THE REVERSIBLE HEAT DENATURATION OF CHYMOTRYPSINOGEN*

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Although the process of the reversible denaturation of proteins is one of the most challenging problems of protein chemistry, this field has been relatively dormant for about 10 years.[1] Recently, the excellent study by Kunitz (1) of the reversible heat denaturation of crystalline soy bean trypsin inhibitor has given rise to a revival of interest in this problem. The present investigation has been made as a part of the general study of the pancreatic proteases which has been carried out in this laboratory.

The reversible heat denaturation of trypsin has been studied by Northrop (2), by Anson and Mirsky (3), and by Kunitz and Northrop (4) and the reversible heat denaturation of chymotrypsin has been studied by Kunitz and Northrop (5). Investigations of reversible denaturation with proteolytic enzymes may be complicated by the fact that over a large portion of the pH scale the native protein is enzymatically active and digests the denatured protein. For this reason, chymotrypsinogen, which has also been found to undergo reversible heat denaturation but which has no known enzymatic activity, was selected for the present study.

Methods

Crystalline chymotrypsinogen was prepared and recrystallized eight times by the method of Kunitz and Northrop (6). The purity of this preparation was tested by measuring its solubility at 25°C. in 0.2 M acetate buffer of pH 5.08 containing 0.5 saturated ammonium sulfate. The technique of Kunitz (7) was used for these measurements. The solubility diagram was that of a single component with a solubility of 0.350 mg. of protein per ml. even in the presence of an eightfold excess of solid phase.

The crystalline protein was dissolved in 3 volumes of water at pH 3.0 and was dialyzed with stirring against frequent changes of 0.001 N HCl until negative tests for both ammonia and sulfate were found in a 12 hour dialysate. At this time no ammonia could be detected in the protein solution by the microdiffusion method of

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[1] Neurath, Greenstein, Putnam, and Erickson (26) have reviewed the literature of the chemistry of protein denaturation up to 1944.

Conway (8). The protein was then dried by evaporation from the frozen state and was stored in a refrigerator.

Protein concentrations were determined from optical density measurements at 282 mμ in the Beckman model DU spectrophotometer. Calibration against semi-micro Kjeldahl determinations yielded 2,070 as the specific extinction coefficient. The nitrogen content of chymotrypsinogen was taken as 15.8 per cent (5, 9).

Measurements of pH were made with a Beckman model G pH meter which was frequently checked against standard buffers.

Since the process of denaturation of proteins which possess no enzymatic activity is most conveniently followed by utilizing the differences in solubility between the native and denatured forms, a search for the optimal conditions for precipitating denatured chymotrypsinogen was undertaken. To this end, 1 ml. samples of a solution

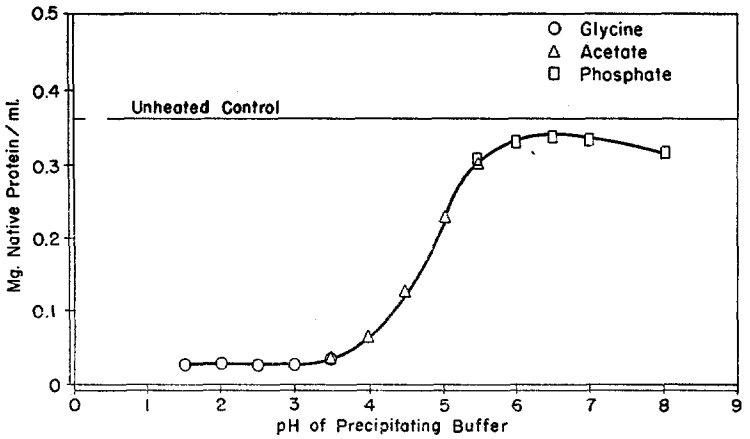

FIG. 1. Effect of pH on the precipitation of reversibly denatured chymotrypsinogen The final ionic strength in each case was 1.0.

of salt-free chymotrypsinogen, containing 2.16 mg. of protein per ml. and adjusted to pH 2 with HCl, were heated to 50°C. for 15 minutes. Five ml. portions of solutions containing 0.1 M glycine, acetate, or phosphate at various pH values plus sufficient NaCl to bring the total ionic strength to 1.2 were then added to the protein solutions. The solutions were mixed by pouring back and forth four times and were allowed to stand at 5–6°C. for 2 hours. The solutions were then centrifuged at 825 × g for 20 minutes and the supernatants decanted for protein determination. The results of this experiment are shown in Fig. 1. In no case was any protein precipitated from unheated control solutions by the addition of the buffer-salt solution.

Further experiments indicated that an increase of total salt concentration to ionic strengths greater than 1.2 has only a very small effect on the precipitation of the denatured protein. The choice of the conditions for precipitating the denatured protein is somewhat arbitrary since no sharp distinction can be drawn between the protein remaining in solution as a result of the solubility of the denatured protein in

the salt solution and that remaining in solution as a result of incomplete denaturation. However, the solution used to precipitate the denatured protein in all subsequent experiments was a pH 3.0 solution of 0.1 M glycine-glycine·HCl in 1.2 M NaCl. This solution was added to the protein solutions in a volume ratio of 5:1.

Native chymotrypsinogen is thus defined by its solubility in a 0.083 M glycine buffer of pH 3.0 containing 1 M NaCl. *Denatured chymotrypsinogen* is the material precipitated when the buffered salt solution is added to a heated solution of chymotrypsinogen. *Irreversibly denatured chymotrypsinogen* is the protein which is still precipitable by this buffer when a previously heated solution of chymotrypsinogen has been allowed to stand for 24 hours at room temperature before the addition of the precipitating buffer, and *reversibly denatured chymotrypsinogen* is defined by the difference

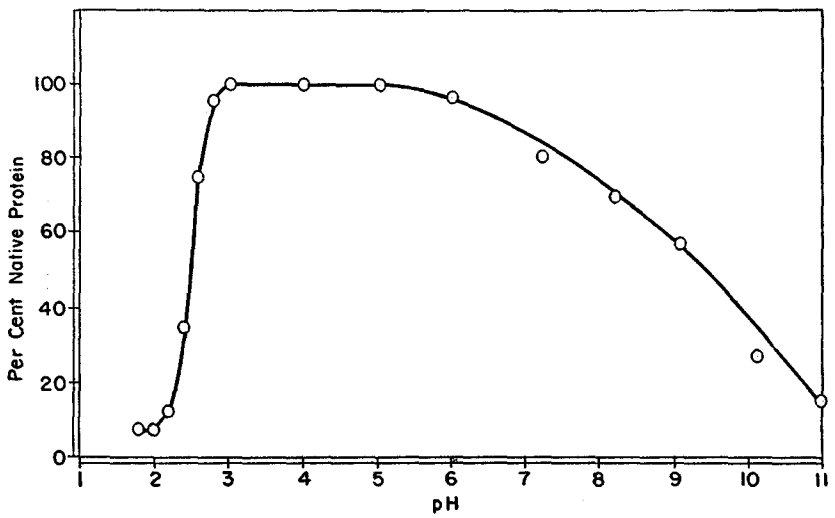

FIG. 2. Effect of pH on the denaturation of chymotrypsinogen at 50°C.

between denatured and irreversibly denatured chymotrypsinogen. These definitions are essentially those which have been previously used in measurements of this type.

EXPERIMENTAL

In order to determine the general effect of pH on the denaturation of the protein, solutions containing approximately one-quarter per cent chymotrypsinogen were adjusted to various pH values by the addition of HCl or NaOH and were warmed to 50°C. for 60 minutes before the addition of the precipitating buffer. The results of this experiment, plotted in Fig. 2, indicate that under these conditions no denaturation occurs between pH 3 and pH 5 while the degree of denaturation increases rapidly at pH values acid to this range and somewhat less rapidly in the pH region alkaline to this range.

The pH range in which reversible denaturation might be found was investigated by heating triplicate sets of solutions, made up as in the preceding experiment, in a

boiling water bath for 2 minutes. The precipitating buffer was added to one set immediately, to the second set after the solutions had been cooled to 5°C. for 5 minutes, and to the third set after the solutions had been kept at 5°C. for 18 hours. As is shown in Fig. 3, complete reversal of the denaturation which occurs under these rather drastic conditions is found only between pH 2 and pH 3. Between pH 4 and pH 6.5 the degree of reversal of denaturation decreases steadily and at pH values greater than 6.5 the protein is completely irreversibly denatured. Between pH 6.5 and pH 9.4 the protein coagulates during the heating process while at all other pH values no visible precipitate was formed. Since the primary purpose of this investigation was the study of the reversible denaturation process, all further measurements were made in the pH range from 2 to 3.

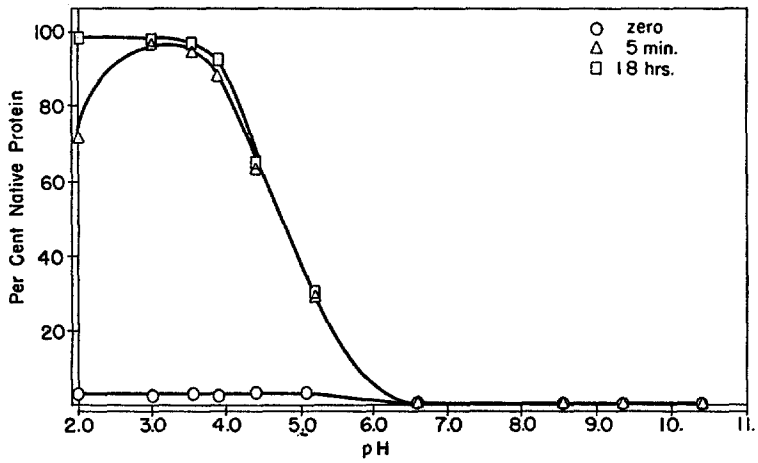

FIG. 3. Effect of pH on the denaturation and reversal of denaturation of chymotrypsinogen. See text for details.

The fact that a true equilibrium between native and reversibly denatured chymotrypsinogen exists in the pH range from 2 to 3 was established by the demonstration that the same amount of native protein remains in solution at a given temperature irrespective of whether the solution was heated to that temperature or cooled to that temperature. Fig. 4 presents typical results obtained by heating one of a pair of duplicate solutions, containing 2.16 mg. of protein per ml. at pH 2.0, to various temperatures between 40 and 45°C. and by cooling the other set from 50°C. to the same temperatures.

If it is assumed that both the forward and backward reactions in the reversible denaturation process follow first order kinetics, the equilibrium constant in this system may be expressed simply as

$$K = \frac{A - a}{a}$$

where: A is the total protein concentration and
a is the concentration of native protein.

The application of this elementary treatment assumes that conditions are so chosen that all the protein present is in either the native or reversibly denatured form. Unless otherwise specified, all experiments were carried out under conditions such that no irreversible denaturation occurred. The variation of these equilibrium constants with temperature at pH 2 and at pH 3 is shown by the van't Hoff plots of Fig. 5.

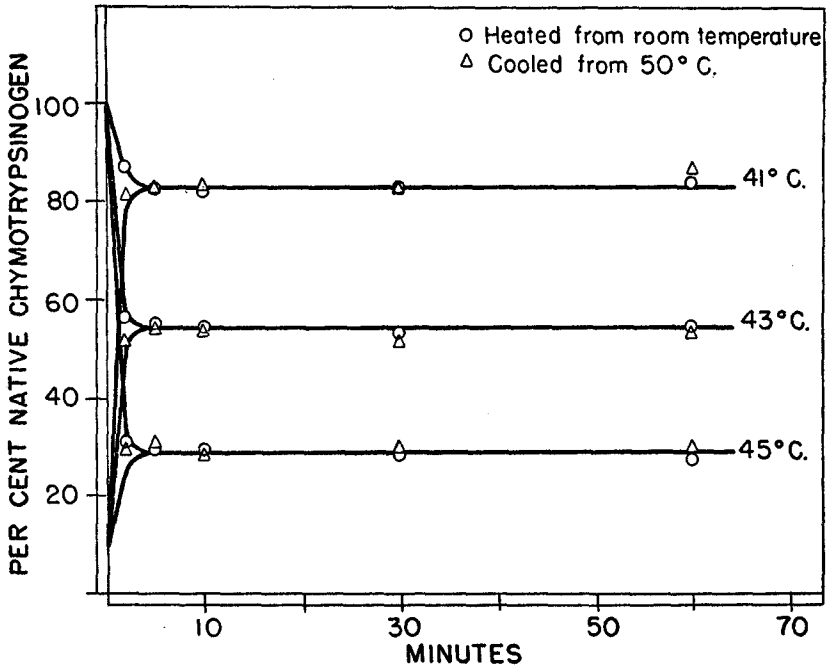

FIG. 4. Curves demonstrating the nature of the equilibrium between native and reversibly denatured chymotrypsinogen.

The effect of variation of pH over the range from pH 2 to pH 3 on the equilibrium between native and reversibly denatured chymotrypsinogen at 50°C. is illustrated by Fig. 6. The inset plot of Fig. 6 indicates that a decrease in pH of only 1 unit causes the equilibrium to shift from completely native protein to almost completely denatured protein. The resemblance of this curve to a titration curve involving several protons is apparent. The linear plot of Fig. 6 was derived from the treatment which Steinhardt used in studying the denaturation of pepsin (10). If it is assumed that an over-all equilibrium exists such that:

$$\underset{(R)}{\text{Reversibly denatured protein}} \rightleftharpoons \underset{(N)}{\text{native protein}} + n\text{H}^+,$$

and if it is further assumed that the dissociation constants for each of the n protons are identical, an equilibrium constant for the dissociation of these protons can be written:

$$nK_a = \frac{(N)(\text{H}^+)^n}{(R)}.$$

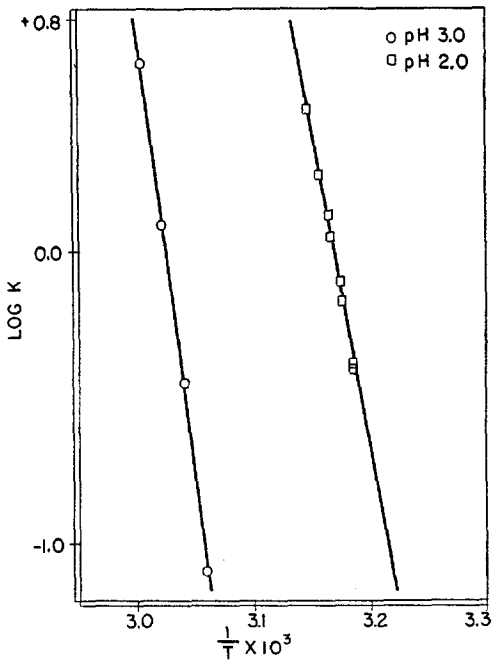

FIG. 5. Van't Hoff plot showing the variation in equilibrium constant for the equilibrium between native and reversibly denatured chymotrypsinogen with absolute temperature at pH 2.0 and at pH 3.0.

Then:

$$\log \frac{(N)}{(R)} = n(\text{pH} - \text{p}K_a).$$

A plot of the logarithm of the equilibrium constant for the equilibrium between native and reversibly denatured protein against pH yields a straight line whose slope is n, the number of protons involved in the equilibrium. In this case the slope is about three (3.16) and the pK_a is about 2.5.

Early attempts to elucidate the effect of protein concentration on the equili-

brium between native and reversibly denatured chymotrypsinogen indicated that at a given temperature a higher percentage of protein remained in the native form in dilute than in concentrated solutions of the protein. Further, it appeared that as the temperature was lowered so that the equilibrium was shifted toward native protein, this discrepancy became more marked. Since all other indications (*vide infra*) pointed to the fact that reversible denaturation is monomolecular with respect to protein, it appeared that this effect might

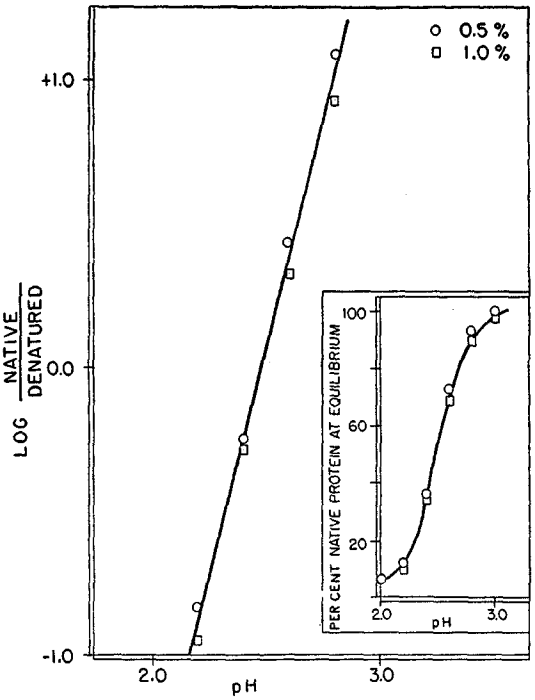

FIG. 6. Variation in equilibrium constant and in per cent native protein at equilibrium with pH for 0.5 and 1.0 per cent chymotrypsinogen.

result from the failure of the precipitating buffer to cause complete flocculation of dilute suspensions of denatured protein and from the failure of the relatively weak centrifugal fields used as routine for separation of the precipitated protein to sediment these suspended particles. Consequently, a second series of determinations was made at pH 2 by the same techniques except that the precipitated protein was centrifuged down at approximately 75,000 $\times$ g for 30 minutes. The results of this experiment are shown in Fig. 7. Although slight discrepancies between the various concentrations are apparent, these are believed to be due to the solubility of the denatured protein in the precipitating

buffer rather than to a real difference in the equilibria at various protein concentrations.

The effects of the addition of small amounts of sodium chloride to the system are shown in Fig. 8. These experiments were run at pH 2.0 and 43°C. The protein concentration was 2.24 mg. per ml. The results shown in Fig. 8, and confirmed by several other experiments, indicate that these are not true equilibria but that native protein continues to disappear from the solutions with increasing time. The amount of protein which does not revert to native protein was found to increase with time, salt concentration, and temperature. The "non-

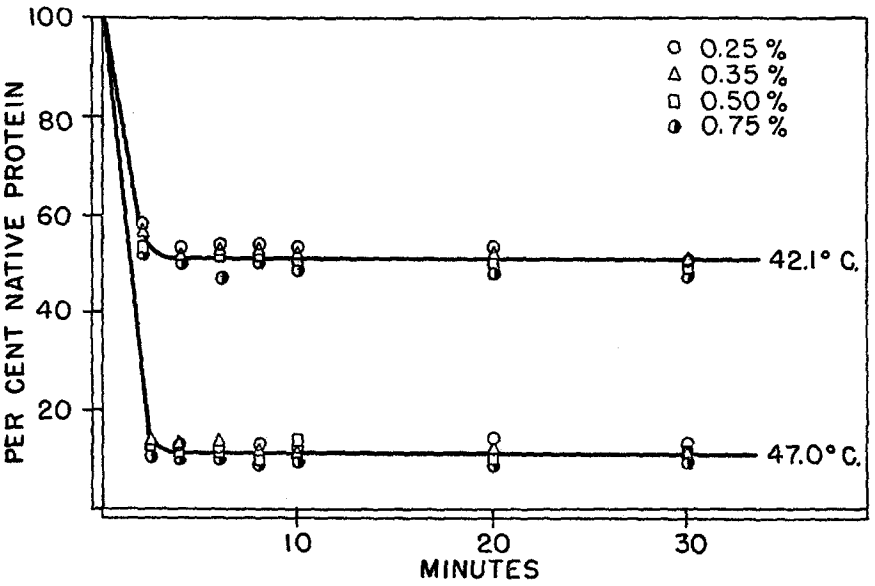

FIG. 7. Plot showing that the equilibrium between native and reversibly denatured chymotrypsinogen is essentially independent of protein concentration.

reversible" protein formed under these conditions, however, is not identical with the irreversibly denatured protein which is formed upon prolonged heating at elevated temperatures or in alkaline solutions, but appears to consist of aggregated reversibly denatured protein. This conclusion is based upon the following evidence:

1. If a solution of chymotrypsinogen containing salt was heated and allowed to stand at room temperature for 24 hours before the precipitating buffer was added, the resulting precipitate could be dissolved in 0.001 N HCl by heating for a few minutes in a boiling water bath. This solution, after being cooled, behaved identically with a solution of native chymotrypsinogen upon heating and upon recooling.

2. When a solution containing 4.42 mg. of chymotrypsinogen per ml. in a 0.05 M glycine buffer of pH 2 was heated for 10 minutes at 52°C. and was then allowed to stand at room temperature for 1 hour, ultracentrifugal analysis revealed the presence of two well defined components. One of these is characterized by a sedimentation constant of 2.38 Svedberg units, which is typical of native chymotrypsinogen in this buffer. $S_{20,w}$ for the heavier component is 16.3 which corresponds to a molecular weight in the region of 5×10^5.

It seemed possible that the effect of pH on the equilibrium between native and reversibly denatured protein (Fig. 6) might be due to the greater ionic

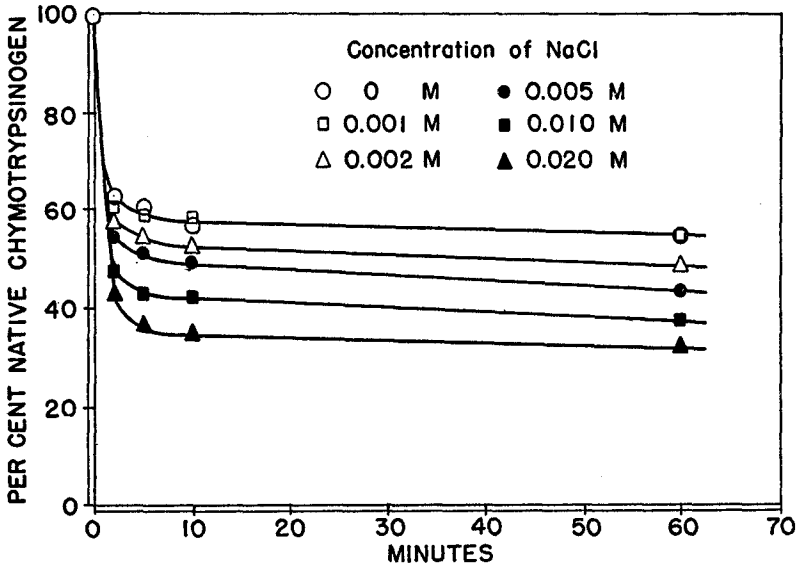

FIG. 8. Effect of added electrolyte on the amount of native chymotrypsinogen remaining in solution after various time intervals at pH 2.0 and 43°C.

strength of the more acid solutions rather than to the hydrogen ion activity of these solutions. This possibility was eliminated by carefully adjusting a solution of salt-free chymotrypsinogen at pH 2.0 to pH 3.0 with NaOH and by comparing this solution with a solution which had been initially adjusted to pH 3.0. When these solutions were warmed to the same temperature, the same percentage of native protein remained in each solution.

The effect of using various mineral acids for the adjustment of the pH of chymotrypsinogen solutions is shown in Table I. From these data it appears that the effect of four of the anions in shifting the equilibrium toward denatured protein at pH 2.0 is in the order:

$$H_2PO_4^- < Cl^- < Br^- < NO_3^-.$$

Although it is suggestive that this is also the order of ion binding by a number of proteins (11, 12), there is no significant difference among the effects of these anions at pH 3.0. In the cases of these four anions the denatured form reverts completely to native chymotrypsinogen when the temperature is lowered to room temperature. On the other hand, when $SO_4^=$ is present, native protein disappears continuously from solution at both pH 2.0 and pH 3.0 and the denatured protein formed during short periods of heating reverts to native protein to only a limited extent while the denatured protein resulting from heating for 30 or 60 minutes is almost wholly irreversibly denatured. Since it

TABLE I

Effect of Mineral Acid Anions on the Denaturation of Chymotrypsinogen

All solutions contained approximately 2.18 mg. of chymotrypsinogen per ml. and were adjusted to the given pH values with the acids given below:

Time, *min.*	Concentration of native protein as per cent total protein				
	H_3PO_4	HCl	HBr	HNO_3	H_2SO_4
			pH 2.0, 44°C.		
2	63	37	32	25	37
5	63	40	35	26	26
10	60	37	31	25	16
30	63	38	33	25	4
60	63	36	31	24	3
			pH 3.0, 56°C.		
2	59	67	63	58	58
5	56	63	—	56	42
10	58	61	56	56	38
30	59	64	59	56	34
60	59	59	59	59	24

seems most unlikely that the effect of H_2SO_4 is due to the contribution of the divalent anion to the ionic strength of these solutions, this effect is tentatively ascribed to a specific effect of the doubly charged sulfate anion.

Identity of the Reversed and Native Chymotrypsinogen.—The criteria which seem to establish a very strong presumption that the renatured chymotrypsinogen is identical with the native protein are as follows:—

(*a*) *Crystallizability of the Renatured Protein.*—240 ml. of a 1 per cent solution of chymotrypsinogen was adjusted to pH 2.0 with 1 N HCl and was heated for 4 minutes in an 80°C. water bath with constant stirring. The solution was rapidly cooled to 0°C. and was left at room temperature for 96 hours. Solid ammonium sulfate was then added to 0.2 saturation and the small precipitate which formed was removed by filtra-

tion. Solid ammonium sulfate was added to the filtrate to 0.85 saturation and the heavy amorphous precipitate was removed by filtration. This precipitate was treated by the usual method for the crystallization of chymotrypsinogen (6) and the product was recrystallized once by the same method. Both crystallizations yielded typical needle-like prisms which were indistinguishable from those of native chymotrypsinogen. The crystalline material was dialyzed free of salt and was lyophilized. 70 per cent of the starting material was recovered by this procedure.

(*b*) *Solubility of the Renatured Protein.*—The solubilities of the native and renatured protein were compared by the "crossed solubility" technique of Kunitz (1). 100 mg. of each of the lyophilized native and renatured proteins were placed in 16 × 125 mm. test tubes and 0.2 M acetate buffer of pH 5.08 containing 0.5 saturated ammonium sulfate was added to each tube until it was nearly full. Two glass beads were added to each tube and the tubes were carefully stoppered to avoid the inclusion of air bubbles.

TABLE II

A Comparison of the Solubilities of Native and Renatured Chymotrypsinogen

	Solubility in mg. protein per ml. supernatant solution	
	Experiment 1	Experiment 2
Last washings	*mg./ml.*	*mg./ml.*
Supernatant 1: Saturated solution of crystals of native protein	0.041	0.042
Supernatant 2: Saturated solution of crystals of renatured protein	0.043	0.042
Crossed		
Supernatant 1: Equilibrated for 48 hrs. with crystals of renatured protein	0.047	0.037
Supernatant 2: Equilibrated for 48 hrs. with crystals of native protein	0.038	0.037

The tubes were attached to a slowly revolving wheel and left for 48 hours at 26°C. At the end of this period the protein in each tube had crystallized. The crystals were centrifuged down and the protein concentration in the supernatants was determined. This process was repeated until three successive supernatants had identical protein concentrations. At this time the supernatants and crystals were crossed and equilibration was again carried out for 48 hours. The results of this experiment are shown in Table II.

(*c*) *Activation of Renatured Protein to δ-Chymotrypsin.*—The activation of chymotrypsinogen to δ-chymotrypsin was carried out by the method of Jacobsen (9). Approximately 100 mg. of native and of renatured chymotrypsinogen were each dissolved in 4.5 ml. of water and the pH was adjusted to 7.4 with dilute NaOH. The solutions were cooled to 0°C. and 0.5 ml. of a solution containing 7 mg. of crystalline trypsin per ml. was added to each solution. After 30 minutes, 1 ml. portions were withdrawn from each solution and were diluted to 200 ml. with 0.001 N HCl.

The activity of the enzyme in the diluted solutions was tested against acetyl-*l*-

tyrosine ethyl ester by the potentiometric titration method previously used in this laboratory (13, 14). Fig. 9 illustrates the results obtained for the hydrolysis of 0.012 M acetyl-*l*-tyrosine ethyl ester in 30 per cent methanol in the presence of 0.01 M phosphate buffer, pH 7.8, at 25°C. The initial slope is identical in each case and is 0.9 mole of substrate split per liter per minute per milligram of enzyme nitrogen per milliliter. This is in fair agreement with the value calculated from the kinetic constants reported by Schwert and Kaufman (15) for this enzyme system.

(*d*) *Sedimentation Rate of the Renatured Protein.*—The sedimentation rate of renatured chymotrypsinogen was determined in the model E analytical ultracentrifuge

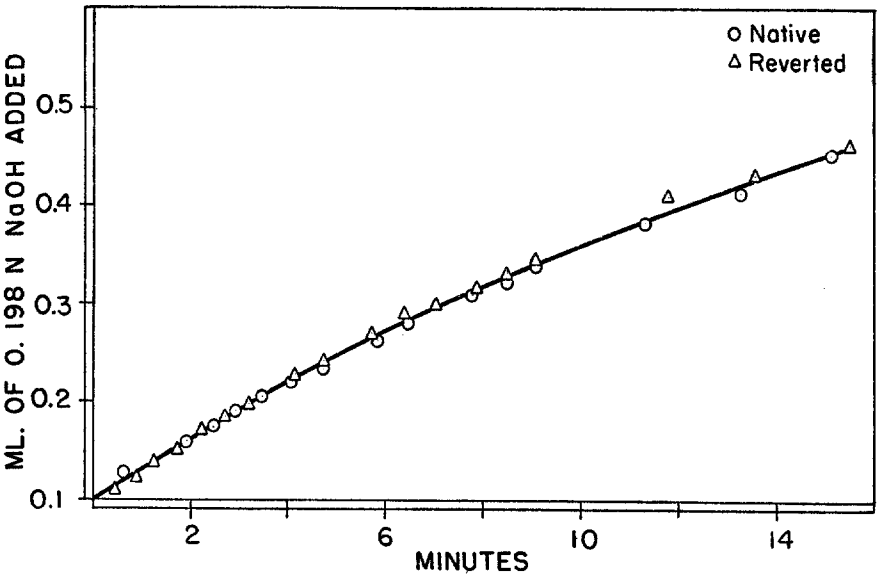

FIG. 9. Rate of hydrolysis of acetyl-*l*-tyrosine ethyl ester in 30 per cent methanol at pH 7.8 and 25°C. by δ-chymotrypsin prepared from native and from reversibly denatured chymotrypsinogen.

built by the Specialized Instruments Corporation by methods previously described (16). $S_{20,w}$ in an acetate buffer of pH 3.86 and ionic strength 0.2 for a protein concentration of 4.53 mg. per ml. was found to be 2.47 Svedberg units. This value is within the range reported for native chymotrypsinogen at this concentration (17).

(*e*) *Behavior of the Renatured Protein on Heating.*—When solutions of native and of renatured protein at pH 2.0 were heated to 45°C., identical equilibria were found with each solution. Further, if these solutions were first heated to 50°C. for 5 minutes and were then cooled to 45°C., identical equilibria were again established in each solution.

Order of the Denaturation Reaction

In view of the slight variations in equilibrium concentrations of native protein with varying total protein concentration, shown in Fig. 7, some doubt as

to the order of the reversible denaturation reaction remained unresolved. The conclusion that the reaction follows first order kinetics is based upon the following findings:

(*a*) If the equilibrium between native and denatured protein is written as:

$$n \text{ Native} \rightleftharpoons \text{denatured},$$

the equilibrium constant for this system is:

$$K = \frac{(\text{Denatured})}{(\text{Native})^n}$$

It has been found that the most satisfactory equilibrium constants are obtained when $n = 1$.

(*b*) If it is assumed that reversible denaturation involves an aggregation of protein molecules, an increased sedimentation rate of the denatured protein with respect to the native protein should be detectable in the ultracentrifuge. If the equilibrium between native and denatured protein is sufficiently slow, a second component should appear. If, however, the equilibrium is established very rapidly, a single boundary with a higher sedimentation rate than that which characterizes the native protein should be observed.

Two determinations of sedimentation rates were made at elevated temperatures at pH 2.0. A solution containing 4.42 mg. of protein per ml. was sedimented at a mean temperature of 43.5°C. and a solution containing 2.21 mg. of protein per ml. was centrifuged at a mean temperature of 45.1°C. The sedimentation constants, corrected to water at 20°, were found to be 1.90 and 2.06 Svedberg units, respectively. As a reference for these determinations a sedimentation rate determination was made on a solution of native chymotrypsinogen at room temperature (27.8°C.) For this determination a solution of chymotrypsinogen containing 4.42 mg. of protein per ml. was adjusted to pH 2.0 with HCl but no salt was added. As was anticipated, the value of $s_{20,w}$ found under these conditions (2.22 Svedberg units) was somewhat lower than that usually found when sufficient salt is present to suppress the sedimentation potential (*ca.* 2.60 Svedberg units).

A single, symmetrical boundary was observed in all three determinations. Although the agreement between these three results is not striking, it is clear that no increase in sedimentation rate occurred in the heated solutions. Probably the discrepancy between these results is due to the uncertainty in the correction used for the viscosity of water at the elevated temperatures.

For the determinations at elevated temperatures the centrifuge rotor was warmed in an oven at 52°C. for 24 hours. The cell, with the center section coated with beeswax to prevent corrosion by the acid solutions, was assembled, placed in a rubber glove, and immersed in a 50°C. bath for 10 minutes. It was then removed, retightened, and replaced in the bath for 5 minutes. The solution to be used was heated for the same period and was introduced into the cell with a warmed syringe. The cell was then

placed in the rotor, and the rotor was replaced in the 52°C. oven for 10 minutes. The rotor was then placed in the centrifuge, the rotor temperature was determined, the rotor chamber was closed, and evacuation of the rotor chamber was begun as quickly as possible.

The principal uncertainty in this procedure is due to the cooling of the rotor during the time which elapsed during the period of evacuation of the rotor chamber and during the acceleration of the rotor to the required speed (59,780 R.P.M.). This lapse of time amounts to 17 to 18 minutes. The temperature drop of the rotor during this interval was approximated by placing the heated rotor in the centrifuge, determining the temperature with a contact thermocouple in the usual way, closing the vacuum chamber, and evacuating the system with the mechanical pump for 17 minutes. At the end of this period air was readmitted to the chamber and the rotor temperature was again determined. The average temperature drop was found to be 3°C. When this correction was subtracted from initial temperature readings subsequently made during actual runs, it was found that the rotor temperature remained essentially constant after speed had been reached.[2]

(*c*) According to the theory of light scattering, the turbidity of a solution, which is a measure of the scattering, is proportional to the mean weight of the particles in a solution (18). Therefore, if aggregation of the protein molecules occurred during reversible denaturation, an increase in turbidity should occur. On subsequent cooling the turbidity should again decrease to that of the unheated solution.

Turbidity measurements were made with the Brice and Speiser light scattering photometer constructed by the Phoenix Precision Instrument Company. Solutions were freed from dust by careful filtration through fine sintered glass filters. Turbidity was determined from the ratio of the galvanometer readings when the photocell was in a position 90° relative to the incident light to those obtained when the photocell was in a position 0° relative to the incident light. For the heated solutions readings were made periodically until the solutions had cooled to room temperature.

Table III illustrates the results of these measurements.[3] As is evident from these data, there is a small change in the turbidity of the system when it is warmed to 52°C. but the change is in the opposite direction to that required by an aggregation of the protein molecules.

Kinetic Measurements

Although both the denaturation reaction and renaturation reaction appeared to have extremely high rates, an attempt was made to evaluate the actual magnitudes of these rates. A typical determination was carried out as follows: a solution of chymotrypsinogen at pH 2.0 was placed in a 30°C. water bath for a sufficient time for thermal equilibration to occur. At the same time an HCl solution of pH 2.0 was warmed

[2] We are indebted to Dr. E. G. Pickels for many suggestions concerning the technique of operating the ultracentrifuge at elevated temperatures.

[3] We are indebted to Dr. Frank Tietze for carrying out these measurements.

in a 60°C. water bath. At zero time, 0.5 ml. of each of the protein and acid solutions was delivered as simultaneously as possible from automatic pipettes (Alfred Bicknell Associates) into a tube which had been previously warmed in a 45°C. water bath and a stop-watch was started. The tube was swirled to mix the solutions and was left in the 45°C. bath. After a desired lapse of time, 5 ml. of cold precipitating buffer was added rapidly from a burette to stop the reaction. The solutions were thoroughly mixed and the system allowed to stand at 5°C. for 2 hours before the precipitate was removed by centrifugation. Protein analyses were made in the usual way.

Although this method afforded reasonable duplication of results, it was found that data for elapsed times of less than 5 seconds were frequently erratic. This is probably the result of imperfect mixing during the addition of the protein and acid solutions. Further, it was frequently found that the equilibrium

TABLE III

Turbidity Measurements on Chymotrypsinogen Solution at pH 2.0 and on the Solvent at 25°, 52°, and during the Cooling Process

System	Galvanometer readings		
	90°	0°	90°/0°
Solvent, HCl solu tion, pH 2.0			
25°C.	6.0	56.5	0.106
52°	6.5	56.5	0.115
Chymotrypsinogen solution			
25°	61.0	32.0	1.91
52°	53.0	32.5	1.63
After 19 min. cooling	56.5	33.0	1.72
After 140 min. cooling	56.5	32.5	1.74

values measured by this technique differed slightly from the values obtained in previous determinations in which the elapsed time was of the order of several minutes. This discrepancy was assigned to small errors in the thermostat settings for the various baths. Since no direct measurement of the temperatures of the solutions after mixing was practicable, the temperature for each set of determinations was defined by the equilibrium attained. The values previously obtained (Fig. 5) were used as references for these equilibria.

Since Kunitz has recently described a kinetic treatment of such systems (1) and has also presented an elegant discussion of the thermodynamic treatment of the data, it is probably sufficient to state that the present results have been treated in the same way. Fig. 10 shows the plots from which the activation energies for the forward and backward reactions were evaluated. The slopes of the lines relating the logarithm of the equilibrium constant, K, to the reciprocal of the absolute temperature are identical with those shown in Fig. 5 since, as

stated above, temperatures were defined in these measurements by the equilibrium constants.

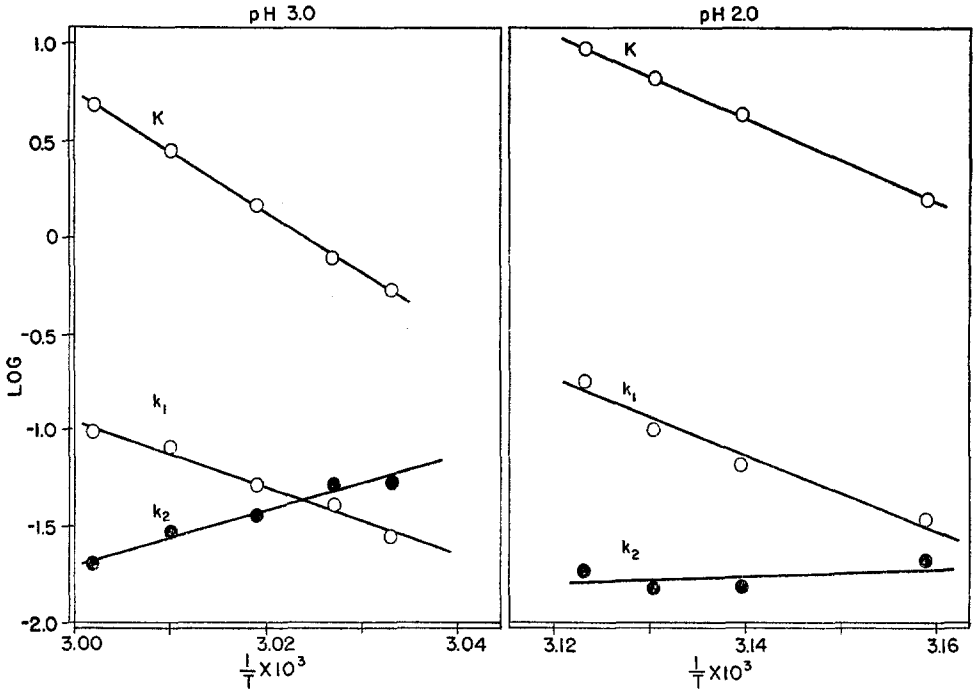

FIG. 10. Variation of the logarithms of equilibrium constants and of rate constants for the denaturation of chymotrypsinogen and for the reversal reaction with the reciprocal of absolute temperature at pH 2.0 and at pH 3.0.

The fit of the reaction constants to the data from duplicate determinations is shown in Fig. 11. The smooth curves in this figure were calculated by substituting various values for t back into the equation:

$$\log \frac{a_0 - a_e}{a - a_e} = \frac{k_1 + k_2}{2.3}$$

where: a_0 = concentration of native protein at zero time.
a_e = concentration of native protein at equilibrium.
a = concentration of native protein at time t.
k_1 = first order reaction constant for the forward reaction,
and k_2 = first order reaction constant for the backward reaction.

The kinetic and thermodynamic results are summarized in Table IV. Since the values of these constants are very sensitive to experimental error, they should be regarded as orders of magnitude rather than as precise values.

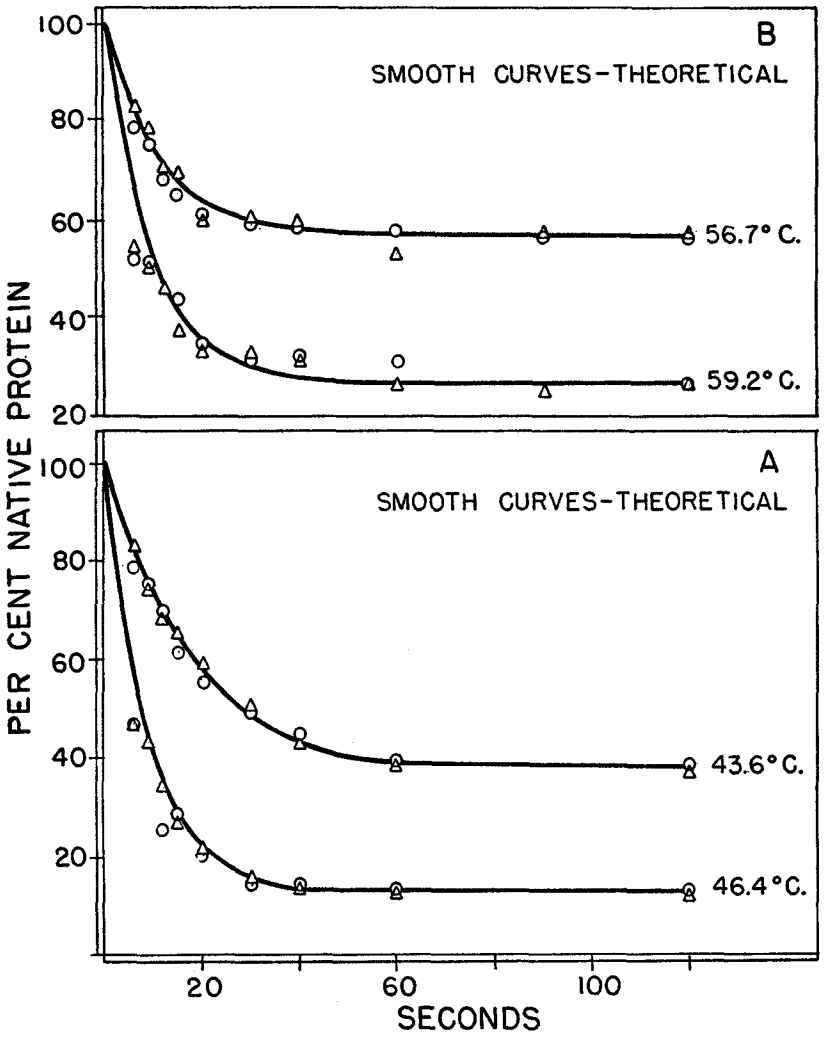

FIG. 11. Curves illustrating the fit of calculated rate curves to experimental points. Graph B is for pH 3.0 and graph A for pH 2.0. The two sets of symbols indicate duplicate determinations.

Irreversible Denaturation

In all the equilibrium and kinetic experiments which have been discussed to this point, conditions were so adjusted that no irreversible denaturation resulted. This restriction limits such studies to a rather narrow range of pH, protein concentration, and temperature. As Fig. 3 indicates, irreversible denaturation increases rapidly as the pH is increased beyond 4. It was also ob-

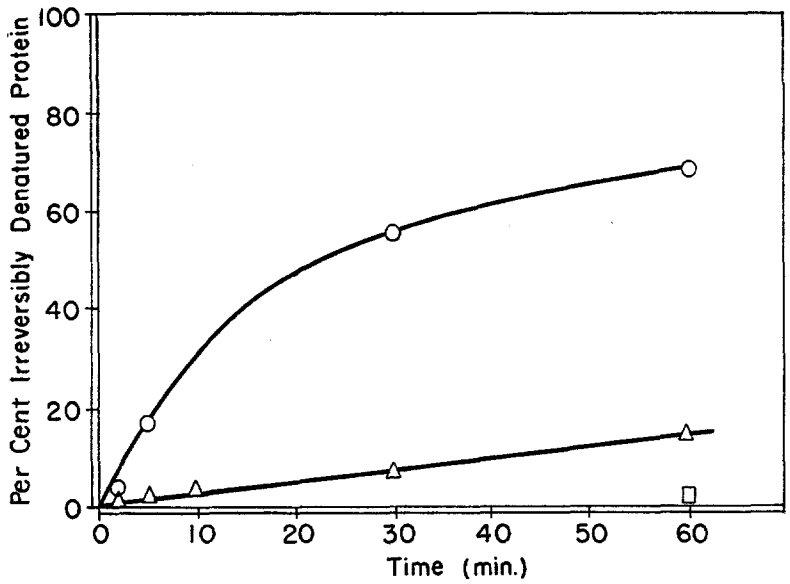

FIG. 12. Effect of protein concentration on the rate of formation of irreversibly denatured chymotrypsinogen. □, 2.16 mg. chymotrypsinogen per ml. △, 4.44 mg. per ml. ○, 8.84 mg. per ml.

TABLE IV

Summary of Kinetic and Thermodynamic Data on the Reversible Denaturation of Chymotrypsinogen

	pH 2.0				pH 3.0				
Temperature	43.6°	45.5°	46.4°	47.2°	56.7°	57.4°	58.2°	59.2°	60.1°
$K_i^u = (100 - a_e)/a_e$	1.61	4.25	6.69	9.64	0.522	0.781	1.46	2.74	4.85
$\Delta H = -4.58\, d \log K/d(1/T)$	← 99,600 →				← 143,000 →				
$\Delta F = -4.58\, T \log K$	−300	−914	−1200	−1440	427	162	−247	−673	−1040
$\Delta S = (\Delta H - \Delta F)/T$	← 316 →				← 432 →				
$k_1 \times 10^2$	3.43	6.64	10.0	18.1	2.76	4.01	5.15	8.01	9.82
$\Delta F_1^* = 4.58T(10.32 + \log T - \log k_1)$	20700	20400	20200	19850	21700	21500	21400	21200	21100
$\Delta H_1^* = E_1 - RT$	← 84,550 →				← 80,200 →				
$\Delta S_1^* = (\Delta H_1^* - \Delta F_1^*)/T$	← 202 →				← 178 →				
$k_2 \times 10^2$	2.13	1.57	1.50	1.88	5.29	5.15	3.54	2.89	2.03
$\Delta F_2^* = 4.58T(10.32 + \log T - \log k_2)$	21000	21300	21400	21300	21300	21300	21600	21800	22100
$\Delta H_2^* = \Delta H_1^* - \Delta H$	← −15,700 →				← −63,700 →				
$\Delta S_2^* = (\Delta H_2^* - \Delta F_2^*)/T$	← −116 →				← −258 →				
E_1	← 85,200 →				← 80,900 →				
E_2	← −15,100 →				← −63,100 →				

served that even at pH 3.0 some irreversibly denatured protein was formed when rather dilute solutions were heated for long periods of time between 50

and 60°C. In Fig. 12 are shown the results of an experiment in which chymotrypsinogen solutions of varying concentration at pH 3.0 were heated to 56°C. for varying lengths of time. It is clear that under these conditions practically no irreversibly denatured protein is formed when the protein concentration is less than 2 mg. per ml.

Similar results were obtained at pH 2.0 except that the chymotrypsinogen concentration could be raised to about 4.5 mg. per ml. before irreversible denaturation was observed.

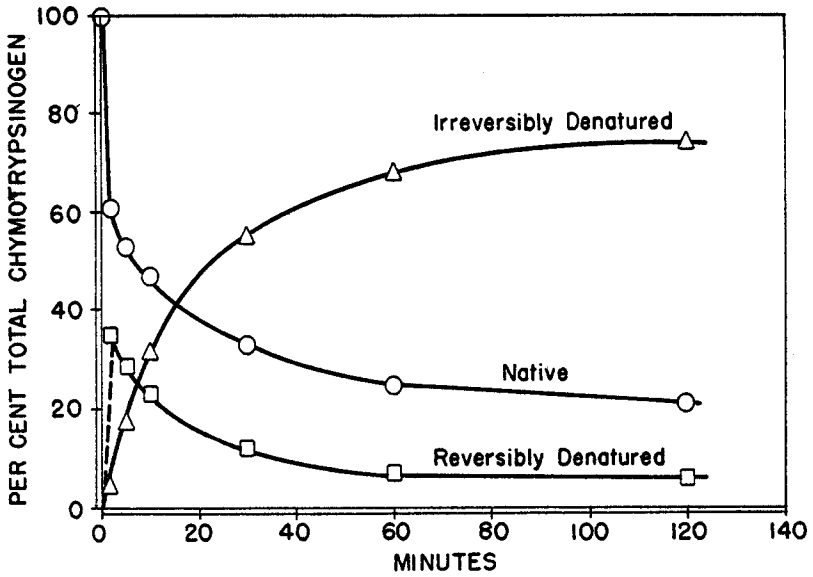

FIG. 13. Typical curves showing the rate of formation of irreversibly denatured chymotrypsinogen and the rate of disappearance of native and of reversibly denatured chymotrypsinogen at the relatively high protein concentration of 8.8 mg. per ml. These determinations were made at pH 3.0 and 56°C.

Fig. 13 shows the changes in concentration of native and reversibly denatured protein with time as irreversibly denatured protein is formed. These curves are for a solution at pH 3.0 containing 8.8 mg. of chymotrypsinogen per ml. which was heated to 56°C. Although the shape of these curves suggests a consecutive reaction of the type:

$$\text{Native} \rightleftharpoons \text{reversibly denatured} \longrightarrow \text{irreversibly denatured},$$

it is also possible that the mechanism is actually:

$$\text{Reversibly denatured} \rightleftharpoons \text{native} \longrightarrow \text{irreversibly denatured}.$$

Similar results were obtained at pH 2.0. The effect of protein concentration on the formation of irreversibly denatured protein was strikingly demonstrated

by an experiment in which an attempt was made to form a saturated solution of chymotrypsinogen at pH 2.0 and 35°C. As the protein was added, the viscosity of the solution increased markedly and finally a gel was formed. Only half the protein in this gel could be recovered in native form, the remainder being wholly insoluble.

The high concentration dependence of the formation of irreversibly denatured protein suggests a highly polymolecular reaction. This hypothesis was confirmed by ultracentrifugal analysis of a solution containing 8.8 mg. of chymotrypsinogen per ml. at pH 3.0 which had been heated for 45 minutes at 56°C. and was then allowed to stand at room temperature for 18 hours. Two components, having widely different sedimentation rates, were observed. The slower sedimenting component had an $s_{20,w}$ of 2.15 Svedberg units (as compared to 2.22 for native chymotrypsinogen in salt-free solution) while the other component had an $s_{20,w}$ of 15.7. It is of interest that the nitroprusside test on the irreversibly denatured protein was negative.

DISCUSSION

Steinhardt (10) and LaMer (19) have considered that the effect of pH on the denaturation of pepsin could be accounted for by the dissociation of five protons from charged amino groups. Since the dissociation of protons from the five critical groups could be promoted both by a decrease in the hydrogen ion activity of the medium and by an increase in temperature, the apparent activation energy for this reaction was too high by the heat of dissociation of the charged groups. Steinhardt found the heat of dissociation of a single group to be 9040 calories so that the total correction was 45,200 calories. When this value was subtracted from the observed activation energy, the true activation energy was only 18,300 calories, a value in the range of activation energies for normal chemical reactions.

In the case of the reversible denaturation of chymotrypsinogen, however, it appears that the only groups which can react with protons in the pH range 2 to 3 are carboxyl groups. Since the heat of dissociation of carboxyl groups is of the order of ± 1500 calories (20), the contribution of the heat of ionization to the heat of activation is of the order of ± 4500 calories. Although this value is of the order of magnitude of the difference between the activation energies at pH 2 and at pH 3 (*cf.* Table IV), it appears that the activation energy of the reversible denaturation reaction is still very large.

The simplest hypothesis which has been found to agree with the observed data is that the reversible heat denaturation of chymotrypsinogen consists largely in a partial dehydration of the protein molecule. This viewpoint is suggested by the following arguments:

1. The very high reaction rates are suggestive of a reaction involving small molecules.

2. The behavior of solutions of reversibly denatured chymotrypsinogen toward dilute salt solutions is parallel to the behavior of lyophobic sols in the presence of dilute salts.

3. If it is assumed that the tendency of a protein molecule to stay in solutions is due to the interactions between water molecules and protein molecules, any condition which decreases this interaction should decrease the solubility of the protein. Further, if the heat of hydration of the protein is sufficiently large, then heating to even moderate temperatures should cause a marked decrease in the interaction between protein and water.

Although the heat of hydration of proteins has not been uniquely determined, there are several indications that it is, in fact, very large. Bull (21) has found the heat of adsorption of the first layer of water between adjacent hydrophilic surfaces of various dry proteins to be between -1060 and -3800 calories per mole of water adsorbed. This amount of adsorption occurs at relative vapor pressures of 0.20 to 0.30. Although it is Bull's opinion that there is no direct relationship between the amount of water bound by solid protein in an atmosphere of water vapor and the amount of water bound by protein in solution, it appears probable that the energetics of hydration are of the same order of magnitude in both cases.

A recent publication by Benson, Ellis, and Zwanzig (22) emphasizes the very high rate of adsorption of water by dry proteins in the absence of air and the large amount of heat evolved in this process. These workers estimate that 6000 calories per mole is an approximate lower limit for the heat of adsorption of water.

Further, although the lattice energy of chymotrypsinogen is probably very large, the heat of hydration of the protein is sufficiently great that the protein has a negative heat of solution. This property has been utilized in the crystallization of chymotrypsinogen (6, 9). A determination of the heat of solution of chymotrypsinogen at pH 3.0 in 0.5 and 0.75 saturated ammonium sulfate yielded values of about $-14{,}000$ calories in each solvent. Jacobsen (9) has reported data from which it can be calculated that the heat of solution of chymotrypsinogen in salt-free solution at pH 5.0 is $-8{,}800$ calories.

4. No definite change could be detected in the properties of the reversibly denatured protein in comparison with the native protein. Although the conditions under which the physical measurements had to be made are not the conditions suitable to fine comparisons, changes of appreciable magnitude seem to be excluded.

5. The large entropy change in the reversible denaturation reaction is in accord with the view that water of hydration is similar to ice in its structure since the fusion of ice involves very large entropy changes. If this viewpoint is adopted and if the heat of fusion of ice is taken to be 1440 calories per mole at elevated temperatures as well as at 0°C. it would appear that, at pH 2 about

69 moles of water must be removed from each mole of chymotrypsinogen while at pH 3 about 99 moles of water must be removed to account for the heat changes in the reaction. If the molecular weight of chymotrypsinogen is taken as 25,000, the percentage dehydration required is 5 and 7, respectively. Since the hydration of proteins in solution seems to be of the order of 20 to 30 per cent (23, 20[4]), this appears to be a conceivable decrease in hydration.

6. Although the differences between the energetics of the reversible denaturation reaction at pH 2 and at pH 3 cannot be accounted for quantitatively, it appears that the changes are in the right direction. It would be anticipated that the repression of the ionization of three carboxyl groups would result in a smaller hydration of the molecule (24) so that less energy would be required at pH 2 than at pH 3 to reduce the hydration of the protein sufficiently that the protein would be precipitated by the addition of salt. The failure of attempts to find a quantitative relation between the results obtained at the two pH values may lie in the fact that the reversibly denatured protein formed at pH 2 is not identical with that formed at pH 3.

This lack of identity is also indicated by the effects of mineral acid anions on the reversible denaturation reaction. It is generally believed (25) that the reactions of anions with proteins occur at positively charged points on the protein surface. Since the chymotrypsinogen molecule carries a high positive charge at both pH 3 and pH 2, and since there is no effect of monovalent mineral acid anions at pH 3 while there is a very definite effect at pH 2, it appears that the effect of anions must be related to the titration of the three carboxyl groups, previously discussed, between these pH values. It is possible that at pH 3 the cations are masked by salt bridges with the charged carboxylic acid groups. The repression of the ionization of these groups at pH 2 would then release the cationic points for the binding of anions.

Although it is recognized that the foundation for these arguments is not as firm as would be desirable, it has seemed proper to call attention to the fact that the changes observed in reversible denaturation may not be due entirely to changes in the protein *per se* but to changes in the environment of the protein. This viewpoint is not wholly original since Neurath, Greenstein, Putnam, and Erickson (26) have written: "In short, if denaturation were to be confined to reactions of and between amino acid side chains, without significantly altering the internal configuration of the protein, such processes may suffice to influence appreciably certain characteristic properties of the native protein and may be reversible, in contrast to any process which affects the state of folding of the polypeptide chains themselves."

It is also recognized that the arguments presented here do not apply to all cases of reversible protein denaturation which have been studied. Although it is true that the reversible denaturation of trypsin (27) and of soy bean trypsin

[4] Oncley, J. L., (20), 560–565.

inhibitor (1) are also characterized by large entropy changes and by small free energy changes, it is also true that in the case of soy bean trypsin inhibitor the reversible denaturation reaction and also the reversal reaction are much slower than in the present case.

We are glad to acknowledge the advice which Dr. Hans Neurath has generously given on many phases of this problem.

SUMMARY

Within a restricted range of pH and protein concentration crystalline chymotrypsinogen undergoes thermal denaturation which is wholly reversed upon cooling. At a given temperature an equilibrium exists between native and reversibly denatured protein. Within the pH range 2 to 3 the amount of denatured protein is a function of the third power of the hydrogen ion activity. The presence of small amounts of electrolyte causes aggregation of the reversibly denatured protein. A specific anion effect has been observed at pH 2 but not at pH 3. Both the reversible denaturation reaction and the reversal reaction have been found to be first order reactions with respect to protein and the kinetic and thermodynamic constants for both reactions have been approximated at pH 2 and at pH 3. Renatured chymotrypsinogen has been found to be identical with native chymotrypsinogen with respect to crystallizability, solubility, activation to δ-chymotrypsin, sedimentation rate, and behavior upon being heated.

Irreversible denaturation of chymotrypsinogen has been found to depend on pH, temperature, protein concentration, and time of heating. Irreversible denaturation results in an aggregation of the denatured protein.

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