The Thermodynamics of Protein Denaturation. III. The Denaturation of Ribonuclease in Water and in Aqueous Urea and Aqueous Ethanol Mixtures

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Abstract: This paper presents results of a thermodynamic study of the denaturation of ribonuclease in water and in aqueous ethanol and aqueous urea mixtures in the acid pH range. The results in water suggest that this unfolding transition is very similar to the transition of chymotrypsinogen, which was studied previously. It is characterized by a large difference in the heat capacity of native and denatured states which, according to the authors' interpretation, can be attributed to the exposure of hydrophobic groups during the unfolding process. The variations in the thermodynamics of denaturation ($\Delta F°$, $\Delta H°$, $\Delta S°$, and ΔC_p), which occur as the composition of the water–ethanol mixture is altered, are very large. Each thermodynamic function exhibits a maximum or a minimum at about 10–15% ethanol. Qualitatively similar extrema are noted to occur in the thermodynamics of solution of small hydrophobic solutes in these same mixtures and in other aqueous mixtures, so that there is the implication that the effects of ethanol on the protein transition arise to some extent from changes in the solvation of exposed hydrophobic side chains in the denatured state. A simple mechanism consistent with the experimental data is suggested. In contrast to ethanol, urea is found to produce only small changes in $\Delta H°$, $\Delta S°$, and ΔC_p, although moderately large changes in $\Delta F°$ of denaturation for ribonuclease. The same pattern of thermodynamic response is not duplicated by any of the customary model compound transfer reactions, so that no firm conclusions relating to the mechanism of urea denaturation are possible.

In earlier work, we have reported detailed experimental results on the conformational stability of native chymotrypsinogen in aqueous solution.[1] The comparison of the experimental thermodynamics with a simple denaturation model suggested[2] that the more prominent aspects of conformational stability are probably associated with the solvation of hydrophobic side chains in the denatured state. More specifically, there is an anomalously large heat capacity associated with the unfolded protein which apparently reflects a striking alteration in the process of accommodation of the exposed hydrophobic side chains in the solvent phase with changing temperature. At low temperature the thermodynamically preferred mode of accommodation is by formation of highly ordered water "clathrates" which will provide solvation for the nonpolar side chains with little or no reduction in the number of water–water hydrogen bonds but at a considerable cost in entropy. These ordered structures appear to be marginally stable, however, and tend to melt as the temperature is increased, leading to a more random mode of accommodation at high temperature. It is this cooperative order–disorder transition in the solvent phase which imparts the previously noted heat capacity effects and gives rise to a temperature of maximum stability (T_{max}) for the native protein such that it may be "cold-denatured" as the temperature is lowered below T_{max} as well as heat-denatured when the temperature is raised above T_{max}.

Since the qualitative interpretation of the experimental thermodynamics is based on fundamental aspects involved in protein solvation and is relatively independent of detailed structural features of native and denatured proteins, it was suggested that most or all major denaturation reactions ought to resemble the chymotrypsinogen reaction when examined closely. Since that time there have been preliminary reports on the denaturations of chymotrypsin and dimethionine sulfoxide chymotrypsin[3] and ribonuclease[4] which would seem to bear this out. One of the purposes of this paper is to present in more detail the thermodynamic results which we have obtained on the ribonuclease transition in water in order that similarities and differences with the chymotrypsinogen transition and others may be carefully noted.

The second purpose of this paper is to provide a more substantial experimental base from which might emerge a better understanding of the conformational stability of proteins in mixed aqueous solvents. There have been numerous suggestions in the literature during the past 10 years about *how* various nonaqueous additives, such as ethanol, urea, dioxane, salts, etc., interact with native or denatured proteins to alter their relative stability. Thus, it has been proposed that certain additives denature proteins primarily by weakening hydrophobic bonds while others do so by interaction with peptide hydrogen bonding valences or by combination with charged sites on the protein or by some composite effect. While it is intuitively clear that differences in mechanism between different additives will unquestionably exist, it is equally clear that in many cases definitive experimental information to support *specific* suggestions is simply unavailable. Up to the present time, the vast majority of thermodynamic studies on denaturation reactions in mixed solvents have been limited to the determination of changes in the free energy of denaturation at a single temperature and pressure as the bulk solvent composition is varied. Information of this sort, although interesting, does not provide the detailed information necessary to distinguish between alternative mechanisms, even when it is supplemented by analogous model compound

(1) J. F. Brandts, *J. Am. Chem. Soc.*, **86**, 4291 (1964).
(2) J. F. Brandts, *ibid.*, **86**, 4302 (1964).
(3) R. Biltonen and R. Lumry, *ibid.*, **87**, 4208 (1965).
(4) J. F. Brandts, *ibid.*, **87**, 2759 (1965).

data. Fundamental differences in the mechanism of denaturation for different nonaqueous additives should be reflected experimentally by differences in the effects of these additives on *all* of the characteristic thermodynamic functions for the denaturation reaction, not only $\Delta F°$ but, perhaps of greater utility, derived functions such as $\Delta H°$, $\Delta S°$, ΔC_p, ΔV, and others. When supplemented by corresponding thermodynamic data on appropriate model compounds in mixed solvents, more definitive interpretations regarding mechanism may be possible. This paper represents an initial effort toward more complete experimental characterization of the denaturation reaction of ribonuclease in mixed solvents.

Results

Analysis of Data. The ribonuclease conformational transition was found to be completely (*i.e.*, better than 99%) reversible in the solvent systems employed in this study and with the relatively low protein concentrations (*ca.* 0.04%) used, so that thermodynamic treatment of the data is justified. The experimental data will be analyzed in the usual way[1,4] assuming a two-state transition, *i.e.*

$$N \rightleftharpoons D$$

where the native and denatured "states" referred to are *macroscopic thermodynamic states*, each of which may consist of a number of available conformational states (*i.e.*, microscopic) and a multitude of solvation states.

The use of two-state formalism in the analysis of protein denaturation is, at best, a suitable approximation. There is at the present time some uncertainty as to the degree of approximation involved for any specific conformational transition. Poland and Scheraga[5] have criticized the use of the two-state analysis for denaturation reactions in general and for the ribonuclease denaturation in particular. More recently, it was shown[6] that the detailed shapes of the transition curves for the thermal denaturation of ribonuclease in water are in agreement with the two-state model and appear to rule out the existence of large concentrations of intermediate states in the transition region. It will be seen that this is also true for the transition curves in aqueous ethanol and, to some extent, in aqueous urea solutions (although, in the latter case, the results do not permit a meaningful test of the two-state approximation at urea concentrations in excess of 1 *M*). Consequently, in terms of the data which are now available and the operational tests for two-state behavior which are pertinent,[6] the application of the two-state model to the ribonuclease transition would not appear to involve severe approximations, and we will proceed under that assumption.

It should be noted, however, that even though the thermal transition may continue to be of the two-state type as the solvent composition is varied, it is likely that the native and denatured states themselves may be quite different in 25% ethanol, for instance, then they are in pure water. Thus, the assumption of a two-state transition over a range of composition for the binary mixtures does not exclude the possibility that there may be structural modifications induced in the native and denatured states as the composition of the solvent is varied and, indeed, it seems certain that such modifications must occur but definitive experiments pertaining to the magnitude and importance of such alterations are not yet available. The necessary information is contained in the thermodynamic parameters which are reported, but the difficult job of separating these effects associated with changes in the average conformation of the two macroscopic states from the effects which arise from solvation changes in existing structures cannot yet be accomplished.

(5) D. Poland and H. Scheraga, *Biopolymers*, 3, 401 (1965).
(6) R. Lumry, R. Biltonen, and J. F. Brandts, *ibid.*, 8, 917 (1966).

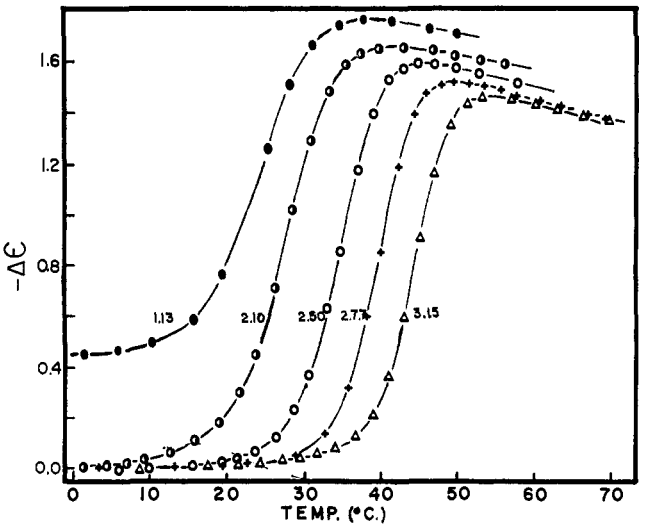

Figure 1. The change in extinction coefficient at 287 mμ for ribonuclease A at five pH values. The samples at pH 2.77 and 3.15 were buffered with 0.04 *M* glycine, while the other samples contain only HCl. Protein concentrations are 0.03–0.04 g/100 ml.

The equilibrium constant for two-state transitions can be calculated from spectrophotometric data as

$$K = \frac{\epsilon(T) - \epsilon_N(T)}{\epsilon_D(T) - \epsilon(T)} = e^{-\Delta F°/RT} \qquad (1)$$

in the case of thermally induced transitions, where ϵ_N and ϵ_D are the extinction coefficients of the native and denatured states, and ϵ is the experimental extinction coefficient of a mixture of the two states in the transition region. The derived thermodynamic functions $\Delta H°$, $\Delta S°$, and ΔC_p may then be obtained from $\Delta F°$ in the usual way.

Results in Water. Typical transition curves for the ribonuclease denaturation are shown in Figure 1 for aqueous solutions of five different pH values. Experimental problems in the application of eq 1 are immediately obvious since ϵ_N and ϵ_D cannot be measured directly in the transition region. This problem has largely been ignored in the past by assuming that these parameters are temperature independent and thereby using values of the extinction coefficient in the immediate pretransition and posttransition region for ϵ_N and ϵ_D, respectively, for the calculation of K values at all temperatures in the transition region. It is evident upon close inspection of the data in Figure 1 in the low- and, in particular, the high-temperature regions of each transition curve that ϵ_N and ϵ_D do depend to a significant extent on temperature and, if accurate values of the equilibrium constant are to be obtained, this must be taken into account by extrapolation of ϵ_N and ϵ_D to the appropriate temperature where K is evaluated. To a very good approximation, the extinction coefficients

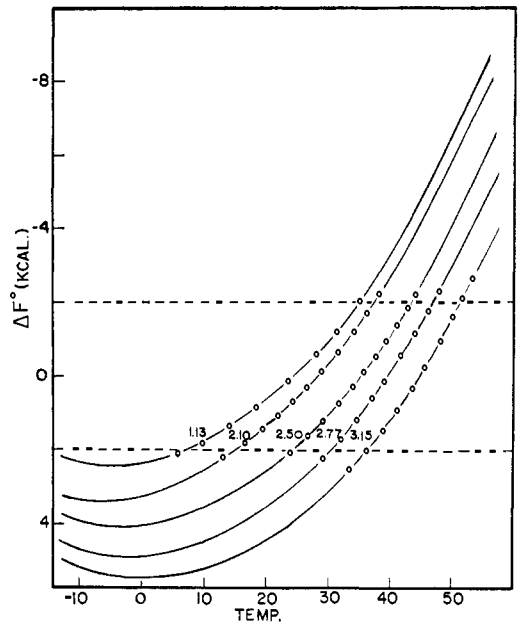

Figure 2. The temperature dependence of the free-energy change for ribonuclease transition at different pH values. The points represent calculated values of $\Delta F°$ from experimental points, and the solid curves correspond to analytical values of $\Delta F°$ calculated from eq 3, using fitting parameters determined from least-squares analysis of the experimental data at each pH. Dashed lines indicate the range of relatively high experimental accuracy.

for the pure states vary linearly with temperature under all conditions used in the present study so that we may work from the following expressions

$$\epsilon_N = A + BT$$
$$\epsilon_D = C + DT \qquad (2)$$

where A, B, C, and D are temperature-independent fitting parameters to be determined from data in the appropriate temperature region for each transition.

Once the above parameters are determined under each set of conditions, experimental values of $\Delta F°$ may be calculated from eq 1. The results of these hand calculations are shown in Figure 2. It should be noted that, at each pH, accurate experimental values of $\Delta F°$ can be obtained over a temperature interval of only about 15 to 20°, because of the sharpness of the transitions brought about by the high degree of cooperativity. Because of the rather large dependence of transition temperature (T_0) on pH, the experimental values of $\Delta F°$ at low pH are therefore not obtained over the same temperature interval as those at high pH. Thus, it is impossible to measure *directly* the variation in thermodynamic parameters as a function of pH *at constant temperature*. The same limitations are encountered if, for instance, one desires to measure the variation in thermodynamic parameters as a function of some other composition variable (*e.g.*, urea concentration) at constant temperature and pH. Therefore, if one wishes to isolate the effects of solution composition from the effects of other variables such as temperature and pressure, it is absolutely necessary to use some form of extrapolation. In the present instance, we will extrapolate along the temperature axis, although extrapolations can be made with regard to any other solution variable.[1,7]

It has previously been shown[2] that the temperature dependence for the free energy of denaturation of chymotrypsinogen is consistent with an expression of the form

$$\Delta F° = E + FT + GT^2(1 + PT) \qquad (3)$$

where E, F, G, and P are temperature-independent parameters which can, in principle, be obtained from experimental values of $\Delta F°$ such as those shown in Figure 2. The precise terms included in this power series were dictated from a consideration of solubility data on small hydrophobic model compounds. As a matter of fact, within the limitations of the model previously used,[2] the value of P can be uniquely determined *a priori* for any protein whose amino acid composition is known, and the value for ribonuclease is -0.00155. Actually, the higher order term containing P plays a relatively minor role in determining the temperature dependence of $\Delta F°$. The procedure will then be to treat only E, F, and G as experimental parameters.

Combining eq 1–3, an expression for the extinction coefficient may be obtained which will be applicable not only in the transition region but also at the extreme temperatures where the pure states exist, *i.e.*

$$\epsilon(T) = \frac{C + DT + (A + BT)\exp\left(\dfrac{E + FT + GT^2(1 + PT)}{RT}\right)}{1 + \exp\left(\dfrac{E + FT + GT^2(1 + PT)}{RT}\right)} \qquad (4)$$

This equation has been applied independently at each pH to *all* of the experimental points shown in Figure 1 to obtain values of the parameters E, F, and G (and, simultaneously, the values of A, B, C, and D) by minimization of the residuals between the experimental extinction coefficients and the analytical expression for ϵ given by eq 4, using a least-squares program based on iteration. The solid lines in Figure 2 show the continuous values of $\Delta F°$ calculated in each case from eq 3 using the best values of the fitting parameters determined at that pH. Thus, using this procedure, thermodynamic parameters may be extrapolated outside the temperature region where direct experimental measurements were possible, and this permits comparison, at constant temperature, as composition parameters such as pH, ethanol concentration, or urea concentration are altered. It will soon become apparent that the temperature effects associated with this denaturation reaction are so large that meaningful comparisons can only be made at constant temperature.

A comparison of the thermodynamic parameters calculated from eq 3 at the five different pH values is shown in Table I at 30°, a temperature which is intermediate with respect to all of the transitions. Thus, although increasing acidity produces quite large decreases in the free-energy parameter, this denaturing action arises from relatively small fractional changes in $\Delta H°$ and $\Delta S°$ and with no evident changes in ΔC_p. This is also apparent in the solid curves of Figure 2, where the most pronounced effect of decreasing pH is simply to shift the entire free energy curve toward more

(7) J. Hermans, Jr., and G. Acampora, *J. Am. Chem. Soc.*, **89**, 1543, 1547 (1967).

Table I. Values of the Molar Free Energy, Enthalpy, Entropy, and Heat Capacity Changes (30°) for the Ribonuclease Thermal Transition, Assuming a Two-State Reaction

pH	$\Delta F°$, cal	$\Delta H°$, kcal	$\Delta S°$, eu	ΔC_p, cal deg^{-1}	Av rel dev,[a] %
1.13	−1085	60.3	202	2072	0.20
2.10	−466	62.3	207	1980	0.25
2.50	912	57.2	186	1985	0.14
2.77	2045	56.6	180	2040	0.13
3.15	3094	53.0	165	1987	0.19

[a] These values represent the average deviation of the experimental extinction coefficients from the analytical values (eq 4) using the best values of the fitting parameters, as compared to the total change in extinction coefficient ($\epsilon_D - \epsilon_N$) for complete denaturation. All experimental points (22 for each transition except that at pH 1.13) were considered and, in general, the deviations in the transition region were not significantly greater than the deviations in temperature regions where the pure states existed.

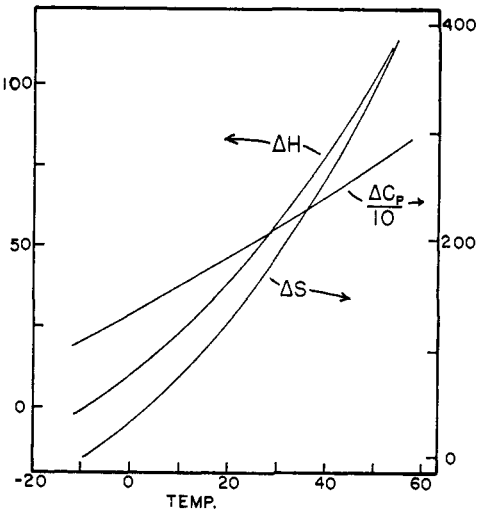

Figure 3. The values of $\Delta H°$, $\Delta S°$, and ΔC_p for the ribonuclease transition. These values have been calculated from eq 3, after appropriate differentiation, using the best values of the fitting parameters determined at pH 2.50.

negative values without altering the temperature dependence to a great extent. Thus, all curves when extrapolated to low temperature indicate a temperature of maximum stability, T_{max}, for native ribonuclease of about 0 to −5°, and this depends very little on the pH of the solution.

The last column in Table I shows the average deviation of the experimental extinction coefficients from the analytical values calculated using the two-state expression (eq 4). The deviations are expressed as a percentage of the total change in extinction coefficient for complete denaturation. The small deviations observed in all experiments are well within experimental errors so that the method of analysis is entirely consistent with the experimental data. The appearance of intermediate states in the transition region would be expected to show up as systematic deviations from the two-state analytical expression,[6] and there is no indication of such deviations in the experimental data.

These results are similar to those previously reported for chymotrypsinogen and also to those obtained more recently on chymotrypsin[3,8,9] and dimethionine sulfoxide chymotrypsin,[3,9] since it was also found for these latter three proteins that $\Delta H°$ and $\Delta S°$ were not strong functions of pH. This fact supports the previous suggestion that the pH dependence of free energy might generally result from the titration of carboxyl groups[1,2] during the denaturation process in the acid pH region since the heat of ionization of carboxyl groups is close to zero.

Another similarity between the transitions of all these proteins is the large temperature dependence of the heat and entropy change, arising from a large difference in heat capacity between native and denatured states. This is seen more clearly in Figure 3, where these thermodynamic parameters are shown as they depend on temperature. These extrapolated values then suggest that $\Delta H°$ and $\Delta S°$ vary from about 10 kcal and 20 eu at 0° to 130 kcal and 415 eu at 60°. The large heat capacity difference, ΔC_p, is also shown in Figure 3. A certain amount of caution must be exercised in extrapolating this function over a large temperature interval, however. Although the experimental data are sufficiently precise to determine an *average* ΔC_p over the temperature interval of a transition, the determination of the temperature dependence of ΔC_p is an order of magnitude more difficult, and the values indicated in Figure 3 are probably significantly in error for temperatures far removed from the transition temperature, since these values will be influenced to a great extent by the precise terms which one uses in the power series representation for $\Delta F°$.

One of the most noticeable differences between these present results on ribonuclease and those on chymotrypsinogen, chymotrypsin, and dimethionine sulfoxide chymotrypsin is that T_{max} occurs at 0° or slightly below for ribonuclease whereas it occurs at about 12° for all three members of the chymotrypsin family.

Results in Aqueous Ethanol Mixtures. Measurements similar to those discussed in the preceding section have been made on the ribonuclease transition in aqueous ethanol solutions in order to learn something of the effect which this organic additive has on the thermodynamics of denaturation. This study involved the measurement of the thermal transition curves for protein solutions of 0, 3.8, 7.6, 15.4, 19.7, and 28.1% ethanol (by weight) and measured pH values of 3.20 ± 0.05. The transition temperatures varied from about 44° for pure water to 36° for the highest alcohol concentration.

The calculated values of free energy for the transitions in three of these solutions are shown in Figure 4. The solid lines again represent continuous values of $\Delta F°$ obtained by the identical least-squares analysis used previously, while the points represent hand calculations. The average residual between the experimental values of the extinction coefficient and the analytical values did not exceed 0.25% of the total change ($\epsilon_D - \epsilon_N$) for any of the six solutions, and thus the transitions all obey the two-state model within experimental error.

The results in Figure 4 show that the effect of ethanol on the temperature dependence of $\Delta F°$ is very different from the effect of acid (Figure 2) and therefore reveal a difference in the thermodynamic mechanism of action of these two agents. The quantity of interest insofar as the denaturing effectiveness of ethanol is concerned

(8) J. F. Brandts, unpublished results.
(9) R. Biltonen, Ph.D. Thesis, University of Minnesota, Minneapolis, Minn., 1965.

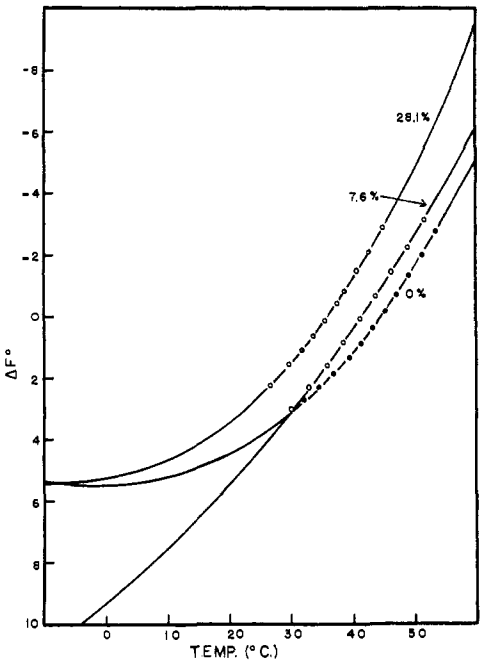

Figure 4. The temperature dependence of $\Delta F°$ for the ribonuclease transition at different ethanol concentrations. The points correspond to experimental values of $\Delta F°$ while the solid curves are analytical values from eq 3, using the best values of the fitting parameters. The measured pH of the solutions is 3.20 ± 0.05, and the solutions were buffered with 0.04 *M* glycine.

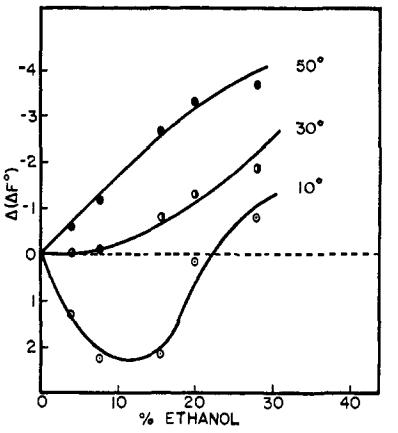

Figure 5. The variation in the free energy of denaturation of ribonuclease as it depends on ethanol concentration at 10, 30, and 50°. The ordinate ($\Delta(\Delta F°)$) corresponds to the difference in $\Delta F°$ in aqueous ethanol and in pure water, the values being compared at the same temperature and pH. Free-energy values are in kcal mole^{-1}.

is the change in free energy brought about by the addition of a certain amount of ethanol, *i.e.*

$$\Delta(\Delta F°) = \Delta F°(c) - \Delta F° \qquad (5)$$

where the first term on the right is the free energy of denaturation in an aqueous ethanol solution containing c% ethanol and the second term on the right is the free energy change in pure water. These two free-energy terms must, of course, be evaluated under identical conditions of temperature and pH[10] if we are to obtain the changes in free energy brought about solely by the presence of ethanol. The points in Figure 5 show the values of $\Delta(\Delta F°)$ calculated by the use of eq 3 using the appropriate fitting constants at each alcohol concentration. Free-energy corrections were made for the small differences in pH of the samples. The results obtained at three different temperatures show a rather striking conclusion. At high temperature (50°) ethanol is quite effective in decreasing the free energy of denaturation in keeping with numerous studies which have shown all alcohols to be rather strong denaturing agents above room temperature. However, at low temperatures (10°) ethanol acts differently even at a qualitative level. Here it behaves as a rather strong *stabilizing agent* for native ribonuclease as long as the concentration of ethanol is below about 20%. Although this conclusion depends to some extent on the validity of the extrapolation (see Figure 4, for instance), since the native protein is too stable at 10° and pH 3.2 to permit direct measurement, the temperature range of extrapolation is short enough and the effect large enough so that this qualitative conclusion appears to be justified. In addition, Skukuya and Schwert[11] found that, for the reversible transition of glutamate decarboxylase, small concentrations of ethanol appear to stabilize the native form at 0° but to denature the protein to a small extent at 25°, suggesting that this protein behaves similarly to ribonuclease. Less complete studies[1,2] on chymotrypsinogen show somewhat the same behavior although the data do not permit a quantitative estimate of the magnitude of the low-temperature stabilizing effect of ethanol. Finally, we shall see later that model compound data suggest that other relatively nonpolar organic additives, previously considered to be only denaturing agents, might show somewhat the same behavior at low temperature so that what is described here for ethanol may be a rather general phenomenon which has gone largely unnoticed because most of the common proteins are too stable at low temperature to permit direct thermodynamic measurements.

A look at higher order thermodynamic functions reveals further interesting information, as is readily anticipated from the complexity in the free-energy curves. The values of $\Delta H°$ as a function of ethanol concentration are shown in Figure 6 at the same three temperatures. The data indicate that this thermodynamic function goes through a maximum, very pronounced at low temperature, as the ethanol concentration is varied. The amplitude of the maximum is nearly 60 kcal in going from 0 to 13% ethanol at 10°. This corresponds to a change in mole fraction of only 0.05. At high temperature, the maximum is considerably less pronounced and is shifted toward higher concentrations of ethanol.

The maximum in $\Delta H°$ (and a corresponding maximum in $\Delta S°$ of about 200 eu at 10°) is accompanied by a minimum in ΔC_p as seen also in Figure 6 for a temperature of 40°, which is close to the transition temperature for all of these solutions. This minimum, which occurs at nearly the same alcohol concentration

(10) Since the addition of ethanol (and urea as well) will shift the pK of carboxyl groups, it would actually be more desirous to evaluate the free-energy change at a constant degree of ionization for both native and denatured states rather than at constant pH. However, this is not a convenient standard state experimentally. These problems have been discussed previously,[2] and the variation in $\Delta(\Delta F°)$ with the choice of standard states was estimated. In the present instance, we are more interested in the temperature dependence of $\Delta(\Delta F°)$ rather than in its absolute magnitude, and this should not depend critically on the choice of standard state in the pH region of carboxyl titration.

(11) R. Skukuya and G. Schwert, *J. Biol. Chem.*, 235, 1658 (1960).

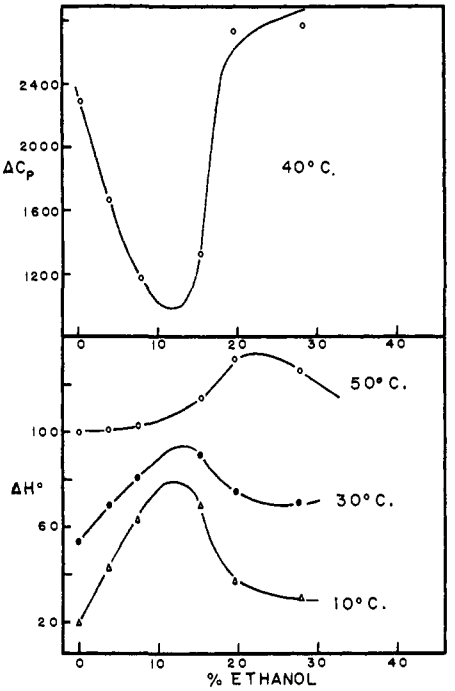

Figure 6. The values for the enthalpy and heat capacity of denaturation of ribonuclease in aqueous ethanol, as they depend upon the amount of ethanol in solution.

as does the maximum in $\Delta H°$, has an amplitude of about 1300 cal deg^{-1} mole^{-1}, and thus is a reflection of quite a sizable alteration in the denaturation process as the ethanol concentration is increased. It will be seen that both the enthalpy maximum and the heat capacity minimum are also observed for transfer reactions which involve the solvation of small nonpolar molecules in aqueous alcohol as the final state, so that the effects outlined above appear to be attributable in part to alterations in the mode of solvation of the hydrophobic side chains of denatured ribonuclease as alcohol is added.

The present results should be compared with the previous results of Schrier, *et al.*,[12] who also studied the effects of ethanol (as well as numerous other alcohols) on the thermodynamics of the ribonuclease transition. One of the conclusions of these authors was that, within their experimental errors, the values of $\Delta H°$ showed no trend with alcohol concentration or with the nature of the alcohol employed and did not differ from the values of the quantities observed for the thermal transition of ribonuclease in water alone. Their results were interpreted in terms of a stoichiometric binding of alcohol molecules to independent sites on the denatured protein. This simple mechanism does not appear to be consistent with the present results since it would always give rise to a negative $\Delta(\Delta F°)$ and it would not, in its simplest form, lead to a maximum in $\Delta H°$ and a minimum in ΔC_p. On the other hand, there appear to be no inconsistencies in the two sets of experimental results. The earlier authors worked with solutions of about pH 7 so that their transition temperatures were much higher than ours, ranging from about 45 to 63° depending on the ethanol concentration. Our data in Figure 6 also suggest a rather small dependence of

(12) E. E. Schrier, R. T. Ingwall, and H. A. Scheraga, *J. Phys. Chem.*, **69**, 298 (1965).

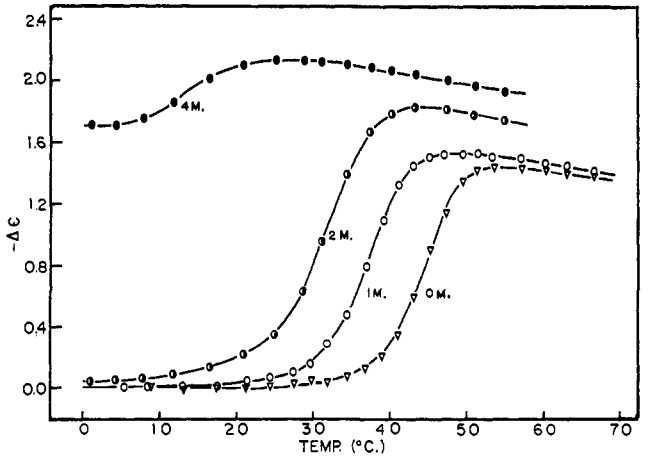

Figure 7. The change in extinction coefficient at 287 mμ for the ribonuclease transition at different urea concentrations. All samples were pH 3.30 ± 0.15 and buffered in 0.04 *M* glycine.

$\Delta H°$ on binary composition at these high temperatures, as Schrier, *et al.*, had previously reported. They did not attempt to estimate the variation in ΔC_p with composition and therefore had no reason to expect that the low-temperature behavior might be quite different from that at high temperature.

Results in Aqueous Urea Solutions. Studies of the thermal denaturation of ribonuclease in aqueous urea solutions of urea concentrations up to 4 *M* have also been completed, and the experimental results are shown in Figure 7. There is an additional complication which arises in the study of the denaturation in urea solutions. In contrast to ethanol, urea is a strong denaturant at all temperatures so that, even in moderate concentrations of urea, the native protein is unstable at low temperatures and is never present to the extent of 100% even at the lowest temperatures in our experimental range. This is true in the 2 and 4 *M* urea cases shown in Figure 7. Thus it is necessary to choose an arbitrary base line for the native state in these two solutions. The data of Figure 7 have been plotted so that the arbitrary base line chosen for the 2 and 4 *M* urea samples coincides with the base lines shown for the 0 and 1 *M* samples. The errors introduced into the thermodynamics of denaturation by an incorrect choice of base line will tend to be small in the case of 4 *M* urea, since all experimental points are considerably closer to ϵ_D than to ϵ_N. However, this arbitrary procedure could introduce quite sizable errors into the thermodynamics for the 2 *M* urea case, particularly at temperatures below 20°. Thus, the thermodynamic parameters obtained at 2 *M* urea must be considered to be less reliable than those at higher and lower urea concentrations.

The calculated values of $\Delta F°$ obtained from these data are shown in Figure 8. In the case of 0 and 1 *M* urea solutions, the solid lines again represent the least-squares fit of the experimental data. No least-squares analysis could be completed for the higher urea concentrations since the computer program is usually not capable of resolving data where the low- or high-temperature base line is missing. Consequently, these data will be used only in the temperature range of about 0–30° where direct measurements were made.

The data in Figure 8 show that urea is a strong denaturant for ribonuclease at all temperatures within the

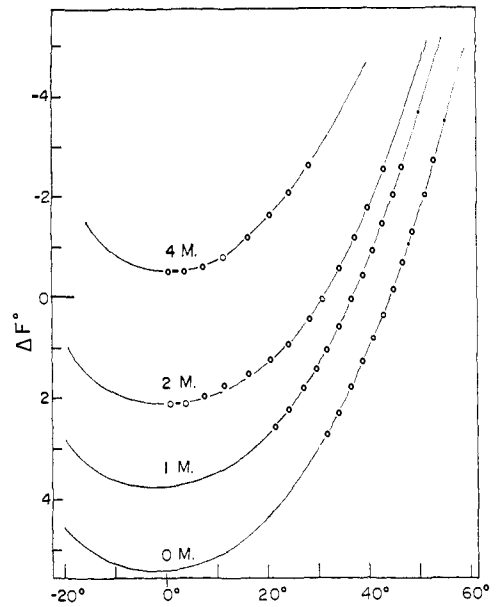

Figure 8. The free energy of denaturation of ribonuclease at different urea concentrations and pH ~3.30. The points are calculated from experimental data and, in the case of 0 and 1 M urea, the solid curves are analytical values of $\Delta F°$ (see text).

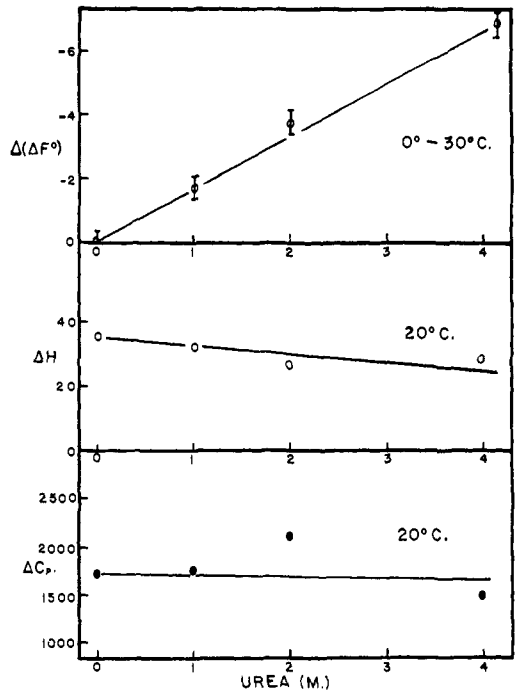

Figure 9. The characteristic thermodynamic parameters for the denaturation of ribonuclease in aqueous urea.

experimental range. It appears that the value of T_{max} for native ribonuclease is very close to 0° up to concentrations of 4 M urea and is thus nearly unchanged from its pure water value.

The effects of urea on the thermodynamics of denaturation are seen more clearly in Figure 9. The change in the free energy of denaturation, $\Delta(\Delta F°)$, is a nearly linear function of urea concentration over the range of conditions studied. The differences between urea and ethanol are emphasized by examining the temperature dependence of $\Delta(\Delta F°)$. Within the limits indicated in Figure 9, there is no detectable difference in the denaturing potency of urea over the temperature interval 0 to 30°. This is in marked contrast to the results with ethanol (Figure 5) where it was found that ethanol was transformed from a denaturing agent to a stabilizing agent as the temperature was lowered through this same temperature interval. This lack of a strong temperature coefficient in the action of urea arises of course because $\Delta H°$ and $\Delta S°$ do not change to a great extent as urea is added. This is seen also in Figure 9, where $\Delta H°$ is shown as a function of urea concentration at 20° and there is, within experimental error, no change in enthalpy up to 4 M urea.

Our data suggest that the large difference in the heat capacity of native and denatured ribonuclease is maintained in aqueous urea solutions. The estimates of the ΔC_p parameter are shown also in Figure 9. Within rather large errors, particularly in the case of 2 M urea, there is no change in ΔC_p with urea concentration. However, the problems involved in the analysis of the urea data are such that small variations in ΔC_p would likely have been overlooked in this study. Nevertheless, it seems significant that there is no indication of the large dependence of ΔC_p on solvent composition as we previously found for the ribonuclease transition in aqueous ethanol mixtures. This comes as somewhat of a surprise since there must be very large differences in the solvation of hydrophobic groups in water and in 4 M urea, and it remains a puzzle as to why this does not show up more prominently in the ΔC_p parameter. It appears possible, however, that these findings on ribonuclease may have some generality with respect to other proteins as well since Tanford and Pace[13] have found a very large ΔC_p (2100 cal deg^{-1} mole^{-1} near room temperature) for the thermal denaturation of β-lactoglobulin in 5 M urea. The value which they found is of the same order of magnitude as might be expected for a protein of this size in pure water.

One final point is worth mentioning in connection with previous studies by Foss and Schellman.[14] They found that in relatively high concentrations of urea, ribonuclease undergoes an "inverted transition" such that the amount of native protein becomes maximal at a particular temperature, which would correspond closely with what we have previously referred to as T_{max}, the temperature of maximum stability. The values of T_{max} which they observed using optical rotation were at somewhat higher temperatures than those indicated in this study. This might be attributable to the fact that they were using solutions of considerably higher pH, and also perhaps to difficulties in determining the base line for the native state in both their experiments and in our experiments. They suggested that this inversion phenomenon was brought about by specific interactions of urea and denatured ribonuclease which act to change $\Delta H°$ as urea is added (although they did not estimate $\Delta H°$ directly). In contrast, the present data suggest that the inverted transition of ribonuclease persists, virtually unmodified, in the absence of urea and therefore might be more a manifestation of protein–water interactions than of protein–urea interactions.

Discussion

Results in Water. The principal conclusion to be drawn from these results is that the thermodynamics

(13) C. Tanford and N. Pace, *Federation Proc.*, **25**, 412 (1966).
(14) J. G. Foss and J. A. Schellman, *J. Phys. Chem.*, **63**, 2007 (1959).

of denaturation of ribonuclease are remarkably similar to those for the chymotrypsinogen and chymotrypsin transitions. This is particularly true if corrections are made for differences in amino acid composition and differences in molecular weight. It was previously shown that, if accurate experimental data are available on the temperature dependence of $\Delta F°$ for protein transitions in pure water, then it is possible to combine this information with theoretical considerations and with model compound data in order to obtain estimates of certain structural parameters for the protein transition involved.[2] The parameters which are of particular interest for the ribonuclease transition are the p parameter (which measures the fraction of the total number of residues which unfold during denaturation), $\overline{\Delta h_H}$ (the enthalpy change for replacing a single peptide–peptide hydrogen bond in the native state with peptide–water hydrogen bonds in the denatured state), and $\overline{\Delta s_C}$ (the change in conformational entropy for unfolding a single residue). The estimates of these parameters obtained independently at each of the five pH values values used in the present study are shown in Table II.

Table II. Empirical Estimates of the Structural Parameters p, $\overline{\Delta h_H}$, and $\overline{\Delta s_C}$ for the Ribonuclease Transition[a]

pH value	p	$\overline{\Delta h_H}$, cal	$\overline{\Delta s_C}$, eu
1.13	0.83	1020	5.5
2.10	0.80	1060	5.6
2.50	0.80	1010	5.4
2.77	0.82	990	5.3
3.15	0.80	970	5.2

[a] In estimating these values, the electrostatic and titration free energy terms in eq 11 of ref 2 have been neglected. Although it is unnecessary to do this, it has already been shown that the temperature dependence of $\Delta F°$ for the ribonuclease transition is nearly independent of pH, so that the estimates of the above structural parameters depend little upon whether or not these two contributions are considered. The explicit consideration of these pH-dependent, free-energy contributions, in a manner analogous to what was done previously for chymotrypsinogen,[1,2] would tend to reduce the estimates of $\overline{\Delta h_H}$ and $\overline{\Delta s_C}$ by 5–10% and leave the values of p unchanged.

Thus, it would appear on the basis of this method of estimation that about 80% of the ribonuclease molecule is involved in the cooperative unit which unfolds during denaturation in water. The values for the hydrogen-bonding enthalpy term and the conformational entropy term are about 1000 cal and 5 eu per unfolded residue. Considering the approximations involved, these latter estimates are in good agreement with independent estimates of the same parameters obtained from model compound studies[15] and from studies of the helix–coil transitions of synthetic polypeptides.[16–18]

It is interesting and perhaps significant that the p parameter does not show large variations with regard to either temperature or pH variations for ribonuclease. This would seem to imply that the average conformation of native and denatured protein does not vary greatly with changes in these solution variables, at least over the limits used in this study. This, if true, is an important conclusion since it suggests that the extremely large variations in $\Delta H°$ and $\Delta S°$ with temperature change arise primarily from changes in the state of solvation of hydrophobic groups as previously indicated also for chymotrypsinogen.

The comparison of the parameters for the ribonuclease transition with those of other transitions which have been looked at carefully is shown in Table III. There are of course quite large differences in the estimates of the fraction of residues involved in each of these cooperative transitions, ranging from a value of 0.51 for dimethionine sulfoxide chymotrypsin to 0.87 for chymotrypsin itself. The values of the hydrogen-bonding and conformational entropy contributions are reasonably constant from one protein to the next as one would anticipate that they should be.

Table III. Estimates of Structural Parameters for the Thermal Transitions of Ribonuclease, Chymotrypsinogen, Chymotrypsin, and Dimethionine Sulfoxide Chymotrypsin

Protein	p	$\overline{\Delta h_H}$, cal	$\overline{\Delta s_C}$, eu	Ref
Ribonuclease	0.80	1000	5.4	...
Chymotrypsinogen[a]	0.55	930	5.4	1, 2
Chymotrypsin[a]	0.87	910	5.4	3, 9
Dimethionine sulfoxide chymotrypsin[a]	0.51	920	5.5	3, 9

[a] In the original treatment[2] of denaturation thermodynamics, the existing data on the solubility of tyrosine in the reference solvent (95% ethanol) indicated the tyrosine side chain behaved significantly differently from the other large hydrophobic side chains, and it was therefore treated as a special case. Since that time, it has been found in this laboratory that these solubility data are significantly in error. Because of this, in the calculation of the above structural parameters, tyrosine has been treated as being identical with the other large hydrophobic side chains (leu, ileu, phe, pro, and trypt). Consequently, the estimates given in this table differ somewhat (*ca.* 10%) from those given in the original references.

Thus, although there are too many uncertainties involved to permit one to attach much quantitative significance to such estimates of structural parameters at this time, they do serve a function in that they permit one to make comparisons between conformational reactions of different proteins and of the same protein under different solution conditions in order that similarities and differences might be noted. In the case at hand, all major differences in the thermodynamics of denaturation of the four proteins in Table III can be adequately accounted for by differences in amino acid composition and differences in the size of the cooperative unit, in accordance with the restrictions in the original model. For instance, one of the apparent differences between the transitions of chymotrypsin, chymotrypsinogen, and dimethionine sulfoxide chymotrypsin and the transition of ribonuclease is that the value of T_{max} for ribonuclease occurs at a temperature about 15° lower than the observed value of T_{max} for the three members of the chymotrypsin family. It was shown previously[2] that T_{max} will depend strongly on the extent of participation of hydrophobic groups in the conformational transitions, such that it would be expected that those proteins

(15) J. A. Schellman, *Compt. Rend. Trav. Lab. Carlsberg, Ser. Chim.*, 29, 223 (1955).
(16) H. Boutin and W. L. Whittemore, *J. Chem. Phys.*, 44, 3127 (1966).
(17) J. Hermans, Jr., *J. Phys. Chem.*, 70, 510 (1966).
(18) W. G. Miller and R. E. Nylund, *J. Am. Chem. Soc.*, 87, 3542 (1965).

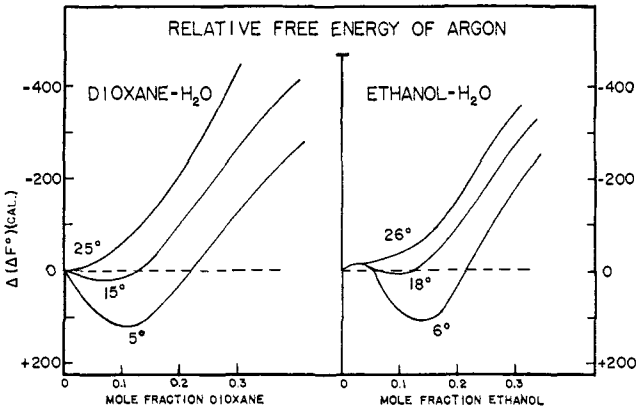

Figure 10. The change in the free energy of solution of argon as it depends on ethanol (or dioxane) concentration in aqueous binary mixtures. The ordinate is the difference in the free energy of solution (calculated from solubility data[21]) in aqueous ethanol (or aqueous dioxane) and in water, compared at the same temperature.

with a high content of large nonpolar side chains would exhibit maximum stability at a higher temperature than those proteins with a small content of large nonpolar groups. Ribonuclease is of course one of the more hydrophilic of the common proteins in terms of amino acid composition, while chymotrypsin is significantly more hydrophobic. Thus, it appears that the observed values of T_{max} are qualitatively consistent with what one might expect on the basis of the amino acid composition of these four proteins.

Results in Aqueous Ethanol. There are innumerable ways in which ethanol might act to stabilize or unstabilize native proteins, and it is difficult to decide *a priori* which interactions will be the more important under any given set of solution conditions. It has been commonly observed that, under the conditions where most studies have been conducted, ethanol acts as a strong denaturing agent for most proteins. This has given rise to the common assumption that ethanol (as well as other alcohols, dioxane, acetone, and many other relative nonpolar organic solvents) denatures proteins primarily by weakening hydrophobic bonds.

The results of this study, which show that ethanol is actually a stabilizing agent for native ribonuclease at low temperatures, might at first hand appear to refute this contention. However, we shall see that it actually provides very specific support for it.

To the extent that ethanol alters the conformational stability of proteins by interacting with the exposed hydrophobic groups of the denatured state, then to this extent should the effects of ethanol on the thermodynamics of denaturation mimic the effects of ethanol on the thermodynamics of solution of small nonpolar model compounds.[2,19,20] We should expect that the analogies between the protein data and the model compound data would extend beyond the primary thermodynamic function $\Delta F°$ to include higher order derivatives as well. There is sufficient model compound data available to show that this is indeed the case. For instance, the curves in Figure 10[21] show the effect of ethanol and dioxane on the free energy of argon, a relatively good analog for nonpolar side chains. The ordinate, $\Delta(\Delta F°)$, is the *change* in free energy of argon which arises from the addition of ethanol (or dioxane) at the temperature in question. This parameter thus corresponds with the same parameter determined from the effects of ethanol on the ribonuclease transition previously shown in Figure 5. It is seen that the predominant effect of both ethanol and dioxane is to *increase* the free energy (*i.e.*, decrease the solubility) of argon at low temperatures up to concentrations of about 0.13 mole fraction (25% by weight for ethanol), whereupon further increases in the amount of the nonaqueous additive act to decrease the free energy of argon. However, at high temperature, the only observable effect of either of these additives is to decrease the free energy of argon. The only major difference between ethanol and dioxane in terms of their effects on argon is that there is a small decrease in free energy when very small amounts of ethanol are added at low temperature, and this is not evident in the dioxane data. A possible explanation for this difference has been suggested,[22] but here we shall be more interested in the large similarities between the effects of ethanol and dioxane rather than the small difference.

The temperature coefficient involved in the effect of ethanol and dioxane on this nonpolar molecule is quite remarkable. The fact that it is seen for two very different additives such as ethanol and dioxane suggests that the effects are controlled to a large extent by the common component in the binary mixtures, *i.e.*, water. Furthermore, comparison of Figures 10 and 5 shows that there is a qualitative similarity between the effects of ethanol on the model hydrophobic compound and the effects of ethanol on the ribonuclease thermal transition. In addition, the extensive calorimetric data of Arnett and colleagues[23] show that the heat of solution of a large number of small hydrophobic molecules (*t*-BuCl, *n*-BuCl, CCl_4, *t*-BuOH, $(n\text{-Bu})_4N^+I^-$, $Na^+BPh_4^-$, and others) in aqueous ethanol solutions depends rather strikingly on the amount of ethanol in the binary mixtures. All of these hydrophobic solutes exhibit strong maxima in their heats of solution, and the positions of the maxima vary from about 22% ethanol ($Na^+BPh_4^-$) to 36% ethanol (CCl_4). The amplitudes of the maxima are as high as 11 kcal ($Na^+BPh_4^-$). In general, the larger the hydrocarbon, relative to the polar or charged portions, the larger the maxima and the closer they lie to pure water at 25°. These strong maxima in the heats of solution of such solutes are observed not only in aqueous ethanol but in aqueous mixtures of *all* organic cosolvents (about a dozen) which have thus far been studied[24,25] which include numerous alcohols, acetone, dioxane, DMSO, and organic amines. They are not observed in nonaqueous binary mixtures,[23,26] which again emphasizes the fact that these effects arise predominantly from the properties of water rather than from specific properties of the nonaqueous cosolvent.

(19) W. Kauzmann, *Advan. Protein Chem.*, 14, 1 (1959).
(20) C. Tanford, *J. Am. Chem. Soc.*, 84, 4240 (1962).
(21) (a) A. Ben-Naim and S. Baer, *Trans. Faraday Soc.*, 60, 1736 (1964); (b) A. Ben-Naim and G. Moran, *ibid.*, 61, 821 (1965).
(22) A. Ben-Naim, *J. Phys. Chem.*, 69, 3420, 3245 (1965).
(23) E. M. Arnett, W. G. Bentrude, J. J. Burke, and P. M. Duggleby, *J. Am. Chem. Soc.*, 87, 1541 (1965).
(24) E. M. Arnett, personal communication.
(25) E. M. Arnett and D. R. McKelvey, *J. Am. Chem. Soc.*, 87, 1393 (1965).
(26) E. M. Arnett, "Correlation of Kinetic, Thermodynamic and Spectral Behavior in Highly Aqueous Binary Solvents," Symposium on Physico-Chemical Processes at Bradford Institute, Bradford, England, May 1966, in press.

Again, the heat effects described here for model hydrophobic compounds are very similar to the effect of ethanol on the enthalpy of denaturation of ribonuclease (Figure 6). The major difference is that, in the latter case, the maximum in $\Delta H°$ occurs at about 12% ethanol which is a somewhat lower concentration than has been observed for any of the small solutes discussed above. This may arise because all of the model compounds are either charged or somewhat more polar than many of the hydrophobic side chains of proteins or it may be simply an indication that there are other nonhydrophobic factors involved in the protein effects which act to shift the maximum in $\Delta H°$ toward the water end of the composition scale.

The amount of available model compound data on heat capacities in binary mixtures is limited. There is a very accurate calorimetric study on the tetraphenylboron anion in *t*-butyl alcohol–water mixtures, however.[27] These data show that the partial molar heat capacity of solution of this solute exhibits a very sharp minimum at about 23% (0.07 mole fraction) alcohol at an average temperature of about 15°. The amplitude of this minimum is nearly 500 cal deg^{-1} $mole^{-1}$. In view of the fact that all molecules containing nonpolar groups exhibit roughly the same enthalpy effects in all aqueous mixtures, it seems possible that similar minima may exist in the heat capacities of solution of hydrophobic solutes in aqueous ethanol. At any rate, the relationship between the heat capacity of denaturation of ribonuclease and the composition of the aqueous ethanol mixtures (Figure 6) appears to be completely analogous to the behavior of at least some hydrophobic solutes in certain aqueous binary mixtures.

In view of the rather complex relationships between the thermodynamic parameters $\Delta F°$, $\Delta H°$, ΔC_p, and the temperature and composition of the solvent, the chances of an accidental correlation between the protein data and the model compound data appear to be very remote. Therefore, it seems entirely possible that *the more prominent changes in the thermodynamics of denaturation of ribonuclease as ethanol is added arise directly from changes in the state of solvation of hydrophobic side chains in the denatured state*. Furthermore, in view of the generality of the findings on model compounds, it is conceivable that other organic additives will be found to show somewhat the same effects on ribonuclease (and on other proteins) as does ethanol when examined carefully. Thus, many of the common "denaturants" should be reexamined at low temperatures and low concentrations since some of these might be strong stabilizing agents for the native protein under these conditions or, at the very least, considerably weaker denaturing agents than previously thought from high-temperature studies.

It is not difficult to speculate on the possible molecular mechanism which brings about the very pronounced relationship between temperature and the effect of organic additives such as ethanol on protein stability (or, equivalently, on the solubility of small nonpolar molecules). The precise mechanism might be closely connected with the "clathrate" structures which form about exposed nonpolar side chains at low temperature and with the clathrate melting transition which occurs as the temperature is increased. The process of interest is schematically illustrated in Figure 11. Although the exact nature of these clathrates is not known, it seems likely that they are very cooperative structures. Consequently, the result of removing a water molecule from a single site in the clathrate lattice, and its replacement with a different type of molecule (*e.g.*, ethanol) which cannot meet the specific hydrogen-bonding requirements of the clathrate, may be the complete disruption of the entire framework. Although it is undoubtedly an exaggeration to imply perfect cooperation of this sort, there will be a strong tendency for this situation to occur because of the interdependence of all sites necessitated by the hydrogen-bond network.

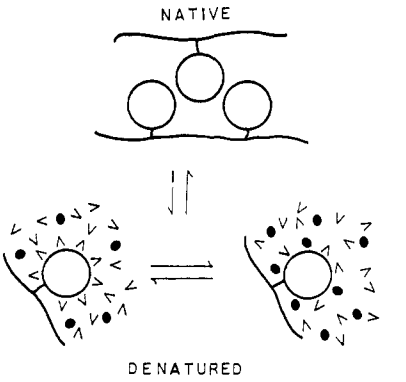

Figure 11. Schematic illustration of the possible relationship between hydrophobic bonding and the "clathrate melting" transition. In the clathrate accommodation of exposed nonpolar groups (bottom left), important at low temperature, only water molecules (V's) will be in the solvation shell while, in "random" accommodation (bottom right), both water and the nonaqueous solvent component (circles) will participate in the solvation process, with the nonaqueous component usually providing preferential solvation. The equilibrium between these two modes of accommodation of nonpolar side chains will therefore be shifted not only by temperature and pressure changes, but by changes in the composition of the solvent as well.

Because of this cooperation in the aqueous solvation shell, the effects arising from the addition of a nonaqueous cosolvent will be rather complex. At *low temperature*, the thermodynamically preferred mode of accommodation of (exposed) nonpolar groups will be by clathrate formation in pure water solutions. The initial effect of adding small amounts of a nonaqueous cosolvent will be to decrease the stability of the clathrate structures simply by decreasing the activity of water. Ultimately, as the activity of the nonaqueous cosolvent is increased further, it becomes entropically favorable for these molecules to enter the solvation shell. Because of the cooperativity in the aqueous solvation shell, the only way in which this can happen is by the isothermal melting of the clathrate structures, as shown in Figure 11. This series of events will lead to an increase in the free energy of the hydrophobic group since the destabilization and ultimate disruption of the clathrates represents the loss of the *best* mode of accommodation of these groups in water at low temperature. It is to be expected that the isothermal melting process will also give rise to an increase in the heat of solvation and a decrease in the heat capacity of solvation of exposed nonpolar groups as the concentration of the nonaqueous cosolvent is increased, thus defining the low concentration segments of the extrema in $\Delta(\Delta F°)$ (10°), $\Delta H°$, and ΔC_p, shown in Figures 5 and 6. Once melt-

(27) E. M. Arnett and D. R. McKelvey, *J. Am. Chem. Soc.*, **88**, 5031 (1966).

ing has occurred, both cosolvents may participate in the solvation shell of the nonpolar groups since cooperative aspects will be much less important. For most aqueous mixtures (with the possible exception of mixtures such as aqueous urea, where the nonaqueous cosolvent has no nonpolar portions and has a strong hydrogen-bonding capacity of its own), it is expected that the nonpolar side chains will be preferentially solvated by the nonaqueous component in the solvent once the ordered water structures have been disrupted. Consequently, further increases in the concentration of nonaqueous cosolvent beyond this point will lead to an increased stabilization of the denatured state because of preferential solvation and this apparently occurs with negative heat changes. The composite result of these two effects, isothermal melting and preferential solvation, is the observed extremum in $\Delta(\Delta F^\circ)$ at low temperature and the maximum in ΔH° at the composition where the positive heat effects associated with melting and the negative heat effects associated with preferential solvation are precisely balanced.

The situation at *high temperature* is considerably less complicated since ordered water of solvation will have been disrupted by thermal melting and the preferred mode of accommodation of nonpolar groups even in pure water is by "random" solvation. Therefore, the important effect of the addition of a second solvent component will be preferential solvation, and this leads to a stabilization of the denatured protein at all concentrations of the additive. Heat effects should be considerably reduced from those observed at low temperature since melting is less important.

Although a mechanism such as this is considerably oversimplified, it may contain elements of truth and represents a rational working hypothesis. It has the advantage that it is consistent with the gross aspects of experimental behavior, relatively nonspecific with respect to both the hydrophobic solute and the nonaqueous solvent component, and in agreement with what is known about the structure of water. A mathematical model, based on the concepts discussed above, will be presented elsewhere.[28]

There is no intent in this discussion to imply that the *only* effects of ethanol on ribonuclease stability arise from changes in the solvation of exposed hydrophobic groups in the denatured state.[29] The system is too complicated to permit such a generalization. The tentative conclusion to be drawn from this study is that there are very prominent effects on the thermodynamics of denaturation brought about by the addition of ethanol which can probably be attributed to changes in the solvation of hydrophobic side chains, irrespective of any other less-prominent (but perhaps equally important) effects ethanol might have on the protein. In addition, the fact that the ribonuclease results correspond closely with what might be anticipated on the basis of model compound studies serves in itself to provide some support for the validity of the two-state analysis for this particular protein transition.

Results in Aqueous Urea. Since the suggested mechanism is relatively nonspecific with regard to the nonaqueous solvent component, it might be anticipated that urea will show some similarities to ethanol in terms of its effects on the thermodynamics of solution of hydrophobic groups. There is fragmentary evidence which suggests that there are certain similarities in their behavior. The data of Wetlaufer, *et al.*,[30] indicate that the solubilities of small aliphatic solutes such as methane, ethane, propane, and butane are all *decreased* by the addition of 7 *M* urea at 0°, but that the solubilities of these same solutes are *increased* by the addition of 7 *M* urea at higher temperatures. The precise temperature where the inversion point occurs is different for different solutes, varying from about 45° for methane to approximately 2° for butane. This behavior arises of course from an *increase in the heat of solution* of these solutes in aqueous urea, as opposed to pure water, such that the enthalpies of ethane and propane are about 2000 cal/mole higher in 7 *M* urea than in water. These results, although fragmentary in the sense that only one urea concentration was investigated, are nevertheless similar to the effects of moderate amounts of ethanol on the heats and free energies of solution of small hydrophobic solutes.

On the other hand, the effects of urea and ethanol on the thermodynamics of denaturation of ribonuclease are very dissimilar. Ethanol acts on the ribonuclease transition by producing very large changes in both ΔH° and ΔS°, and these enthalpic and entropic changes compensate one another almost completely near room temperature to give rise to small positive or negative changes in ΔF° which are markedly temperature dependent. Urea acts to produce small noncompensating changes in ΔH° and ΔS° which lead to fairly large negative changes in ΔF° which are not extremely temperature dependent. The apparent discrepancy then between the effects of urea on model hydrophobic solutes and on ribonuclease, particularly with regard to temperature changes, seems to imply one of two things. (1) The effects which urea has on the solvation of nonpolar side chains in denatured ribonuclease is somewhat "anomalous" and cannot be predicted from model compound studies. This might be true for instance if the domain of the denatured protein were too small to

(28) J. F. Brandts in "Biological Macromolecules," Vol. 1, Marcel Dekker, Inc., New York, N. Y., in press.

(29) Other possible interpretations of the ribonuclease data in aqueous ethanol do exist. In particular, J. M. Luck and co-workers published a number of papers in the 1940's (see E. L. Duggan and J. M. Luck, *J. Biol. Chem.*, **172**, 205 (1948), for instance) which showed that various detergents, fatty acids, and other long-chain hydrophobic compounds stabilize serum albumin (and other proteins) when added in small amounts to the aqueous protein solutions. These same additives act as denaturants when added in large enough amounts. Thus, there exists a qualitative similarity between the detergent effects and the effects of ethanol on ribonuclease. Their results were interpreted in terms of a preferential binding of detergent molecules to the native state at low concentrations of detergent and a preferential binding of detergent to the denatured state at high concentrations. Such a differential binding mechanism cannot explain the effects of ethanol on model compounds (since, for transfer reactions of the type

hydrophobic solute (water) $\rightleftharpoons$ hydrophobic solute (aqueous ethanol)

ethanol can bind only to the state represented by the right-hand side), but it is conceivable that the ribonuclease results could contain a contribution from the differential binding of ethanol in a way analogous to that discussed above for the long-chain compounds. Unfortunately, the data of Luck and co-workers do not permit any conclusions on the temperature dependence of their observed effects, and this is probably critical if any meaningful comparison is to be made between their results and our results on ribonuclease. Nevertheless, the possibility that such a mechanism might be important cannot be eliminated with the data at hand.

(30) D. B. Wetlaufer, S. K. Malik, L. Stoller, and R. L. Coffin, *J. Am. Chem. Soc.*, **86**, 508 (1964).

permit complete solvent penetration, in which case the model compound studies, which are carried out effectively at infinite dilution of the nonpolar solute, would not be applicable. (2) The effect which urea has on the solvation of nonpolar side chains is basically what would be predicted from model compound studies but these hydrophobic effects are covered up to some extent in the over-all thermodynamics of denaturation by other interactions of urea with different portions of the protein. Of these two alternatives, the second seems more attractive in view of the fact that the hydrophobic effects of ethanol on ribonuclease are not at all "anomalous." As a matter of fact there seems to be a very logical, although inconclusive, argument which can be made along the lines of the second suggestion above. Robinson and Jencks[31] have made an excellent study of the effect of urea on the solubility of acetyltetraglycine ethyl ester (ATGEE), a model compound which contains four peptide groups. They found that although urea solubilizes ATGEE at all temperatures from 0 to 40°, the solubilizing effect at low temperature is much greater than at high temperature and, accordingly, the enthalpy of solution of ATGEE is about 2000 cal/mole *smaller* in 6 *M* urea than in pure water. Thus we might expect that the enthalpy of a single exposed peptide group in a denatured protein is lowered by approximately 500 cal by the addition of 6 *M* urea.

Thus, the model compound results lead us to expect that there will be quite a large cancellation in the effects of urea on the thermodynamics of denaturation of proteins due to the fact that urea produces quite different changes in the thermodynamics of solvation of exposed peptide groups and exposed hydrophobic groups. The enhanced effect of urea in decreasing the free energy of exposed peptide groups of the denatured protein at low temperature, as opposed to its effect on these same groups at high temperature, will tend to be at least partially offset by the fact that urea increases the free energy of exposed hydrophobic groups at low temperature. Thus, it is conceivable that the result of this could be a $\Delta(\Delta F°)$ of denaturation which is nearly temperature independent for some proteins, as has been suggested for ribonuclease. On a semiquantitative level, it was shown that the effect of urea (6–7 *M*) in increasing the enthalpy of a typical exposed hydrophobic group is roughly four times as great as its effect in decreasing the enthalpy of an exposed peptide group. Since the frequency of occurrence of hydrophobic side chains is roughly one-third or one-fourth as great as the frequency of occurrence of peptide groups and peptide-like side chains in ribonuclease, it is conceivable that heat effects will cancel almost completely.

Previous studies on the effect of urea on the free energy of denaturation of chymotrypsinogen[1,2] indicated that urea was a somewhat better denaturing agent at 39° than at 0° for this protein, although the data were not as complete as in the case of ribonuclease. This is, however, the trend to be expected since the chymotrypsinogen molecule is significantly more hydrophobic than is ribonuclease and this should tend to lead to a temperature coefficient similar to that observed for chymotrypsinogen, relative to the observations on ribonuclease.

(31) D. R. Robinson and W. P. Jencks, *J. Am. Chem. Soc.*, **87**, 2462 (1965).

Although one is able to rationalize the observed lack of a strong temperature coefficient in the denaturing action of urea in terms of a compensating mechanism such as suggested above, this simple picture does not tell the entire story. An additional dilemma arises if one attempts to account quantitatively for the *absolute value* of $\Delta(\Delta F°)$ for urea at any single temperature and composition. For instance, at 25° the net effect of urea on all hydrophobic groups must be such as to make a negative contribution to $\Delta(\Delta F°)$ for ribonuclease, although perhaps a small contribution at this fairly low temperature. Therefore, if the total experimental $\Delta(\Delta F°)$ is divided by the effect of urea on one exposed peptide group (estimated from the data of Robinson and Jencks), this should give an upper limit to the number of peptide groups exposed during denaturation. For the ribonuclease transition in 4 *M* urea, this leads to a maximum of about 50 "peptide" groups which are exposed. The estimate which we have obtained in pure water from the *p* factor is about 100, and this would be a minimum estimate since polar side chains which might possibly behave like peptide groups in terms of their interactions with urea have not been counted. Thus, it seems possible that the absolute effect of urea on ribonuclease stability is actually considerably smaller than one would estimate from its effects on *both* hydrophobic model compounds and peptide model compounds. This same problem has already been noted by Tanford[32] from a consideration of thermodynamic data on several proteins including ribonuclease. He explained this apparent discrepancy by suggesting that perhaps the unfolding transitions of these proteins are not two-state transitions but actually involve a series of partial unfolding steps. If this is the case, the experimental values of $\Delta(\Delta F°)$ will be smaller than the true value for the entire protein. It is not certain that this is the correct explanation for ribonuclease since existing data suggest that the two-state approximation may not be far in error. It seems equally possible that there are other factors involved in the action of urea in addition to those which have thus far received the bulk of attention, and we must understand these before quantitative agreement can be expected.

Along these same lines, one very important factor involved in the effect of additives such as urea and ethanol has been ignored in this discussion. Our comments regarding the effects of these agents were always prefaced with the assumption that exactly the same conformational changes occurred in the binary solvents as occurred in water. This is probably not the case. For instance, many denatured proteins, including ribonuclease and chymotrypsinogen, have intrinsic viscosities in 8 *M* urea which are nearly twice as large as the intrinsic viscosities of these same proteins when they are denatured in pure water, under conditions where no apparent aggregation takes place.[28] Similiar large differences have been noted in optical rotatory properties for denatured proteins in the two solvents. Therefore, it would appear that the time-averaged conformation of the denatured state may be different in aqueous urea than in water, and this may be attributed to changes in the probability of occurrence of the various microscopic species which constitute this state. These results

(32) C. Tanford, *ibid.*, **86**, 2050 (1964).

might be interpreted as indicating that denatured proteins in aqueous urea are more extensively unfolded than in water. It is also conceivable that similiar changes in the average conformation of native proteins might occur as urea is added, although we are not suggesting this as necessarily a likely possibility. If changes in the average conformation of one or both of the macroscopic states should occur as urea (or ethanol) is added, this would not preclude the possibility that the thermally induced transition is still of the two-state type in aqueous urea. What it would mean, however, is that the modifications in the average conformations of the two states, brought about by the presence of urea, might in themselves cause significant changes in the thermodynamics of denaturation. Thus, the addition of a second solvent component may produce variations in the conformational as well as in the solvation characteristics of the transition. Our results here were discussed only with respect to variations in solvation, insofar as these can be measured by model compound transfer reactions. The problems with respect to conformational variations are considerably more difficult to handle, since no suitable model compound studies are pertinent, and that is the reason they have not been treated explicitly in this discussion. The neglect of this very important factor necessitates that any conclusions to be drawn from these data must be regarded as somewhat tentative in the absence of more specific experimental information pertaining to conformational changes in mixed solvent systems.

In the particular case of the urea studies, the only point which we would like to emphasize is that the total effect of urea on the ribonuclease transition cannot be adequately accounted for by *any single effect* which has thus far been observed in model compound transfer reactions, since the temperature dependence is incorrect.

In summary, these results indicate that there are significant differences in the thermodynamic response of ribonuclease to the addition of ethanol and urea, presumably reflecting differences in the way in which these additives interact with the protein and with the water of solvation. It was suggested earlier, and emphasized by these studies, that certain prominent features of the thermodynamics of denaturation of proteins in pure water can perhaps be attributed to the thermal order–disorder transition which we have referred to as "clathrate melting." The additional suggestion which comes from these studies is that equally prominent thermodynamic changes may result when isothermal clathrate melting is forced by the addition of a nonaqueous solvent component.[33]

Experimental Section

Materials. The chromatographically homogeneous ribonuclease A used in these experiments (batch no. 4535 and 7654) was purchased from Nutritional Biochemicals Corp. (Cleveland, Ohio). The urea was reagent grade (Baker and Adams) and recrystallized twice from 50% ethanol prior to use. The ethanol was 95% U.S.P.

Methods. The procedures employed in making difference spectra measurements were nearly identical with those previously used.[1] The Cary 14 spectrophotometer which was used was checked periodically for long-time stability on the 0.1 slidewire. The relative temperatures of the samples were known to approximately 0.02°. Thermal equilibration, usually the slow step in total equilibration, was about 99% complete in 10 min. The pH values given in the text are indicated values (Radiometer pH meter 4) at room temperature and were not corrected for electrode errors in aqueous ethanol or aqueous urea. All extinction coefficients were corrected for changes in concentration arising from density changes with changing temperature. Extinction coefficients were calculated according to per cent by weight.

In the case of aqueous urea solutions, some decomposition of urea took place at elevated temperatures. This was most apparent in the pH changes which took place after samples were run over the desired temperature range. These pH changes were as large as 0.35 pH unit (1.0 *M* urea sample) after cooling to room temperature, which corresponds to a negligible loss in total urea concentration for these solutions of low buffering capacity. However, the pH changes themselves might be cause for concern if they occurred in the temperature region of transition. It is felt, however, that most of the urea decomposition and therefore most of the pH change took place in the extreme high temperature region above the point where the transitions were completed. We looked for, and could not find, any indication of slow drifts in the optical density of thermally equilibrated solutions which might arise from pH changes and corresponding shifts in the denaturation equilibria. We also conducted experiments in solutions of the same buffering capacity in order to deduce time–temperature–pH relationships. It was found that, for the time intervals involved, pH changes are very small below 45°, the temperature at which all transitions in this study were complete (Figure 7).

However, because of pH changes brought about by urea decomposition at higher temperatures and because of the fact that in the 2.0 and 4.0 *M* urea solutions the protein was in a partially denatured state at the low temperature used for reversibility check, these samples exhibited an apparent reversibility in excess of 100%, and we attributed this to the fact that the pH upon reversal was slightly higher than the pH before heating.

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(33) NOTE ADDED IN PROOF. Since this paper was written, the results of a calorimetric study of the ribonuclease thermal transition in water have been published (R. Danforth, H. Krakauer, and J. Sturtevant, *Rev. Sci. Instr.*, **38**, 484 (1967)). The calorimetric estimates of $\Delta H°$ and ΔC_p, along with our two-state estimates at the same temperature (45°), are as follows: $\Delta H°$ (kcal) = 86 ± 5 (calorimetric), 88 (two-state); ΔC_p (cal deg^{-1} mole^{-1}) = 2300 ± 400 (calorimetric), 2500 (two-state). The good agreement between these two sets of results provides some additional support for the method of data analysis employed in this paper.