior is more marked, the higher the protein concentration and is caused by the gradual aggregation of the unfolded protein molecules. The factors affecting this aggregation were studied in two ways: (1) by observing the rate of gelation at high ovalbumin concentrations, and (2) by studying the effect of protein concentration on the viscosity change induced by urea. The effect on aggregation of electrolytes, *p*H, urea concentration and various oxidizing and reducing agents have been investigated. In accordance with the results of Huggins, Tapley and Jenson on serum albumin, it was found that the reaction between sulfhydryl and disulfide groups is an important cause of aggregation. Other factors (electrostatic charges and hydrogen bonds) also seem to play an important role, especially at lower urea concentrations. When the sulfhydryl-induced aggregation is suppressed by *p*-chlormercuribenzoate, the optical rotation and viscosity change at nearly the same rates. Values of the intrinsic viscosity of the unassociated, denatured ovalbumin molecule have been obtained under various conditions. The behavior of the viscosity subsequent to changes in both the protein concentration and the urea concentration has also been studied.

In Part I[3] an account has been given of the kinetics of the change of the optical rotation of ovalbumin in urea solutions. This change is undoubtedly a consequence of the unfolding, or denaturation, of the ovalbumin molecule and it therefore offers a convenient means of studying this interesting process. The structural changes which accompany denaturation are, however, probably very complex and as Neurath, *et al.*,[4] have pointed out, it is therefore desirable to investigate denaturation by studying simultaneously the changes in different properties which depend on the structure of the protein in different ways. The optical rotation depends on the position of the groups in the vicinity of the asymmetric carbon atoms in the protein. A convenient complementary property is the viscosity increment of the protein solution, which is sensitive to the over-all molecular shape and compactness.[5] Whereas a change in the optical rotation of a protein indicates a change in the internal relationships of the atoms, a change in the viscosity of a protein solution indicates a change in the shape of the external envelope of the molecule.

Many workers have noted and studied the very considerable increase in the viscosity of ovalbumin and serum albumin solutions in the presence of urea.[6-11] The increase is often at least partially time-dependent.[11] It furnishes very strong evidence for the generally accepted view that denaturation involves uncoiling of the relatively tightly folded native protein molecule to a more open and extended form.

Using ovalbumin and serum albumin in urea we have found that the viscosity invariably continues to increase with time long after the optical rotation has reached a steady value (compare Fig. 2 of this paper with Fig. 1 of Part I). This paper and the next one are concerned with a detailed examination of this difference in behavior and with its explanation.

There are two possible ways of accounting for such a result: (1) the initial unfolding of the molecule might be followed by a gradual aggregation involving only a few points of contact between molecules; or (2) the initial unfolding might produce a molecule consisting of flexible loops held together by a few intramolecular cross links which then gradually rupture, permitting the molecule to take on more extended shapes (Fig. 1). Neither of these possible secondary changes should affect the optical

(1) This article is based upon the theses submitted by H. K. Frensdorff and M. T. Watson in partial fulfillment of the requirements for the degree of Doctor of Philosophy at Princeton University.

(2) U. S. Public Health Service Predoctoral Research Fellow of the National Cancer Institute, 1951–1952.

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

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

(5) H. Mark and A. V. Tobolsky, "Physical Chemistry of High Polymer Systems," 2nd ed., Interscience Publishers, Inc., New York, N. Y., 1950, Chap. IX.

(6) M. L. Anson and A. E. Mirsky, *J. Gen. Physiol.*, **15**, 341 (1932).

(7) S. C. Liu, *Chinese J. Physiol.*, **7**, 107 (1933).

(8) H. Neurath and A. M. Saum, *J. Biol. Chem.*, **128**, 347 (1939).

(9) H. Neurath, G. R. Cooper and J. O. Erickson, *ibid.*, **142**, 249 (1942); *J. Phys. Chem.*, **46**, 203 (1942).

(10) H. B. Bull, *J. Biol. Chem.*, **133**, 39 (1940).

(11) J. M. Luck, *J. Phys. Colloid Chem.*, **51**, 229 (1947).