[CONTRIBUTION FROM THE DEPARTMENT OF CHEMISTRY, UNIVERSITY OF MASSACHUSETTS, AMHERST, MASSACHUSETTS]

The Thermodynamics of Protein Denaturation. I. The Denaturation of Chymotrypsinogen

BY JOHN F. BRANDTS

RECEIVED MARCH 30, 1964

The thermodynamics for the reversible denaturation of chymotrypsinogen have been studied by the difference spectrum technique. The results are analyzed to provide continuous values of the standard free energy, enthalpy, and entropy terms over the temperature range from 0 to 65° and over the pH range from 0.5 to 3.7. The heat and entropy changes are shown to be very strong functions of temperature, indicating a difference in the heat capacities of the native and denatured forms of several thousand cal. deg.$^{-1}$ mole^{-1}. This large difference in heat capacity leads to an inversion in the sign of both $\Delta H°$ and $\Delta S°$ at low temperature and, consequently, to a maximum in the stability of the native protein at about 10°. The effect of ionic strength on the equilibrium between the native and denatured forms indicates that salt may act as either a stabilizing agent or a denaturing agent, depending on the pH of the solution. The effectiveness of ethanol and urea as denaturing agents for chymotrypsinogen has been measured by observing the change in $\Delta F°$ brought about by the addition of these agents.

Introduction

There has been a great deal of work recently on the problem of determining the nature of the forces which help to stabilize the folded conformation of globular proteins in solution and on obtaining quantitative estimates of the strength of the more important interactions. One of the most promising methods of approach to this problem is to measure the thermodynamics for reactions of small model compounds resembling protein groups and then to extrapolate to obtain information regarding protein stability. This method has already led to important tentative conclusions regarding the strength of peptide hydrogen bonds and the strength of interaction of nonpolar side chains.[1-5] The extrapolation from data on simple model compounds to complex protein molecules is rather uncertain, however, and beset with difficulties. For instance, it has been concluded[1,2] on the basis of model-compound studies that one of the most important factors which determines the stability of the folded conformation of proteins is the tendency of nonpolar side chains to remove themselves from contact with solvent and to preferentially locate in the protein interior. However, the choice of different model systems and a different line of reasoning has led to the opposite conclusion[3] that contact between solvent and nonpolar groups is necessary and helps to stabilize the folded conformation. Similar inconsistencies[4,5] have also arisen in regard to hydrogen bonding so that the estimate of peptide hydrogen bond strength depends largely on personal prejudices regarding the suitability of particular model systems.

The resolution of these difficulties must come in part from direct studies on proteins themselves. It must be shown ultimately that the thermodynamics of protein-unfolding reactions are, at the least, *consistent* with the choice of model systems which one uses to describe intramolecular interactions in the protein. The present studies have been undertaken with the idea of providing suitable experimental data so that such consistencies can be demonstrated. Although there is an extremely large amount of data available on any number of denaturation reactions, no single protein has been thermodynamically characterized to the extent that it will provide a sufficiently rigorous test for various models used to describe unfolding reactions.

The first paper in this series reports the results of thermodynamic measurements on the denaturation of chymotrypsinogen which include nearly the entire range of temperature, ionic strength, and pH where the transition can be made to occur reversibly. The comparison of these results with simplified models of denaturation reactions is made in the companion paper.

Results

Thermodynamic Calculations.—It was found in earlier work[6] that the changes in several physical properties of chymotrypsinogen during denaturation parallel each other. The simplest and most reasonable interpretation of this parallelism is that the reversible denaturation reaction involves a transition between only a single native and a single denatured state, *i.e.*

$$\text{N(native)} \rightleftharpoons \text{D(denatured)}$$

$$K = (\text{D})/(\text{N})$$

where K is the thermodynamic equilibrium constant.

All of the thermodynamic data to be presented will be calculated from spectrophotometric data obtained by the difference spectrum technique. It has been found that Beer's law is obeyed by both the native and denatured protein over the narrow concentration ranges used in these experiments. This being the case, the equilibrium constant can then be calculated from the relation

$$K = \frac{\epsilon - \epsilon_N}{\epsilon_D - \epsilon}$$

where ϵ_N is the extinction coefficient for the native protein, ϵ_D that of the denatured protein, and ϵ is the extinction coefficient of the solution in the transition region. Since the difference spectrum technique yields raw data suitable for obtaining only differences in extinction coefficient, the calculation of the equilibrium constant will ordinarily be made from the equivalent relation

$$K = \frac{\Delta\epsilon - \Delta\epsilon_N}{\Delta\epsilon_D - \Delta\epsilon} \qquad (1)$$

where all of the extinction coefficients have been re-

(1) W. Kauzmann, *Advan. Protein Chem.*, **14**, 1 (1959).
(2) C. Tanford, *J. Am. Chem. Soc.*, **84**, 4240 (1962).
(3) I. M. Klotz, *Science*, **128**, 815 (1958).
(4) J. A. Schellman, *Compt. rend. trav. lab. Carlsberg, Ser. chim.*, **29**, 223 (1955).
(5) I. M. Klotz and J. Franzen, *J. Am. Chem. Soc.*, **84**, 3461 (1962).
(6) J. F. Brandts and R. Lumry, *J. Phys. Chem.*, **67**, 1484 (1963).

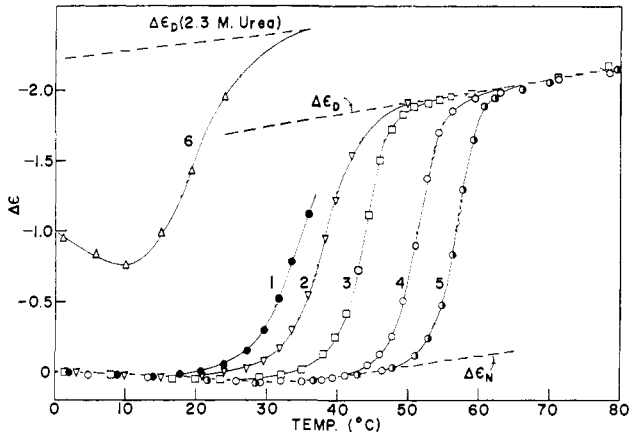

Fig. 1.—The temperature dependence of the extinction coefficient at 293 mμ: curve 1, pH 1.11; curve 2, pH 1.71; curve 3, pH 2.07; curve 4, pH 2.56; curve 5, pH 3.00; curve 6, 2.3 *M* urea, pH 1.55; all solutions contain only HCl (no added salt).

duced by the same amount by subtracting the extinction coefficient of some arbitrary standard solution.

The standard free energy of denaturation will then be

$$\Delta F^\circ = -RT \ln K \qquad (2)$$

while the enthalpy change may be obtained from a van't Hoff plot by the usual relation

$$\Delta H^\circ = -R \frac{\mathrm{d}(\ln K)}{\mathrm{d}\left(\frac{1}{T}\right)} \qquad (3)$$

Reversibility.—If the thermodynamic constants are to have validity, it must be established that the equilibrium is completely reversible. Eisenberg and Schwert[7] have shown that between pH 2 and pH 3.5, an apparently reversible equilibrium exists between native and denatured chymotrypsinogen if the ionic strength is kept low. This same behavior was noted later by Brandts and Lumry.[6] At high ionic strength, however, reversibly denatured chymotrypsinogen aggregates into a very high molecular weight species,[8] and this reaction is not readily reversed.

The results to be presented will delineate more clearly the exact conditions under which reversibility can be expected. It was found that aggregation of the sample could be detected by the spectrophotometric measurements. Whereas reversible denaturation gives rise to rapid decreases in optical density at 293 mμ, the onset of aggregation was characterized by slow increases in optical density. Under conditions of reversibility, sample equilibration usually required less than 15 min. In the presence of aggregation, equilibration was incomplete even after several hours. It was not ascertained whether the increase in optical density occurring with aggregation was brought about by changes in absorption or by increases in the amount of scattered light.

The experimental points included in the following were taken from samples which showed rapid equilibration and, by the criteria discussed above, exhibited no aggregation. In addition, reversibility was routinely checked by lowering the temperature of the samples to a point where the amount of reversibly denatured protein should no longer be significant. Reversibility was then assumed if the extinction coefficient of the solution reverted to that of the native protein.

Temperature Data.—The effect of temperature on the absorption spectrum of chymotrypsinogen at acid pH has been studied previously.[6] The total difference spectrum for denatured chymotrypsinogen measured against native chymotrypsinogen shows three minima, the largest occurring at 293 mμ. In studying the temperature dependence of the difference spectrum, the sample solutions were compared at 293 mμ with a reference solution of chymotrypsinogen of the same concentration and a pH of approximately 3. The reference solution was at ambient temperature and thus entirely in the native form. The temperature of the sample solution was varied and, at each temperature, the optical density was recorded until equilibration was complete. In the calculation of the extinction coefficients, the change in concentration of the sample solutions resulting from thermal density changes has been taken into account.

All samples shown in Fig. 1 were run from about 0° through the reversible transition region. The extinction coefficient of native chymotrypsinogen at 293 mμ was found to be 14.3 at 0° and independent of pH below pH 3.0. The data in Fig. 1 show the change in ϵ as the temperature is increased above 0°. The dashed line at the bottom shows the differential extinction coefficient of native chymotrypsinogen, $\Delta\epsilon_N$, as it depends on temperature. To an excellent approximation, this temperature dependence was the same at all pH values represented in the first five curves. Many additional points falling on this line were obtained but have been omitted for clarity. It is seen that $\Delta\epsilon_N$ increases slightly with increasing temperature from 0 to 32° and decreases above this temperature. Previously, it had been thought that the small decreases in extinction coefficient between 32 and 50° at pH 3.0 (curve 5) resulted from the onset of denaturation.[6,8] A more careful analysis, using the precipitation technique of Eisenberg and Schwert, has shown that the amount of denatured protein is insignificant below 50° at pH 3.0. Consequently, the decreases in extinction coefficient in this temperature region must result from a change in the temperature dependence of $\Delta\epsilon_N$, as has been indicated in Fig. 1.

The differential extinction coefficient of the denatured protein, $\Delta\epsilon_D$, is shown as the dashed line at the top of curves 1 through 5. This actually represents a small oversimplification of the data since it was found that $\Delta\epsilon_D$ could not be reproduced as precisely from one experiment to the next as could $\Delta\epsilon_N$. Consequently, in the calculation of the equilibrium constant from eq. 1, the $\Delta\epsilon_D$ used was obtained by the best fit of the data from each individual sample rather than from the composite $\Delta\epsilon_D$ shown in Fig. 1.

The pH 1.11 sample (curve 1) exhibited aggregation above 36°, and the high temperature half of the transition could not be obtained. In this case, the extrapolated $\Delta\epsilon_D$ obtained from the higher pH curves was used in the calculation of the thermodynamic data.

The qualitative effect of lowering the pH is to unstabilize the native protein and shift the transition to lower temperatures. Significant amounts of denatured

(7) M. A. Eisenberg and G. W. Schwert, *J. Gen. Physiol.*, **34**, 583 (1951).
(8) J. F. Brandts, Doctoral Thesis, University of Minnesota, 1961.

protein cannot be obtained below 20° simply by lowering the pH of the solution, however. Even at a pH value of 0.3, it was found that the protein exists almost entirely in the native form at these low temperatures. This situation arises because the equilibrium constant becomes nearly independent of pH below pH 1, as will be seen shortly. Thus, accurate thermodynamic data cannot be obtained at low temperature in pure water solutions.

In order to extend the data to low temperature, in a semiquantitative fashion at least, we have added a small amount of urea, 2.3 *M*, to a chymotrypsinogen solution of pH 1.55 in order to unstabilize the native protein and make the transition occur at low temperature. The resultant changes in extinction coefficient with temperature are seen in curve 6 of Fig. 1. It will be shown later that the extinction coefficient for both the native and denatured protein are very sensitive to urea concentration. The data in the urea solution have been adjusted so that the differential extinction coefficient for the native protein in 2.3 *M* urea corresponds to the dashed line drawn for the pure water solutions. The differential extinction coefficient of the denatured form is then shown as the dashed line at the top of the curve. It should be noted that, in 2.3 *M* urea, the amount of native protein in the solution is at a maximum at about 10° and that further denaturation can be achieved by *either* raising or lowering the temperature. This interesting behavior and its relevance to the stability of chymotrypsinogen in pure water solutions will be discussed later.

A close inspection of all the transition curves in Fig. 1 shows that none are completely symmetrical about the midpoint of the transition. The high temperature half of each transition is somewhat sharper than the low temperature half. In addition, it can be readily seen that in going from high pH to low pH the transitions become considerably broadened. At pH 3.0 (curve 5) it requires an increase of only 8° to shift the equilibrium from 10% denatured protein to 90% denatured protein while it requires about 14° temperature change to achieve the same shift in equilibrium at pH 1.71 (curve 2). This transition broadening was the same phenomenon observed by Eisenberg and Schwert and led them to conclude that the thermodynamic changes associated with the denaturation reaction were considerably smaller at pH 2.0 than at pH 3.0. However, it does not necessarily follow that the large changes in thermodynamics are associated with the pH changes *per se* since, as the pH of the solution is lowered, the thermodynamic measurements must be made at a lower temperature. Consequently, the important variable influencing the thermodynamics may be the temperature and not the pH. An unequivocal answer to this question may be obtained by close inspection of the thermodynamic data obtained from Fig. 1.

The equilibrium constant may be calculated from the above data using eq. 1. In Fig. 2, $R \ln K$ is plotted against descending values of the reciprocal of the absolute temperature. Accordingly, the slope of these curves at any particular temperature and pH will be equal to $\Delta H°$ (from eq. 3). It may be noted that there is a distinct increase in slope with increasing temperature (decreasing $1/T$) at all pH values, leading to

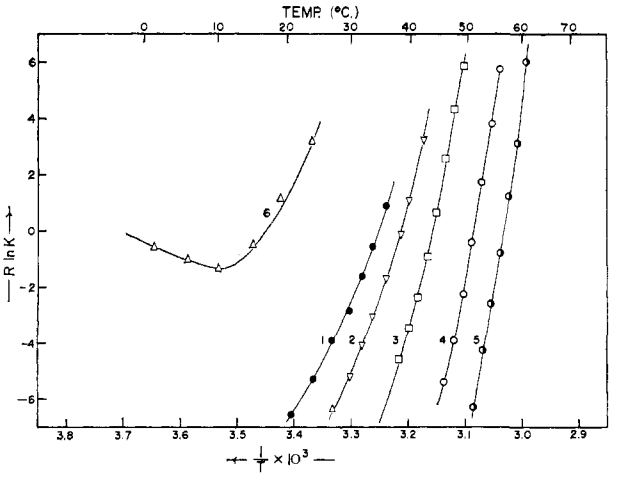

Fig. 2.— Van't Hoff plots: values of $R \ln K$ have been calculated from data of Fig. 1.

the conclusion that $\Delta H°$ is a very strong function of temperature. Furthermore, since each of curves 1–5 overlaps at least one other curve in a common temperature interval, the slopes may be compared at constant temperature and different pH to see if there is any variation in $\Delta H°$ with pH. The curves in these common temperature intervals have all been drawn with the same slopes for curves 1–5 to facilitate comparison. Thus, curves 1 and 2 have identical slopes from 27 to 36°, curves 2 and 3 have identical slopes from 38 to 43°, etc. It is evident from the fit of the data that, within experimental error, $\Delta H°$ is independent of pH when compared at the same temperature.

It is very clear then that the large changes in $\Delta H°$, attributed by Eisenberg and Schwert to changing the pH of the solution, are in fact due primarily to the different temperatures at which the measurements were made. The failure of the above authors to detect the curvature in the van't Hoff plots must have been due to the short temperature interval, about 3.5°, over which their equilibrium constant was measured at each pH value.

The van't Hoff plot for the data in 2.3 *M* urea is also shown as curve 6 in Fig. 2. The pronounced curvature leads to a change in the sign of $\Delta H°$ from positive values above 12° to negative values below this temperature.

pH Dependence of the Free Energy.—The data of Fig. 1 show that decreasing the pH of a chymotrypsinogen solution shifts the equilibrium toward the denatured form. In order to determine more precisely the effect of pH on the standard free-energy change we have studied in detail the shift in extinction coefficient brought about by changing the pH at constant temperature and ionic strength. These data are summarized in Fig. 3. The three lowest temperature curves, at 30.0, 34.0, and 42.6°, contain only HCl and KCl and the total chloride ion concentrations are 0.22, 0.10, and 0.020 *M*, respectively. The only exceptions to this are the two points, at pH 1.2 and 1.4, at the top of the 42.6° curve which were taken from solutions containing no added KCl but which had a higher chloride concentration than 0.02 *M* from the addition of HCl alone. These points were used only to determine the extinction coefficient of the denatured protein, however, and this is independent of the chloride ion concentration. The two high temperature

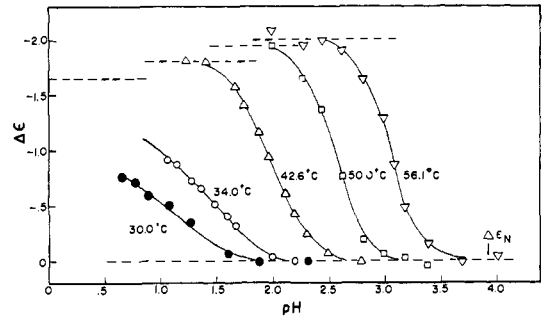

Fig. 3.—pH dependence of the extinction coefficient: see text for details.

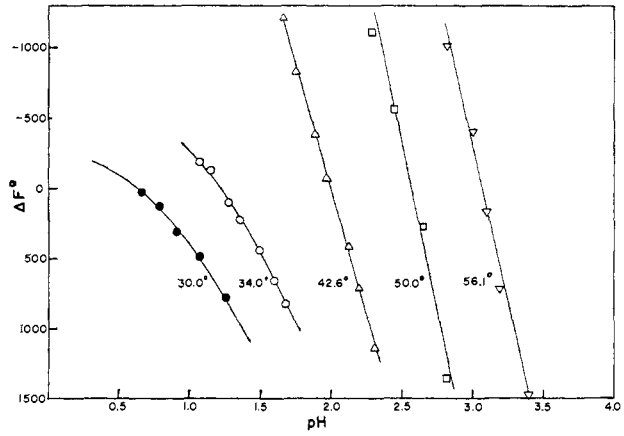

Fig. 4.—The standard free energy as a function of pH.

curves at 50.0 and 56.1° are buffered with 0.01 *M* glycine with added HCl and KCl sufficient to bring the total chloride ion concentration to 0.015 *M*.

The reference extinction coefficient was chosen so that $\Delta\epsilon_N$ for each solution has a value of zero. The value of $\Delta\epsilon_D$, indicated as the dashed lines at the top of each curve, is then slightly different at different temperatures. The high ionic strengths employed in the two low temperature curves promoted aggregation and prevented extending the measurements into the upper half of the transition so that $\Delta\epsilon_D$ for these solutions was obtained by extrapolation of $\Delta\epsilon_D$ in Fig. 1 back to 32°.

The $\Delta F°$ values calculated from these data are shown in Fig. 4. It is seen that the free-energy dependence on pH is very pronounced at high pH. This decreases markedly below pH 2 but even at pH 0.7, the native protein is unstabilized to a small extent by decreasing the pH at constant ionic strength.

The Effect of Chloride Ion.—In addition to its effects on the reversible equilibrium, salt is also very potent in promoting irreversible aggregation of the reversibly denatured protein. Studies of the salt dependence of $\Delta F°$ are therefore hampered by irreversibility effects. We have, however, been able to obtain a limited amount of reversible data by taking advantage of the difference in rate of the reversible and irreversible processes.

At low salt concentration the aggregation reaction does not proceed to a significant extent during the time required for the reversible equilibrium to be set up. All solutions used for thermodynamic analysis indicated no detectable change in optical density for at least a 15-min. period after rapid reversible equilibration was complete. In addition,

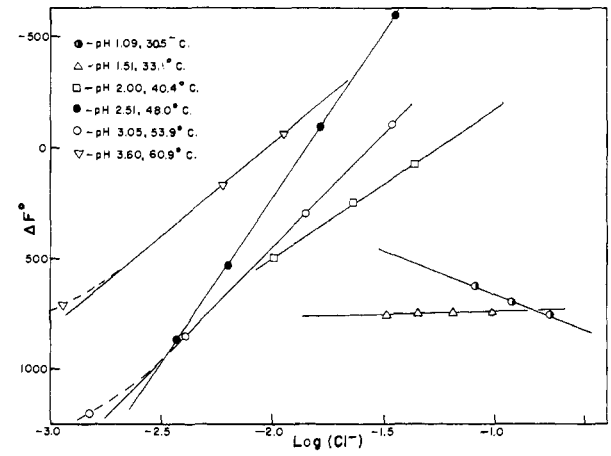

Fig. 5.—The dependence of $\Delta F°$ on chloride ion concentration.

reversibility was routinely checked by lowering the temperature below the transition region. Using these criteria, the amount of salt (KCl) which could be added before rapid aggregation occurred varied with the pH of the solution. At pH 1.09, no detectable aggregation occurred up to total chloride ion concentrations of 0.2 *M*, whereas at pH 3.6 a chloride concentration greater than 0.01 *M* was sufficient to produce rapid aggregation.

Figure 5 summarizes all of the reversible data which we were able to obtain. The $\Delta F°$ values have been obtained from spectrophotometric data in much the same manner as previously discussed in connection with the pH data. These data show that, to a very good approximation, $\Delta F°$ varies linearly with the logarithm of the chloride ion concentration, at least when the concentration is greater than 0.003 *M* (log (Cl^-) greater than −2.5). Although this linear relation holds approximately at all pH values, the slopes are seen to be quite dependent on pH. At the four pH values above pH 1.51, $\Delta F°$ becomes more negative as the chloride concentration is increased, indicating a rather large unstabilization of the native protein. At pH 1.09 the opposite effect is observed so that chloride ion acts as a stabilizing agent at low pH.

The linear dependence of $\Delta F°$ on the logarithm of the concentration suggests that rather strong binding of chloride ion is occurring. If this is so, the denaturation reaction in the presence of chloride ions may be written as

$$N + \nu Cl^- = D$$

where ν may be considered to represent the excess number of chloride ions bound to the denatured form as compared to the number bound to the native form. Neglecting activity coefficients, the equilibrium constant for the above reaction will be

$$K^* = \frac{(D)}{(N)(Cl^-)^\nu} = \frac{K}{(Cl^-)^\nu}$$

where K is the usual equilibrium constant for denaturation. Since K^* will be independent of chloride ion concentration, neglecting changes in activity coefficients, we have the approximate relation

$$\Delta(\Delta F°)_{Cl^-} = -\nu RT \ln \frac{(Cl^-)}{(Cl^-)'} \qquad (4)$$

where $\Delta(\Delta F^\circ)_{Cl^-}$ is the change in standard free energy of denaturation when the chloride ion concentration is changed from $(Cl^-)'$ to some other concentration, (Cl^-), at constant temperature and pH.

Using eq. 4, one may obtain experimental values of ν from the slopes of the curves in Fig. 5. These values are shown in Fig. 6. A maximum positive value of ν, indicating excess binding of chloride ion to the denatured protein, is attained at pH 2.5. Below pH 1.5, ν becomes negative indicating that the native form has a greater affinity for chloride ions. Thus, it is seen that the quantitative effect of chloride ions on the denaturation reaction is a rather complicated function of pH. It may act as a stabilizing or an unstabilizing agent for native chymotrypsinogen, depending on the pH of the solution. It will be seen later that it is rather difficult to rationalize these data if it is assumed that the predominant effect of salt arises from screening of long-range electrostatic interactions in the native protein.

Composite Temperature, pH, and Ionic Strength Data.—Unfortunately, the standard free-energy change for denaturation reactions can only be measured accurately in the range from about 1800 to −1800 cal., corresponding to values of the equilibrium constant from 0.05 to 20. This experimental limitation greatly restricts our knowledge of the denaturation process. Under conditions of neutral pH and low temperature, where most folded proteins are highly stable, we have only the vaguest idea of the amount of free energy which must be overcome to unfold the protein. In the past, workers have measured ΔH° and ΔS° at high temperature and assumed these were temperature independent in order to calculate ΔF° at low temperature. In view of the marked temperature dependence found in this study, this would seem to be a particularly poor approximation.

In order to provide continuous values of the thermodynamics of denaturation over fairly wide ranges of experimental conditions, it is then necessary to use an indirect method. The combined data discussed in the preceeding sections offers such a method.

It was previously mentioned that, within the limits of experimental error, ΔH° appears to be independent of pH as long as the temperature is maintained constant. Although the accuracy of the data is such that small changes in ΔH° would not be detected, there is some independent evidence which also tends to substantiate this conclusion. Sturtevant and co-workers have shown[9] that the calorimetric heat change for the fast reversible acid denaturation of bovine serum albumin is independent of pH, within 0.5 kcal., from pH 4 to pH 3. They found the situation completely identical for the reversible denaturation of ferrihemoglobin in the same pH range.[10] It seems then that the lack of a significant pH dependence of ΔH° may be characteristic of reversible acid denaturations in general and that the effect of pH on ΔF° arises from changes in ΔS°.

Analysis of the effects of chloride ions on ΔF° indicates that this also is predominantly an entropy effect. In the concentration range employed in this study, the logarithmic dependence suggests that the entropy of dilution of chloride ions is the important factor, as indicated in eq. 4. This being the case, the correction of all of the free-energy values indicated in Fig. 2 to the same solution conditions is greatly facilitated. Since the ordinate in Fig. 2, $R \ln K$, is simply $-\Delta F^\circ/T$, then the difference in ordinate values for two successive curves compared at the same temperature will be equal to the difference in ΔS° under the two sets of conditions if we regard ΔH° as being constant. A close comparison of curves 1 and 2 in the common temperature region from 27 to 36°, for instance, shows that ΔS° is 3.8 e.u. less positive under the conditions of curve 2 (pH 1.71, no salt) than under the conditions of curve 1 (pH 1.11, no salt). Therefore, all of the ΔF° values measured under the conditions of curve 1 may be corrected to the pH and chloride ion concentration of curve 2 simply by adding the appropriate free-energy correction (*i.e.*, $3.8T$ cal.) to the experimental values from 20 to 36°. The composite curve 1–2 obtained in this manner may then be corrected to the conditions of curve 3 by a similar analysis of data in the temperature region of overlap between curves 2 and 3. Proceeding in this manner, all of the data in curves 1–5 of Fig. 2 may be corrected to the same pH and chloride ion concentration.

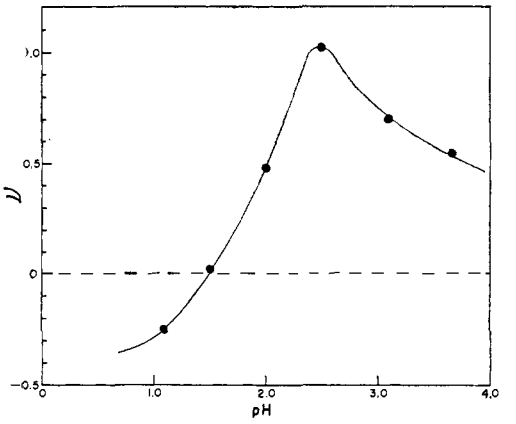

Fig. 6.—The effect of pH on ν.

The points in Fig. 7 show the result of this procedure when all of the data have been corrected to pH 3.0 and 0.01 M Cl^-. The data obtained in 2.3 M urea, curve 6 of Fig. 2, have also been included in Fig. 7. Comparison of these data with those of curve 1 in the region of temperature overlap indicated that in this case both ΔH° and ΔS° were significantly different than those obtained in pure water. These differences were taken into account in an attempt to correct the data in aqueous urea to water at the appropriate pH and salt concentration. This extrapolation between two rather dissimilar solvents must be regarded as very approximate, however, and the author will not rely heavily on the quantitative significance of the data obtained in aqueous urea. These data have been included because they illustrate rather nicely that chymotrypsinogen does show a maximum in stability at low temperature and this cannot be shown directly in pure water solutions because the equilibrium constant is too small to measure.

It is desirable to obtain a mathematical expression for the standard free-energy change and the most convenient way of doing this is to use a power series involving the absolute temperature. In order to accurately express all of the data of Fig. 7, at least four terms must be used in the power series. The solid

(9) P. Bro and J. M. Sturtevant, *J. Am. Chem. Soc.*, **80**, 1789 (1958).
(10) W. W. Forrest and J. M. Sturtevant, *ibid.*, **82**, 585 (1960).

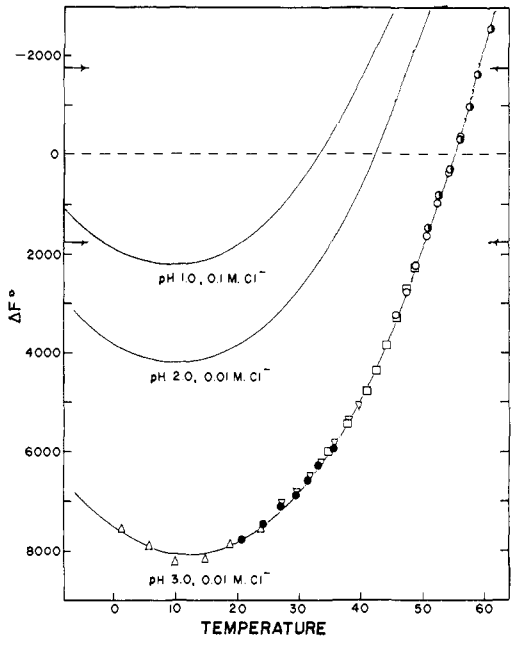

Fig. 7.—The temperature dependence of ΔF° under various conditions; the points have been calculated from the data in Fig. 1; the symbols used are consistent with the notation in Fig. 1.

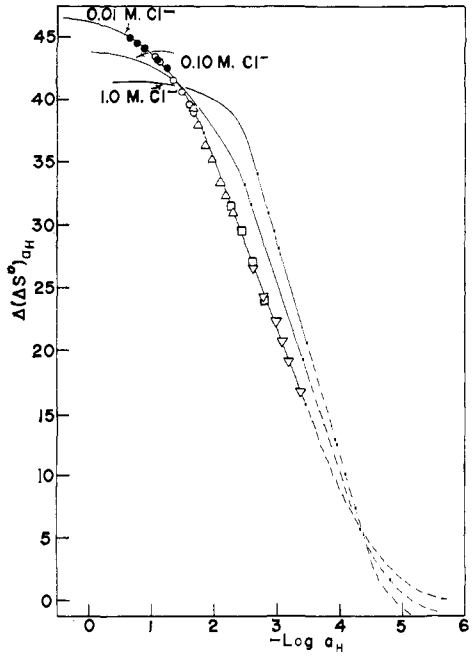

Fig. 8.—The change in the standard entropy of denaturation with pH at three chloride ion concentrations (calculated from the data in Fig. 4); the designation of experimental points is consistent with the notation of Fig. 4.

line passing through the points at pH 3.0 represents the continuous values of ΔF° given by the expression

$$\Delta F^\circ = 121{,}700 - 2248T + 11.57T^2 - 0.01783T^3 \quad (5)$$

It should be noted that the individual coefficients of this power series have not been obtained independently by curve fitting but have been partially dictated by theoretical considerations, to be discussed in detail in the second paper of this series. In fitting the mathematical expression to the experimental data, the points obtained in 2.3 M urea and corrected to pure water, as indicated above, were neglected because of the uncertainty in the extrapolation. It is seen, however, that the smooth curve obtained by fitting the points obtained in pure water from 20 to 60° passes also through the points obtained in urea from 0 to 25°. Thus it appears that the corrections from urea to water are not greatly in error.

These data show rather strikingly that ΔF° is not a linear function of temperature as has been assumed for other denaturations. The temperature dependence is much more nearly parabolic with the vertex located at the temperature of maximum stability, about 10° in the case of chymotrypsinogen. The maximum stability indicated at pH 3.0 and 0.01 M Cl^- is about 8000 cal. The estimate which would be obtained if one were to measure the standard entropy and enthalpy of denaturation at the transition temperature, 54° in this case, and assume them to be independent of temperature would be in excess of 20,000 cal. at temperatures of 10° or below! This method will always lead to drastically high estimates of ΔF° at low temperatures and is, therefore, unsuitable even as a semiquantitative approximation.

The significance of the two additional curves in Fig. 7 will be discussed shortly.

The pH data of Fig. 4 may be analyzed in much the same way as the temperature data, again taking advantage of the lack of dependence of ΔH° on pH. These data may all be converted to the same chloride ion concentration by eq. 4 and the resulting free-energy values can be analyzed in terms of variations in ΔS° with pH. If the separate curves are then made to coincide in the overlap region, a continuous representation of ΔS° as a function of pH at constant temperature and chloride ion concentration may be obtained. The result of this procedure is shown in Fig. 8. The ordinate, $\Delta(\Delta S^\circ)_{a_H}$, represents the variation in the standard entropy of denaturation as the hydrogen ion activity, symbolized as a_H, is varied at constant chloride ion concentration. For reasons which will be obvious in a moment, we have adjusted $\Delta(\Delta S^\circ)_{a_H}$ to an arbitrary value of 44 e.u. at pH 1 and 0.01 M Cl^-.

The general form of the curve indicated by the experimental points at 0.01 M Cl^- resembles that of a titration curve. It will be shown in the succeeding paper that these data are consistent with the hypothesis that chymotrypsinogen contains three anomalous carboxyl groups with a pK of 1.3 in the native protein. However, we are at the moment interested only in providing a mathematical representation of $\Delta(\Delta S^\circ)_{a_H}$. The solid line passing through the points at 0.01 M are the calculated values obtained from the equation

$$\Delta(\Delta S^\circ)_{a_H}^{0.01} = -R \ln \left(\frac{1 + a_H/5.0 \times 10^{-2}}{1 + a_H/3.2 \times 10^{-5}} \right)^{3.18} \quad (6)$$

and it is seen that this adequately represents all of the experimental data. The value of $\Delta(\Delta S^\circ)_{a_H}$ at chloride concentrations other than 0.01 M may be obtained quite easily by adding to $\Delta(\Delta S^\circ)_{a_H}^{0.01}$ the corresponding entropy change resulting when the chloride concentration is changed from 0.01 M to some other concentration (Cl^-) at a hydrogen ion activity of a_H. This latter correction is obtained quite easily from

eq. 4 so that the general expression for the pH and salt dependent entropy term becomes

$$\Delta(\Delta S^\circ)_{a_H} = -R \ln \left(\frac{1 + a_H/5.0 \times 10^{-2}}{1 + a_H/3.2 \times 10^{-5}} \right)^{3.18} + \nu R \ln \frac{(Cl^-)}{(0.01)} \quad (7)$$

where ν is evaluated at a_H and may be obtained by interpolation of the data in Fig. 6. The values of $\Delta(\Delta S^\circ)_{a_H}$ at chloride concentrations of 0.1 and 1.0 M Cl^- have been calculated from this equation and are shown as the two additional solid lines in Fig. 8. The extrapolation to concentrations as high as 1.0 M by assuming linearity of the plots in Fig. 5 might justifiably be questioned since the experimental data were restricted to concentrations below 0.3 M Cl^-. However, the qualitative conclusions to be drawn from these data at high salt concentration will not be dependent upon quantitative uncertainty.

The general effect of increasing the chloride concentration is to reduce the maximum value of the entropy term at low pH and to displace the entire curve to higher pH. The curves all cross at a pH of 1.5, leading to the conclusion that increasing the salt concentration will stabilize the native protein below pH 1.5 while unstabilizing it at higher pH. At pH higher than 2.5, the curves begin to approach each other again and the dashed line extrapolations beyond the point where reversible data was obtained indicates that there may be another crossover point at a pH slightly below 5. Chervenka's kinetic studies[11] of the effect of KCl on the urea denaturation of chymotrypsinogen also show a second crossover point of activation free energies at a pH of about 4.5 so that the dashed line extrapolations to high pH may be qualitatively correct. If this is true, we may conclude that salt has a stabilizing effect on native chymotrypsinogen at those pH values where the free energy of denaturation is independent of or only weakly dependent on pH but has a rather strong unstabilizing effect at those pH values where ΔF° is strongly dependent on pH.

It is now possible to formulate a general expression for the standard free energy of denaturation of chymotrypsinogen which will apply to all conditions of temperature, pH, and chloride concentrations encompassed in the experimental results. The value of ΔF° at any general set of conditions [T, a_H, (Cl^-)] will be equal to the value at conditions [T, 10^{-3} M H^+, 10^{-2} M Cl^-] (given by eq. 5) plus the change in ΔF° resulting when the hydrogen ion activity and chloride ion concentration are changed from 10^{-3} and 10^{-2} M to a_H and (Cl^-), respectively, at temperature T. This latter term is readily calculated from eq. 7 since ΔH° is independent of pH and chloride concentration. We then have

$$\Delta F^\circ = 121{,}700 - 2226T + 11.57T^2 - 0.01783T^3 + RT \ln \left(\frac{1 + a_H/5.0 \times 10^{-2}}{1 + a_H/3.2 \times 10^{-5}} \right)^{3.18} - \nu RT \ln \frac{(Cl^-)}{(0.01)} \quad (8)$$

(11) C. Chervenka, *J. Am. Chem. Soc.*, **83**, 473 (1961).

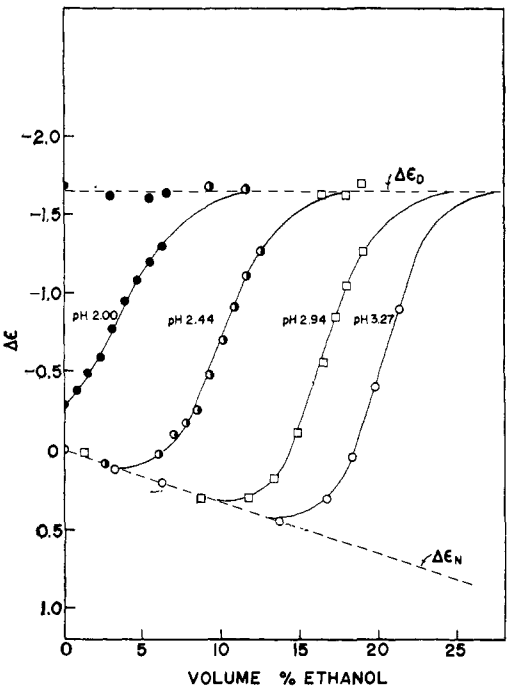

Fig. 9.—The influence of ethanol on the extinction coefficient of chymotrypsinogen solutions at 39.1°; solutions contain only HCl with no added salt.

Equation 8 has been plotted as a function of temperature at several pH values in Fig. 7. The effect of pH on the denaturation is readily seen from these curves. Increasing the hydrogen ion activity shifts the values of ΔF° in the negative direction without altering the temperature dependence of ΔF° to a great extent. The transition temperature (that temperature where ΔF° is zero) changes markedly with acidity, decreasing from 54.5 to 42.5 to 33.7° as the pH is changed from 3.0 to 2.0 to 1.0 at the chloride concentrations indicated.

Even at pH 1.0 and 0.1 M Cl^-, native chymotrypsinogen still has a stability of 2200 cal. at 10°. It is not possible to unstabilize the protein further solely by the addition of HCl since, in order to decrease the pH below 1.0, we must also increase the chloride ion concentration above 0.1 M. At this low pH, the stabilizing effect brought about by increasing the chloride concentration outweighs the unstabilizing effect brought about by increasing the hydrogen ion concentration. Consequently, chymotrypsinogen cannot be denatured at low temperatures by the addition of HCl; *i.e.*, it has no "acid" denaturation.

The arrows on the ordinate indicate the approximate range in which the thermodynamics can be measured experimentally. It is obvious then that the thermodynamics cannot be measured below about 20° if we are to restrict ourselves to pure water solutions containing only HCl and KCl. With the addition of urea, however, the curves at any particular pH are shifted toward more negative values of ΔF° and may be brought into the transition region even at low temperatures. This is the procedure which was used to obtain approximate thermodynamics at low temperatures.

The Effects of Ethanol.—The data in Fig. 9 show the change in the extinction coefficient at 293 mμ for chymotrypsinogen solutions of different pH as the concentration of ethanol is increased at 39.1°. The differential extinction coefficient of the denatured protein was obtained by heating solutions of different

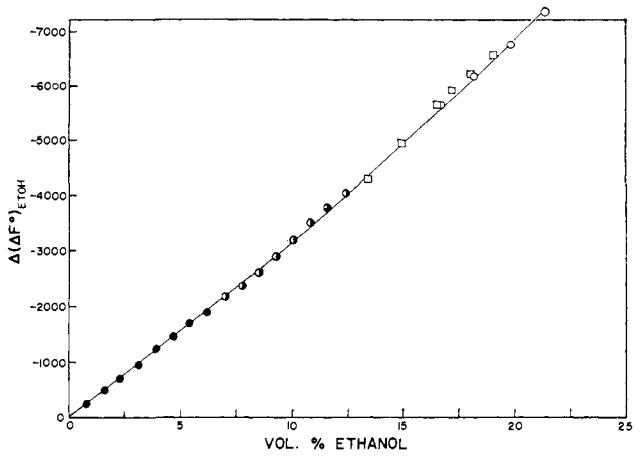

Fig. 10.—The dependence of $\Delta(\Delta F^\circ)_{EtOH}$ on ethanol concentration at 39.1°. The designation of experimental points is the same as in Fig. 9.

ethanol concentrations to several temperatures above the transition region where denaturation was essentially complete and then extrapolating these values back to 39.1°.

The reversibility of denaturation in ethanol was checked carefully because it had been found previously that reversibly denatured chymotrypsinogen forms a low molecular weight aggregate, possibly a dimer, in the presence of ethanol.[6] This reaction is slow though and requires several hours before an appreciable amount of the heavy species forms. Each experimental point in Fig. 9 was taken from a separate protein solution and each required less than 0.5 hr. for complete equilibration in the transition region. Under these conditions the amount of aggregation should not be appreciable. All of the samples up to the highest ethanol concentrations included, 22%, showed complete reversibility when cooled below room temperature. Above 22%, ethanol irreversibility was detected and these data could not be used for thermodynamic analysis.

In the past the analysis of the effects of denaturing agents such as ethanol on protein stability has been quite restricted since little was known about the free energy of stabilization in the absence of the denaturant. With the data and the analysis which has preceded we are in a better position to evaluate quantitatively the effects of these reagents. The quantity of interest in this case is the change in the standard free energy of denaturation brought about by the addition of a given amount of ethanol. This may be expressed as

$$\Delta(\Delta F^\circ)_{EtOH} = \Delta F^\circ(c) - \Delta F^\circ \qquad (9)$$

where the first term on the right is the standard free energy of denaturation in an aqueous ethanol solution containing c% ethanol and the second term on the right is the standard free-energy change in water. These terms must, of course, be evaluated under identical conditions of temperature, hydrogen ion activity, and chloride ion concentration if we are to ascertain the changes in free energy brought about solely by the presence of ethanol.

The $\Delta F^\circ(c)$ term of eq. 9 may be evaluated in the usual way at different ethanol concentrations from the measured extinction coefficients in the transition curves shown in Fig. 9. The ΔF° values in water under equivalent conditions cannot be measured directly in most cases because the stability of the native protein is too great. Consequently, we shall use eq. 8 to calculate the values in water so that an accurate estimate of $\Delta(\Delta F^\circ)_{EtOH}$ may be obtained.

The resulting values of $\Delta(\Delta F^\circ)_{EtOH}$ are shown in Fig. 10. The changes in ΔF° brought about by the addition of ethanol are seen to be approximately a linear function of the concentration of added ethanol at 39.1°. At concentrations above 10% there does appear to be a slight upward curvature but this is not very marked. It is encouraging that the $\Delta(\Delta F^\circ)_{EtOH}$ values obtained from solutions of different pH and ionic strength fall approximately on the same smooth curve. This provides a great deal of confidence in the accuracy of eq. 8 and also suggests that the effect of ethanol on the stability of the protein is reasonably independent of pH and ionic strength. Since the net positive charge on chymotrypsinogen varies by nearly a factor of two[12] over the range of conditions used in studying the ethanol dependence, we may also tentatively conclude that the effect of ethanol is nearly independent of the net protein charge. Thus the predominant effect in this case does not appear to arise from the change in the dielectric constant of the solvent as ethanol is added and from the corresponding effect which this might have on the electrostatic free-energy differences between the native and denatured forms. Although the electrostatic differences will likely make some contribution, particularly at high ethanol concentrations, the predominant effect under the conditions employed here would appear to be on the nonionizable groups in the protein. This is in keeping with current opinion which holds that the effect of organic solvents on protein stability arise to a large extent from a weakening of hydrophobic interactions between nonpolar and slightly polar side chains in the native protein.

Another very qualitative observation should be mentioned at this point. An attempt was made to study the effects of ethanol at 0°. Even after the addition of 20% ethanol to a chymotrypsinogen solution of pH 1.5, the solution still exhibited no detectable denaturation. Since ΔF° in water at this temperature and pH is about 2700 cal., we may conclude that the change in ΔF° on adding 20% ethanol is somewhat less than -2000 cal. It is seen from Fig. 10 that 20% ethanol alters the stability by about -6800 cal. at 39.1°. It thus appears that the effects of ethanol on protein stability are a great deal smaller at lower temperatures, which means that ΔH° and ΔS° are much larger for the denaturation occurring in ethanol-water mixtures as compared to water at the same temperature. This conclusion will be investigated in more detail at a later time.

The precise value of $\Delta(\Delta F^\circ)_{EtOH}$ will depend to some extent on the reference state which one uses to evaluate the ΔF° term in pure water. We have here chosen to evaluate this term at the same pH at which the free energy was measured in the aqueous ethanol solution. Since ethanol has the effect of increasing the intrinsic pK of carboxyl groups, the protein will exist in a different state of ionization in the aqueous ethanol and pure water solutions at constant pH. Consequently, the $\Delta(\Delta F^\circ)_{EtOH}$ values plotted in Fig.

(12) From unpublished titration data obtained in this laboratory.

10 will contain a rather trivial contribution which arises from titrating the protein in aqueous ethanol to a different state of ionization than that existing in pure water. In order to eliminate this effect, one should calculate the ΔF° term on the right of eq. 9 at a pH such that the state of ionization of *both* the native and denatured protein in water is the same as in the aqueous ethanol solution under the conditions where $\Delta F^\circ(c)$ was measured. It is unlikely that this precise situation will ever occur, but it can best be approximated by choosing conditions such that the *native* protein has the same average number of protonated groups in both solvents. Even to treat this situation accurately, it is necessary to have rather complete titration data covering the entire range of solvent composition. This problem of the reference state will be treated more fully in a subsequent paper but, for the moment, we shall be content to consider the effect of ethanol at constant pH. The correction for trivial titration effects will be significant but not so large as to interfere with the qualitative conclusions to be drawn from these data in the second paper. For instance, we can estimate that, if the effect of ethanol on the pK of the acidic groups in chymotrypsinogen is the same as its effect on the pK values of small carboxylic acids, the values of $\Delta(\Delta F^\circ)_{\mathrm{EtOH}}$ evaluated under conditions of constant charge will be about 15% greater in absolute value than those given in Fig. 10 at constant pH. The same general conclusions apply as well to the urea data presented below.

The Dependence of Free Energy on Urea.—The same type of analysis used for ethanol is satisfactory to study the effect of urea on protein stability. Figure 11 shows the variations in extinction coefficient with urea concentration at four pH values and 30.4°. The change in $\Delta\epsilon_N$ with urea concentration is exceedingly large. Whether this is indicative of conformational changes occurring in the "native" protein cannot be ascertained. Although $\Delta\epsilon_D$, shown approximately as the dashed line at the upper portion of the curves, increases appreciably with urea concentration also, the changes are not so marked and therefore the total change in $\Delta\epsilon$ for complete denaturation increases as the urea concentration is increased.

In the low pH curve, pH 1.62, aggregation occurred above the midpoint of the transition so that the value of $\Delta\epsilon_D$ used in the calculation of the standard free energies at this pH had to be obtained by the extrapolation of the data at pH 2.00 to lower urea concentrations.

In this study the final protein solutions were obtained by adding various amounts of 9 M urea to the stock protein solution at the particular pH desired. Before addition, the pH of the 9 M urea was adjusted with HCl to the point where the addition of urea to the stock protein solution had only a very small effect on the pH of the solution. The pH values shown in Fig. 11 are the average values for all the points on a single curve. The differences in pH between samples never exceeded several hundredths of a pH unit, however, and these differences were taken into account in the calculation of the free energies, as will be seen.

Although no salt was added in these urea studies, the chloride ion concentration was still considerably greater than the hydrogen ion concentration because of the titration of urea. In view of the relatively high dissociation constant of 0.67[13] for urea, only a relatively small fraction of the urea molecules was protonated even at the most acid pH, but this nevertheless had an appreciable effect on the total chloride ion concentration.

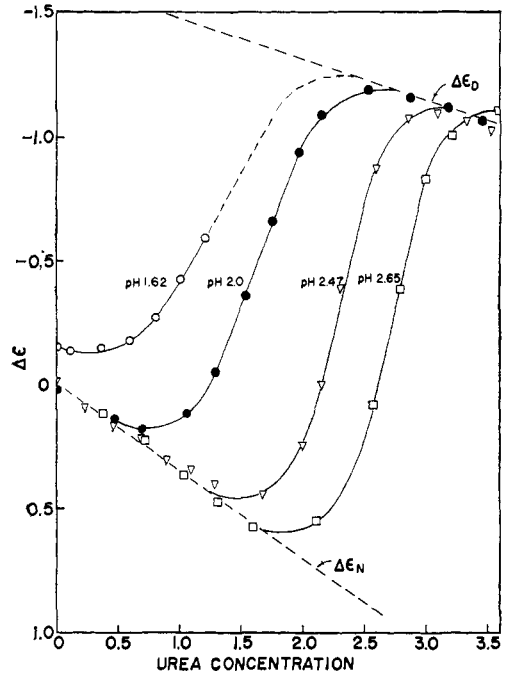

Fig. 11.—The variation in extinction coefficient of chymotrypsinogen with urea concentration at 30.4°; solutions contain no added salt.

Again, the quantity most suitable for measuring the potency of urea as a denaturing agent is the change in the standard free energy produced by the addition of urea, all other solution variables being held constant. This will be represented as

$$\Delta(\Delta F^\circ)_{\mathrm{urea}} = \Delta F^\circ(\mathrm{urea}) - \Delta F^\circ \qquad (10)$$

where the first term on the right is the standard free-energy change at a specified urea concentration and the second term is the standard free-energy change in the absence of urea but under identical conditions of pH and ionic strength. The standard free-energy change in the presence of urea can be calculated from the extinction coefficients shown in Fig. 11 using the two-state analysis while the corresponding term in the absence of urea can be calculated from eq. 8. The calculations of ΔF° had to be made separately for each point in Fig. 11 because of the small variations in pH and chloride ion concentration resulting from the addition of urea, noted previously.

The values of $\Delta(\Delta F^\circ)_{\mathrm{urea}}$ as a function of urea concentrations are shown in Fig. 12. The adequate fit of all data obtained at different pH and ionic strength to a single curve suggests that urea, as well as ethanol, acts primarily on nonionizable groups in the protein.

It is obvious in this case that the standard free energy of denaturation does not change linearly with the urea concentration. The situation is little different if the activity of urea is used rather than the concentrations. The implications of the observed concentration dependence will be discussed in the accompanying paper.

(13) "Handbook of Chemistry," N. A. Lange, Ed., Handbook Publishers, Inc., Sandusky, Ohio, 1952.

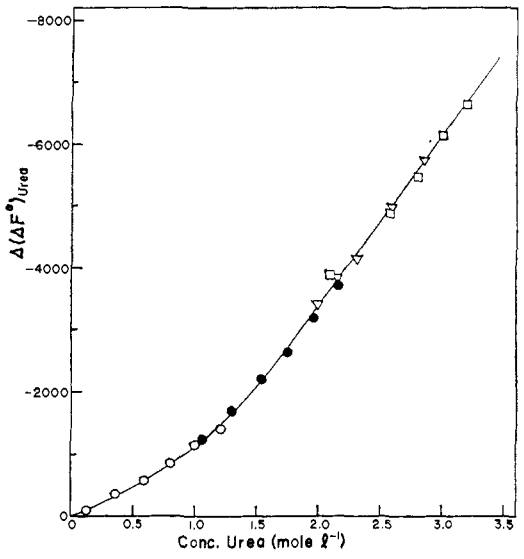

Fig. 12.—The dependence of $\Delta(\Delta F^\circ)_{urea}$ on urea concentration at 30.4°; the designation of experimental points is the same as in Fig. 11.

The results (Fig. 1) at low temperature and pH 1.55 show that the value of $\Delta(\Delta F^\circ)_{urea}$ is about -2500 cal. at 2.3 M urea and 0°. The value at this same urea concentration and 30.4° is seen in Fig. 12 to be -4200 cal. Thus the effect of urea as a denaturing agent increases markedly as the temperature is increased, as was previously found to be the case for ethanol. Consequently, we may conclude that the average values of ΔH° and ΔS° are considerably larger when the denaturation occurs in urea than when it occurs in water in the temperature range from 0 to 30°. The average ΔS° is, for instance, about 50 e.u. more positive in 2.3 M urea than in water in this temperature range.

Discussion

The results of all of the thermodynamic measurements in water are summarized concisely in the expression for ΔF° given by eq. 8. Well over 100 measurements of ΔF° in water have been reported and, almost without exception, these agree within 100 cal. of the values calculated by eq. 8. Thus the calculated values are nearly within the expected experimental error under conditions where the calculated and experimental values can be compared directly. The consistent results obtained on the effects of urea and ethanol, where eq. 8 was used to calculate the standard free energies outside the transition region, suggest that the accuracy may be equally good under conditions where direct comparison with experiment is not possible.

The validity of the analysis used depends principally on two points which are not subject to precise experimental verification. The first of these is that the standard enthalpy of denaturation does not depend significantly on the pH of the solution. The present results enable one to rule out a strong pH dependence, but changes in ΔH° of the order of 5 kcal. per pH unit would probably have gone undetected. Calorimetric studies of other reversible denaturations in acidic media show the pH dependence of ΔH° to be less than 1 kcal. per pH unit, as mentioned previously, and this permits one to feel reasonably comfortable about the quantitative nature of this approximation.

We have also assumed throughout this discussion that the denaturation reaction involved a transition between a single native and a single denatured species. The interpretation of the denaturation reaction given in the second paper depends quite strongly upon the validity of this assumption. Certainly as one adds ethanol or urea to the protein solution, there probably are at least minor variations in the structure of the native and/or denatured protein. In particular, in the case of urea, the total change in extinction coefficient as well as the change in rotatory dispersion parameters[6] are somewhat greater than occur in pure water. Although these differences could possibly arise from the solvation of the protein by urea, we cannot discount the possibility that the conformational changes during denaturation become significantly altered in the presence of these additives.

In pure water the situation appears to be a little more clear cut. It has been shown previously[14] that the optical rotation of native chymotrypsinogen is nearly independent of pH from pH 2 to pH 11. Likewise, the ultraviolet absorption spectrum is unchanged by pH below pH 3. Although there are small changes in both optical rotation and extinction coefficient of native chymotrypsinogen with temperature, these are not of the proper magnitude to be attributed to conformational changes. Thus it appears certain that the conformation of native chymotrypsinogen is, to a good approximation, the same under all conditions employed in this study.

The same general conclusions apply also to reversibly denatured chymotrypsinogen. The absorption spectrum, optical rotation, and specific viscosity of denatured chymotrypsinogen all show a small temperature dependence,[6] but none seem to indicate any appreciable conformational changes. Thus, we may tentatively conclude that the denaturation reaction occurs between essentially the same two conformational states of the protein at all temperatures and pH values in the reversible region.

In the past, temperature variations in the heat and entropy change for denaturation reactions have been interpreted as signifying complex reaction mechanisms involving more than one structurally distinct native or denatured species.[15,16] If this interpretation is to be rejected for chymotrypsinogen, we must seek an alternative explanation to account for the extreme dependence of thermodynamics on temperature.

The data in Fig. 13 illustrate more clearly the magnitude of the temperature dependence of both ΔH° and ΔS°. The solid lines were calculated from eq. 8 at pH 3.0 and 0.01 M Cl^- using the thermodynamic relations

$$\Delta H^\circ = \frac{\delta(\Delta F^\circ/T)}{\delta\frac{1}{T}}$$

$$\Delta S^\circ = -\frac{\delta\Delta F^\circ}{\delta T}$$

(14) H. Neurath, J. A. Rupley, and W. J. Dreyer, *Arch. Biochem. Biophys.*, **65**, 243 (1956).

(15) D. N. Holcomb and K. E. Van Holde, *J. Phys. Chem.*, **66**, 1999 (1962).

(16) J. Hermans, Jr., and H. A. Scheraga, *J. Am. Chem. Soc.*, **83**, 3283 (1961).

At 65° both $\Delta H°$ and $\Delta S°$ are very large and positive, being about 177 kcal. and 543 e.u., respectively. As the temperature is lowered, both decrease abruptly and become zero in the region of 10° and negative below this temperature.

If the transition is envisioned as occurring between only two structurally distinct forms of chymotrypsinogen, then these large variations in $\Delta H°$ and $\Delta S°$ must result from a difference in the heat capacity of the two forms. This may also be calculated from eq. 8 and the relation

$$\Delta C_p = -T \frac{\partial^2 \Delta F°}{\partial T^2}$$

which gives

$$\Delta C_p = -23.14T + 0.1070T^2$$

This equation is plotted in Fig. 13. The value of ΔC_p increases in a slightly nonlinear fashion from 1650 at 0° to 4150 cal. mole^{-1} deg.$^{-1}$ at 60°. Although these values may seem quite large at first glance, even at the low temperatures, they are nevertheless in good agreement with calorimetric determinations of ΔC_p by Sturtevant and co-workers[9,10] for other proteins. They have reported values of 9800 cal. mole^{-1} deg.$^{-1}$, for native and acid denatured ferrihemoglobin and 8000 cal. mole^{-1} deg.$^{-1}$ for native and acid denatured serum albumin at about 20°. If these are converted to specific heat capacities for comparison purposes, the values are 0.14 for ferrihemoglobin, 0.12 for serum albumin, and 0.10 cal. g.$^{-1}$ deg.$^{-1}$ for chymotrypsinogen at 20°. The value for chymotrypsinogen, based on a two-state analysis, is then quite comparable to the values for other proteins obtained from calorimetric measurements at similar temperatures.

To the best knowledge of the author, this study represents the first instance in which a temperature dependence of ΔC_p has been noted for a denaturation reaction. This provides a sufficiently detailed criteria which any model proposed to describe the denaturation of chymotrypsinogen, and possibly other denaturations as well, must take into account.

In this same connection, it is significant that native chymotrypsinogen shows a maximum in stability in the experimentally accessible temperature range. This maximum occurs, of course, at the temperature where $\Delta S°$ (*i.e.*, $-\delta\Delta F°/\delta T = 0$) is zero so that the precise temperature depends to a small extent on the pH of the solution but is always close to 10° below pH 3.5 (see Fig. 7). It is not possible to make any definite conclusion regarding the prevalence of this behavior, but it seems certain that at least several other proteins show stability maxima at low temperature. The kinetic measurements of Simpson and Kauzmann[17] show clearly that there is a large maxima in the activation free energy for ovalbumin denaturation in urea at 20°, and this is probably indicative of a similar maxima in the thermodynamic free energy. Also, the calorimetric heat change for the reversible denaturation of ferrihemoglobin[10] changes from positive to negative in going from 25 to 15°, indicating a maximum in free energy at some in-between temperature. The measurements of optical rotation[18] and calorimetric heat

(17) R. B. Simpson and W. Kauzmann, *J. Am. Chem. Soc.*, **75**, 5139 (1953).
(18) J. F. Foster and J. T. Yang, *ibid.*, **77**, 389 (1955).

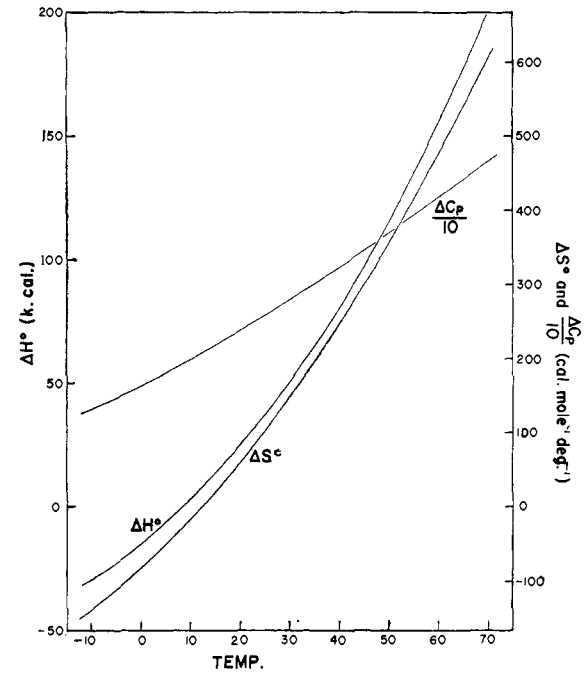

Fig. 13.—The temperature dependence of $\Delta H°$, $\Delta S°$, and ΔC_p for chymotrypsinogen denaturation; the $\Delta S°$ values refer to solutions of pH 3.0 and 0.01 *M* Cl$^-$.

change[9] for the denaturation of serum albumin suggest that the stability of the protein is maximal at 15° but the evidence in this case is not conclusive. We may add to this list other proteins[19] which have been reported to be denatured by either increasing or decreasing the temperature. Although this does not exhaust the list, it does serve to show that the behavior of chymotrypsinogen at low temperature is by no means unique. In view of this, the popular concept which regards temperature solely as a denaturing agent would seem to need revision in order to accommodate these low temperature inversions resulting from heat capacity effects.

Experimental

Materials.—The α-chymotrypsinogen used in these experiments was from batch 685-690, Worthington Biochemicals Corp. (Freehold, N. J.). The urea was Merck reagent grade and was recrystallized once from 50% ethanol prior to use. All other reagents were of the best grades available and used without further purification.

Methods.—The preparation of protein solutions and the determination of protein concentration are described elsewhere.[5] The measurements of pH were made on a Radiometer pH meter 4 and were reproducible to 0.01 unit. The readings of pH in solutions containing ethanol were corrected for the glass electrode error by the expression[20] $\Delta pH = 1.1 \ln a_{H_2O}$, where ΔpH is the electrode error and a_{H_2O} is the activity of water in the ethanol solutions, determined from the partial pressure.[21]

The difference spectra were measured on the Cary 14 spectrophotometer using the sensitive 0.0-0.1 slidewire and 1.0-cm. quartz cells. All solutions were centrifuged immediately prior to use to remove undissolved protein and other impurities. Other details of experimental procedure are given in the text.

Acknowledgment.—The author wishes to acknowledge financial assistance from the Public Health Service, Grant GM-11071, during part of this investigation.

(19) A. Rosenberg, Abstracts, 47th Annual Meeting of the Federation of American Societies for Experimental Biology, Atlantic City, N. J., April, 1963, p. 346.
(20) M. Dole, *J. Am. Chem. Soc.*, **54**, 3095 (1932).
(21) "International Critical Tables of Numerical Data," McGraw-Hill Book Co., Inc., New York, N. Y., 1926–1933.