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The Kinetics of Protein Denaturation. I. The Behavior of the Optical Rotation of Ovalbumin in Urea Solutions[1]

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The optical rotation of ovalbumin changes with time in the presence of urea. The change does not follow a simple first-order law. The half time of the change at 30° is inversely proportional to the fifteenth power of the urea concentration and depends only slightly on the ovalbumin concentration. The reaction is about one-tenth as fast at 20° as at either 0 or 40°. Among the other factors whose effects on the reaction have been studied are the following: hydrostatic pressure, *p*H, salts, detergents, sulfhydryl reagents, various organic substances, and changes of temperature and urea concentration during the reaction. The principal conclusions which can be drawn from the observations are these: (1) the change in optical rotation is caused by an intimate attack on the protein by the urea; the mere presence of urea in high concentration in the solvent around the protein does not affect the rotation. (2) The failure to give a first-order law is due either to the existence of a series of stages in the denaturation, or to the inhomogeneity of the native protein. (3) The action of urea is caused by complex formation between ovalbumin and urea rather than by a less localized, non-stoichiometric interaction of the solvent with the electric fields around the protein. (4) It is therefore theoretically justifiable to regard the denaturation of ovalbumin as a true fifteenth-order reaction with respect to urea at 0°. (5) The explanation given by Hopkins for the unusual temperature dependence of the rate can be given quantitative expression. The numerical values of the constants required by this analysis do not seem entirely reasonable if, as is usually assumed, urea acts by breaking hydrogen bonds within the protein. There may be a fundamental difference in the mechanisms of heat and urea denaturation. (6) Many processes in addition to the rupture of hydrogen bonds may control the rate of unfolding of ovalbumin in urea.

There are many reasons for believing that most of the remarkable biological and chemical properties of proteins result in some way from the special patterns in which polypeptide chains are folded in native proteins. If this be true, then one of the major problems of protein chemistry is the understanding of the principles of protein folding. This difficult problem is as yet hardly touched by experiment in spite of much speculation. One obvious approach to it is the study of the process of unfolding the protein molecule—a process which is undoubtedly responsible for much of the somewhat ill-defined group of phenomena called "denaturation."[2-5] We have therefore investigated this process, giving particular attention to its kinetics and following simultaneously the changes in different properties which depend in different ways on the structure of the protein molecule—a procedure recommended by Neurath, Greenstein, Putnam and Erickson in their review of denaturation.[4] In this paper we report the results of the study of the optical rotation. The results of studies with other properties will be reported in later papers.

The optical rotation is sensitive to relatively subtle changes in structure[6-9] and is readily measured without interfering with the course of a chemical reaction. Therefore it seems an ideal property with which to investigate the kinetics of denaturation. Studies of the behavior of the optical rotation of proteins on denaturation have been made by Young,[10] who observed an increase in the specific rotation on denaturation by exposure to sunlight. Holden and Freeman[11] and Pauli and co-workers[12] observed that the optical rotations of proteins generally increase on denaturation by heat, urea,

(1) This article is based upon a thesis submitted by Richard B. Simpson in partial fulfillment of the requirements for the degree of Doctor of Philosophy at Princeton University.

(2) H. Wu, *Chinese J. Physiol.*, **5**, 321 (1931).

(3) A. E. Mirsky and L. Pauling, *Proc. Nat. Acad. Sci. U. S.*, **22**, 439 (1936).

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

(5) M. L. Anson, *Advances in Protein Chem.*, **2**, 361 (1945).

(6) W. Kuhn, in K. Freudenberg, "Stereochemie," Berlin, 1932-1933, p. 317.

(7) W. Kuhn and K. Freudenberg in Eucken, "Hand- und Jahrbuch der Chemischen Physik," Vol. 8, No. 3, 1936.

(8) W. Kauzmann, J. Walter and H. Eyring, *Chem. Revs.*, **26**, 339 (1940).

(9) E. Hückel, *Z. Elektrochem.*, **50**, 13 (1944).

(10) E. G. Young, *Proc. Roy. Soc. (London)*, **B93**, 253 (1922).

(11) H. F. Holden and M. Freeman, *Australian J. Exp. Biol. Med. Sci.*, **7**, 13 (1930).

(12) (a) W. Pauli and R. Weiss, *Biochem. Z.*, **233**, 381 (1931); (b) W. Pauli and W. Kolbl, *Kolloid-Beih.*, **41**, 417 (1935); (c) W. Pauli and L. Hoffmann, *ibid.*, **42**, 34 (1935).

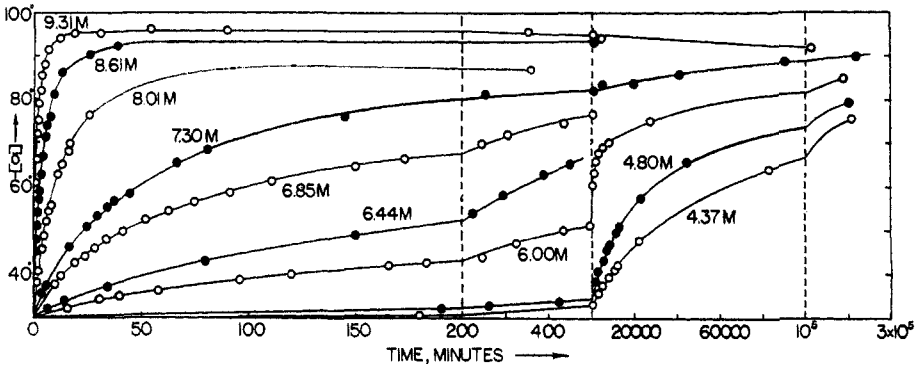


Fig. 1.—Change in the optical rotation of ovalbumin in different concentrations of urea at 30°. Buffer: 0.035 *M* sodium phosphate containing 9 parts dibasic salt and 1 part monobasic salt (*p*H 7.7–8.0, depending on the urea concentration). Protein concentration, 1.5 to 3.5 g./100 ml. solution. Numbers give the molarity of urea. Time scale changes at 200, 500 and 100,000 minutes.

acid or alkali. Barker[13] made a study of the effects of heat denaturation on the optical rotation of ovalbumin. Aten, *et al.*,[14] have found that the increase in the levorotation of serum albumin caused by urea and by heat are reversed on renaturing the protein. The optical rotation has been used by Christensen[15] and by Groves, Hipp and McMeekin[16] to follow the denaturation of β-lactoglobulin, and recently Jirgensens and co-workers[17] have studied the behavior of the optical rotation of a large number of proteins during denaturation. Christensen[15b] gives other references to early work in which the optical rotation was used to study denaturation.

Materials and Methods.—Reagent grade urea was used in most of the experiments. There was no detectable difference in behavior when recrystallized urea was employed. When the *p*H of a buffer mixture is given, it is the *p*H of the 0.2 *M* buffer in water.

The ovalbumin was prepared by the methods of Sørensen and Høyrup,[18] LaRosa,[19] Heidelberger,[20] and Kekwick and Cannan.[21] Most of our work was with protein prepared by the last method. The protein was crystallized from three to five times, dried in air at room temperature, and stored in a desiccator. In preparing a stock solution the dried crystals, which contained a large amount of salt, were dissolved and dialyzed against tap water followed by distilled water. Electrodialysis was employed in two instances with no apparent advantage; simple dialysis is evidently sufficient to reduce the free sulfate to a concentration at which it has no effect on the reaction rate.

In much of the work the *p*H of the stock solution was adjusted by adding sodium hydroxide in one of the following ways: a small dialysis bag containing 1 *N* NaOH was dipped into the protein solution until the *p*H had reached the desired value; or 1 *N* NaOH was added to the solution through a fine capillary which was used as a stirrer. Neither of these methods was felt to be entirely satisfactory, however, and in later work (including all work reported in subsequent papers in this series) the *p*H was brought to the desired value before each measurement by adding buffers.

The protein content of the stock solutions was determined from the optical rotation assuming $[\alpha]_D$ −30.0° for ovalbumin. In some instances it was also checked by determining the dry weight of protein in a given volume of solution.[22] The stock solutions were kept in a refrigerator under saturated toluene vapor.

Stock solutions of the proteins and solutions of the reagents were brought to the reaction temperature before mixing. The first reading of the optical rotation could easily be made within one minute of mixing.

A Schmidt and Haensch polarimeter was used. The monochromator was set to the wave length of the sodium D-line.

In experiments with ovalbumin at temperatures other than 0°, water from a constant temperature bath was circulated through a jacketed polarimeter tube. The temperature of the protein solution in the tube was constant to within 0.1° or less during most of the experiments. In experiments at 0° an unjacketed polarimeter tube packed in ice was used.

All urea and buffer concentrations are expressed on a molar basis. The necessary density data for the urea solutions were obtained from the International Critical Tables and from the data of Gucker, Gage and Moser[26] with the further assumption that the partial specific volumes of the buffers and protein are the same in urea solutions as in water.

Results

The optical rotation of ovalbumin changes very considerably with time in concentrated solutions of urea (Fig. 1). The change seems to take place in two fairly distinct stages, which will be called the primary and secondary reactions. For instance, in 7.0 *M* urea (Fig. 1) the primary reaction would be said to be over after about 500 minutes, while the secondary reaction consists in the smaller change which continues for more than 10,000 minutes. In most of what follows we shall be concerned with the behavior of the primary reaction, since the secondary reaction is frequently so slow that it is rather difficult to study, and since complications

(13) H. A. Barker, *J. Biol. Chem.*, **103**, 1 (1933).

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

(15) (a) L. K. Christensen, *Nature*, **163**, 1003 (1949); (b) L. K. Christensen, *Compt. rend. trav. lab. Carlsberg, Sér. chim.*, **28**, 39 (1952).

(16) M. L. Groves, N. J. Hipp and T. L. McMeekin, THIS JOURNAL, **73**, 2790 (1951).

(17) J. Jirgensens, *J. Polymer Sci.*, **3**, 635 (1948); **4**, 545 (1949); **5**, 179 (1950); **6**, 477 (1951); *Arch. Biochem.*, **39**, 261 (1952).

(18) S. P. L. Sørensen and M. Høyrup, *Compt. rend. trav. lab. Carlsberg, Ser. chim.*, **12**, 213 (1917).

(19) W. LaRosa, *Chemist Analyst*, **16**, No. 2, 3 (1927).

(20) M. Heidelberger, "An Advanced Laboratory Manual of Organic Chemistry," Chemical Catalog Co., N. Y., 1932, p. 83.

(21) R. A. Kekwick and R. K. Cannan, *Biochem. J.*, **30**, 232 (1936).

(22) The value of −30.0° for the specific rotation of ovalbumin is slightly lower than that given in the literature. Young[23] gives −30.0° and cites other values from −28.6° to −31.6°. Jessen-Hansen[24] showed that the protein concentration as well as the *p*H have only a slight effect on the rotation and gave values which agree well with those of Young. Almquist and Greenberg[25] also report rotations between −30 and −31°. Our measurements indicate a specific rotation within a few tenths of a degree of −30.0°.

(23) E. G. Young, *Proc. Roy. Soc.* (*London*), **B93**, 15 (1922).

(24) H. Jessen-Hansen, *Compt. rend. trav. lab. Carlsberg*, **16**, 10 (1927).

(25) H. J. Almquist and D. M. Greenberg, *J. Biol. Chem.*, **105**, 579 (1934).

(26) F. T. Gucker, F. W. Gage and C. E. Moser, THIS JOURNAL, **60**, 2583 (1938).

are likely to arise in its interpretation because of the slow hydrolysis of urea. There is, however, no reason to believe that the secondary reaction is basically different from the primary one.

With urea concentrations up to at least 7.3 *M* the optical rotation immediately after mixing the protein with the urea is identical with that of the native protein in water ($[\alpha]_D$ −30°).

A final rotation for the primary reaction, $[\alpha]_f$, usually can be estimated because of the marked difference in rates of the primary and secondary reactions. When log ($[\alpha]_f - [\alpha]$) is plotted against t ($[\alpha]$ being the specific rotation measured at time t), straight lines are not obtained (Fig. 2). Thus even during the primary reaction the change in rotation does not follow a simple first-order law.

As a measure of the reaction velocity it is convenient to define a "half time for the primary reaction" in the following way: A provisional value of $[\alpha]_f$ is estimated for the primary reaction. The total change in rotation in the primary reaction is thus ($[\alpha]_f + 30$). The time required for the rotation to change by half this amount is then found. This gives a provisional value of the half time of the primary reaction (denoted by $t_{1/2}$). A new provisional value of $[\alpha]_f$ is now taken as the rotation reached at a time equal to twenty times the above value of $t_{1/2}$. From this new value of $[\alpha]_f$ a new half time is found. The process is repeated until steady values of $[\alpha]_f$ and $t_{1/2}$ are obtained; the "convergence" is found to be very rapid except perhaps at low urea concentrations, where the primary and secondary reactions are more difficult to distinguish and where, therefore, the procedure is admittedly somewhat more arbitrary. In general, however, nearly the same values of $t_{1/2}$ are obtained by choosing $[\alpha]_f$ at multiples of $t_{1/2}$ differing from twenty by several-fold.

Figure 2 shows plots of log ($[\alpha] - [\alpha]_f$) against the ratio of the elapsed time to the half time for various conditions of denaturation. The shapes of the resulting curves are in most instances nearly the same, the exceptions being the curves obtained at 0° and at 65° (which deviate less from the simple first-order straight line than do the normal curves), and those obtained in the presence of detergent and iodine (which deviate from simple first-order behavior considerably more than the others).

Because of the constancy of both the rotation at the start of the reaction and the shapes of the reaction curves, the half time and the final rotation provide an adequate and convenient means of describing the effects of most changes in conditions on the kinetics of denaturation.

Variations between Different Albumin Preparations.—As a convenient standard set of conditions for comparing various stock ovalbumin solutions we have used 7.30 *M* urea, 2 to 3% protein, 0.035 *M* sodium phosphate buffer (*p*H 7.6), and a temperature of 30.0°. These conditions give a half time of about 30 minutes, which allows accuracy of measurement and yet is not inconveniently long. Thirty-seven such runs were made with ten different stock solutions prepared from six batches of crystalline ovalbumin. It was found that the mean deviation of the half times for runs with a given

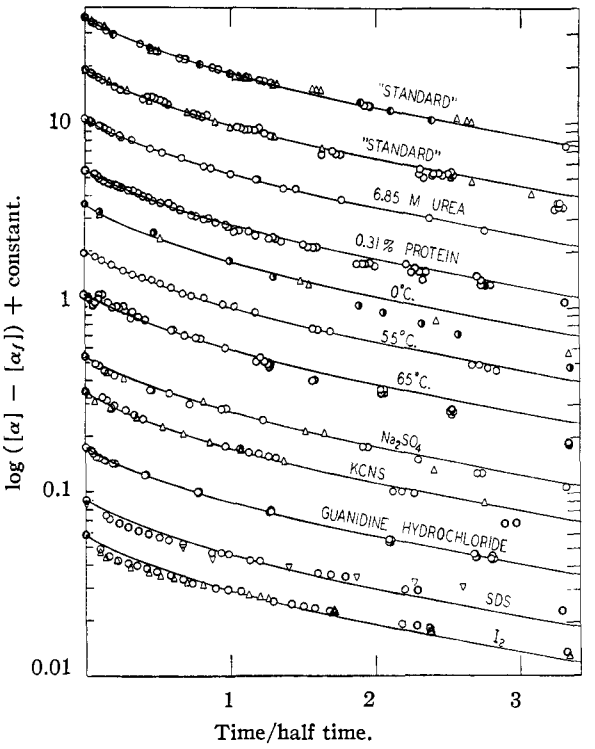


Fig. 2.—Effect of variations of the conditions of denaturation on the kinetics of the change in optical rotation of ovalbumin. Except where otherwise indicated, the urea concentration is 7.3 *M*, the protein concentration is 2 to 3%, the temperature is 30°, and the buffer is the same as in Fig. 1. The curves are all drawn with the same shape: 0°, 6.08 *M* urea (◑) and 5.58 *M* urea (△); 55°, 4.44 *M* urea; 65°, 1.52 *M* urea; Na_2SO_4, 0.3 *M* Na_2SO_4 in 8.86 *M* urea (△) and 0.6 *M* Na_2SO_4 in 10 *M* urea (○); KCNS, 0.1 *M* KCNS in 6 *M* urea (△) and 0.3 *M* KCNS in 6 *M* urea (○); guanidine hydrochloride; 2.47 *M* guanidine hydrochloride, no urea; SDS, 0.00134 *M* sodium dodecyl sulfate (SDS) in 6 *M* urea (▽) and 0.00537 *M* SDS in 6 *M* urea (○).

stock solution from the average for that stock solution was only about 3%. There was, however, as much as a 30% variation in the half times of different stock solutions, showing that in the preparation of these solutions there is some source of irreproducibility. A similarly consistent but much smaller variation in $[\alpha]_f$ also was observed. Therefore, in comparing half times and final rotations in experiments with different stock solutions we have multiplied the results from one stock solution by the appropriate ratio of the average half times and average final rotations of the stock solutions under standard conditions. Although this procedure is rather arbitrary, it seems to improve slightly the consistency of the results. It might, however, have been dispensed with without affecting the conclusions we shall draw from this work.

No significant or consistent changes in half time or final rotation were ever observed when stock solutions were allowed to stand a month or more, even when a luxuriant growth of microörganisms had occurred between measurements.

This variation may have been caused by the mode of addition of sodium hydroxide to the stock solutions.

Effect of Urea Concentration on the Rate.—The half time of the primary reaction shows an extremely strong dependence on the urea concentration (see Fig. 3). Thus, when the urea concentration is increased from 7.3 to 8.16 *M* (a change of only 18%) the half time at 30° decreases by a factor of ten. The dependence is somewhat less strong at higher temperatures.

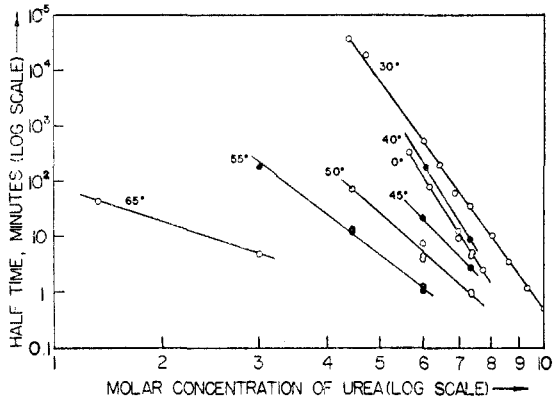


Fig. 3.—Effect of urea concentration on the half time for the change in the optical rotation of ovalbumin in 7.3 *M* urea at different temperatures. Buffer as in Fig. 1.

The rate of the primary reaction is somewhat more sensitive to the urea concentration than is that of the secondary reaction. As a result, at low urea concentrations (below 6 *M* at 30°) the two processes become difficult to separate and the measurement of the half time and final rotation of the primary reaction becomes perhaps objectionably arbitrary.

Effect of Temperature.—The half time for denaturation in 7.30 *M* urea is plotted against the temperature in Fig. 4, and the temperature dependence of the final rotation, $[\alpha]_f$, is shown in Fig. 5. The strong maximum in the half time at about 20° is very striking.[27]

Effect of Protein Concentration.—The values of $[\alpha]_f$ and of the half time both appear to increase slightly with decreasing concentration. The increase in the half time in 7.30 *M* urea at 30° amounts to about 50% for a twenty-fold decrease in protein concentration (from 6.0 to 0.3 g./100 ml.). The final rotation became more negative by about 5 degrees for the same change in protein concentration. Barker[13] found that the final specific rotation of heat denatured ovalbumin became *less* negative with decreasing protein concentration, the effect being considerably larger than that which we have observed.

Effect of *p*H.—Ovalbumin has its maximum stability toward urea denaturation near *p*H 8.[29] The exact *p*H of the minimum denaturation rate is somewhat difficult to determine, however, since during the urea denaturation of unbuffered ovalbumin the *p*H drifts toward approximately *p*H 7.5

(27) The urea–ammonium cyanate equilibrium and the subsequent hydrolysis of cyanate to ammonia and carbon dioxide may have some effect on the rate at higher temperatures. It may be deduced from the data of Warner,[28] however, that at most only a few per cent. of the urea will hydrolyze in a day even at 65°.

(28) R. C. Warner, *J. Biol. Chem.*, **142**, 705 (1942).

(29) H. Wu and E. Yang, *Chinese J. Physiol.*, **5**, 301 (1931).

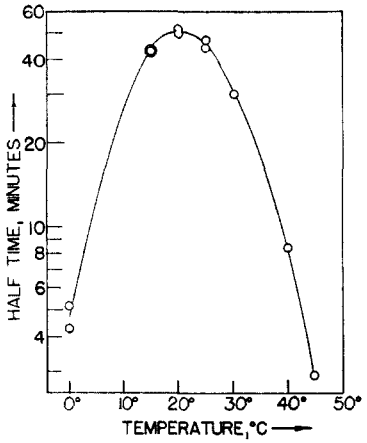


Fig. 4.—Effect of temperature on the half time for the change in optical rotation of ovalbumin in 7.3 *M* urea. Buffer as in Fig. 1.

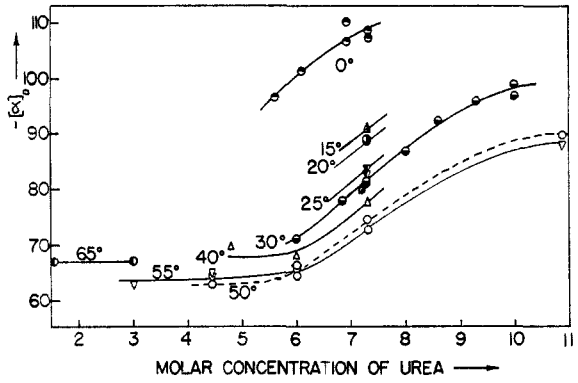


Fig. 5.—Effect of temperature and urea concentration on the final optical rotation of ovalbumin. Buffer as in Fig. 1.

while buffers have specific effects on the rate.[29] The precise significance of *p*H measurements in urea solutions may also be questionable.[30]

The results of some experiments on the effect of *p*H on the half time in buffered and unbuffered solutions are shown in Table I. In the unbuffered solutions the *p*H was measured simultaneously with the rate of change of optical rotation and a mean *p*H value was estimated; these results are of only semi-quantitative significance.

TABLE I

DEPENDENCE ON *p*H OF THE HALF TIME FOR THE CHANGE IN OPTICAL ROTATION OF OVALBUMIN IN 7.30 *M* UREA AT 30.0°

Buffer	*p*H of buffer in water	*p*H of urea soln.	Half time,[a] min.
None	...	5.5	3
None	...	6.0	7
None	...	6.6	15
None	...	7.3	22
Phosphate	6.8	7.3	17
Phosphate, borate, veronal	7.6	8.0	20
p-Nitrophenol	8.0	8.4	22
Borate	8.8	9.2	18

[a] Extrapolated value for zero buffer concentration.

The half times in buffered solutions were obtained by making measurements at several buffer concen-

(30) N. F. Burk and D. M. Greenberg, *J. Biol. Chem.*, **87**, 197 (1930).

trations and extrapolating to zero buffer concentration. Three different buffers were studied at *p*H 8.0, and although they had very different effects on the rate, the extrapolated half times at zero buffer concentration were identical.

These results show that the rate of urea denaturation is almost independent of the *p*H between *p*H 7 and 9 at 30°. Experiments at 0° showed that the rate in 6.25 *M* urea is the same at *p*H 7.0 and 7.8.

Chick and Martin[31] and Lewis[32] found that the velocity constants for the thermal coagulation rate of ovalbumin varied in a similar way with the *p*H, except that the minimum rate was at *p*H 6.76 and the dependence of the rate on *p*H at either side of the minimum was much greater. Since the addition of urea to an albumin solution of *p*H 6.76 shifts the *p*H in the direction of *p*H 8, the *p*H of minimum denaturation rate is apparently quite similar for urea and heat denaturation.

Lewis[32] showed that the decrease in the first-order rate constant with time during heat denaturation in the absence of buffer could be accounted for quantitatively by the change in *p*H during denaturation. In urea denaturation, however, the similar decrease in the first-order rate constant which we have observed is too great to be accounted for by the change in *p*H. Furthermore, the rate constant decreases with time in almost the same way in unbuffered solutions and in buffered solutions, for which there is very little change in *p*H during denaturation.

The final rotation depends rather strongly on the *p*H. In 7.3 *M* urea at 30° $[\alpha]_f$ is −70° at *p*H 5.5, −80° at *p*H 6, −86° at *p*H 7, and −89° at *p*H 9. A similar though smaller increase in levorotation with *p*H was also observed by Pauli and Kölbl.[12b]

The work reported in this paper is concerned only with the reaction in the range over which the rate is independent of the *p*H. Studies also have been made at more acid and more alkaline *p*H, but these will be discussed elsewhere.

Effect of Pressure.—The effect of pressure on the course of the urea denaturation of ovalbumin was studied by exposing solutions of ovalbumin in 6.25 *M* urea to 610 atmospheres of hydrostatic pressure for various lengths of time at 0° and at 40°, and then releasing the pressure and following the subsequent changes in optical rotation.[33] The results are shown in Fig. 6. The effect of pressure at 0° is very different from that at 40°; at 0°, 610 atmospheres accelerate the reaction by a factor of approximately five (equivalent to a contraction in going to the activated state amounting to 60 ml. per mole),[34] and somewhat higher rotations are reached. At 40° pressure is practically without effect.[35]

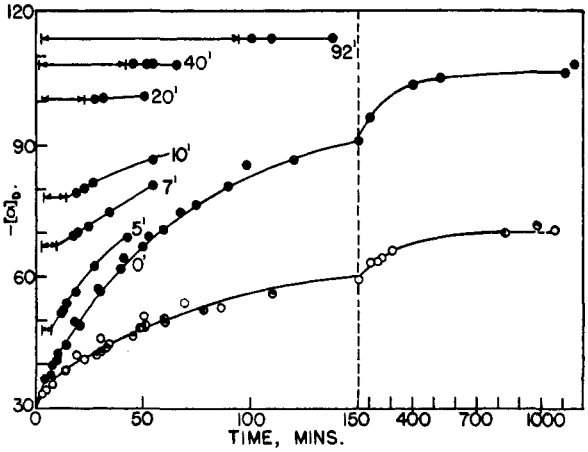


Fig. 6.—Effect of pressure on the rate of change of the optical rotation of ovalbumin in 6.25 *M* urea at 0° and at 40°. 0.05 *M* sodium phosphate buffer of same composition as in Fig. 1. Hydrostatic pressure of 9000 lb./sq. in. applied to solution for the intervals indicated by numbers and horizontal arrows, subsequent changes in optical rotation measured. Solid points (●), results at 0°. Open and partially filled circles, results at 40°: no pressure (○); 9000 lb./sq. in. between 3′ and 23′ (◓); 9000 lb./sq. in. between 4′ and 44′ (◐). Time scale changes after 150 min.

Johnson and Campbell[37] have observed that the heat coagulation of ovalbumin in water at 65° is retarded by the application of pressure. The absence of any effect at 40° may therefore be a result of competing positive and negative effects of pressure at this intermediate temperature. It is interesting in this connection that there is an over-all decrease in volume on urea denaturation at 30° as measured in a dilatometer. Christensen[15b] has observed a decrease in volume of 612 ml. per mole during the urea denaturation of β-lactoglobulin. One of us (WK), working at the Carlsberg Laboratory,[38] observed that in the denaturation of ovalbumin by 6 to 8 *M* urea at 30° there was a decrease in volume amounting to about 300 ml. per mole which took place concurrently with the change in the optical rotation. Evidently the over-all volume change is much greater than that which occurs in going to the activated state, and at higher temperatures it may even be opposite in sign.

Effect of Salts.—Both the rate of change of the optical rotation and the final rotation are affected by salts, but the shape of the rotation *vs.* time curves is generally unaffected (Fig. 2). It was found with sodium sulfate, sodium phosphate (*p*H 7.6), potassium phosphate (*p*H 6.8), sodium borate (*p*H 8.0 and 8.8), *p*-nitrophenol (*p*H 8.0), and veronal (*p*H 8.0), that plots of the logarithm of the half time against the salt concentration gave straight lines. The slopes of these lines (along with the slopes estimated from measurements at single concentrations of other substances, assuming that these

(31) H. Chick and C. J. Martin, *J. Physiol.* (*London*), **40**, 404 (1910); **45**, 61 (1912).

(32) P. S. Lewis, *Biochem. J.*, **20**, 978 (1926).

(33) The equipment used in this experiment was generously provided by Prof. F. H. Johnson. We also wish to thank Elizabeth Flagler Kauzmann for technical assistance in this part of the work.

(34) See S. Glasstone, K. Laidler and H. Eyring, "Theory of Rate Processes," McGraw-Hill Book Co., New York, N. Y., 1941, p. 470.

(35) Phosphate buffers were used in these experiments. Since the reaction $H_2PO_4^- \rightarrow HPO_4^= + H^+$ is accompanied by a decrease in volume of 24 ml. per mole,[26] there is a decrease in the *p*H of the buffers amounting to 0.3 unit when 610 atmospheres of pressure are applied. The experiments on the effect of *p*H on the rate showed, however, that the rate is nearly independent of the *p*H around *p*H 8. Therefore, the pressure effect is not caused by the change in the phosphate dissociation constant.

(36) K. Linderstrøm-Lang and C. F. Jacobsen, *Compt. rend. trav. lab. Carlsberg, Sér. chim.*, **24**, No. 1, p. 28 (1941).

(37) F. H. Johnson and D. H. Campbell, *J. Cellular Comp. Physiol.*, **26**, 43 (1945).

(38) The generosity of Prof. Linderstrøm-Lang in providing facilities at the Carlsberg Laboratory is very gratefully acknowledged.

also obey the logarithmic law) are given in Table II. These slopes give a quantitative measure of the relative effects of salts on the rate of urea denaturation. There appears to be a tendency for multiply charged anions to inhibit the reaction and for multiply charged cations to accelerate it, but this general charge effect is superimposed on much larger specific effects of some of the ions. Sulfate and dibasic phosphate are particularly strong inhibitors, while thiocyanate, magnesium, calcium, veronal and *p*-nitrophenol are even stronger accelerators.

TABLE II

EFFECT OF SALTS ON THE RATE OF CHANGE OF OPTICAL ROTATION OF OVALBUMIN IN UREA AT 30°

(c = salt concentration in moles per liter, $t_{1/2}$ = half time for change in optical rotation, c_m = highest concentration of salt used. *p*H is about 7.6 except where otherwise stated.)

Salt	c_m	$\frac{d \log_{10} t_{1/2}}{dc}$
Sodium sulfate	0.6	4.0
Sodium phosphate (*p*H 7.6)	.25	3.8
Potassium phosphate (*p*H 6.8)	.2	2.8
Potassium ferrocyanide	.03	1.2
Sodium acetate	.3	0.7
Sodium borate (*p*H 8.8)	.25	.4
Sodium borate (*p*H 8.0)	.25	.2
Sodium chloride	.27	.2
Potassium oxalate	.1	.1
Sodium citrate	.05	.0
Sodium nitrate	.3	− 1.8
Barium chloride	.1	− 3.7
Potassium iodide	.3	− 3.7
Magnesium chloride	.1	− 4.2
Sodium barbiturate (*p*H 8.0)	.05	− 4.5
Potassium thiocyanate	.3[a]	− 4.8
Calcium chloride	.1	− 5.4
Sodium *p*-nitrophenolate (*p*H 8.0)	.08	−11.1

[a] At potassium thiocyanate concentrations above 0.3 M, log $t_{1/2}$ does not vary linearly with concentration, the slope in 0.6 M being only about −2.5.

These results parallel those of Burk[39] on the effect of salts on the appearance of sulfhydryl groups in the urea denaturation of ovalbumin. A strong inhibitory effect of both sodium sulfate and sodium chloride on the heat denaturation of ovalbumin was observed by Chick and Martin.[31]

The inhibition by anions and acceleration by cations might suggest that the activated complex is more negatively charged than the native protein. The absence of an effect of the *p*H on the rate in the neighborhood of *p*H 8, however, indicates that such a charge could not arise from the removal of protons. It is possible that protons are shifted from one part of the molecule to another in the activated state, or that a positive charge other than a proton is liberated. We shall see, though, that electrostatic factors do not play an essential role in the urea denaturation of ovalbumin.

The final value of the levorotation is reduced by all the salts studied except KI and $NaNO_3$, which have practically no effect on $[\alpha]_f$, and KCNS, which increases $[\alpha]_f$ slightly. The change in final rotation is linear in the salt concentration and is of the order of 25° mole^{-1} liter for uniunivalent salts and 50° mole^{-1} liter or more for salts containing multivalent ions.

The cause of the depression of the final rotation by salts is complex, as is indicated by the following experiment. Some ovalbumin was denatured in 7.30 M urea at 30° and a final specific rotation of −82° was reached. After 5 days a solution of sodium sulfate in 7.30 M urea was added, bringing the sulfate concentration to 0.62 M. The specific rotation immediately after adding the sulfate was −70° and there was no subsequent change. If ovalbumin were denatured in 7.30 M urea with 0.62 M sodium sulfate present from the very start, the final rotation would be −40°, and even in 10 M urea the final rotation is only −57° in 0.6 M sodium sulfate. Thus, although sodium sulfate causes some change in the rotation of ovalbumin which has already been denatured, it does not have the same effect as if it were present during the denaturation reaction. Therefore, we must conclude that sulfate acts by preventing certain changes in the albumin altogether, or by making possible alternative changes, as well as by changing the properties of the denatured product.

Effect of Detergents.—Desreux and Fabry[40] investigated the effect of Texapon (a mixture of alkyl sulfates with ten to eighteen carbon atoms in the alkyl group) on ovalbumin and found that this detergent increases the optical rotation by an amount independent of the detergent concentration if this is greater than 0.5 g. per gram of ovalbumin. We have found a similar behavior with sodium dodecyl sulfate (SDS) (see Fig. 7).[41] On the addition of more than about 0.3 g. of SDS per g. of ovalbumin at 30° in the absence of urea there is an instantaneous change in the rotation from −30 to −42°, followed by a slow further change of a few degrees over a period of a day.

The effect of SDS in the presence of urea at 30° is unlike the effects of other reagents thus far considered in that the shape of the reaction curves indicates the occurrence of two reactions of widely different rates (Figs. 2 and 8). There is a fast reaction, which one might be tempted to ascribe mainly to the detergent, and a slow reaction, presumably attributable chiefly to urea. These two reactions are not, however, the same as the corresponding reactions in the absence of urea and SDS, respectively, for the initial increase in levorotation here is greater than the increase caused by the detergent alone, and the rate of the subsequent slow increase in levorotation is much greater than the rate in the presence of urea alone.

The value of $[\alpha_f]$ at a given concentration of urea becomes less negative on increasing the detergent: protein ratio (Fig. 7). This decrease also can be brought about by adding SDS to solutions of oval-

(39) N. F. Burk, *J. Phys. Chem.*, **47**, 104 (1943).

(40) V. Desreux and C. Fabry, *Bull. soc. chim. biol.*, **28**, 478 (1946).

(41) Although we have not studied the effect of changing the protein concentration here, we have given our results in terms of the detergent: protein ratio, since it has been shown by Putnam and Neurath[42a] and by Lundgren, Elam and O'Connell[42b] that this ratio rather than the absolute detergent concentration determines the effect on the protein as far as the electrophoretic behavior and the solubility are concerned.

(42) (a) F. W. Putnam and H. Neurath, THIS JOURNAL, **66**, 692 (1944); (b) H. P. Lundgren, D. W. Elam and R. A. O'Connell, *J. Biol. Chem.*, **149**, 183 (1943).

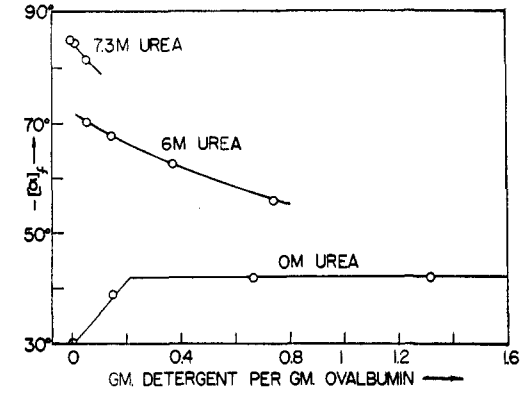


Fig. 7.—Effect of sodium dodecyl sulfate on the final optical rotation of 2.6% ovalbumin in urea at 30°. Buffer as in Fig. 1.

bumin in urea in which the rotation has reached its final value. Addition of 1.8 g. SDS/g. ovalbumin to a solution of ovalbumin which has been standing in 7.3 *M* urea for 1600 min. resulted in an immediate change in the specific rotation from −82.5 to −62.5°. It is possible that this behavior is related to the tendency, observed by Duggan and Luck,[43] for detergents to counteract the denaturing action of urea on serum albumin.

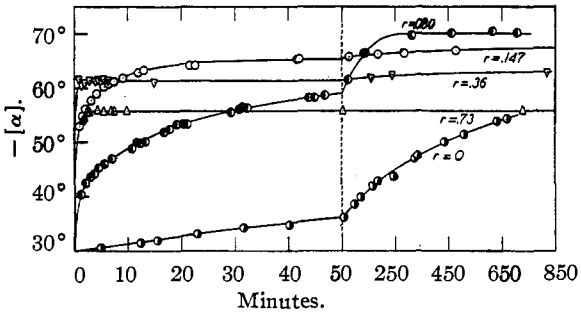


Fig. 8.—Effect of sodium dodecyl sulfate on the change of optical rotation of 2.6% ovalbumin in 6.0 *M* urea at 30°. Buffer as in Fig. 1. The weight ratio of detergent to ovalbumin present in the solution is indicated by *r*. Time scale changes after 50 min.

Effect of Various Organic Substances.—As with the salts mentioned above, it was found that the logarithm of the half time was proportional to the concentration of sucrose. It is reasonable to assume that this logarithmic law is generally true of organic substances, at least at low concentrations. In Table III the effect of various organic substances is expressed in terms of this law for purposes of comparison. (In most instances, however, the effect was investigated at only one concentration.) It is seen that molecules having a large fraction of hydroxyl groups (sucrose, glycerol) inhibit the reaction, while those with more non-polar character (dioxane, ethanol, acetone) accelerate it. The inhibition by sucrose is particularly striking. Beilinsson[44] showed that sucrose and glycerol inhibit the heat denaturation of ovalbumin, and Ball, Hardt and Duddles[45] found that many sugars inhibit the appearance of sulfhydryl groups on heating.

TABLE III

EFFECT OF VARIOUS ORGANIC SUBSTANCES ON THE RATE OF CHANGE OF THE OPTICAL ROTATION OF OVALBUMIN IN UREA

(c = concentration of organic substance in moles per liter, c_m = highest concentration of organic substance used, $t_{1/2}$ = half time of change in rotation, *p*H about 7.6 except where otherwise stated.)

Substance	c_m, *M*	Urea concn., *M*	Temp., °C.	$\frac{d \log_{10} t_{1/2}}{dc}$
Sucrose	0.2	8.9	30	2.6
Glycerol	1.7	8.9	30	0.5
Ethylene glycol	2.4	8.9	30	−0.05
	4.2	7.3	30	−0.08
	4.75	6.0	30	−0.2
	4.75	6.0	0	−0.01
Propylene glycol	1.7	7.3	30	−0.44
Methanol	1.6	7.3	30	−0.88
Ethanol	1.2	7.3	30	−1.55
	1.2	6.4	30	−1.7
	1.2	6.4	0	−0.87
	1.1	6.0	30	−1.9
	1.1	6.0	0	−0.85
Acetone	1.0	6.4	30	−2.1
Veronal (*p*H 8.0)	0.08	7.3	30	−4.5
p-Nitrophenol (*p*H 8.0)	0.08	7.3	30	−11.1

The effect of ethanol and ethylene glycol in accelerating denaturation seems to be greater, the more dilute the urea. Their effect is also much smaller at 0° than at 30°. This result may be related to the well-known fact that the stability of proteins in alcohol is markedly increased at low temperatures.

Effect of Sulfhydryl Reagents.—Various reagents known to react with sulfhydryl groups were added to a 1.5% solution of ovalbumin in 7.5 *M* urea at 30° and *p*H about 7.1 (0.05 *M* phosphate buffer containing equal amounts of mono- and dibasic salts). Sodium cyanide, potassium ferricyanide and sodium sulfite at a concentration of 0.02 *M* had little or no effect on the rate or extent of the change in optical rotation. 0.02 *m* sodium iodoacetate had no effect on the half time but increased the final levorotation by 15°. 0.02 *M* cysteine decreased the half time by 50% and increased the final levorotation by about 10°. When iodine was added to the protein at *p*H 5 in an amount just sufficient to react with all of the sulfhydryl groups, the half time for the change in rotation in urea was reduced by about 50% and there was no change in the final rotation. *p*-Chloromercuribenzoate (PCMB) in amounts just sufficient to react with all of the sulfhydryl groups caused a fourfold increase in the rate of change of the optical rotation but also had no effect on the final rotation.

Of the above-mentioned reagents, it is known that ferricyanide[46] and iodoacetate[47] (which have no effect on the rate of denaturation) react with fewer than half of the sulfhydryl groups of native ovalbumin, while iodine[48] and PCMB[49] (which accelerate

(43) E. L. Duggan and M. Luck, *J. Biol. Chem.*, **172**, 205 (1948).
(44) A. Beilinsson, *Biochem. Z.*, **213**, 399 (1929).
(45) C. D. Ball, D. T. Hardt and W. J. Duddles, *J. Biol. Chem.*, **151**, 163 (1943).
(46) F. G. Hopkins, *Nature*, **26**, 328, 383 (1930).
(47) M. L. Anson, *J. Gen. Physiol.*, **23**, 321 (1940).
(48) M. L. Anson, *Advances in Protein Chem.*, **2**, 369 (1945).
(49) L. R. MacDonnell, R. B. Silva and R. E. Feeney, *Arch. Biochem.*, **32**, 288 (1951).

the rate of urea denaturation) react more or less quantitatively with these groups. Apparently, therefore, the state of the sulfhydryl groups in the native protein may have some effect on the denaturation rate in urea. On the other hand, if either iodine or PCMB is added in excess of the amounts needed to react with all of the sulfhydryl groups, there is a considerable further increase in the rate of change in rotation in urea. This shows that the reaction of these reagents with sulfhydryl groups is not the only means by which they can influence the rate of denaturation. It is even possible that sulfhydryl groups play no part at all in determining the rate of urea denaturation; at most their role seems not to be a predominant one.

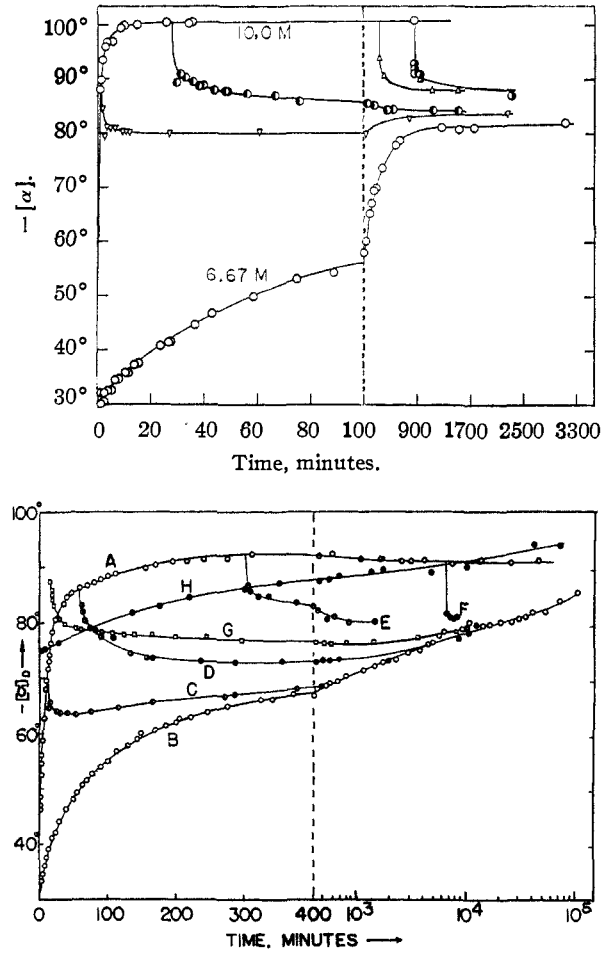


Fig. 9.—Effect of changes in the urea concentration during denaturation at 30°. (a, upper) Dilution from 10 to 6.67 *M* urea after various times. Unbuffered solutions, *p*H 7.5–7.9. Upper and lower curves show the normal behavior in 10 and 6.67 *M* urea. Time scale changes after 100 min. (b, lower) Solutions with 0.05 *M* sodium phosphate buffer (equal parts mono- and dibasic salts; *p*H about 7.1). Time scale linear below 400 min. and logarithmic beyond 400 min.: A, behavior in 8.5 *M* urea; B, behavior in 7.0 *M* urea; C, diluted to 7.0 *M* after 12 min. in 8.5 *M* urea; D, diluted to 7.0 *M* after 60 min. in 8.5 *M* urea; E, diluted to 7.0 *M* after 300 min. in 8.5 *M* urea; F, diluted to 7.0 *M* after 6600 min. to 8.5 *M* urea; G, diluted to 7.0 *M* after 12 min. in 10.0 *M* urea; H, urea concentration increased to 8.5 *M* after 1200 min. in 7.0 *M*.

Effect of Variation of the Urea Concentration during and after Denaturation.—Information concerning the degree of reversibility of the denaturation of ovalbumin is obtainable from a study of the optical rotation on diluting the urea–protein mixtures after various times. In Fig. 9 are shown the results of experiments at 30° in which the urea concentration was changed from 8.5 to 7.0 *M* and from 10 to 6.67 *M*. Similar results were also found on dilution from 10 to 5 *M*. The normal curves obtained at the respective initial and final urea concentrations are also included in the figure.

These experiments show that on dilution the optical rotation tends to revert to values fairly close to those normally found at the lower urea concentration. This means that as far as the arrangements of atoms in the immediate vicinity of the asymmetric centers are concerned, most of the changes brought about in urea denaturation can be reversed by dilution of the urea. The well-known irreversibility of the denaturation of ovalbumin (as compared with, say, serum albumin) therefore depends on changes in the relationships between atoms which are too far apart to affect the optical rotation.

On the other hand, the process of reversing the optical rotation change is complex. For instance, after 12 minutes in 8.5 *M* urea a rotation of −70.5° is reached. On dilution to 7.0 *M* the rotation decreases at first very rapidly, then more slowly, going through a minimum of −63.5° at about 40 minutes after dilution. It then slowly increases, merging with the normal curve obtained in undiluted 7.0 *M* urea after several hundred minutes and eventually reaching a rotation of −80°. Apparently the protein in 8.5 *M* urea having a rotation of −70.5° is not in the same state as the protein having the same rotation in 7.0 *M* urea. Similarly, after one minute in 10 *M* urea a rotation is reached which is much more negative than any value ever reached in 6.67 *M* urea. Yet dilution to 6.67 *M* urea results in a decrease in levorotation followed by a further slow increase, showing that some of the processes which occur in 6.67 *M* urea have not yet taken place after one minute in 10 *M* urea in spite of the high rotation. In both instances some change which had occurred at the high urea concentration seems to be partly reversed on dilution, while other changes continue to go on, though much more slowly, at the lower concentration.

In one experiment the urea concentration was increased to 8.5 *M* after 1200 minutes in 7.0 *M* (at which time the rotation had reached −72.0°). There was an instantaneous increase in the levorotation to −75°, followed by a very slow increase eventually reaching −92°, the usual final rotation in 8.5 *M* urea. The change in rotation on increasing the urea concentration was very much less rapid than that which occurs after the rotation has reached −75° with ovalbumin treated from the start with 8.5 *M* urea. Once again we seem to have a situation in which ovalbumin is in different states even though the optical rotations of these states are the same.

In addition to these indications of complexity during the primary reaction, there is evidence that

further, largely irreversible changes occur on long standing in 10 M urea. These changes are, however, not accompanied by any changes in the optical rotation in 10 M urea. Thus, the optical rotation in 10 M urea is constant after 20 minutes. Yet the rotation eventually reached on dilution of the urea from 10 to 6.67 M is $-84°$ on dilution after 28 minutes, and $-88°$ on dilution after 200 minutes. Similar results were obtained on dilution from 10 to 5 M urea after a series of times between 11 minutes and 49 days.

Effect of Temperature Changes during Denaturation.—Jacobsen and Christensen[50] have made interesting observations on the effect of temperature on the rate of the urea denaturation of β-lactoglobulin as measured by the production of material insoluble in magnesium sulfate solutions. We have performed similar experiments with ovalbumin in urea using the optical rotation instead of solubility to follow the reaction. Solutions of ovalbumin in 7.3 M urea were prepared at 0°. After standing various lengths of time at this temperature they were poured into a jacketed polarimeter tube at 30.0°. The resulting changes in the optical rotation are shown in Fig. 10. Curves for experiments conducted entirely at 0 and 30° are also given. There is a superficial similarity to the results of Jacobsen and Christensen in that part of the increase in rotation at 0° is lost on warming to 30°. It is clear, however, that the denatured ovalbumin produced at 0° is nearly the same as that produced at 30° because if the reaction is permitted to go nearly to completion at 0° and if the solution is then warmed to 30° the rotation is found to be close to its final value for this temperature as well.

In one experiment a solution of the above composition was prepared at 0°, warmed to 30° after 10 minutes, then returned to 0° after 72 minutes. A rotation of about $-85°$ was reached, and this subsequently changed much more slowly than it would with protein which had reached this rotation at 0° without having first been warmed to 30°. This behavior is very similar to that described above, in which, when the urea concentration was increased to 8.5 M after 1200 minutes in 7.0 M, the rotation changes abnormally slowly. In both instances, exposure of the protein to conditions causing less rapid denaturation seems to slow down the changes which occur when more severe conditions are imposed.

Discussion

The Magnitudes of the Initial and Final Rotations.—The optical rotation of ovalbumin immediately after mixing with urea is identical with that of the native protein in water, up to urea concentrations at least as large as 7.3 M (Fig. 1). In 7.3 M urea, 39% by weight of the solution is made up of urea, so the protein is dissolved in a solvent considerably different from water. That the optical rotation of the protein is not changed in this solvent is rather surprising and quite significant. It means that the mere presence of urea in the vicinity of the protein, even in high concentration, is not enough to have any influence on the rotation. The subsequent change in rotation with time must result from a more intimate attack of urea on the protein molecule, and unfolding of the molecule by urea is just such a process.

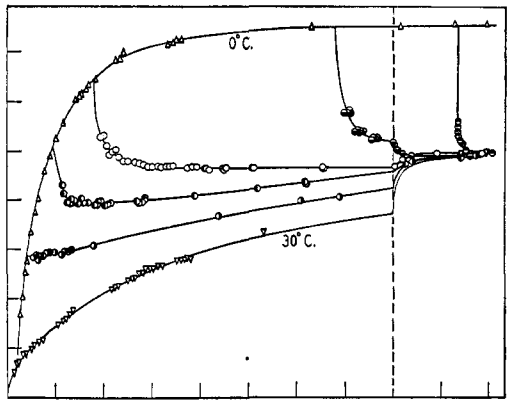


Fig. 10.—Effect of increasing the temperature to 30° after various times at 0°. Urea concentration, 8.3 M. Buffer as in Fig. 1. Upper and lower curves show normal behavior at 0 and 30°, respectively.

When the final rotation is plotted against the urea concentration, an S-shaped curve is obtained which seems to approach definite limiting values at low and high urea concentrations (Fig. 5). As the temperature is increased the curve is shifted to the right and the two limits move closer together. This probably means that the denatured protein can bind urea to give complexes whose optical rotations are considerably greater than that of the denatured protein in water. The changes in rotation associated with the binding are largely (though not entirely) reversible with respect to changes in urea concentration and temperature. At higher temperatures the complexes apparently tend to dissociate and greater urea concentrations are required to form them. The structures of the complexed and uncomplexed denatured protein molecules resemble one another more and more closely, the higher the temperature. This behavior is typical of that generally observed for solvent effects on the optical rotation at high temperatures.[51]

Barker[13] found that the optical rotation of heat denatured ovalbumin is more negative, the greater the protein concentration. It is possible that this is caused by a urea-like action of the peptide links of ovalbumin, an increase in the protein concentration having the same effect on the optical rotation of the denatured protein as an increase in the urea concentration.

Significance of the Failure to Follow Simple First-order Kinetics.—It was found (Fig. 2) that a semilogarithmic plot of the change in optical rotation with time fails to give the straight line expected of a reaction which is of the first order in the protein concentration. Several explanations are possible.

(1) Since the reaction appears to slow down in its later stages, it may be of an order higher than the first with respect to protein. This possibility is ruled out by the fact that neither the shape nor the half time of the reaction curve changes very much when the total protein concentration is changed by a large factor.

(50) C. F. Jacobsen and L. K. Christensen, *Nature*, **161**, 30 (1948).

(51) W. Kauzmann and H. Eyring, *J. Chem. Phys.*, **9**, 41 (1941).

(2) Depletion of a reactant other than protein would also cause the reaction to slow down in its later stages. This is ruled out by the fact that the other possible reactants, *i.e.*, urea and water, are present in large excess, especially at the lowest protein concentrations employed. The possibility that one of the rate-controlling reactants is an impurity in the protein (as was found by Lauffer[52] in the urea denaturation of tobacco mosaic virus) is ruled out by the small effect of the protein concentration on the kinetics.

(3) The decrease in the rate as the reaction proceeds might be caused by an inhibiting effect of the products of denaturation. This was shown not to be so by adding a solution of ovalbumin which had been completely denatured in 7.3 *M* urea to a freshly prepared solution of native ovalbumin in 7.3 *M* urea. The rate of change of the rotation was identical with that normally found at this urea concentration.

(4) The only plausible explanation of our result is that the primary process is a complex of simultaneous or successive reactions, each of which is of the first order with respect to protein. Two alternatives are possible here: (a) the native ovalbumin may consist of a mixture of components, P_1, P_2, P_3, etc., which denature independently at different rates

$$N_1 \xrightarrow{k_1} D_1, N_2 \xrightarrow{k_2} D_2, N_3 \xrightarrow{k_3} D_3, \text{etc.} \quad (I)$$

or (b) the change in optical rotation is caused by a sequence of first-order reactions

$$N \xrightarrow{k_1} D_1 \xrightarrow{k_2} D_2 \xrightarrow{k_3} D_3 \longrightarrow \text{etc.} \quad (II)$$

It is possible to distinguish between these alternatives experimentally by exposing protein to urea until most of it has been denatured, then recovering the undenatured protein and comparing the rate of change of optical rotation of the recovered material in urea with that of the original protein. If it behaves in the same way as the original protein we must accept alternative (b). If its rotation changes less rapidly, then alternative (a) must operate. This experiment has been performed with ovalbumin. As we hope to report in a later paper, the result seems to support alternative (b), although not without some interesting complications.[53]

If the specific rotations of the native and denatured forms of the protein are independent of the protein concentrations (as is highly likely, from our experiments at different protein concentrations), the optical rotation will vary with the time as

$$[\alpha] = [\alpha_f] + ae^{-k_1 t} + be^{-k_2 t} + ce^{-k_3 t} + \ldots . \quad (1)$$

where there are as many exponential terms as there are different forms of the protein, and the rate constants k_1, k_2, etc., are those of the reactions indicated in (I) and (II).[54] We have found that the curves in Fig. 2 can be fitted with three exponential terms. At least two more exponential terms are required to describe the secondary reaction, so that altogether at least five different native or denatured forms of ovalbumin seem to be involved here.

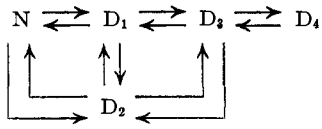
Significance of the Variation of the Rate with Urea Concentration.—The rate of change of the optical rotation of ovalbumin depends very strongly on the urea concentration. (Doubling the urea concentration at 30° increases the rate by a factor of about 30,000.) A similar though somewhat less extreme dependence of the denaturation rate on the urea concentration has been observed by Lauffer[52] with tobacco mosaic virus, by Mihalyi[58] with fibrinogen, by Deutsch[59] on erythrocyte catalase and by Christensen[15b] with β-lactoglobulin. Behavior of this kind is also often found when proteins and living systems are exposed to small molecules (for instance the work of Johnson and co-workers on the effects of drugs on enzymes and on bacterial luminescence[60]). There are a number of ways of interpreting these effects but, as we shall now show, each of the interpretations is equivalent to one or the other of two mutually exclusive points of view, depending on the nature of the forces which act between the protein and the small molecules. Only one of the two points of view can be correct for a particular system, and it is important to decide which of them should be used in each instance.

Small molecules may interact with proteins by means of either short range forces or long range forces. If the forces act over only a short distance the small molecules must be located close to definite "sites" on or within the protein molecule in order to have any effect on it. The number of these sites is presumably fixed and there is a definite upper limit to the number of small molecules which can interact with each site. We can therefore speak of a series of "complexes" or "compounds" of the protein and the small molecules, each compound having a definite composition. On the other hand, if the forces in question act over long distances it is unreasonable to speak of compounds having a definite composition. Such an interaction is better

(52) M. Lauffer, THIS JOURNAL, **65**, 1793 (1943).

(53) The deviation from first-order kinetics was observed with ovalbumin whose *p*H had been adjusted by buffers as well as by direct addition of alkali to the stock solution. Therefore, this behavior cannot be explained by the momentary exposure of part of the ovalbumin to a high *p*H, as may have occurred with our methods of adding alkali.

(54) If we deal with successive reactions for which some of the rate constants happen to be equal, the dependence of the specific rotation on the time would also involve some terms containing powers of the time multiplied by exponentials. If the possibility of reversal of some or all of the partial reactions and of "branching" of the reaction path is taken into account, as in the scheme



then the specific rotation (or any other additive property) will still depend on the time as a sum of as many exponentials as there are denatured forms, but the coefficients of the time in the exponents will be complicated functions of the rate constants of the various partial reactions. The kinetics of these types of reaction sequences are discussed by Rakowski[55] and by Zwolinski and Eyring,[56] and by Tambs-Lyche.[57]

(55) A. Rakowski, *Z. physik. Chem.*, **57**, 321 (1907).

(56) B. J. Zwolinski and H. Eyring, THIS JOURNAL, **69**, 2702 (1947).

(57) R. TambsLyche, *Kgl. Norske Videnskab. Selskabs, Skrifter*, No. 4 (1928).

(58) E. Mihalyi, *Acta Chem. Scand.*, **4**, 317 (1950).

(59) H. F. Deutsch, *ibid.*, **5**, 1074 (1951).

(60) F. H. Johnson, H. Eyring, R. Steblay, H. Chaplin, C. Huber and G. Gherardi, *J. Gen. Physiol.*, **28**, 463 (1945); D. Fraser, F. H. Johnson and R. S. Baker, *Arch. Biochem.*, **24**, 314 (1949); F. H. Johnson, E. A. Flagler, R. Simpson and K. McGeer, *J. Cellular Comp. Physiol.*, **37**, 1 (1951).

called a "solvent effect"—a term which implies a non-stoichiometric influence.

These two alternatives will now be discussed in detail with reference to the urea denaturation of ovalbumin. We shall show that the first alternative may be analyzed in terms of a reaction order with respect to urea, while the second is best discussed in terms of the dielectric constant of the solution. Urea denaturation will be shown to involve "complex formation" rather than a "solvent effect." This result is significant because the solutions we have used contained as much as 50% by weight of urea, so that the protein is dissolved in a solvent very different from water. We might, therefore, have expected that the denaturing action of urea could occur in some way through this change in solvent rather than by the direct attack of urea on the protein. This is not the case.

Alternative 1.—Urea might be bound to the protein through short range forces (*e.g.*, van der Waals–London forces, hydrogen bonds or chemical bonds) to form a series of compounds which then unfold, each at its own rate, to give the denatured protein. A "mechanism" can then be written for the process

$$\begin{aligned} \mathrm{P} &\xrightarrow{k_0} \mathrm{D} \\ \mathrm{P} + \mathrm{U} &\underset{}{\overset{K_1}{\rightleftharpoons}} \mathrm{PU} \xrightarrow{k_1} \mathrm{D} \\ \mathrm{P} + 2\mathrm{U} &\overset{K_2}{\rightleftharpoons} \mathrm{PU_2} \xrightarrow{k_2} \mathrm{D} \qquad \text{(III)} \\ &\cdots\cdots\cdots\cdots \\ \mathrm{P} + n\mathrm{U} &\overset{K_n}{\rightleftharpoons} \mathrm{PU}_n \xrightarrow{k_n} \mathrm{D} \\ &\cdots\cdots\cdots\cdots \end{aligned}$$

where P stands for native protein, D for denatured protein, U for urea, and PU_n for the nth intermediate complex. K_n is the association constant for the nth complex and k_n is the specific rate constant for denaturation of this complex. (If the protein unfolds in a series of similar steps, as in the reaction sequence (II), mechanism (III) would presumably apply to each step in the sequence.) This mechanism leads to the following expression for the observed specific rate of denaturation

$$\bar{k} = \frac{1}{P}\frac{\mathrm{d}D}{\mathrm{d}t} = \Sigma K_n k_n U^n / \Sigma K_n U^n \qquad (2)$$

P and U here should, strictly, be taken to be protein and urea activities rather than concentrations.

$$\frac{\mathrm{d}\ln \bar{k}}{\mathrm{d}\ln U} = \bar{n}' - \bar{n} = n^* \qquad (3)$$

where

$$\bar{n}' = \Sigma n K_n k_n U^n / \Sigma K_n k_n U^n \qquad (4a)$$

$$\bar{n} = \Sigma n K_n U^n / \Sigma K_n U^n \qquad (4b)$$

$\bar{n}'$ is the mean number of bound urea molecules in the activated states of the denaturation reactions and $\bar{n}$ is the mean number bound by the native protein. The difference, n^*, may be called "the apparent order of the reaction with respect to urea," and corresponds to an effective rate-controlling reaction

$$\mathrm{PU}_n^{\ddagger} + n^*\mathrm{U} \longrightarrow \mathrm{D} \qquad \text{(IV)}$$

Alternative 2.—Urea might act through less specific long range forces which do not result in compounds of definite composition such as are postulated above—a mode of action which we have called a "solvent effect." The only chemically important forces capable of acting over large distances are, however, the electrostatic fields of charged groups, dipoles and multipoles; the interaction of a non-conducting solvent such as urea–water mixtures with these fields is best described in terms of the solvent's dielectric constant. Therefore, "solvent effects" can be limited to those associated with changes in the dielectric constant of the solvent. Solvents with high dielectric constants will accelerate reactions in which the electric field surrounding the activated complex is larger and penetrates further into the solvent than that surrounding the normal molecule.[61,62]

Urea solutions have considerably higher dielectric constants than water (8 M urea at 25° has a dielectric constant of 97 as compared with 78.5 for water).[63] If, therefore, urea acts simply through a "solvent effect," any substance which increases the dielectric constant of water should be a denaturing agent. In particular, glycine, whose solutions have considerably larger dielectric constants than those of urea,[64] should be a more powerful denaturant than urea. This is not observed; a 2.1 M glycine solution (dielectric constant 126) has no effect on the optical rotation of ovalbumin and does not produce material insoluble in water at the isoelectric point. In fact, glycine in the presence of urea seems to inhibit the change in optical rotation of ovalbumin.[65]

Furthermore, it can be shown that according to alternative (2), electrolytes should be even more effective denaturants than urea. Scatchard's theory[61] shows that a change, ΔD, in the dielectric constant, D, of a solvent changes the free energy of activation by an amount

$$\Delta F_{\mathrm{D}} = -A(\Delta D/D)(1/rD) \qquad (5)$$

while the effect of the addition of electrolytes on the free energy of activation is

$$\Delta F_{\mathrm{e}} = -A'\sqrt{I}/3D \qquad (6)$$

where r is the effective radius of the protein molecule in ångström units (at least 30 Å. for ovalbumin) and I is the ionic strength. Insofar as the theory is correct, the constants of proportionality, A and A', have the same numerical value. Although it is a gross over-simplification to apply this theory to proteins, it is reasonable to expect that A and A' should at least be of the same order of magnitude. In 8 M urea, $\Delta D = 18.5$ and $D = 80$, giving $\Delta F_{\mathrm{D}} = -10^4 A$ as compared with pure water. If A and A' were the same, ΔF_{e} would have this value in an aqueous electrolyte solution of ionic strength 10^{-3} or 10^{-4}. Thus dilute salt solutions should be about as effective as 8 M urea in causing denaturation. This, of course, is not the case; some electrolytes (*e.g.*, sodium sulfate) even act as inhibitors of denaturation.

The absence of electrostatic effects of this kind makes it very unlikely that "salt links" play an important role in maintaining native ovalbumin in its folded state. Jacobsen and Linderstrøm-Lang[65a] have reached the same conclusion from other evidence.

The Apparent Order of the Reaction with Respect to Urea.—The urea solutions which we have employed are very concentrated, so the reaction order must be determined with reference to the thermodynamic activity of the urea rather than its concentration. If the reaction involves, directly or indirectly, a slow step equivalent to IV, then according to the absolute theory of reaction rates[66] the rate of disappearance of native protein, P, is

$$-\mathrm{d(P)}/\mathrm{d}t = k(\gamma_{\mathrm{p}}/\gamma^*)(\mathrm{P})U^{n*} \qquad (7)$$

where γ_{p} and γ^* are the activity coefficients of the native protein and the activated complex and U is the activity of the urea. In the presence of a large excess of urea the ratio of the activity coefficients as well as the activity of the urea may be assumed to be constant throughout the course of the reaction. This leads to the expression

$$-\log t_{1/2} = \frac{\log k\gamma_{\mathrm{p}}}{\log 2\gamma^*} + n^* \log U \qquad (8)$$

(61) G. Scatchard, *Chem. Revs.*, **10**, 229 (1932).

(62) K. Laidler and H. Eyring, *Ann. N. Y. Acad. Sci.*, **39**, 303 (1940).

(63) J. Wyman, THIS JOURNAL, **55**, 4116 (1933).

(64) J. Wyman and T. L. McMeekin, *ibid.*, **55**, 908 (1933).

(65) Observation by Dr. B. Levedahl in this Laboratory.

(65a) C. F. Jacobsen and K. Linderstrøm-Lang, *Nature*, **164**, 411 (1949).

(66) S. Glasstone, K. J. Laidler and H. Eyring, "The Theory of Rate Processes," McGraw-Hill Book Co., Inc., New York, 1941, p. 402.

It seems reasonable to assume that the effect of urea on the activated complex will be nearly the same as its effect on the native protein especially in the narrow range of urea concentrations employed by us, so that γ_p/γ^* will be nearly independent of the urea concentration and the first term on the right in eq. 8 will be a constant at constant temperature when the urea concentration is varied. By plotting log $t_{1/2}$ *vs.* log U a line should be obtained whose negative slope is n^*, the apparent reaction order with respect to urea.

The activity of urea in water at 25° is accurately known from the vapor pressure studies of Scatchard, Hamer and Wood.[67] In order to find the activities at other temperatures the careful studies of the heat of dilution of urea by Gucker and Pickard[68] and of the partial molal specific heat by Gucker and Ayres[69] can be utilized. Their measurements only extend to a urea concentration of about 8 M and to a temperature of 40°, however, so that at higher concentrations and temperatures a somewhat uncertain extrapolation must be made.

In Fig. 11 the logarithm of the half time is plotted against the logarithm of the activity of the urea at temperatures from 0 to 50°. Straight lines are obtained with slopes corresponding to $n^* = 15.0 \pm 0.8$ at 0°, 12.5 ± 0.1 at 30°, 12.5 at 40°, 8.3 at 45°, and 7.8 ± 1.0 at 50°. A similar plot using the urea concentration instead of the activity (Fig. 3), but including data at still higher temperatures, gives straight lines with slopes equivalent to $n^* =$ 15 at 0°, 14.5 at 30°, 15 at 40°, 9.5 at 45°, 8.5 at 50°, 7 at 55°, and 3.2 at 65°. Evidently the activity correction makes a small but noticeable difference in the apparent order of the reaction.

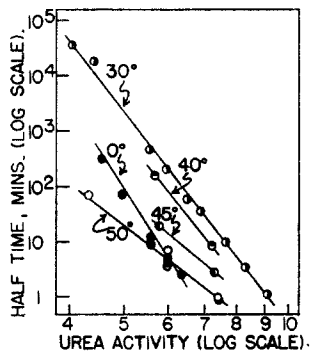


Fig. 11.—Dependence of rate of change of optical rotation of ovalbumin on activity of urea at various temperatures. Same data as Fig. 3.

The slight dependence of the rate on the protein concentration could easily be caused by the dependence of the activity coefficient ratio γ_p/γ^* in equation 7 on the protein concentration. Scatchard and co-workers[70] have found from osmotic pressure measurements on serum albumin that contributions to the activity coefficient due to protein–protein interactions can amount to more than a factor of two in a 5% albumin solution even at the isoelectric point. A difference in the activity coefficients of the native protein and of the activated state amounting to 50% at a protein concentration of 5% as compared with a concentration of 0.3% is therefore not inconceivable.

Significance of the Dependence of the Rate on the Temperature.—The strong minimum in rate in 7.3 M urea at 20° and pH (Fig. 4) is strikingly similar to the behavior observed by Lauffer[52] in the urea denaturation of tobacco mosaic virus. Our result is somewhat different from that observed by Hopkins[46] with ovalbumin in 7 M urea at pH 6, however. Hopkins made rough measurements of the rate of production of material insoluble at the isoelectric point on dilution of the urea with ammonium sulfate solution. He observed a much slower rate at 22° than at 0°, but the rate at 37° appeared to be slower than that at 22° (*i.e.*, there was a negative temperature coefficient but there appeared to be no minimum in rate at 20°). Hopkins' work has been repeated in this Laboratory by Dr. John Schellman, who has found that in 7.3 M urea at pH 7.6 the half time for the production of ovalbumin insoluble in 0.5 M $MgSO_4$ buffered with 0.1 M acetate (6:4 acetic acid:sodium acetate) has the same pronounced maximum at about 20° as was found under the same conditions by means of the optical rotation. The time required to precipitate half of the protein was, however, about 50% greater than the half time for the change in optical rotation at all temperatures, indicating that some change probably takes place in the protein before it becomes insoluble.

The rates of urea denaturation of proteins frequently have very low or even negative temperature coefficients between 0° and room temperature. This has been observed with diphtheria antitoxin by Wright and Schomaker,[71] with erythrocyte catalase by Deutsch,[59] and with β-lactoglobulin by Jacobsen and Christensen.[50] It was not, however, observed with fibrinogen by Mihalyi.[58]

Hopkins suggested that the unusual effect of temperature on the rate was the result of the formation of a readily denatured complex between the protein and urea, the complex being dissociated when the temperature is raised. Lauffer attempted to give this interpretation a quantitative formulation but was unable to apply it to his results because of the strong dependence of his rates on the pH—a complication with which we do not have to contend, at least in the range pH 7 to 9. The Hopkins–Lauffer hypothesis fits very nicely into the reaction scheme III, and it is interesting to treat our results quantitatively from this point of view.

We suppose that the portion of the molecule involved in initiating the unfolding (or a stage of unfolding, if it occurs in independent steps) has N equivalent sites for the adsorption of urea and that each adsorbed urea molecule increases the denaturation rate by a constant factor, a. (This is equivalent to Lauffer's assumption that each adsorbed urea molecule lowers the free energy of activation by a constant increment.) Suppose that each site has an association constant, K, which is the same

(67) G. Scatchard, W. J. Hamer and S. E. Wood, THIS JOURNAL, **60**, 3061 (1938).

(68) F. T. Gucker and H. B. Pickard, *ibid.*, **62**, 1470 (1940).

(69) F. T. Gucker and F. D. Ayres, *ibid.*, **59**, 2152 (1937).

(70) G. Scatchard, A. C. Batchelder and A. Brown, *ibid.*, **68**, 2320, 2610 (1946); see also J. T. Edsall, H. Edelhoch, R. Lontie and P. R. Morrison, *ibid.*, **72**, 4641 (1950).

(71) G. C. Wright and V. Schomaker, *ibid.*, **70**, 356 (1948).

for all sites. Then the association constant for the nth complex, PU_n (see reactions III), is

$$K_n = \frac{N!}{n!(N-n)!} K^n \quad (9)$$

and the specific rate constant for denaturation of this complex is

$$k_n = a^n k_0 \quad (10)$$

where k_0 is the rate in the absence of urea. The over-all specific rate of denaturation obtained by substituting these into eq. 2 is

$$\bar{k} = \left(\frac{1 + aKU}{1 + KU}\right)^N k_0 \quad (11)$$

where U is the activity of the urea. The average numbers of urea molecules adsorbed on the normal and activated molecules are, from eq. 4, 9 and 10

$$\bar{n} = \frac{KU}{1 + KU} N \quad (12a)$$

$$\bar{n}' = \frac{aKU}{1 + aKU} N \quad (12b)$$

and the apparent order of reaction with respect to urea is

$$n^* = \bar{n}' - \bar{n} = \frac{(a-1)KU}{(1 + aKU)(1 + KU)} N \quad (13)$$

The apparent heat of activation is

$$\Delta H^* = RT^2 \left(\frac{\partial \ln k}{\partial T}\right)_U = \Delta H_0 - n^*(H + h) - \bar{n}h \quad (14)$$

where ΔH_0 is the heat of activation for denaturation in the absence of urea, H is the heat of adsorption of a urea molecule onto the protein, and h is the reduction in the heat of activation for denaturation when a single urea molecule is adsorbed. That is

$$a = A \exp(h/RT) \quad (15a)$$

$$K = B \exp(H/RT) \quad (15b)$$

$$k_0 = C \exp(\Delta H_0/RT) \quad (15c)$$

These equations have been applied to our results, with the additional important assumption that k_0 is the rate of heat denaturation of ovalbumin observed by Lewis[32] at the pH giving the minimum rate. A reasonably good reproduction of our data is obtained with the following set of constants

$$N = 16$$
$$H + h = 10 \text{ kcal.}$$
$$\Delta H_0 = 130 \text{ kcal.}$$
$$\log_{10} C = 79.1$$
$$\log_{10} AB = -0.7$$

The quantity KU was too small (less than about 0.2) to be determined with sufficient accuracy to give the temperature dependence of K. Therefore, separate values of H and h and of A and B could not be determined.

No combination of constants differing by more than about 10% from the above set can give even a roughly adequate fit to the data. If the basic model on which the above treatment is based is true, then urea can attack something like 16 "potential weak points" in the ovalbumin molecule. Each urea contributes an effective total of about 10 kcal. toward the reduction of the activation energy of denaturation, and the urea molecules can, and especially at low temperatures do, coöperate in bringing about unfolding.

It is generally supposed that urea unfolds proteins by rupturing the hydrogen bonds between the NH and CO groups of the peptide chains. In the absence of urea, water acts in the same way but requires a higher temperature because the reaction

$$2H_2O + NH \ldots OC \longrightarrow NH \ldots OH_2 + CO \ldots H_2O$$

is more endothermic than that in which urea replaces water.[72]

Qualitatively this picture fits into the Hopkins–Lauffer mechanism very nicely. The "potential weak points" are the interpeptide hydrogen bonds which in the absence of urea are broken by water. (If neither water nor urea is present, it is much more difficult to unfold the molecule—which explains the stability of dried proteins.)

The ability of urea molecules to coöperate in promoting denaturation would mean that the folds in the polypeptide chain cannot be opened unless several hydrogen bonds are broken simultaneously. This conclusion has some interesting connotations. If folds contained free chain ends exposed to attack by urea, we should expect that they could be unwrapped by breaking one hydrogen bond at a time, in the fashion of a zipper. Since this does not seem to be the mechanism of unfolding in ovalbumin, we must conclude that the ends of the polypeptide chains in ovalbumin are buried inside the molecule or are inaccessible to urea for some other reason.[74] Instead, the folds must be attacked in the middle of the chains, and they can apparently only be opened if several bonds are first broken simultaneously. In terms of the recent Pauling–Corey[77] spirally folded polypeptide structure this might mean that in order for unfolding to occur, one turn of the spiral would first have to be pried loose. This would require the rupture of about 8 hydrogen bonds. The value, $N = 16$, might mean that two urea molecules were utilized in rupturing each bond. Once a single loop of the spiral had been forced out of place in this way, the rest of the spiral could be unfolded by breaking single hydrogen bonds one after another, as in a zipper.

Quantitatively, on the other hand, this interpretation in terms of hydrogen bonds is not very

(72) Urea can be regarded as two pure peptide bonds. The deviations of solutions of urea in water from ideality suggest a tendency of urea to form dimers and higher polymers, presumably because of this stability in water of the NH...OC hydrogen bond. Dr. John Schellman[73] has analyzed the thermodynamic data of Scatchard[67] and Gucker[68,69] from this point of view. He finds that for the reaction

$$(\text{urea})_2 + 2H_2O \longrightarrow 2(\text{urea}\cdot H_2O)$$
$$\Delta F^0 = -1470 \text{ cal. (at } 25^\circ)$$
$$\Delta H^0 = 1000 \text{ cal.}$$
$$\Delta S^0 = 8.3 \text{ e.u.}$$

the concentration units being mole fractions. The number of NH...CO hydrogen bonds in a urea dimer may be anything from one to four, so that insofar as urea represents a peptide bond, the heat content of a protein is increased by between 250 and 1000 cal. for each hydrogen bond ruptured on denaturation in water.

(73) J. Schellman, private communication.

(74) This conclusion is interesting in connection with Porter's finding[75] that no polypeptide chain ends could be detected in ovalbumin with 2,4-dinitrofluorobenzene. Steinberg,[76] however, has found that terminal amino acids can be hydrolyzed from ovalbumin by carboxypeptidase.

(75) R. Porter, *Biochem. J.*, **46**, 304 (1950).

(76) D. Steinberg, THIS JOURNAL, **74**, 4217 (1952).

(77) L. Pauling and R. B. Corey, *Proc. Nat. Acad. U. S.*, **37**, 205, 235 (1951).

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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[CONTRIBUTION FROM THE FRICK CHEMICAL LABORATORY, PRINCETON UNIVERSITY]

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).