bonds are in non-helical portions and their apparently increased strength may be due to stabilization from side-chain hydrogen bonds[41] or other interactions, *viz.*, the Arg 39–Cys 40 bond by the proximity of positive charges on Lys 37 and Arg 39, the Lys 66–Asp (NH_2) 67 bond by a Lys 66–Asp (NH_2) 67 hydrogen bond, and the Tyr 97–Lys 98 bond by the coöperative hydrogen-bonding interaction involving Tyr 97, Asp (NH_2) 101, and Lys 98. Those peptide bonds near the ends of the helical portions probably are not stabilized as much as those in interior portions of the helices. Presumably, the breakdown of the secondary and tertiary structure (*e.g.*, in oxidized ribonuclease) enhances the susceptibility to enzymatic attack.[41]

A variety of other observations on the effect of various chemical agents on the configuration of ribonuclease has been reported.[38,43] These observations seem to be compatible with the proposed model. Also, numerous interactions, not listed here in detail, exist between the side-chain groups in the model.

There are several ways to envision the association of the enzyme and substrate; for example, the substrate could be attached parallel to H_1 and lying across H_2, H_5 and H_6. If serine and histidine are involved in the "active center," several such pairs are close together, *viz.*, His 16–Ser 13, His 105–Ser 90, His 105–Ser 87 and His 119–Ser 123. However, whereas His 119 may be involved,[10] Ser 123 cannot be since this group can be removed by carboxypeptidase without loss of activity.[9] Obviously further experiments are required to locate the "active center" in the molecule.

In conclusion a model has been constructed which seems to be in accord with the available chemical evidence. Its importance lies in the fact that it provides a basis to plan experiments for the investigation of interactions between side-chain groups, along lines indicated elsewhere.[31] It may also be of help in Fourier analyses of X-ray data on ribonuclease crystals.

Acknowledgment.—I am indebted to Mrs. Joanne Widom for assembling the model.

(43) K. U. Linderstrøm-Lang and J. A. Schellman, "The Enzymes," Ed. P. D. Boyer, H. Lardy and K. Myrbäck, Vol. I, Academic Press, Inc., New York, N. Y., 1959, p. 443.

[CONTRIBUTION FROM THE DEPARTMENT OF CHEMISTRY, UNIVERSITY OF SOUTHERN CALIFORNIA, LOS ANGELES 7, CALIFORNIA]

The Effect of Water upon the Rate of Heat Denaturation of Egg Albumin[1]

BY ROBERT L. ALTMAN[2,3] AND SIDNEY W. BENSON

RECEIVED DECEMBER 21, 1959

The rate of heat denaturation of solid egg albumin has been found to be extremely sensitive to the water content of the protein. This sensitivity makes it extremely difficult to obtain precise rate data in the system. This is further complicated by the small amounts of hysteresis observed in the sorption process and the slow approach to sorption equilibrium. Despite these difficulties it is possible to interpret the data in terms of a 12th order dependence (± 2) on sorbed H_2O and an apparent activation energy of 75 ± 10 kcal. These values cannot be interpreted in terms of a simple breaking of hydrogen bonds leading to an unfolding of the peptide chain, nor can they be interpreted in terms of a homopolar fission of a covalent bond. They do indicate that some high energy process involving compensating solvation energy is involved in the denaturation process.

Introduction

Early studies of the heat denaturation of solid egg albumin showed that the denaturation process was greatly accelerated by the presence of water vapor.[4,5] Barker[6] reinvestigated this process in a rather crude manner, measuring the rate of heat denaturation of solid egg albumin as a function of the relative humidity of water. He found the heat denaturation rate exponentially related to the concentration of water vapor. His method, however, did not admit of very precise control or knowledge of the amount of water sorbed by the protein nor did they give any detailed information on the order of the reaction with respect to protein. To further elucidate both of these points the present study was undertaken.

The experimental approach followed in this research was to expose initially dry egg albumin to water vapor at a specific constant relative humidity (P/P_0) and temperature and determine the extent of insolubilization with time as a function of water vapor pressure and temperature. Initial work on determining the effect of relative humidity upon the denaturation rate was undertaken by using salt hydrates as the method of maintaining constant humidity.[7] Vapor pressure tables of those salts useful in the temperature range 80 to 100° appear in the International Critical Tables[8] and the Landolt-Börnstein Tabellen.[9]

From this work using hydrates or saturated solutions of NaCl, NaI, KBr and KI, it was soon found that the rate of denaturation was very sensitive to changing vapor pressure. But while internal re-

(1) This work has been supported by a grant (G-3541) from the U. S. Public Health Service of the National Institutes of Health.

(2) The material in this paper has been included in a dissertation submitted by R. L. Altman to the Graduate School of the University of Southern California in partial fulfillment of the requirements for the degree of Doctor of Philosophy.

(3) University of California, Berkeley, California.

(4) S. Lewith, *Arch. Exp. Pathol. Pharmakol.*, **26**, 341 (1890).

(5) H. Chick and C. J. Martin, *J. Physiol.*, **40**, 404 (1910); **43**, 1 (1911); **45**, 61, 261 (1912).

(6) H. A. Barker, *J. Gen. Physiol.*, **40**, 404 (1933).

(7) Barker, ref. 6.

(8) "International Critical Tables," Vol. I, McGraw-Hill Book Co., New York, N. Y., 1926, p. 67.

(9) Landolt-Bornstein, "Physikalisch-Chemische Tabellen," Vol. I, 5th Auflage, Julius Springer, Berlin, 1923, pp. 1288, 1907.

producibility (*i.e.*, removing two samples simultaneously) was good to ±2%, the entire run could never be reproduced again at the same temperature and vapor pressure. Such results can be attributed to the lengthy time required to establish vapor pressure equilibrium using hydrates and the sluggish response of such hydrates to changes in temperature.[10,11]

It became apparent that saturated solutions were not a very good method of maintaining constant vapor pressure in this kinetic system. Therefore, a manostat to meet these requirements was developed from a similar instrument invented by Zahn.[12] A full description of the experimental technique developed with this instrument is presented in the experimental part of this paper.

Experimental

Materials and Reagents.—The powdered egg albumin used in these denaturation studies was obtained from: (1) Armour Research Division, Chicago, Ill., Lot E-81116 of 15 g.; (2) Mann Research Laboratories, New York, N.Y., Lot 533 of 15 g.; (3) General Biochemicals, Inc., Chagrin Falls, Ohio, Lots 35587, 35451, 33059 of 10, 10 and 15 g. Preliminary experiments were conducted with the Armour material. After initial lyophilization, the dry powdered egg albumin was divided into two parts by sieving through wire screens of mesh number 10, 30 and 60.[13] Both the fine (30–60) and coarse (10–30) cuts were then evacuated and exposed to saturated sodium chromate. It was found that the finer cut tended to sorb slightly more water than the coarse and that it also tended to denature slightly more rapidly. However, our techniques did not permit us to obtain sieved samples of reproducible particle size or surface area and for the coarser samples the differences appeared to be small. Hence all the results presented in this paper have been obtained with both the General Biochemicals and the Mann Research egg albumin used just as prepared by the supplier. Each lot received was taken just as it came from the bottle and mixed together with what was left from the previous supply.

The source of water vapor at constant relative humidity (P/P_0) is the manostat illustrated in Fig. 1. It was operated in the following manner.

(1) Stopcocks A, B, F, H and X shut off; A and X opened to pump out mercury manometer, M.

(2) Stopcocks A and E (capillary leak) shut off; C (three-way T-capillary stopcock) turned into ⊢ position; B and H opened to vacuum line, V, to evacuate CGN section.

(3) Container, Z, filled with distilled water and connected to C; Stopcocks B and H turned off and then C turned counter-clockwise 90° to ⊥ position; B opened to vacuum gently, and air pumped out of ZCB by alternate torching and intermittent pumping for about an hour, until water in Z thought free of air.

(4) B cut off, and vapor pressure of water measured with manometer, M, together with the temperature of the surrounding air space. If the manometric reading fell within one millimeter of that rated for pure water at the measured temperature,[14] the water in vessel Z was then judged air free.

(5) C turned clockwise 90° to ⊢ position and H opened again to V; Z turned upside down on ball-joint and C turned again clockwise 90° to ⊤ position; water run into CGN until above bulb G in capillary tubing on both sides.

(6) C turned 90° counterclockwise back to ⊢ position and B opened to vacuum until water level in both capillaries was the same.

(7) B and H cut off.

The apparatus for exposing a thermostated sample of dry egg albumin to a given partial pressure of water is illustrated in Fig. 2. The sample of protein was used in the following manner.

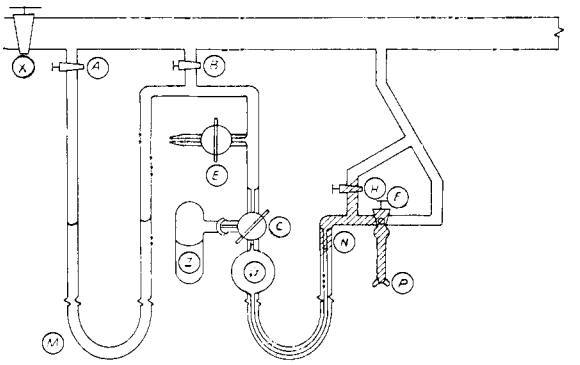

Fig. 1.—Vapor pressure manostat: A, B, C, E, F, H, X, stopcocks; G, water reservoir; M, mercury manometer; N, water meniscus, heated by nichrome wire; P, ground joint leading to protein samples; Z, distilled water supply for outgassing. Crosshatched sections were heated by nichrome wire.

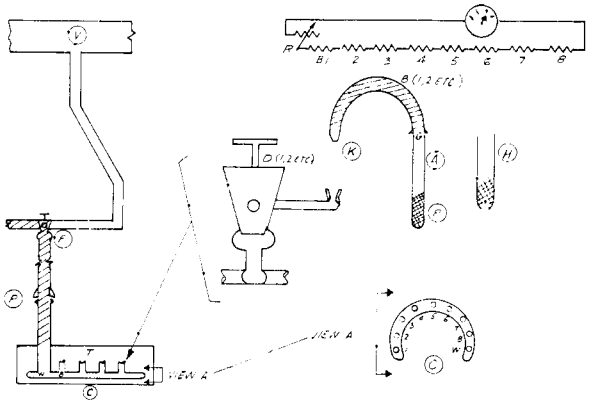

Fig. 2.—Apparatus for protein denaturation: V, vacuum system leads; F, silicone grease lubricated stopcock, heated by nichrome elements; P, spherical ground joint leading to thermostatted manifold; D, silicone lubricated stopcocks for admitting vapor to individual protein samples to vapor manifold C; B, heated gooseneck adapter for attaching protein samples through stopcock D to manifold C *via* joint K; C, top and side views of horseshoe shaped manifold. Top view shows points of attachment of individual stopcocks D; G, glass wool plug; A, test tubes with attached glass joints for holding protein samples P; B1...B8, nichrome heating elements. Crosshatched areas heated by nichrome wires.

Eight male 12/30 ground joints were sealed off about three inches from the neck; each one being numerically marked to identify it (Fig. 2, part A). A particular tube was oven-dried, cooled and weighed; some powdered protein put in and the total weighed again. The protein weight was of course determined by difference and sufficient protein was put in so that the difference amounted to about a decigram.

But since this protein already contained water adsorbed from the air, a further weight-correction was required. A large amount of this same protein taken from the same bottle was put into a small test tube, weighed before and after (with a glass-wool plug) and pumped out overnight on the vacuum line, then weighed again next morning. It was found that the air-adsorbed water generally amounted to about 5% of the initial "wet" protein weight and this same procedure was repeated with each new run. The loss of weight once determined was applied to each of the samples

(10) P. LeClerc, *Silic. Ind.*, **19**, 237 (1954); *C.A.*, **48**, 13186i (1954).

(11) A. Wexler and S. Hasegawa, *J. Research Natl. Bur. Standards*, **53**, 19 (1954).

(12) C. T. Zahn, *Rev. Sci. Instr.*, **1**, 299 (1930).

(13) The numbering and hole size of these sieves are the specifications of the National Bureau of Standards LC-584.

(14) "International Critical Tables," Vol. III, p. 212.

in a given run. The protein-containing tube, A, was plugged with glass wool, G, and connected to another 12/30 female joint of semicircular shape, B, wrapped with nichrome wire. The protein tube, A, was attached to the semicircular part, B, with sealing wax because it had been found that the glycerol heating-bath fluid often succeeded in creeping into silicone high-vacuum grease, the other sealing material tested.

The glass cane-like tube, AB, then was sealed at K with this silicone grease to one of a series of stopcocks, D, connected through the bottom to a horseshoe-shaped glass tube, C, having a vacuum outlet, W, at one end. This whole set-up, about eighteen inches in diameter and six inches high, was placed in a glass cylindrical trough, T, containing a glycerol–ethylene glycol–water mixture which served as the heat transferring agent from centrally-immersed Cenco knife heaters. The fluid was sufficiently agitated by an air-driven stirrer so as to keep the bath-temperature in the 80 to 100° range isothermal to within 0.1°. The whole apparatus was connected to the water-vapor apparatus at stopcock F, as described in Fig. 2, through ball-joint P and operated in the following manner.

After the ABCD set-up was placed in the bath, T, stopcocks D and F were opened to the vacuum line, V, and the protein samples pumped upon for at least 12 hours, generally for 24 hours. Upon completion of this evacuation, a run was ready to be performed.

(1) The heaters and stirrer were started in bath, T, to heat up the tube, A, to say 90°, stopcocks D and F still open to the vacuum line.

(2) The series of eight nichrome resisters (B1, B2. . . B8) were connected serially to another eight-lead variable resister, R, both connected through a Variac to supply heating electricity generally of about 20 volts, the purpose of which was to prevent the condensation of water in B.

(3) Nichrome wire, NHFP, (Fig. 1), was simultaneously electrified to heat up the glass and, therefore, the water in capillary N. At the same time, air leak, E, was opened and sufficient air was admitted to obtain an arbitrary pressure in the mercury manometer, M. This forced the water up higher into the heated capillary, N, and it boiled and receded until the vapor pressure of the water was equal to that of the air in the manometer, M.

If the water level in both capillaries was not the same in this steady state, the hot wire at N was loose enough to be moved slightly either up or down to obtain an equal water level in both tubes. The air pressure read by the mercury manometer under these conditions was taken as the vapor pressure of water for the run to follow and this air pressure was read at successive time intervals during the run. These pressures were found to vary within one or two millimeters but because the total pressures were always about half an atmosphere, the variation in pressure produced only a very small error. The run itself was ready to start, once the bath was in a thermal steady state, say 90 ± 0.1°, read on the bath thermometer.

(4) Water vapor at the pressure of interest was admitted to the protein samples by turning stopcock F counter-clockwise 180 degrees, the timer simultaneously being started.

(5) At a series of arbitrary time intervals, an AB-cane was removed from the bath in the following manner. Stopcock D (D1, D2, etc.) was turned 180 degrees so as to cut off the water vapor source from the protein, once a water vapor pressure reading had been taken. The resistance winding, B (B1, B2, etc.), was taken out of the series, and the variable resister, R, adjusted so as to maintain the same current and heating effect as before. Then the AB set-up was pulled out of stopcock D (D1, D2, etc.), protein sample made ready for analysis.

Since the initial protein weight was known, the analytical problem was to determine the weight of insoluble protein produced as an effect of heat and water vapor. Distilled water, itself, was the solvent used to separate the native protein from the denatured portion. It had been previously found that the use of a sodium acetate–acetic acid buffer solution to hold the *p*H of the wash to that of the isoelectric point, *p*H 4.8, produced identical results within the experimental errors of weighing. But a potassium acid phthalate–phthalic acid buffer solution at the same *p*H did produce somewhat higher results. This appears to be due to the adsorption of phthalic acid by the denatured protein since repeated washing with distilled water yielded the NaAc-HAc results.

(6) For analysis, the powder in the tube was removed by adding distilled water, containing a wetting agent, Tween 20, and scraping the solid–liquid mixture out into a 100 ml. beaker by the use of a wooden policeman. More water was added to tube A and the process repeated until no more protein was left in A. Enough water was added to the beaker, similarly numerically marked, to immerse the solid in plenty of water. The beaker was left standing for several hours, covered by a watch glass, and the finally-remaining solid protein was filtered in the following manner.

It was found extremely difficult to filter these solutions through conventional Gooch crucibles or sintered glass crucibles. Therefore, a suggestion of Dr. Ryden Richardson of these Laboratories was used to develop a glass wool type of Gooch crucible which worked well. A $2^1/_2$'' piece of 10 mm. Pyrex tubing, H (Fig. 2), was narrowed at one end and a glass wool plug pushed down into this narrowed end. The whole set-up was then oven-dried and weighed.

(7) The protein contents of the beaker were drained into this filter by suction through a specially built funnel. After all the water passed through the glass wool, more water was added and suctioned in order to remove any soluble protein adsorbed by the denatured protein from the solution. This whole set-up, H, plus glass wool plus insoluble protein, was then evacuated overnight on the vacuum line, V, to remove the water and 24 hours later the tube was weighed again to determine the weight of insoluble dry protein produced by denaturation.

Results

Duplicate samples of protein taken from the same batch and denatured in the same experiment gave reproducibility of about ±2% for the amounts of denaturation. However the reproducibility of successive samples was much less, the plots of amount of denaturation against time showing a scatter of as much as ±50% about a mean value. Some of this scatter is undoubtedly due to differences in particle size and method of sample preparation but this cannot account for all of the scatter.

To trace the origin of this scatter several control experiments were run. In one the rates for coarse and fine mesh samples were compared in the same experiment. It was found that the finer mesh protein particles denatured about 10 to 30% more rapidly than the coarse mesh. While no effort was made to correlate mesh size with surface area, it is probable that the finer mesh has the larger surface area. Since the rate of denaturation is exponentially dependent on sorbed water and independent measurements[15] of the sorption, isotherms of both native and denatured proteins have shown a higher water sorption (up to 10% in some cases) for more finely divided material, it appears likely that particle size may have been responsible for most of the scatter. However, there was about the same scatter shown by some samples of the same mesh size. Examination of the coarse particles showed a considerable heterogeneity in structure and it appeared that mesh size might not have been a good criterion of uniformity of surface area. In the absence of any method of controlling homogeneity of particle, we turned to mixing our total samples in the hope of at least getting a reproducible distribution. This is not wholly satisfactory and is complicated by settling of finer particles in the mixture and an "aging" effect in the coarse particles which tends to increase their superficial density.

It has been shown that the rate of attainment of sorption equilibrium[16] is controlled by the diffusion

(15) R. L. Altman and S. W. Benson, in press; see also ref. 16.

(16) S. W. Benson, D. A. Ellis and R. W. Zwanzig, THIS JOURNAL **72**, 2102 (1950)

of heat through the protein sample and for the apparatus used here, the half-life may be expected to be of the order of about 30 minutes. To see whether this had any effect on the scatter, experiments were made in which samples were permitted to sorb up to 85% of their equilibrium water.[17] This would not result in significant denaturation under the conditions employed. However, such samples showed just as much scatter as the untreated samples and about the same mean value.

Plots of the amount of denaturation against time usually showed an initially erratic rate of denaturation followed by a region which was linear on a semi-log plot. Examples of such plots are shown in Fig. 3. Some runs showed the behavior illustrated in this figure where an initially rapid rate is followed by an apparent first order region. Induction periods can, of course, be rationalized in terms of a slow approach to sorption equilibrium. However, a rapid initial rate is not so easily accounted for. It is tempting to say that in very heterogeneous mixtures the large amount of heat liberated by the protein during water sorption accelerated the rate of denaturation of the "fine" part of the mixture for which the heat effect is largest. Compensating for this heating effect is a lowered H_2O sorption which would tend to reduce the rate. In the absence of a control on particle uniformity it is difficult to check such speculations.

A further complication arises from the small amount of hysteresis in the sorption isotherms of the proteins. This introduces an uncertainty into the actual H_2O sorption which can account readily for the spread in rates which were observed. Since the amount of hysteresis will depend on the path for the process which in turn will depend on the superficial density and particle size distribution of the protein, in the present work it was not entirely controllable.

For purposes of comparison it was decided to plot the results semi-logarithmically as shown in Fig. 4 and to take the slope corresponding to the apparent first order region as representative of the rate of denaturation. This was done because we did not know just where in the sorption period the denaturation process began. The half-lives obtained in this manner are not the times necessary for 50% denaturation of initially native egg albumin. Rather they represent the time required for an arbitrary amount of native egg albumin to be reduced by one-half at some time after the time required to complete the water sorption. For example, in Fig. 3, the half-life reported for 80° is the time required for a sample containing 60% native egg albumin to be reduced to one containing only 30%. Similarly, in the 90° run, it is the time required for an 80% sample to be reduced to 40%.

As to the reproducibility of these results, six separate runs were made at 90° with a vapor pressure of 360 mm. ($P/P_0 = 0.685$). The average of the half-lives obtained is 2.2 ± 0.6 hours. Because the denaturation half-life is extremely sensitive to the amount of sorbed water, Fig. 4 shows that this half-life range results when the amount of water sorbed is 10.0 ± 0.2 grams of water/100 grams of protein. Thus a root-mean-square deviation of 25% in the half-life could be accounted for by a 2%

(17) This was done by sorbing at a lower vapor pressure and then raising the vapor pressure to the final equilibrium value.

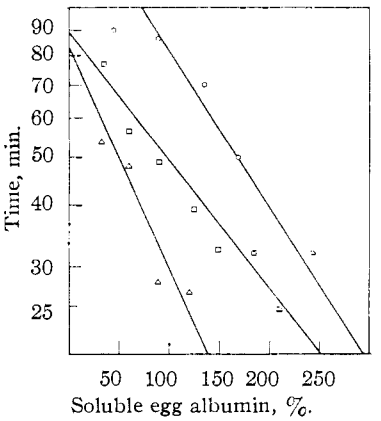

Fig. 3.—Rate of denaturation of egg albumin in steam at 80 (△), 90 (○) and 100° (□). Lines are representative of apparent first order regions. The △ data points were obtained at a vapor pressure of 252 mm. which results in the sorption of 11.7 grams of water per hundred grams of protein at 80°. The apparent half life is 1.1 hours. The ○ data points were taken at 366 mm. which results in the sorption of 10 grams of water at 90°. The apparent half life is 1.7 hours. The □ data points were obtained at 511 mm. which results in the sorption of 8.3 grams of water at 100°. The apparent half life is 1.9 hours.

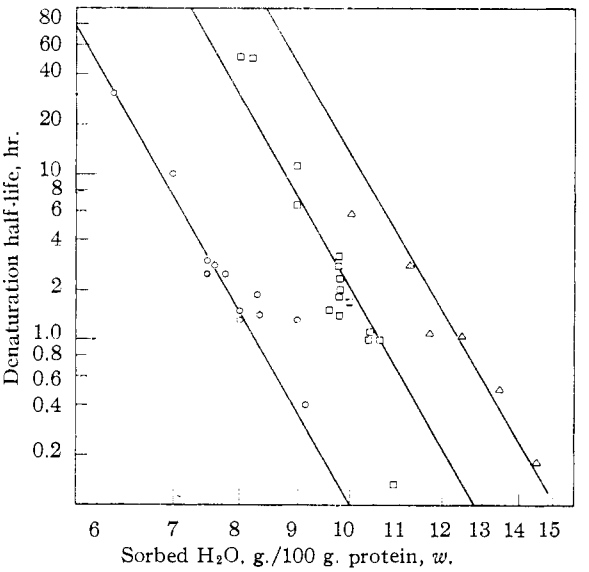

Fig. 4.—Denaturation half life of egg albumin in steam at 80 (△), 90 (□) and 100° (○). A straight line that obeys the equation

$$\log T_{1/2} = -12.1 (\log W) + 13.2$$

has been drawn through all the 80° points. They have a root-mean-square error of ±0.45 hour relative to this line. The straight line represented by

$$\log T_{1/2} = -12.1 (\log W) + 12.4$$

has been drawn through all the 90° points. They have a root-mean-square error of ±0.68 hour relative to this line. The straight line represented by

$$\log T_{1/2} = -12.1 (\log W) + 11.1$$

has been drawn through all the 100° points. They have a root-mean-square error of ±1.08 hours relative to this line.

spread in the amount of sorbed water.[18] First order behavior is of course to be expected if denaturation proceeds molecule by molecule or particle by particle.[19] A more sophisticated method of treatment of the data would not change the present results with regard to the effect of H_2O significantly and is not justified in the absence of control of the the particle size. In solution, the bulk of egg albumin denaturation data are interpreted as first order relative to protein although there has appeared some data to the contrary.[18,20] It is quite possible that much of the uncontrollable scatter and the deviation from apparent first order behavior is a reflection of molecule–molecule interaction.

Figure 4 summarizes our data on the effects of amount of sorbed water on rates of apparent first order denaturation (as defined above) at 80, 90 and 100°. The plots are log–log and the straight lines which have been drawn have been deliberately chosen to be the best straight lines through the data for all temperatures. If the best lines are drawn at each temperature the slopes vary by a maximum of about 25% while the root mean square error is not much improved over the present plots.

The rate constants at the three temperatures can be represented algebraically as:

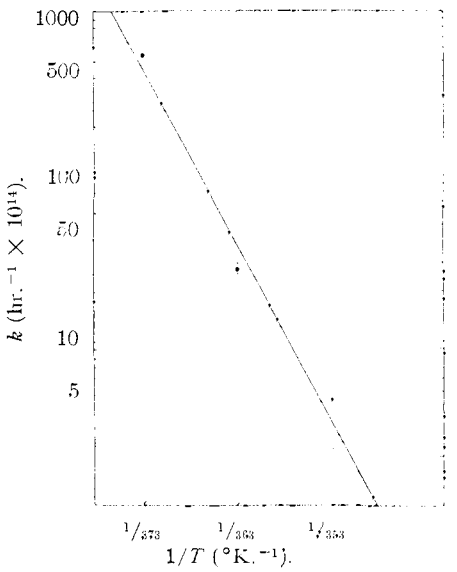

Fig. 5.—Determination of the activation energy: the rate constants obtained from Fig. 4 have been plotted semilogarithmically. The lengths of the characters indicate the estimated error in these rate constants. The straight line has a slope of 75 ± 10 kcal./mole which is, therefore, the activation energy of these denaturation experiments.

T (°C.)	k
80	$4.4 \times 10^{-13}(W)^{12}$
90	$2.8 \times 10^{-13}(W)^{12}$
100	$5.5 \times 10^{-12}(W)^{12}$

where W is the weight in grams of water sorbed[21] per hundred grams of protein. Despite the large spread in the apparent first order rate constants which in absolute magnitude extends over a factor of 4, the power of the rate dependence in sorbed water, 12, is good to within about 15% or two units. In fact it can be argued that the rather large spread in the rate constants is attributable to this high order of the reaction with respect to water. Figure 5 is a plot of log k *vs.* $1/T$ and the denaturation reaction activation energy computed in this fashion has the value of about 75 ± 10 kcal./mole.

As heat was initially evolved in the sorption process, these experiments may be questioned with regard to how isothermal the denaturation runs were. Denaturation does not occur at a measurable rate until the relative humidity exceeds 0.60. But already better than half the water necessary to induce rapid denaturation has been sorbed at this low partial pressure and much heat has been liberated.[20]

Therefore, to test whether essentially isothermal conditions prevailed, the sorption process was lengthened by adding the water vapor in small P/P_0 increments to the dry native egg albumin samples. Figure 6 shows the results of a usual run (Δ data points) at a relative humidity of 0.685, in which no attempt to delay the sorption process was made. As soon as the steady state pressure was attained, a zero time sample was taken with no denaturation resulting. Sorption was also extended over a ten minute period (O, data points) at the end of which time the protein was 22% denatured, and over a thirty minute period (□ data points) in which time 33% denatured.

If the heat release had a significant influence upon the denaturation velocity, the slower sorption process should produce an increased half-life. But Fig. 6 shows that, within the difficulties of this experimental technique, the heat of sorption exerts no significant influence upon the denaturation velocity greater than the range of experimental error (Fig. 4). In summary it appears that the denaturation of solid egg albumin over the range 80 to 100° can be represented by a simple equation which is approximately first order in protein and twelfth order in sorbed water. The sensitivity to temperature at constant water sorption is indicated by the following half-lives taken from the smoothed graphs in Fig. 4. At 10 g. H_2O per 100 g. dry protein the half-life goes from 6 hours at 80° to 1.5 hours at 90° and 0.1 hour at 100°. The combined sensitivity to sorbed water content and temperature is such that we have been able to take almost no data at our different temperatures corresponding to the same water content. The value of 75 kcal./mole for the activation energy is of comparable magnitude to that reported for solution denaturation which ranges from 87 to 128 kcal.[22]

Discussion

Despite the low precision of the data, the present results indicate that the rate of denaturation of egg

(18) It is interesting to note that in the urea-catalyzed denaturation of egg albumin (R. B. Simpson and W. Kauzmann, THIS JOURNAL, **75**, 5139 (1953)) it was found that the rate was not quite 1st order with respect to protein and that different stock solutions of protein, although giving similar rate curves, yielded absolute rates varying by some 30%.

(19) J. Hume and J. Colvin, *Phil. Mag.*, **8**, 590 (1929), find that the rate of conversion of monoclinic to rhombic sulfur is approximately first order for uniform particle size systems.

(20) I. N. Bufanski and N. A. Shabanova, *Ukrain. Biochem. Zhur.*, **26**, 235 (1954); *C. A.*, **49**, 9705h (1955).

(21) Sorption data calculated from relative humidity and the isotherms of R. L. Altman, Ph.D. Thesis, University of Southern California (1958).

(22) H. Eyring and A. Stearn, *Chem. Revs.*, **24**, 253 (1939).

albumin is extraordinarily sensitive to the amount of sorbed water. At sorptions corresponding to relative humidities (r.h.) of about 0.6 the rate of denaturation is almost too slow to measure while at an r.h. of 0.7 the rate is too fast to measure. A crude quantitative estimate of this sensitivity is given by the conventional rate equation which shows a 12th order dependence of the denaturation rate on water content.

At these values of r.h. the concentration of H_2O in the solid protein corresponds to about 7 moles/liter or about 250 moles H_2O per mole protein. Since the protein contains, per mole, about 96 moles of acid and base residues and about 400 peptide bonds, this is about one water molecule per two amino acid residues.[23] From the work of Mellon, Korn and Hoover[24] it is likely that the bulk of this water is attached to the zwitterion residues and only a small fraction is attached to the peptide residues. If it is proposed that the denaturation process involves the uncoiling of the α-helix, then it is likely that the role of water in this process is to facilitate the cleavage of the hydrogen bonds in the helix by providing a compensating hydration energy. In terms of such a model it seems quite reasonable that the coöperative action of a large number of water molecules is required. There are an average of 3.7 peptide hydrogen bonds per turn of the helix and it seems likely that one must provide at least one water molecule for each member of the pair forming a hydrogen bond. This would amount to 7.5 H_2O molecules per turn of the helix and would represent a lower limit if there were more than one H_2O molecule required per peptide linkage freed. On the basis of such a picture one would then imagine that the barrier to denaturation is the unravelling of a single turn of the helix whereupon the further unravelling is a fast process. Unfortunately the activation energy for such a process cannot amount to more than 20 to 30 kcal./mole.

While such a model makes plausible the necessity for the coöperative participation of a large number of water molecules in the denaturation act, it leaves vague the precise way in which the uncoiling of the protein molecule occurs. It also leaves unanswered the relation between uncoiling and subsequent insolubility of the protein which has been shown to involve S–S and S–H bonds.[25] Kauzmann and co-workers[18,26] have shown that changes in optical rotation and viscosity such as one might expect from the uncoiling of a rigid helix precede precipitation of denatured protein.

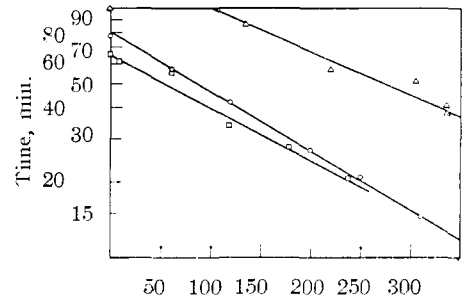

Fig. 6.—Rate of denaturation of egg albumin in steam at 90° and 360 mm. water vapor pressure. Lines are representative of apparent first order regions. This vapor pressure results in the sorption of 9.8 grams of water per hundred grams of protein at this temperature. The Δ data points were obtained when no attempt to delay the sorption process was made and the apparent half life of this run is 2.8 hours. The O data points were obtained when the sorption time was lengthened to ten minutes and the apparent half life of this run is 2.0 hours. The □ data points are the result of lengthening the sorption time to thirty minutes and the apparent half life of this run is 2.3 hours.

The present results on the effect of H_2O cannot be interpreted in any reasonable way in terms merely of the homopolar rupture of covalent bonds. They make it appear much more likely that the denaturation act involves considerable changes in solvation energy which would be compatible with the relative displacement of ionized groups or the cleavage of polar hydrogen bonds. However, such displacements would not by themselves be expected to result in denaturation and it appears that bond rupture is a necessary further step in the sequence, as Kauzmann has suggested. The large activation energy of 75 kcal./mole which is observed would be compatible with a bond breaking process or with separation of charges but not with a simple rupture of hydrogen bonds.

The combined evidence thus supports Kauzmann's inference that denaturation does not involve merely an unfolding of the peptide chain but must involve some significant bond breaking as well. The possibility that simple charge separation is responsible for denaturation is rendered unlikely by the observation that in water solution, where charges can move quite readily, the activation energy is still quite high.

(23) F. Haurowitz, "Chemistry and Biology of Proteins," Academic Press Publ., New York, N. Y., 1950, p. 32.

(24) E. F. Mellon, A. H. Korn and S. R. Hoover, THIS JOURNAL, **71**, 2761 (1949).

(25) A review of this aspect of the subject recently has been presented by E. V. Jensen, *Science*, **130**, 1319 (1959).

(26) H. K. Frensdorff, M. T. Watson and W. Kauzmann, THIS JOURNAL, **75**, 5157 (1953).