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The Kinetics of Denaturation of Conalbumin[1]

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The kinetics of denaturation of conalbumin in the *p*H interval 3.2 to 4.2 at temperatures of 0, 5, 10 and 28.7° has been studied by following changes in the solubility of the protein under two sets of conditions. The data are best described at a given *p*H by the scheme A ⇄ B ⇄ C. The variation with *p*H of the kinetic parameters for these two successive, reversible reactions has been analyzed in terms of their dependence on the ionization of certain critical groups. Thermodynamic functions and activation analogs have been calculated. Instability is introduced when 10 and 18 protons have been bound by a set of 18 basic groups. It is concluded that sixteen of these groups are carboxylate ions.

Changes in the solubility and electrophoretic behavior of conalbumin at moderate temperatures as the *p*H is lowered below 4 were first described by Longsworth, Cannan and MacInnes.[3] Cann and Phelps[4] have also investigated the properties of conalbumin denatured under these conditions. The finding that a time dependent appearance of titratable basic groups accompanies these changes[5] demonstrates a close resemblance of the denaturation of conalbumin to that of hemoglobin.[6] In this investigation the kinetics of these reactions have been studied as a function of *p*H and temperature.

Steinhardt[7] first proposed that the successive ionization of only a limited number of critical groups (five, in the case of pepsin) produced species with distinctly different rates of denaturation, the contribution of each species to the observed rate being weighted by its hydrogen-ion equilibrium concentration. This theory, generalized by Levy and Benaglia,[8] accommodated the denaturation kinetics of ricin over an enormous variation in *p*H, temperature and rate. A number of subsequent studies have used this approach.[9-12] The action of urea in protein denaturation has been treated using essentially the same assumptions.[13] The critical groups deduced by this method often do not resemble the typical prototropic groups found in proteins. Hydrogen bonding may account for both their unusual properties and their role in stabilizing the protein structure.[14]

Experimental

Conalbumin was prepared as in the preceding paper.[5]

The denaturation experiments were performed in ten formate buffers (final ionic strength, 0.100) at *p*H's between 3.2 and 4.2, at 0.00, 4.95, 9.95 and 28.65°. The final *p*H, measured at 25.0°, was corrected to the experimental temperature by using the data of Harned and Robinson[15] for the temperature variation of the pK_0 of formic acid.

The criterion of denaturation used in this work was insolubility in a stopping buffer[5,9] having the composition: $(NH_4)_2SO_4$, 2.68 *M*; KH_2PO_4, 0.0555 *M*; KH_2PO_4, 0.278 *M*.

Two sampling procedures were used, depending on the rate of denaturation. For relatively slow reactions (half over in 200 seconds or more), 5 ml. of formate buffer was rapidly pipetted into 5 ml. of 1% conalbumin stock, both solutions being at bath temperature. Time of half-delivery was about 2 seconds. Since there is no danger of surface denaturation by shaking,[16] the tube was swirled violently to ensure mixing. At intervals (recorded to 1 second), 0.332 ml. of reaction mixture was transferred rapidly by a Carlsberg pipet[17] into 0.9998 ml. of cold stopping buffer in a centrifuge tube. Two sets of samples were taken (see below). In one set the samples were kept at about −1° for 3 to 15 minutes, then centrifuged in the cold in a Servall SS-1 centrifuge for 5 minutes at 3/4 speed. As soon as possible, aliquots of the supernatants were removed and diluted. The second set was stored at room temperature for 24 hr. with infrequent shaking, then cooled and treated as above. Fourteen or fifteen samples of each type were taken. The protein concentration in the supernatants was determined with a Beckman DU spectrophotometer at 278 mμ.

For reactions half over in less than 200 seconds, a discontinuous technique was used. 0.332 ml. of formate buffer was blown into a like volume of conalbumin solution, delivery from the straight-tip Carlsberg pipet taking less than 0.2 second. The reaction was stopped by adding 2.00 ml. of ice-cold stopping buffer rapidly by pouring from a test tube. Proper dilution was assured by pouring the mixture back and forth between the two tubes 3 or 4 times. The sample was then split in half, one part kept cold and centrifuged within 3 to 15 minutes after stopping, the rest kept at room temperature for 24 hr., both sets being treated as above.

Many of the runs were done in duplicate, some, to check new batches of stock conalbumin, were repeated a third time.

Some explanation is necessary regarding the procedure of centrifuging some samples in a run immediately and waiting 24 hr. to centrifuge the rest. Experiments to determine a proper stopping buffer had used "well-denatured" material —conalbumin brought to *p*H slightly below 3.0 with HCl and allowed to stand at room temperature until used. Under these conditions essentially all the protein was precipitated no matter how long one waited before centrifuging. However, in trial kinetic runs the apparent half-time was increased when the samples were allowed to stand longer before centrifugation. The obvious answer seemed to be that some of the precipitate was going back into solution. By partially denaturing several larger samples of conalbumin, stopping the reaction, distributing samples into 5 or 6 tubes and centrifuging at intervals, it was shown that resolution

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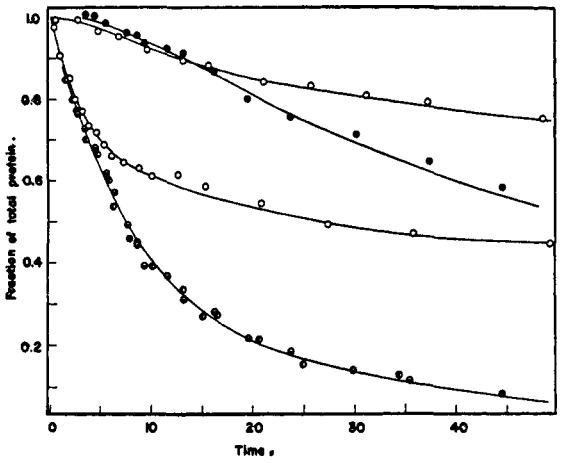

Fig. 1.—Typical denaturation experiments. Curves are calculated from derived constants; points are experimental; *p*H 3.20, 5°, time in sec.: ◐ and ◒, $A(t)/A_0$ (two expts., three months apart); ●, $1 - C(t)/A_0$. *p*H 3.95, 5°, time in hundreds of sec.: O, lower, $A(t)/A_0$; O, upper, $1 - C(t)/A_0$.

had occurred, rapidly at first, then more slowly with time. This material was shown not to be occluded native protein. Moreover, the amount of re-solution did not depend on the amount of precipitate but varied for the same amount of initial precipitate depending on the specific denaturing conditions—*p*H, time and temperature.

In view of Levy and Warner's[9] conclusion that the fastest reaction is the best, efforts were made to minimize re-solution. Varying the stopping *p*H was unsuccessful. An entire run of 15 points was subjected to re-solution analysis, one experimental point giving 5 re-solution points. It was found that re-solution had essentially stopped after 24 hr. for all points, that it was possible to extrapolate reasonably well to zero time in the stopper and that with time in the *denaturing* medium the net amount of re-solution went through a maximum and fell to zero. This was highly suggestive of consecutive reactions, or branched reactions with equilibria, and the amount of re-solution seemed to be an important quantity in itself. Subsequently, it was found that if the stopped reaction was kept at −1 to 0° and centrifuged cold, no re-solution occurred for the interval 3 to 15 minutes after stopping. Since this procedure is less tedious (30 points against 75) and more satisfactory otherwise than extrapolation, it was adopted as standard technique. Several runs by both methods gave reasonable agreement.

All of the kinetic experiments were performed in the presence of 0.1 *M* formate ion. The substitution of other ions at constant ionic strength has a profound effect on the reaction. The rate is greatly increased in the presence of nitrate, trichloroacetate, thiocyanate and to a small degree by chloride and acetate. Some difference between sulfate and chloride has been noted in electrophoretic experiments by Phelps and Cann.[4]

Results

Kinetics.—Each kinetic run gave the relative concentrations of three species as functions of time: $A(t)$, the native protein; $B(t)$, the redissolved protein; and $C(t)$, the residual precipitate. The mechanism that describes the data with the greatest economy of assumptions is $A \rightleftarrows B \rightleftarrows C$. The choice of this mechanism was based on the following considerations. An irreversible branched reaction $C \leftarrow A \rightarrow B$ was eliminated immediately since in many cases $B(t)$ goes through a maximum. $A \rightarrow B \rightarrow C$ is also unsatisfactory because log $A(t)$ against t is not linear. The falling off of the apparent first order rate constant could not be explained by reactions of higher order since the rate was independent of concentration over a four-fold change in A(0). A graph of $1/A(t)$ against t is not linear and the deviation is in the direction of lower order. In this connection Phelps and Cann[4] found no evidence of aggregation under conditions of *p*H, ionic strength and time of exposure similar to those used here. We have examined the sedimentation properties of protein isolated from the supernatant solutions of kinetic experiments. Material equivalent to A and A + B behaved identically with native conalbumin. These results taken together indicate that the mechanism must involve only unimolecular reactions and equilibria. The simplest schemes below *p*H 3.7 consist of a rapidly established equilibrium between A and B with a gradual displacement of the equilibrium position by the formation of C from A or B. At higher *p*H's it is necessary to include the reversal of the reaction which forms C. The simplest alternatives thus become (a) $A \rightleftarrows B \rightleftarrows C$ and (b) $C \rightleftarrows A \rightleftarrows B$. The differential equations were solved by the methods outlined by Frost and Pearson.[18] The experimental data usually showed the characteristic sigmoid shape of $C(t)$ predicted from (a). It was also evident that $C(t)$ depends on $B(t)$ as in (a) rather than on $A(t)$. The best fit using integrated equations for (a) was always better than the best fit from (b). The kinetic data therefore have been evaluated for the scheme

$$A \underset{k^{(21)}}{\overset{k^{(12)}}{\rightleftarrows}} B \underset{k^{(32)}}{\overset{k^{(23)}}{\rightleftarrows}} C.$$

For some reactions $k^{(32)}$ may be negligibly small. The appropriate expressions are

$$\frac{A}{A(0)} = \frac{k^{(21)}k^{(32)}}{\lambda_2\lambda_3} + \frac{k^{(12)}(\lambda_2 - k^{(23)} - k^{(32)})e^{-\lambda_2 t}}{\lambda_2(\lambda_2 - \lambda_3)} + \frac{k^{(12)}(k^{(23)} + k^{(32)} - \lambda_3)e^{-\lambda_3 t}}{\lambda_3(\lambda_2 - \lambda_3)} \quad (1a)$$

$$\frac{B}{B(0)} = \frac{k^{(12)}k^{(32)}}{\lambda_2\lambda_3} + \frac{k^{(12)}(k^{(32)} - \lambda_2)e^{-\lambda_2 t}}{\lambda_2(\lambda_2 - \lambda_3)} + \frac{k^{(12)}(\lambda_3 - k^{(21)})e^{-\lambda_3 t}}{\lambda_3(\lambda_2 - \lambda_3)} \quad (1b)$$

$$\frac{C}{C(0)} = \frac{k^{(12)}k^{(23)}}{\lambda_2\lambda_3} + \frac{k^{(12)}k^{(32)}e^{-\lambda_2 t}}{\lambda_2(\lambda_2 - \lambda_3)} + \frac{k^{(12)}k^{(32)}e^{-\lambda_3 n}}{\lambda_3(\lambda_2 - \lambda_3)} \quad (1c)$$

$$\lambda_2 = (p + q)/2;\ \lambda_3 = (p - q)/2$$

$$p = k^{(12)} + k^{(21)} + k^{(23)} + k^{(32)}$$

$$q = [p^2 - 4(k^{(12)}k^{(23)} + k^{(12)}k^{(32)} + k^{(21)}k^{(32)})]^{1/2}$$

As a first approximation, $k^{(12)}$ was calculated from the initial rate, $k^{(12)} = \dot{A}(0)/A(0)$ and $k^{(23)}$ at about maximum $C(t)$ where $k^{(23)} = \dot{C}/B$. At low *p*H, $k^{(21)}$ was estimated from the maximum $B(t)$ where $\dot{B} = 0$. At high *p*H, $k^{(21)}$ and $k^{(32)}$ were estimated from the values of $k^{(12)}$ A/B and $k^{(23)}$ B/C, respectively, at periods five to ten times the interval for completion of the rapid phase of the reaction. These values then were used to calculate A/A(0), B/A(0) and C/A(0) at five or six times. When necessary the rate constants were readjusted by inspection to obtain a better fit. Two to six recalculations usually were made. Two representative runs selected from opposite ends of the available *p*H range are shown in Fig. 1. Most of the experiments showed as good a fit.

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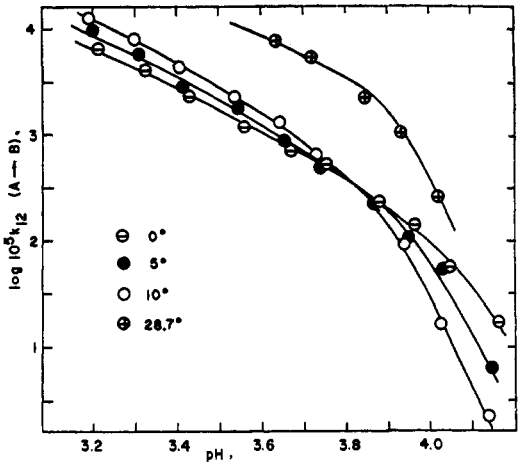

Fig. 2 —The effect of pH on the reaction A → B, log $k^{(12)}$. The curves are theoretical plots based on constants given in Table I.

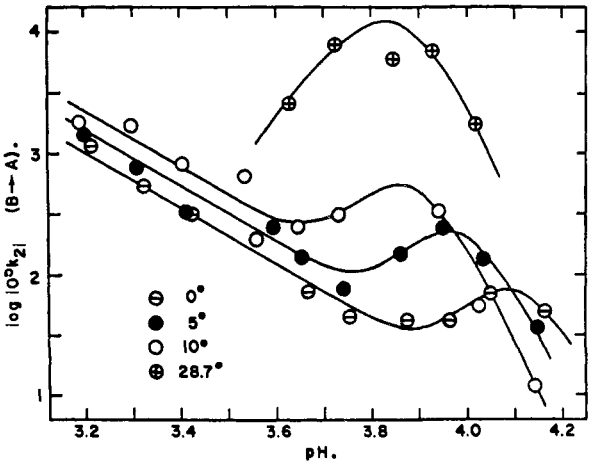

Fig. 3.—The effect of pH on the reaction B → A, log $k^{(21)}$. The curves are theoretical plots based on constants given in Table I.

The derived rate constants are given as the points in Figs. 2 to 5.[18a] The reaction is essentially controlled by $k^{(12)}$. This constant is therefore the most sensitive and probably is good to 2 or 3%. Other constants are necessarily less precise.

*p*H Dependence.—The purpose of the analysis of the rate as a function of pH is to gain insight into the ionization steps that introduce critical instabilities into the molecule. In the limited pH range accessible to investigation, from pH 4.2 where the equilibrium favors native protein and the reactions take days, to pH 3.2 where denaturation is complete in seconds, the computed rate constants change by a factor of 10^4. Features to note in Figs. 2 to 5 include the slope of −8 for log $k^{(12)}$ and log $k^{(21)}$ in the interval pH 3.9 to 4.2 (highest pH dependence of denaturation yet reported); the maxima in log $k^{(21)}$ and log $k^{(32)}$; and the integral

(18a) The original data on which the figures are based have been deposited as Document number 6525 with the A.D.I. Publications Project, Photoduplication Service, Library of Congress, Washington 25, D. C. A copy may be secured by citing the Document number and by remitting in advance $1.25 for photoprints, or $1.25 for 35 mm. microfilm, payable to Chief, Photoduplication Service, Library of Congress.

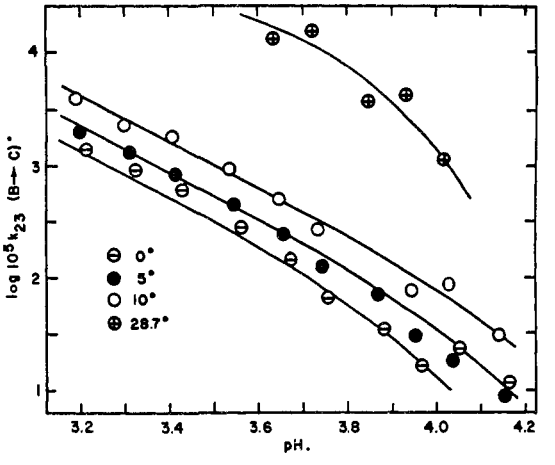

Fig. 4.—The effect of pH on the reaction B → C, log $k^{(23)}$. The curves are theoretical plots based on constants given in Table I.

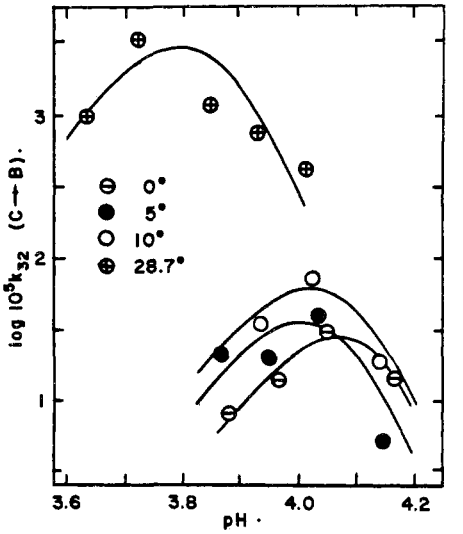

Fig. 5.—The effect of pH on the reaction C → B, log $k^{(32)}$. The curves are theoretical plots based on the constants given in Table I.

limiting slope of −2 in the lower pH regions. The latter is a characteristic feature of Steinhardt–Levy theory.[7,8]

In the formulation of Levy and Benaglia[8] there are $n + 1$ critical species corresponding to n dissociations of the type

$$PH_\nu = PH_{\nu-1} + H^+ \quad K_{n-\nu+1} = (PH_{\nu-1})(H^+)/(PH_\nu)$$

where $\nu = 1, \ldots, n$

The total protein, P_T is equal to $\Sigma_\nu(PH\nu)$ and the observed rate constant, k, equals $\Sigma_\nu k_{n-\nu+1}(PH_\nu)/P_T$. The problem in these terms reduces to finding the kinetically significant species and estimating the specific rate and equilibrium constants that will account for the observed dependence of rate on pH. With respect to log $k^{(12)}$, the slope of −8 is taken to mean that at least eight proton associations are involved in reaching the least stable species and the limiting slope of −2 to mean that the average charge of the critical groups is now only two proton associations away from that of the unstable form.[8] Log $k^{(21)}$ which also increases

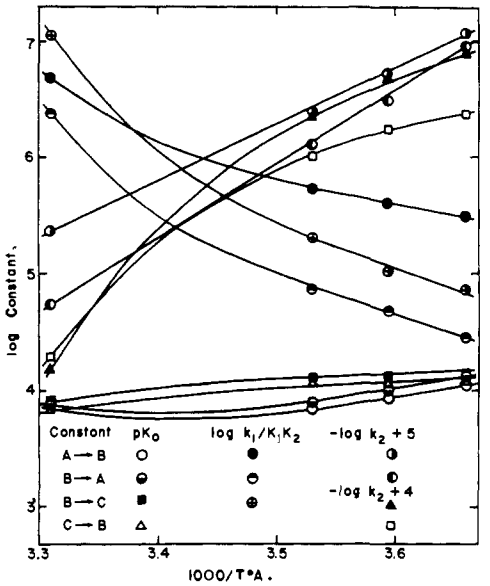

Fig. 6.—Arrhenius plots of the activation parameters for conalbumin denaturation.

initially with a slope of -8 goes through a distinct maximum (not a plateau) before the limiting slope of -2 is attained. This behavior requires that at least two species of B of rather different charge type are capable of reverting to the native protein, A, at finite rates. More than two such species would broaden the maximum. To satisfy the principle of detailed balancing (which imposes a stringent requirement discussed below), it must be assumed that two subspecies of A reach equilibrium with the corresponding subspecies of B. Log $k^{(23)}$ and log $k^{(32)}$ resemble log $k^{(12)}$ and log $k^{(21)}$, respectively.

A given observed rate constant, $k^{(ij)}$, will then depend on two species: $k^{(ij)}P_T = k_1^{(ij)}(PH_n) + k_2^{(ij)}(PH_{n-m})$. The general equation of Levy and Benaglia now reduces to

$$k^{(ij)} = \frac{k_1^{(ij)}h^n + k_2^{(ij)}h^{n-m}K_1^{(ij)} \ldots K_m^{(ij)}}{h^n + h^{n-1}K_1^{(ij)} + \ldots + K_1^{(ij)} \ldots K_n^{(ij)}} \quad (2)$$

$h = a_{H^+}$

ij = 12, 21, 23, 32

With the restriction that K_1 and K_2 are larger than h (to get a limiting slope of -2), a few computations showed that little flexibility was possible in assigning n and m. The values chosen were n = 18 and m = 8. The slope on the high pH side is governed by $n - m$ and must reach -8 very close to the maximum in Fig. 3. On the acid side of the maximum the slope, governed by $m - 2$, must reach + 3 in a short span. It was also assumed that $K_3 = \ldots = K_{18} = K_0$. Making $K_3 > K_4 > \ldots > K_{18}$, especially if the difference among the first few (K_3 to K_8) is made large, would require larger values of n and $n - m$. The pH range does not cover the region where $K_1 \ldots K_{18}$ is the predominant term in the denominator. Thus the assumption that $K_3 = \ldots = K_{18}$ does not preclude small differences in the last few dissociation constants since the limiting slope of -10 on the high pH side is never reached in this work. The relationship of the ionic species which now have been assumed to determine the rate is indicated in equations 5 and 9 which are discussed below.

For the purposes of computation the quantities in equation 2 were expressed in terms of K_0 and dimensionless parameters defined by $K_1 = \alpha K_0$, $K_2 = \beta K_0$, $h = \gamma K_0$, and $\delta = \alpha\beta k_2/k_1$. Since $K_3 = \ldots = K_{18} = K_0$, equation 2 becomes

$$\log k^{(ij)} = \log \frac{k_1^{(ij)}}{K_1^{(ij)}K_2^{(ij)}} + 2 \log K_0^{(ij)} + \log f(\gamma) \quad (3)$$

where

$$f(\gamma) = \frac{\gamma^{18} + \delta\gamma^{10}}{\gamma^{18}/\alpha\beta + \gamma^{17}/\beta + \gamma^{16} + \gamma^{15} + \ldots + 1}$$

The function $f(\gamma)$ was computed at ten values of γ for a number of values of α, β and δ for each rate constant and the resulting curves of log $f(\gamma)$ against log γ fitted over the experimental points. Log $k^{(12)}$, log $k^{(21)}$ and log $k^{(23)}$ each fit the same curves at 0, 5 and 10° to within a translation, but different parameters are required at 28.7°. The best fit of the theoretical curves to the experimental points is shown in Figs. 2 to 5. Values are thus obtained of the dimensionless parameters which account for the dependence of rate on pH. From these the constants $pK_0^{(ij)}$, log $k_1^{(ij)}/K_1^{(ij)}K_2^{(ij)}$ and log $k_2^{(ij)}$ were obtained from equation 3 and the relation $h = \gamma K_0$ by substituting log $k^{(ij)}$ and h at the point at which $\gamma = 1$. The equations arranged for this purpose are

$$pK_0^{(ij)} = p\text{H} \quad (4a)$$

$$\log k_1^{(ij)}/K_1^{(ij)}K_2^{(ij)} = \log k^{(ij)}(1) + 2\,pK_0^{(ij)} - \log f(1) \quad (4b)$$

$$\log k_2^{(ij)} = \log k^{(ij)}(1) + \log \delta - \log f(1) \quad (4c)$$

where $k^{(ij)}(1)$ refers to $k^{(ij)}$ and $f(1)$ to $f(\gamma)$ when $\gamma = 1$. The results are listed in Table I. It was not possible to determine α and only limited indication of the value of β could be obtained. K_1 and K_2 are thus not known, and it is necessary to describe the results in terms of the mixed constant $k_1/K_1/K_2$.

For C → B only $k_2^{(32)}$ and $pK_0^{(32)}$ could be evaluated from the kinetic data since $k^{(32)}$ is not available for the lower pH interval. For B → C the graph has so little curvature at 0, 5 and 10° that $k_2^{(23)}$ and $pK_0^{(23)}$ are not very reliable at these temperatures.

The constraint imposed by detailed balancing may now be discussed. The reaction A ⇄ B can be written schematically as

$$\begin{array}{ccccc} AH_{18} & = & AH_{10} & + & 8H^- \\ k_1^{(12)} \downarrow \uparrow k_1^{(21)} & & k_2^{(12)} \downarrow \uparrow k_2^{(21)} & & \\ BH_{18} & = & BH_{10} & + & 8H^+ \end{array} \quad (5)$$

From the kinetic and mass action relations at equilibrium it may be shown readily that

$$\log \frac{k_1^{(21)}}{K_1^{(21)}K_2^{(21)}} = \log \frac{k_1^{(12)}}{K_1^{(12)}K_2^{(12)}} + \log \frac{k_2^{(21)}}{k_2^{(12)}} - 6\,(pK_0^{(21)} - pK_0^{(12)}) \quad (6)$$

All the quantities on the right side of equation 6 are known. If log $k_1^{(21)}/K_1^{(21)}K_2^{(21)}$ is computed in this way the proper temperature dependence is obtained, but the derived values are 0.7 unit higher than the experimental values. It would be comforting to know whether this factor of five is

TABLE I

THERMODYNAMIC AND ACTIVATION PARAMETERS OF CONALBUMIN DENATURATION

	A → B (ij = 12)				B → A (ij = 21)			
Parameters[a]	0°	5°	10°	28.7°	0°	5°	10°	28.7°
$pK_0^{(ij)}$	4.048	3.931	3.828	3.840	4.130	4.006	3.900	3.869
Log $k_2^{(ij)}$	−2.006	−1.721	−1.386	−0.359	−1.961	−1.491	−1.096	+0.270
Log $k_1^{(ij)}/K_1^{(ij)}K_2^{(ij)}$	5.486	5.597	5.726	6.668	4.454	4.678	4.859	(6.373)
$K_2^{(ij)}/K_0^{(ij)}$	10	10	10	2		$K_2 \gg K_0$		
$\Delta H_2{}^{*b}$	22.03	22.02	22.01	21.96	28.29	28.28	28.27	28.22
$\Delta F_2{}^{*b}$	18.52	18.43	18.33	18.16	18.39	18.13	17.96	17.30
$\Delta S_2{}^{*b}$	12.8	12.9	13.0	12.6	36.2	36.5	36.4	36.2
$\Delta H_1{}^{*c}$	7.99	7.98	7.97	26.16	13.85	13.84	13.83	....
$\Delta F_1{}^{*c}$	9.08	9.12	9.12	8.46	10.37	10.28	10.25	(7.51)
$\Delta S_1{}^{*c}$	−4.0	−4.1	−4.1	58.7	12.7	12.8	12.6	(Large)?
$\Delta H_{ion}{}^{0d}$	9.26	7.78	5.67	0	8.20	8.20	8.20	0
$\Delta F_{ion}{}^{0d}$	5.06	5.00	4.96	5.30	5.16	5.10	5.05	5.34
$\Delta S_{ion}{}^{0d}$	15.4	10.0	2.5	−17	11.1	11.1	11.1	−17
	B → C (ij = 23)				C → B (ij = 32)			
$pK_0^{(ij)}$	4.15	4.12	4.11	3.88	4.11	4.04	4.06	3.83
Log $k_2^{(ij)}$	−2.90	−2.68	−2.38	−0.18	−2.37	−2.23	−2.00	−0.29
Log $k_1^{(ij)}/K_1^{(ij)}K_2^{(ij)}$	4.86	5.02	5.30	7.04	....	....	....	....
$\Delta H_2{}^{*b}$	21.8	21.8	21.8	63.0	13.0	13.0	13.0	54.0
$\Delta F_2{}^{*b}$	19.6	19.6	19.6	17.9	18.9	19.1	19.1	18.0
$\Delta S_2{}^{*b}$	8.1	7.9	7.8	149	−21.6	−21.9	−21.5	119
$\Delta H_1{}^{*c}$	17	17	17	55				
$\Delta F_1{}^{*c}$	9.9	9.8	9.7	7.9				
$\Delta S_1{}^{*c}$	26	26	26	156				
$\Delta H_{ion}{}^{0d}$	1.4	1.4	1.4	5.5	3.8	3.8	3.8	3.8
$\Delta F_{ion}{}^{0d}$	5.19	5.24	5.32	5.35	5.1	5.1	5.3	5.3
$\Delta S_{ion}{}^{0d}$	−13.9	−13.9	−13.8	+0.5	−4.7	−4.8	−5.2	−4.9

[a] ΔH and ΔF are given in kcal./mole and ΔS in e.u. [b] Calculated from k_2. [c] Calculated from k_1/K_1K_2. [d] Calculated from K_0.

trivial or fundamental, but the source of this discrepancy is unknown. A similar anomaly appears to be found for hemoglobin.[6,19]

The Effect of Temperature.—The thermodynamic functions ΔH^0, ΔF^0, and ΔS^0, and their analogs ΔH^*, ΔF^* and ΔS^*, in the absolute reaction rate theory were computed from the equations[20]

$$\Delta H^0 = -4.574 \times 10^{-3}\, \mathrm{d}(\log K)/\mathrm{d}(1/T) \quad (7a)$$

$$\Delta F^0 = -4.574 \times 10^{-3}\, T \log K \quad (7b)$$

$$\Delta S^0 = (\Delta H^0 - \Delta F^0)/T \quad (7c)$$

$$\Delta H^* = -4.574 \times 10^{-3}\, \mathrm{d}(\log k)/\mathrm{d}(1/T) - RT \times 10^{-3} \quad (8a)$$

$$\Delta F^* = 4.574 \times 10^{-3}\, T\,(10.319 + \log T - \log k) \quad (8b)$$

$$\Delta S^* = (\Delta H^* - \Delta F^*)T \quad (8c)$$

The values determined from k_1/K_1K_2, k_2 and pK_0 for each reaction are given in Table I and Arrhenius plots are presented in Fig. 6.

It is immediately apparent that the activation parameters at 0, 5 and 10° are actually quite small. The reactions at 28.7° seem almost qualitatively different. For these, the data from $k_2^{(12)}$ and $k_2^{(21)}$ agree with the low temperature results (the Arrhenius plots are linear) while $k_2^{(23)}$ and $k_2^{(32)}$ show unusual behavior, suggesting an enormous difference between the heat capacity of the activated complex and that of the corresponding ground states of $BH_{10} \rightleftarrows CH_{10}$ ($\Delta C_p{}^*$ of several kcal. per mole per degree). However, the heat and entropy of activation at 28.7° for $AH_{16} \rightarrow BH_{16}$ and $BH_{16} \rightarrow CH_{16}$ (from k_1/K_1K_2) also seem large. In these cases the increased curvature of the $k^{(12)}$ and $k^{(23)}$ graphs at 28.7° requires a decrease in $K_2^{(12)}$ from $10K_0$ to $2K_0$ so that $k_1^{(12)}$ itself may not be unusual. The anomaly also occurs in the second ionization.

It should be noted that by choosing the concentrations of the unstable species as $(PH_{n-m}) = (PH_n)K_1 \ldots K_m/h^m$ it is assumed implicitly that PH_{n-m} is that species for which any 6 of the total of 16 identical groups may be dissociated. To specify that a *specific* set of 6 of the identical groups must be ionized and a specific set of 10 groups protonated (PH_{n-m}) must be reduced by the binomial coefficient $(^{16}/_{10})$, and consequently $k_2^{(12)}$ or $k_2^{(21)}$ increased by the same factor. This would not change $\Delta H_2{}^*$, but it would decrease $\Delta F_2{}^*$ by the amount $4.574 \times 10^{-3}\, T \log (^{16}/_{10}) = 5.1$ kcal. and increase $\Delta S_2{}^*$ by $4.574 \times 10^{-3} \log (^{16}/_{10}) = 18$ e.u. There is obviously no way of distinguishing between these two extremes and the actual situation may have some intermediate distribution.

It is also apparent from the data (Figs. 2 to 5) that an attempt to compute activation parameters for a standard state of constant pH, as Lumry and Eyring[21] suggest, would be unreasonable. The dis-

(19) J. Steinhardt, E. M. Zaiser and S. Beychok, *J. Am. Chem. Soc.*, **80**, 4634 (1958).

(20) S. Glasstone, "Textbook of Physical Chemistry," D. Van Nostrand Co., New York, N. Y., 1946, p. 827 ff., p. 1103.

(21) R. Lumry and H. Eyring, "Implications of the Chemical Kinetics of Some Biological Systems," in "The Present State of Physics," American Association for the Advancement of Science,

$$\begin{array}{ccccccc}
 & \overset{K_1^{(12)}K_2^{(12)}}{\cdots\cdots} & & \overset{(K_0^{(12)})_6}{\cdots\cdots} & & \overset{(K_0^{(12)})^{10}}{\cdots\cdots} & \\
AH_{18} = & & = AH_{16} + 2H^+ = & & = AH_{10} + 8H^+ = & & = A + 18H^+ \\
k_1^{(12)}\downarrow\uparrow k_1^{(21)} & & & & k_2^{(12)}\downarrow\uparrow k_2^{(21)} & & \\
 & \overset{K_1^{(21)}K_2^{(21)}}{\cdots\cdots} & & \overset{(K_0^{(21)})^6}{\cdots\cdots} & & \overset{(K_0^{(21)})^{10}}{\cdots\cdots} & \\
BH_{18} = & \underset{K_1^{(23)}K_2^{(23)}}{\cdots\cdots} & = BH_{16} + 2H^+ = & \underset{(K_0^{(23)})^6}{\cdots\cdots} & = BH_{10} + 8H^+ = & \underset{(K_0^{(23)})^{10}}{\cdots\cdots} & = B + 18H^+ \\
k_1^{(23)}\downarrow\uparrow k_1^{(32)} & & & & k_2^{(23)}\downarrow\uparrow k_2^{(32)} & & \\
 & \overset{K_1^{(32)}K_2^{32}}{\cdots\cdots} & & \overset{(K_0^{(32)})^6}{\cdots\cdots} & & \overset{(K_0^{(32)})^{10}}{\cdots\cdots} & \\
CH_{18} = & & = CH_{16} + 2H^+ = & & = CH_{10} + 8H^+ = & & = C + 18H^+
\end{array} \quad (9)$$

tinction made by LaMer,[22] Steinhardt[7] and with modification by Eyring and Stearn[23] must be retained. By choosing a standard state of constant pH, not only are the ΔH^0 and ΔS^0 of ionization included in the activation parameters, but also the entropy of dilution of H^+ from the usual standard state of pH 0. It is recognized that the ionizing groups may be unusual[8] and that the ionization reactions may involve rearrangements as well. However, it does not seem reasonable to include in ΔS^* the ΔH^* dilution term when discussing for example the number of rotational degrees of freedom gained in the activated complex.

Discussion

The analysis of the kinetics of denaturation of conalbumin has been based on the sequence of equilibria summed up in the diagram shown.

Two sets of values for the equilibrium constants associated with the B species are indicated. One set, $K^{(21)}$, has been derived from the reaction B → A and one, $K^{(23)}$, from B → C. If the identical species of B are involved in both reactions, the two sets of constants should be the same. The differences apparent in Table I may be within the error of the analysis of the B → C reaction. However, it is possible that the species going to A and going to C are somewhat different, particularly if the differences arise as discussed above from the consequences of a requirement that a specific set of groups must be ionized rather than only a given number.

It may now be considered how much can be inferred about the nature of the ionizing groups and about the mechanism by which a constellation formed by the ionization of exactly 6 out of 16 identical groups produces a uniquely unstable species.

The chemical nature of the 16 groups corresponding to the ionization constant K_0 is obvious. They are carboxyl groups. At ionic strength 0.1 in the pH region in which pK_0 is determined (3.8 to 4.2) the electrostatic contribution to pK, $-0.87\ Zw$, is about -0.7 and no longer strongly dependent on Z.[5] The intrinsic pK's all lie between 4.5 and 4.8. The normal heat and entropy of carboxyl dissociations are 0 to 1 kcal. and -18 to -21 e.u. per mole, respectively. ΔH^0 for the critical groups is high (1 to 9 kcal.) and the corresponding entropies, even after subtracting the 3 e.u. contributed by neglecting the electrostatic term, are also high. It is interesting to note that the average values of ΔH^0 and ΔS^0 for all the carboxyl groups, computed from the titration curves, are 2.1 kcal. and -13 e.u., respectively. This is the behavior expected if there are 16 homologous single-bond interactions[14] and 68 normal carboxyls, with pK_0 estimated by extrapolation from the alkaline end of the titration.

The reactivity of a PH_{10} species may reflect destabilization of this charge type or stabilization of both adjacent species. The latter would require separate mechanisms at low pH, such as COOH . . . . COOH bonds, and at high pH, perhaps COO^-X^+ interactions. A possible mechanism may be considered based on the stronger COOH . . . . COO^- interaction.[5] The uniqueness of 10 COOH and 6 COO^- may represent a species in which this type or bond is maximally formed. On this view, successive protonations starting with a stable high pH species would decrease the electrostatic repulsions in a group of closely packed carboxylates so that maximum hydrogen bonding would occur when one particular species, PH_{10}, was reached. Further protonation would result in breaking these hydrogen bonds. This process yields a single species, PH_{10}, in which bonding is greater than in the adjacent species, PH_9 and PH_{11}. Presumably the strong bonding distorts another portion of the molecule which then undergoes rearrangement. The values obtained for pK_0 were computed close to the point of equal numbers of carboxyl and carboxylate groups. ΔH^0 and ΔS^0 for further ionization would include positive contributions for breaking the COOH . . . . COO^- bonds. It may be supposed that such a mechanism would depend on one or only a few of the possible microscopic configurations of the PH_{10} species as discussed above.

It must be emphasized that the net changes are small. If the thermodynamic functions are calculated for those equilibria for which there are data, the following somewhat surprising results are obtained: For $AH_{16} = BH_{16}$ (*via* AH_{18} and BH_{18}, from k_1/K_1K_2) $\Delta F^0 = -1.2$ kcal., $\Delta H^0 = -6$ kcal., $\Delta S^0 = -16.8$ e.u.; for $AH_{10} = BH_{10}$, $\Delta F^0 = +0.1$ to 1.0 kcal., $\Delta H^0 = -6$ kcal., $\Delta S^0 = -23$ e.u.; for $BH_{10} = CH_{10}$, $\Delta F^0 = 0.5$ kcal., $\Delta H^0 = +9$ kcal., $\Delta S^0 = 30$ e.u.

Laskowski and Scheraga[14] estimate ΔH and ΔS for hydrogen-bonding of the NH . . . O type as

Washington, D. C., 1954, pp. 189–210. "LaMer has criticized the high activation parameters calculated for thermal inactivation of pepsin on the grounds that since these quantities vary with pH, a standard state of constant acid dissociation is preferable to that of constant pH, on which the original calculations were based. Use of the standard state he suggests would result in subtracting, from activation values, the very large contributions made by proton dissociation. The important process is the transition from folded to unfolded form at constant pH, so that the standard state of constant pH is certainly more useful. Indeed, it is probably the only meaningful state."

(22) V. K. LaMer, *Science*, **86**, 614 (1937).

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

-6 kcal. and -20 e.u. Schellman's[24] analysis of the properties of urea as modified by Kauzmann[25] leads to $\Delta H = -1.5$ kcal. and ΔS (unitary) $= -3.3$ e.u. Values of $\Delta H = -4$ kcal. and $\Delta S = -10$ e.u. for similar hydrogen bonds in synthetic polynucleotides were obtained by Warner and Breslow.[26] The transitions A → B and B → C would thus correspond to the *formation* of one to two and disruption of two or three such bonds respectively.

In a molecule the size of conalbumin the observed phenomena may be the resultant of many interactions. In particular, Kauzmann[25] has suggested that the transfer of a non-polar residue from the interior of a protein molecule to the aqueous interface would have a ΔH of -2.5 kcal. and ΔS of -18 e.u. A denatured species, probably C, is known to expand[4,5] with hydration, increasing from 75 to 200%. Transfers and bond-breaking could proceed together, with the net change in ΔH and ΔS remaining small.

The kinetic data do not permit further refinement. It has been concluded that the changes in the molecule are of a limited nature. This seems reasonable in a general sense from the fact that the reactions are reversible, with substantial amounts of all species present at equilibrium, and because the solubility discrimination into three species rationalizes the kinetics.

Comparison of the data on rates and equilibrium positions of the reactions discussed here with the conditions under which the two-component electrophoretic patterns were observed has permitted the identification of the slower component as A + B and the faster as C.[5] The increase in net positive charge and expansion of the protein are thus associated with B → C.

An altogether different, non-specific electrostatic theory for the *p*H dependence of the rate of denaturation has been suggested by Kauzmann.[27] This treatment may explain, qualitatively, the greater stability of proteins near their isoelectric points. However, the behavior of conalbumin, particularly that illustrated by Figs. 2 and 3, like that of lysozyme[12] and bovine plasma albumin,[9,10] shows that the detailed variation in rate of denaturation with *p*H cannot be accounted for by simple functions of the net charge.

(24) J. A. Schellman, *Compt. rend. trav. lab. Carlsberg*, **29**, 223 (1955).

(25) W. Kauzmann, *Advances in Protein Chem.*, **14**, 1 (1959).

(26) R. C. Warner and E. Breslow, "Proceedings of the Fourth International Congress of Biochemistry, IX," Pergamon Press, London, 1959, p. 157.

(27) W. Kauzmann, in W. D. McElroy and B. Glass, "A Symposium on the Mechanism of Enzyme Action," The Johns Hopkins Press, Baltimore, Md., 1954, pp. 76–79.

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The Hydrogen-ion Equilibria of Conalbumin[1]

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Titration curves of conalbumin have been obtained at 5, 15 and 25° at ionic strengths of 0.01, 0.03 and 0.1. Time dependent proton equilibria near *p*H 4 have been observed with the rapid flow titration technique. A *p*H dependent transition between two species of conalbumin in this *p*H range has been investigated by electrophoresis. These have been correlated with the species indicated by kinetic studies to be formed during denaturation. The mobilities of these species and the electrostatic interaction factors obtained in the forward and reverse titrations indicate that the native and denatured forms are initially compact but expandable. The behavior of the tyrosyl residues indicates that 7 are buried (*i.e.*, not in hydrogen ion equilibrium) and that a large portion of the rest, possibly the 6 involved in chelating Fe^{+++} to form the iron complex, are hydrogen-bonded. Specific local electrostatic interactions are indicated in the entire *p*H range. The implications of the data for a model of the conalbumin molecule are discussed.

Hydrogen ion equilibria, electrophoretic mobility and denaturation kinetics depend, in different but related ways, on the charge, the charge distribution and the underlying structure of the protein molecule. These properties of conalbumin were the object of the present studies.[3]

The Linderstrøm-Lang model,[4] derived from Debye–Hückel theory applied to a sphere the net charge of which is smeared over its surface, has long been used as the point of departure for the discussion of protein titration curves[5–7] although deviations already were observed in its first application.[5] Scatchard[8] showed that the combinatorial terms used by Cannan[5] could be neglected when the number of identical groups exceeded four. His result, generalized to several types of groups, has been used in the extensive studies from Tanford's laboratory.[9–16] The final equations are

(1) Taken in part from a thesis submitted by Arnold Wishnia in partial fulfillment of the requirements for the degree of Doctor of Philosophy at New York University, June, 1957. An account of this work was presented at the Biocolloid Symposium, "Transformations of Proteins in Acidic Media," 132nd Meeting, American Chemical Society, New York, N. Y., September, 1957. A. Wishnia and R. Warner, *Abstr.*, p. 18-I. This work was supported by a research grant, H-1642, from the National Heart Institute, Public Health Service.

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(3) A. Wishnia and R. C. Warner, *J. Am. Chem. Soc.*, **83**, 2065 (1961).

(4) K. Linderstrøm-Lang, *Compt. rend. trav. lab. Carlsberg*, **15**, No. 7 (1924); K. Linderstrøm-Lang and S. O. Nielsen, in "Electrophoresis," Ed. M. Bier, Academic Press, Inc., New York, N. Y., 1959, p. 35.

(5) R. K. Cannan, A. C. Kibrick and A. H. Palmer, *Ann. N. Y. Acad. Sci.*, **41**, 243 (1941).

(6) R. K. Cannan, A. H. Palmer and A. C. Kibrick, *J. Biol. Chem.*, **142**, 803 (1942).

(7) R. K. Cannan, *Chem. Revs.*, **30**, 395 (1942).

(8) G. Scatchard, *Ann. N. Y. Acad. Sci.*, **51**, 660 (1949).

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(10) C. Tanford and G. L. Roberts, *ibid.*, **74**, 2509 (1952).

(11) C. Tanford and J. Epstein, *ibid.*, **76**, 2163 (1954).

(12) C. Tanford and M. K. Wagner, *ibid.*, **76**, 3331 (1954).