Acknowledgments.—We are indebted to Dr. O. K. Behrens of the Eli Lilly Co. for generous gifts of crystalline beef zinc insulin, to Dr. M. L. McFadden for the preparation of insulin methyl ester and for the identification of the heptapeptide produced by tryptic hydrolysis of insulin and to Dr. L. Gruen for carrying out the zinc analyses.

ITHACA, NEW YORK

[CONTRIBUTION FROM THE PALO ALTO MEDICAL RESEARCH FOUNDATION]

The Urea Denaturation of Chymotrypsinogen as Determined by Ultraviolet Spectral Changes. The Influence of *p*H and Salts[1]

BY CHARLES H. CHERVENKA

RECEIVED AUGUST 7, 1959

The rate of change of the spectrum of chymotrypsinogen in urea solutions is strongly influenced by the ionic environment. At low *p*H the rate is proportional to the third power of the hydrogen ion concentration, and this dependence varies with increasing *p*H in a manner which can be correlated with changes in the ionic form of the protein. In general, salts increase the rate of change below *p*H 4.8 and decrease it above this *p*H, and these alterations are proportional to the ionic strength. Calcium and strontium chlorides have an additional and more specific effect which is associated with binding of the metal ion in a 1:1 ratio with the protein molecule, probably to an imidazolyl side chain or the α-amino terminal group.

Several recent reports describe changes in the ultraviolet absorption spectra of proteins during urea treatment.[2-5] These spectral shifts are related to changes in bonding or other factors in the environment of the side chains of certain of the amino acid residues,[6,7] so that they are inherently a reflection of the configurational changes occurring during the general process of denaturation of proteins. Thus a new means of studying this process was suggested.

Previous work had indicated that such a method could be applied to the pancreatic protein, α-chymotrypsinogen.[4] When treated with a concentrated solution of urea, this protein shows a large shift in its ultraviolet spectrum, with a maximum change at 293 mμ. Calcium chloride was found to inhibit the spectral change at neutral *p*H. In the present study this effect was investigated more fully, particularly because the stabilizing action of certain divalent metal ions, notably calcium, upon the enzyme chymotrypsin[8,9] suggested a possible common mechanism. A study of the influence of *p*H upon the rate of urea denaturation was also undertaken so that this factor could be separated from the salt effect; also very little such data are available in the literature.[10] The changes of optical rotation of chymotrypsinogen in urea have been investigated previously,[11] but the effects of variation in *p*H upon the rate were not reported.

Experimental

α-Chymotrypsinogen was purchased from the Worthington Biochemical Laboratory, Freehold, N. J. as the once crystallized filter cake. The material was recrystallized five times from ammonium sulfate,[12] dialyzed thoroughly against 0.001 *M* hydrochloric acid and lyophilized. Fresh solutions in 0.001 *M* hydrochloric acid were prepared as needed.

Both of two brands of commercially available reagent grade urea were found to contain colloidal material which interfered with their use in the spectrophotometer. Before use, a warm, concentrated solution of urea in 30% ethanol was filtered through a Seitz bacterial filter and allowed to crystallize in the cold. The crystals were collected and dried at room temperature. Fresh stock solutions of this material were made up daily. Other chemicals were either C.P. or reagent grade and were used with no further purification.

Ultraviolet absorption measurements were made in the Beckman model DU Spectrophotometer. Temperature control was obtained by circulating water of constant temperature through the thermospacers. Although the cuvette compartment of the instrument could be maintained at 25 ± 0.1° easily, the temperature variation of samples in cuvettes during reaction times was greater, ±0.2°.

The urea solution and the required amount of hydrochloric acid or sodium hydroxide and salt were equilibrated at 25°. In some instances the salt was dissolved in the chymotrypsinogen solution. At zero time the protein sample was added and mixed thoroughly. The final total volume in the cuvette was 3.0 ml. Readings of the difference in absorbance at 293 mμ between the test cuvette and a reference cuvette containing an equal concentration of protein in 0.001 *M* hydrochloric acid were made with time; the initial reading was made at 30 sec. and subsequent readings were made at 15 sec. intervals or longer. The final absorbance value was the same for all reactions which went to completion.

The first-order rate constants were calculated from the change in absorbance by the usual graphical method and are expressed in reciprocal seconds. The molar extinction coefficient of the spectral change at 293 mμ is 7.6×10^3, using a molecular weight of 25,000 for chymotrypsinogen.[13]

Measurements of *p*H were made using the Radiometer model 22 *p*H meter. In the presence of urea the measurements were recorded as taken, and no attempt was made to correct for the effect of urea on the system.

(1) This investigation was supported by research grant numbers C-2289(C4) and A-2800 from the National Institutes of Health, Public Health Service. A report of the preliminary results of this investigation was made at the annual meeting of the Pacific Slope Biochemical Conference at Los Angeles in December, 1958.

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Results

Order of Reaction.—At all *p*H values used the urea denaturation of chymotrypsinogen follows the kinetics of a first-order reaction with respect to time, as indicated for example by the straight lines of Fig. 1. The reaction is also first order with respect to protein concentration at *p*H 5.4 to 5.6, as can be seen by the parallel slopes of the plot for four different concentrations of chymotrypsinogen.

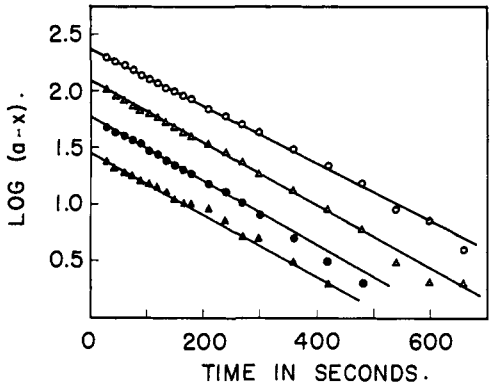
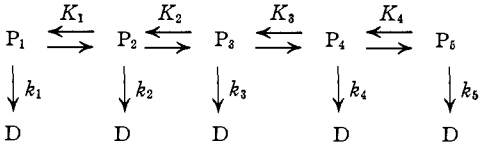


Fig. 1.—First-order plots for rates of change of absorbance at 293 mμ in 7.9 *M* urea at *p*H 5.4 to 5.6. Concentration of chymotrypsinogen × 10^5 *M*; O, 3.2; △, 1.6; ●, 0.8; ▲, 0.4.

Influence of *p*H.—Figure 2 illustrates the profound effect of the hydrogen ion concentration upon the rate of urea denaturation of chymotrypsinogen. The experimental data support the general theory of Levy and Benaglia[14] describing the *p*H dependence of denaturation rates. In 7.9 *M* urea below *p*H 4 the rates are too fast to measure by the manual spectrophotometric method. Using lower concentrations of urea, measurements were made down to *p*H 3 (Fig. 2), where the rate was found to be proportional to the third power of the hydrogen ion concentration. Thus there are at least three proton dissociations, whose *pK*'s are well below the experimental range used here, which influence the rate of denaturation. A fourth dissociation of *pK* 7.1 is indicated by the downward bend in the curve of Fig. 2. Using the notation of Laidler,[10] these dissociations can be represented as



where P_1, P_2, etc., represent different ionic forms of the protein resulting from successive losses of one proton, and D is the denatured form of the protein. The special case of Levy and Benaglia's general equation which has been found to fit the experimental data for chymotrypsinogen is

$$\bar{k} = \frac{k_1 + \frac{k_2K_1}{H} + \frac{k_3K_1K_2}{H^2} + \frac{k_4K_1K_2K_3}{H^3} + \frac{k_5K_1K_2K_3K_4}{H^4}}{\frac{K_1K_2K_3}{H^3} + \frac{K_1K_2K_3K_4}{H^4}} \quad (1)$$

(14) M. Levy and A. E. Benaglia, *J. Biol. Chem.*, **186**, 829 (1950).

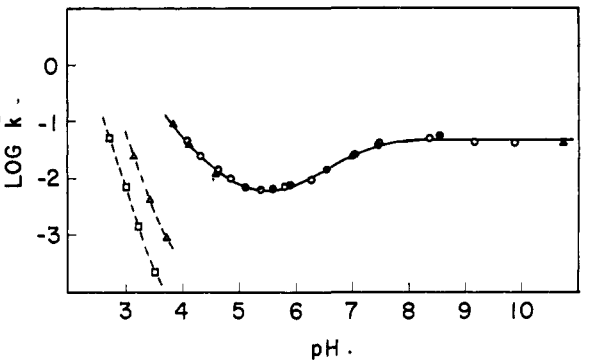


Fig. 2.—Variation of the logarithm of the rate constant with *p*H for 1.6 × 10^{-5} *M* chymotrypsinogen in urea. Concentration of urea: O, ●, ▲, 7.9 *M*; △, 5.0 *M*; □, 4.0 *M*. For 7.9 *M* urea the different symbols give results of separate experiments. The solid line was calculated from eq. 2 in the text.

where *H* is used to represent hydrogen ion concentration.

This equation simplifies to

$$\bar{k} = \frac{\frac{k_1H^3}{K_1K_2K_3} + \frac{k_2H^2}{K_2K_3} + \frac{k_3H}{K_3} + k_4 + \frac{k_5K_4}{H}}{1 + \frac{K_4}{H}} \quad (2)$$

The values of the constants required to fit this equation to the experimental data were found empirically and are given in Table I. In the *p*H region employed with 7.9 *M* urea the first term of the numerator is insignificant.

TABLE I

APPARENT KINETIC CONSTANTS FOR DENATURATION[a]

Salt	Concn., *M*	k_4 × 10^3	k_5 × 10^2	$\frac{k_2}{K_2K_3}$ × 10^{-5}	$\frac{k_3}{K_3}$	K_4 × 10^7
None	..	4.2	5.3	1.3	420	0.8
Potassium	0.01	3.8	3.5	3.8	470	.9
chloride	.10	1.7	1.5	13	530	.7
	.30	1.3	0.74	29	360	.9
Magnesium	.001	4.2	4.2	..	420	.9
chloride	.01	2.6	2.5		650	.7
	.10	1.3	0.6	11	530	1.0
Potassium sulfate	.033	1.3	1.5	7.7	380	0.8
Magnesium	.0025	2.7	2.8	..	470	.5
sulfate	.025	1.3	1.1	6.7	420	.8
Calcium	.001	4.2	0.67	2.3	470	56
chloride	.01	..	.048	4.8	600	..
	.10	..	.0055	17.5	87	..
Strontium	.0033	3.2	1.7	..	630	2.0
chloride	.033	1.5	0.22	9.3	750	13

[a] Dimensions: *k* in reciprocal seconds, *K* in moles/liter.

Influence of Salts.—The case of the general effect of salts is illustrated in the data for potassium chloride, shown in Fig. 3, with the corresponding empirical constants for eq. 2 given in Table I. With magnesium chloride, cadmium chloride, potassium sulfate, magnesium sulfate and zinc sulfate a similar behavior was observed. With the exception of cadmium chloride, the extent of apparent change in the constants such as k_5 brought about by these salts is directly proportional to the ionic strength. Cadmium chloride is slightly more

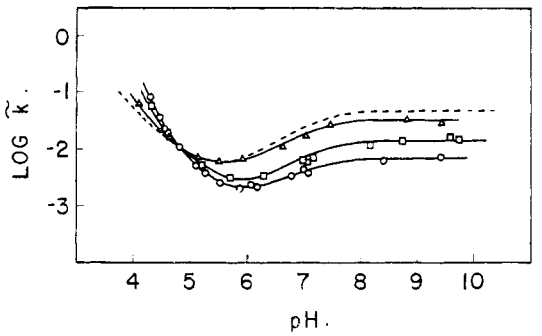


Fig. 3.—Influence of potassium chloride on the logarithm of the rate constant of 1.6×10^{-5} *M* chymotrypsinogen in 7.9 *M* urea. Dashed line from Fig. 2; solid lines calculated from eq. 2. Potassium chloride concentration: △, 0.01 *M*; □, 0.10 *M*; ○, 0.30 *M*.

effective than solutions of the other salts of equal ionic strength, possibly because of the tendency of cadmium to form ionic complexes in solution.

In Fig. 4 the more specific effect of calcium chloride is shown, with the constants required to fit the experimental data given in Table I. In this

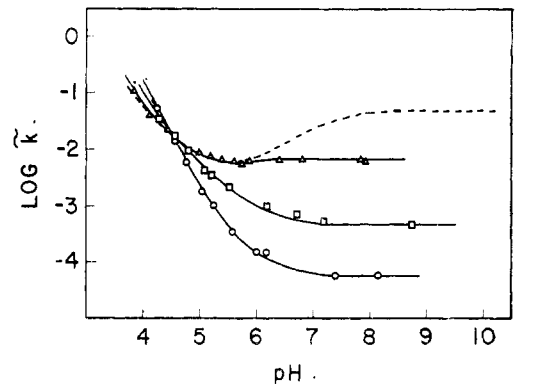


Fig. 4.—Influence of calcium chloride on the logarithm of the rate constant of 1.6×10^{-5} *M* chymotrypsinogen in 7.9 *M* urea. Dashed line from Fig. 2; solid lines calculated from eq. 2. Calcium chloride concentration: △, 0.001 *M*; □, 0.01 *M*; ○, 0.10 *M*.

case, and in the case of strontium chloride, the apparent changes in the specific rate constants indicate a direct binding of one metal ion per protein molecule. If in the presence of certain metal ions the following equilibrium occurs

$$PM_n \overset{K_I}{\rightleftharpoons} P + nM \qquad (3)$$

where PM_n is the metal–protein complex, P is the unbound protein, M is the metal ion and n is the number of ions bound per protein molecule, then

$$K_I = \frac{[P] \times [M]^n}{[PM_n]} \qquad (4)$$

If the rate of denaturation v equals $k[P]$ and the rate of denaturation of PM_n is zero, then the decrease in rate as metal ion is added is

$$\Delta v = k[PM_n] \qquad (5)$$

and

$$K_I = M^n \frac{v}{\Delta v} \qquad \text{Log}\left(\frac{\Delta v}{v}\right) = n \log M - \log K_I \qquad (6)$$

A plot of log $\Delta v/v$ *versus* $-\log M$ has a slope equal to $-n$ and an intercept of pK_I. Such a plot for calcium chloride at *p*H 6.5 is shown in Fig. 5 and has a slope of -1 and an intercept at 3.1. Between *p*H 4.8 and 9.0 the slope remains one, while the intercept increases with *p*H to a maximum of 4.0. For strontium chloride the slope of such a plot is also unity, but the maximum pK_I attained is only 2.7, so that the binding of strontium ion is less complete than that of calcium ion. Similar behavior was expected for barium, the third element of Group IIA of the periodic table, but conclusive data could not be obtained because of the reduced solubility of the barium–chymotrypsinogen complex.

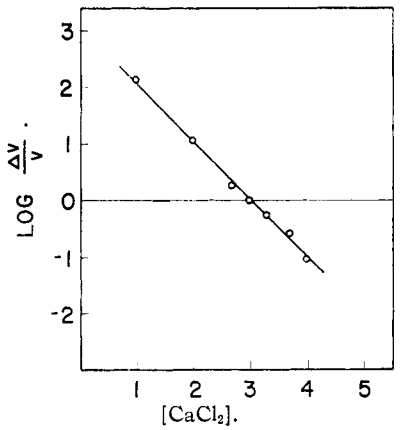


Fig. 5.—Variation of log $\Delta v/v$ with concentration of calcium chloride; *p*H 6.5.

This relation between the concentration of calcium or strontium ion and the decrease in rates shows that the values of k_5 given in Table I are only apparent constants, because the decrease in rates is due to a decrease in the concentration of the form of chymotrypsinogen susceptible to denaturation, rather than a change in the intrinsic rate constant.

When the $\Delta v/v$ data were plotted for the metal salts previously listed other than calcium and strontium chloride, the slopes obtained were variable and less than one. Thus in these cases there is no evidence that the inhibition is a result of 1:1 binding of metal ion.

From Table I it can be seen that K_4 is not greatly affected by changes in salt concentration except when the salt is calcium or strontium chloride. In these two instances the value of K_4 increases rapidly with increasing salt concentration; thus the ionization which has the dissociation constant K_4 is shifted far to the right due to the displacement of protons by metal ions. In the limit it is possible that $K_4 = K_3$, and the species P_4 disappears entirely, while P_5 is all in the form of a calcium complex.

Discussion

From the fit of the experimental data to the general theory of Levy and Benaglia[14] it appears that variations in the rate of urea denaturation of α-chymotrypsinogen with *p*H can be adequately explained in terms of varying amounts of different ionic forms which react at different rates. The

two rate constants which can be determined separately, k_4 and k_5, both are apparently decreased by all the salts employed. With calcium and strontium chlorides the large decrease in rates in the neutral and alkaline *p*H regions can be correlated with a decrease in the concentration of the labile form of the protein. With other salts the effect is a function of ionic strength and cannot be explained in terms of specific ion binding. Similarly, changes in the value of K_4 alone could not account for the decrease in experimental rates in the alkaline region. In the acid region, however, where the effect of calcium and strontium chlorides is qualitatively the same as that of the other salts, it would appear that the increases in rates noted result in decreases in one or more of the constants K_1, K_2 and K_3. The data in Table I indicate that a change in the value of K_2 is most likely. In the absence of salt these three constants must have values well above 10^{-3} M, and probably as in the case of ricin[14] are too acid to be dissociation constants of the carboxyl groups. The nature of these very acid groups is unknown.

The prototropic group of chymotrypsinogen which could have a pK of 7.1 in urea solution is most likely one of the imidazolium side chains[15] or the α-ammonium group of the terminal cystine residue.[16] Since an imidazole side chain has been postulated to be an essential part of the active site of chymotrypsin,[17] and since calcium chloride increases the activity and reduces the autolysis of this enzyme,[4,8,9] the possibility arises that the calcium binding reported here occurs at the group in the precursor which becomes a part of the active center of the enzyme. Further experiments are planned in order to investigate this possibility.

In a study of the urea denaturation of ovalbumin, Simpson and Kauzmann[18] found, for example, that calcium and magnesium ions accelerated the rate of change of the optical rotation at *p*H 7.6, while anions such as sulfate were found to be inhibitory. This pattern is quite different from the results of the present study, where no salt tested increased the rate of denaturation at neutral *p*H. This difference may represent a fundamental difference in key structural features of the two proteins or may mean that the two methods measure quite different phases of the over-all process of denaturation.

Initially the experiments described here were attempted using 6.6 M urea solution. At this concentration the reactions are considerably slower, and the extent of spectral change was found to be a function of *p*H. This was interpreted as an indication that the reaction measured was reversible and that equilibrium values of the absorbance change were obtained rather than final values. Because the equilibrium is attained only slowly and the *p*H of unbuffered urea solutions drifts slowly toward *p*H 7.5,[18] it was found necessary to use stronger solutions of urea so that reactions were complete within 15 to 20 minutes. In some cases, for example when the high concentrations of calcium chloride were used, this was not possible because of the very slow rates even in 7.9 M urea. However, the kinetics and the results of long time experiments indicate that in these cases the reactions still go to completion, so that the effect of salt is not that of shifting the equilibrium point. In the future it is hoped that the difficulties involved in the study of these equilibrium reactions can be overcome, so that equilibrium and rate constants of reverse reactions can be determined.

Acknowledgment.—The author wishes to acknowledge the support and helpful suggestions of Dr. B. H. J. Hofstee during the course of this work.

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PALO ALTO, CALIFORNIA

[CONTRIBUTION NO. 1531 FROM THE STERLING CHEMISTRY LABORATORY, YALE UNIVERSITY]

The Heat of Denaturation of Ferrihemoglobin in Acid Solution[1]

BY W. W. FORREST AND J. M. STURTEVANT

RECEIVED FEBRUARY 19, 1959

The reversible acid denaturation of horse ferrihemoglobin in formate buffer has been subjected to calorimetric study. At 25° in formate buffer a heat absorption of 10.0 ± 0.3 kcal./mole (mol. wt. 68,000) is observed, the kinetics of heat absorption agreeing fairly well with the kinetics of denaturation reported by Steinhardt and co-workers.[2] At 15°, a heat evolution of 76.0 ± 1.6 kcal./mole is observed; at this temperature the calorimetric kinetics deviate considerably from the denaturation kinetics given by Steinhardt, *et al.* The large influence of temperature on the heat of the reaction is indication of the complexity of the reaction. The results obtained are discussed in connection with the nature of the reaction.

The acid denaturation of horse ferrihemoglobin has recently received detailed study by Steinhardt[2] and his co-workers. The reaction is reversible (although accompanied by a considerably slower irreversible denaturation), and it takes place at a measurable rate, which increases with decreasing *p*H, when the *p*H of a ferrihemoglobin solution is taken below about *p*H 4.5 (at 25°). The reaction is accompanied by a loss of solubility at the isoelectric point, a large decrease in absorp-

(1) This research was aided by grants from the National Science Foundation (G179) and the United States Public Health Service (RG3996).

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