# THE STABILITY OF GLOBULAR PROTEINS

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## I. INTRODUCTION

Most proteins have a compact, globular conformation that is essential for their biological function. This conformation, which we will refer to as the native state and denote N, is only marginally stable under physiological conditions and exists in equilibrium with various unfolded states of the molecule, which we will denote D. This equilibrium

$$N \rightleftharpoons D \qquad (1)$$

is the subject of this review. In the absence of a denaturant the concentration of unfolded states is small, and they are difficult to measure or characterize. Consequently, most studies of this equilibrium are carried out in the presence of a denaturant. Fortunately, it is often possible to draw conclusions about the equilibrium under physiological conditions from these studies.

For most of the proteins that will be discussed in this review, the complete structure of the native state has been determined. The denatured states are more difficult to characterize, but considerable progress has been made in this area in recent years. In Section II we summarize studies of the denatured state under various conditions. Our understanding of the mechanism by which proteins fold and unfold has been improved in the past few years by a variety of experimental studies. The findings that are of interest to this review are discussed in Section III.

The free-energy change in the absence of a denaturant for (1), $\Delta G_D^{H_2O}$, is a useful measure of the stability of N. Most estimates of $\Delta G_D^{H_2O}$ have come from an analysis of denaturation curves for the protein. A few estimates have been reached by studying the rate of hydrogen exchange of N. Also, very recently, some estimates of $\Delta G_D^{H_2O}$ have been calculated using results from calorimetric studies. In Section IV we examine these approaches. Reasonable estimates of $\Delta G_D^{H_2O}$ are available for several of the smaller globular proteins. In addition, some interesting results are now available on the difference in stability between homologous proteins and on the effect of various types of chemical modification on the stability of globular proteins. In Section V we summarize and discuss the estimates of $\Delta G_D^{H_2O}$ that have been obtained to date.

A recent review on protein denaturation by Tanford provides detailed information on many topics treated only briefly here.[1,2] Other reviews

on protein denaturation have been written by Kauzmann,[3] by Brandts,[4] and by Lumry and Biltonen.[5] For historical aspects of protein denaturation, two earlier reviews by Neurath et al.[6] and by Putnam,[7] or a book by Joly[8] may be consulted. Reviews by Englander et al.,[9] by Willumsen,[10] and by Hvidt and Nielson[11] on the hydrogen exchange of proteins are also available.

## II. DENATURED STATES

The most frequently used protein denaturants are acid, heat, urea, and guanidine hydrochloride (GdnHCl).[a] For most of the papers discussed in this review, one or more of these denaturants were used to promote unfolding of specific proteins. In this section we summarize studies that characterize the denatured state produced by these denaturants. We also discuss the denatured states most likely to exist in the absence of a denaturant.

The popularity of urea and GdnHCl as protein denaturants rests on the time-tested observation that no other denaturant gives a greater extent of unfolding. In fact, studies by Tanford's group have shown that the extent of unfolding is complete.[12-14] Proteins in 6*M* GdnHCl with their disulfide bonds broken have been shown to closely approach a randomly coiled conformation. Even with the disulfide bonds intact, polypeptide chains appear to be as completely unfolded as possible, given the restraints imposed by the disulfide bonds.[b,13] For urea, higher concentrations of denaturant are required to complete unfolding, but the resulting denatured state is similar to that found in GdnHCl solutions.[17] This is illustrated in Figure 1, which shows the urea and GdnHCl denaturation curves for lysozyme and ribonuclease.[15] The optical rotatory properties are similar after urea or GdnHCl denaturation, indicating that denatured states with similar conformations are formed.

Urea denaturation does not always lead to complete unfolding. Superoxide dismutase[18] and lysozyme,[19] for example, are not completely unfolded in 10*M* urea at neutral pH. GdnHCl denaturation almost always leads to complete unfolding, but a proteolytic enzyme from *Streptomyces griseus* has been shown to retain activity in 6*M* GdnHCl.[20] The stability results because the native enzyme has a good binding site for GdnHCl.[21] Uricase is quite resistant to GdnHCl denaturation for the same reason.[22]

More potent denaturants than GdnHCl are known.[23,24] Generally these are compounds related to GdnHCl in structure or simply different salts of guanidine. One of the best-characterized is guanidine thiocyanate, which acts at a significantly lower concentration than GdnHCl for some proteins.[23] There is some evidence, however, that the extent of unfolding in guanidine thiocyanate may be less than it is in GdnHCl.[26] We will see in Section IV (Part B3) that solubility studies on protein model compounds in urea and GdnHCl solutions are useful in interpreting protein denaturation curves. Similar studies are now available for guanidine thiocyanate.[25]

In most cases, the products of acid or thermal denaturation are less completely unfolded than those of urea and GdnHCl denaturation.[1] For ribonuclease,[27,28] lysozyme,[27,29] chymotrypsinogen,[27,30] myoglobin,[31] carbonic anhydrase B,[32] and other proteins, there is good evidence that the product of acid or thermal denaturation can be further unfolded by adding urea or GdnHCl. This is illustrated for lysozyme in Figure 2. The viscosity data of Kugimiya and Bigelow[29] in Figure 3 show that the difference in the extent of unfolding may be large; the viscosity of urea- or GdnHCl-denatured lysozyme is twice as large as the viscosity of the thermally denatured protein.

For some proteins, acid denaturation does appear to lead to a randomly coiled conformation. Cytochrome *c* is an interesting example.[33] The acid denaturation curves for horse and cow ferricytochrome *c* are shown in Figure 4. For horse ferricytochrome *c*, acid and GdnHCl denaturation lead to products with identical optical rotations, whereas for cow ferricytochrome *c* the rotations

[a] There is no agreement on the abbreviation to be used for guanidine hydrochloride (guanidinium chloride). *Biochemistry* uses Gdn·HCl; *J. Biol. Chem.* uses GmHCl; and the most frequently used abbreviation in the past has been GuHCl. We will use GdnHCl, which is the abbreviation currently recommended by the office of Biochemical Nomenclature, Dr. Waldo E. Cohn, Director.

[b] This conclusion is based almost entirely on studies of the optical rotatory properties and should be investigated further. There is evidence from denaturation studies[15] and from solvent perturbation studies[16] that the presence of disulfide bonds may in some cases significantly decrease the accessibility of portions of the polypeptide chain to solvent. This decreased accessibility need not, of course, indicate the presence of ordered structure.

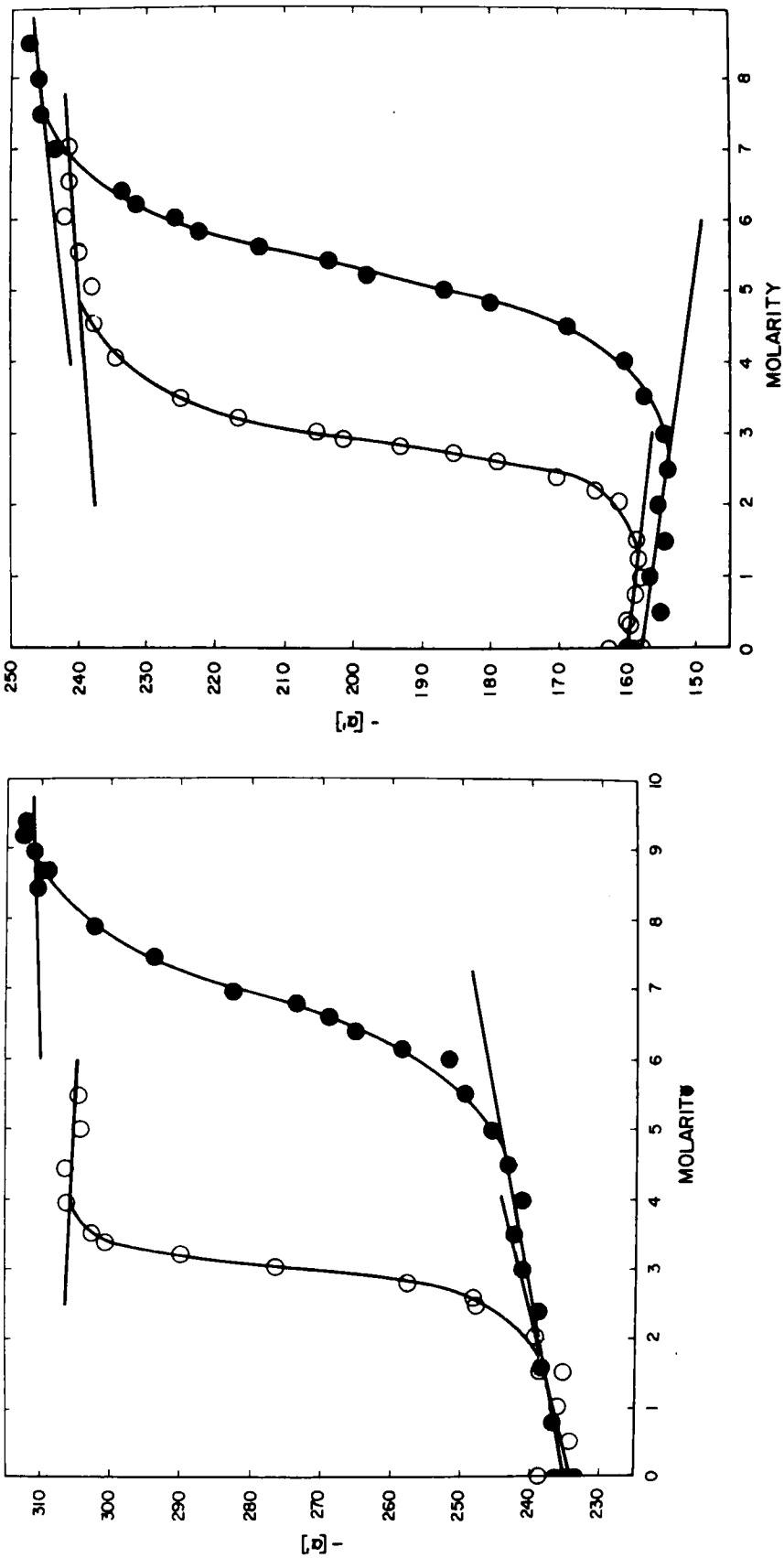



FIGURE 1. Urea (•) and GdnHCl (○) denaturation curves of ribonuclease (pH 6.6), left, and lysozyme (pH 2.9), right, at 25°C. [α'] is the reduced specific rotation at 365 nm. (Reproduced from Green, R. F., Jr., and Pace, C. N., *J. Biol. Chem.*, 249, 5388, 1974. With permission.)

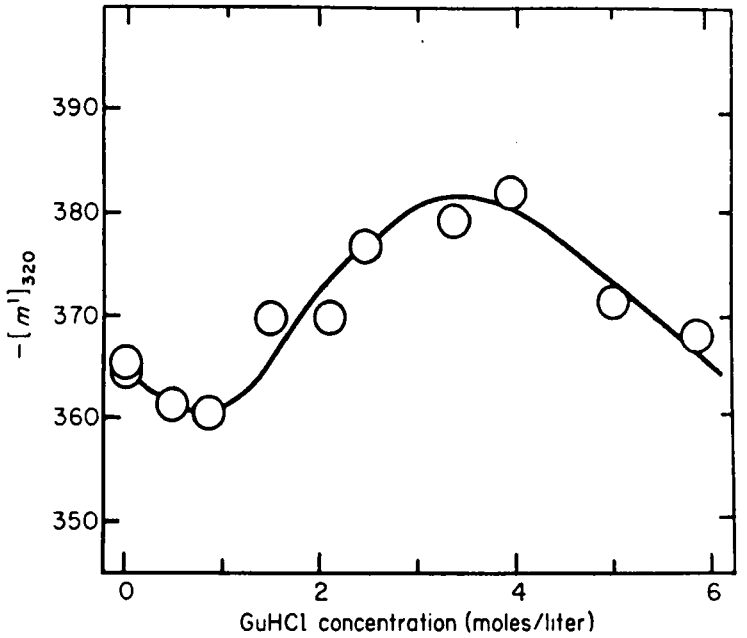



FIGURE 2. Denaturation of thermally denatured lysozyme (60.5°C, pH 1.65) by GdnHCl. [$m'$] is the reduced mean residue rotation at 320 nm. (Reproduced from Aune, K., et al., *J. Biol. Chem.*, 242, 4486, 1967. With permission.)

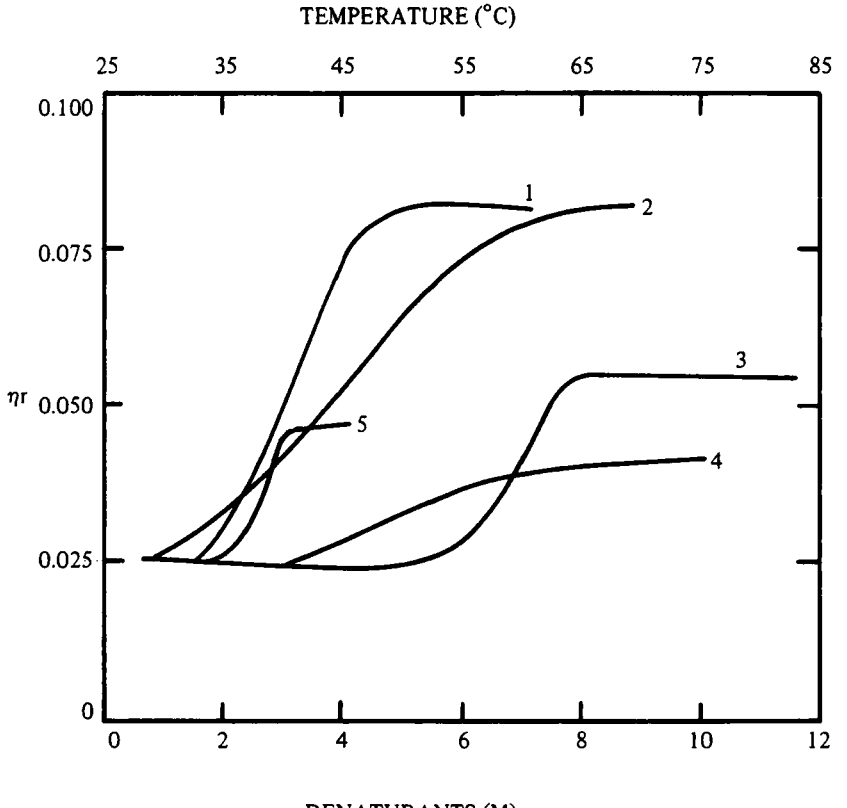



FIGURE 3. Dependence of the reduced viscosity of 1% solutions of lysozyme on the concentration of denaturant or the temperature. (1) GdnHCl; (2) urea; (3) lithium chloride; (4) temperature; (5) lithium perchlorate. (Reproduced by permission of the National Research Council of Canada from Kugimiya, M. and Bigelow, C. C., *Can. J. Biochem.*, 51, 581, 1973.)

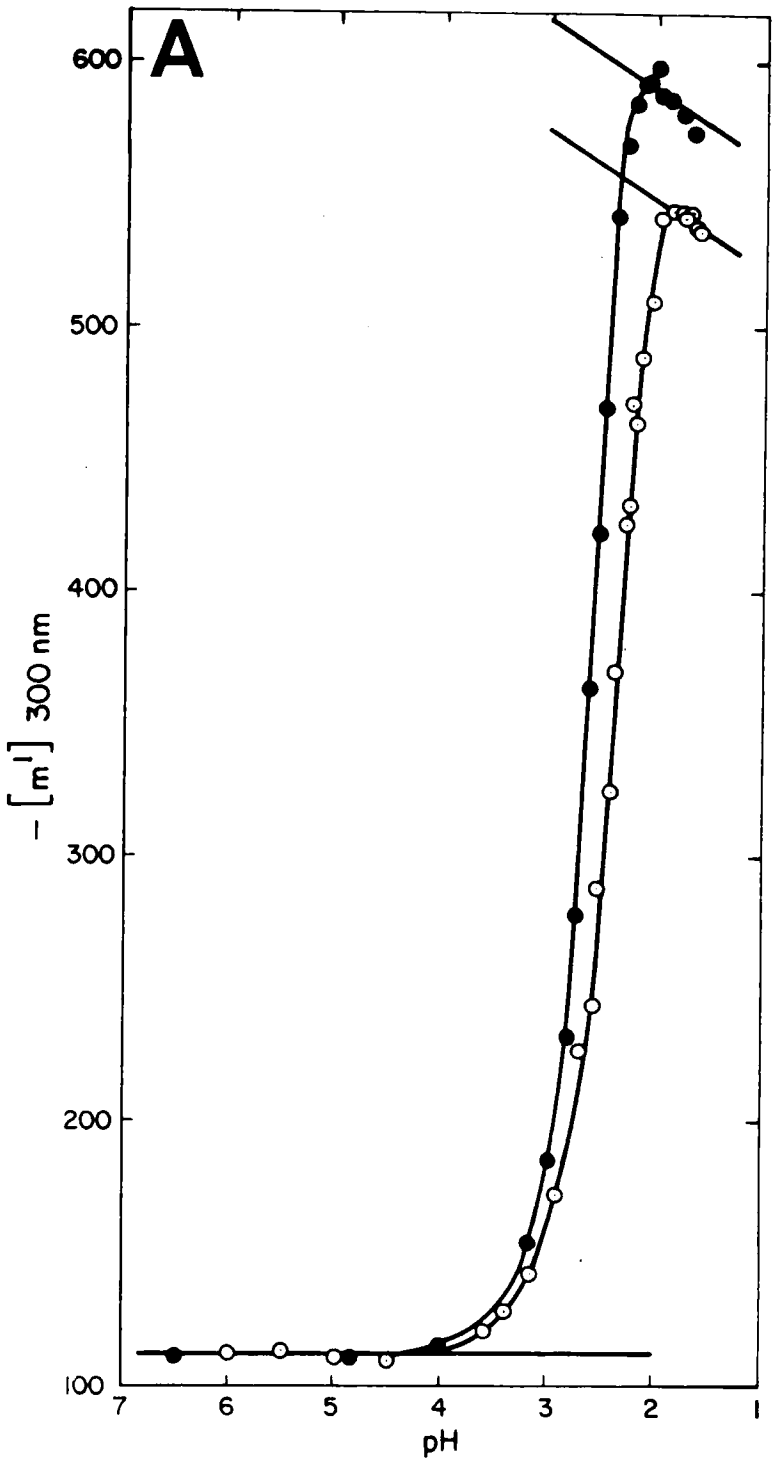



FIGURE 4. Acid denaturation curves of horse (•) and cow (○) ferricytochrome *c* at 25°C. [*M'*] is the reduced mean residue rotation at 300 nm. (Reprinted from Knapp, J. A. and Pace, C. N., *Biochemistry*, 13, 1289, 1974, © 1974 by the American Chemical Society. Reprinted by permission of the copyright owner.)

differ. It appears that acid denaturation leads to complete unfolding for horse ferricytochrome *c* but not for cow ferricytochrome *c*. This is a remarkable result, considering that proteins differ by only three residues in amino acid sequence. Another example is glyceraldehyde-3-phosphate dehydrogenase, which appears to unfold as completely at low pH as it does in urea.[34]

The difference between a protein such as horse ferricytochrome *c* and a protein like ribonuclease, where acid denaturation does not lead to complete unfolding, is further illustrated by the intrinsic-viscosity measurements given in Table 1. If ribonuclease were randomly coiled after acid-thermal denaturation, a value of $[\eta]$ much larger than 9.1 cc/g would be expected, because of a stiffening of the coil through electrostatic repulsion at low pH. This is exactly what is observed in the case of horse ferricytochrome *c*.

It is difficult to study the unfolded states in equilibrium with N in the absence of a denaturant, because they are present at such low concentrations. (An interesting exception is β-casein, a protein that appears to be randomly coiled under physiological conditions.[35]) On the basis of the discussion above, it seems likely that the denatured states that exist will generally be less than completely unfolded. This appears to be so in cases where proteins have been unfolded through chemical modification. For example, if 23 residues are removed from the C-terminal end of staphylococcal nuclease (leaving 126 residues), the enzyme unfolds to give a product with an intrinsic viscosity of 9.8 cc/g, whereas a value around 17 would be expected if it were randomly coiled.[36] An exceptional case is oxidized ribonuclease, a derivative in which the disulfide bonds have been broken. Here most,[1,38] but not all[37] evidence indicates that oxidized ribonuclease is randomly coiled in aqueous solution.

Studies of the rates of hydrogen exchange under native-state conditions also suggest that several partially unfolded denatured states will predominate over a randomly coiled conformation in the absence of a denaturant.[40] Further support comes from studies of the kinetics of folding and unfolding globular proteins. In general, the results show that the mechanisms are more complex in the absence of a denaturant, indicating the presence of higher concentrations of intermediate states.[39] Both of these topics are discussed in more detail below.

The picture that emerges, then, is that urea or GdnHCl denaturation generally leads to complete unfolding of the polypeptide chain, whereas acid or thermal denaturation generally leads to a product that retains some ordered structure. Thus, under physiological conditions the denatured state in equilibrium with N will generally be a mixture of partially unfolded conformations.

## III. MECHANISM OF PROTEIN DENATURATION

The mechanism by which proteins fold and unfold has been actively investigated during the past five years. It is obvious that a protein molecule will assume various partially folded conformations in the course of folding or unfolding. For a denaturation study the concentration of these intermediates at equilibrium in the transition region is of considerable importance. If the concentration of intermediate states is low, the analysis in terms of a two-state mechanism is straightforward. If the mechanism is more complex and if appreciable concentrations of intermediates exist, the denaturation curve can be analyzed only in special cases. Thus, our main interest in the mechanism of protein denaturation is in the number, the properties, and the concentrations of intermediate states that exist at equilibrium, rather than in the details of the mechanism of folding and unfolding.

TABLE 1

**Intrinsic Viscosities of Ribonuclease and Cytochrome *c* in Various Denaturants**

| | $[\eta]$ (cc/g) | | | |
|---|---|---|---|---|
| | 8*M* Urea | 6*M* GdnHCl | pH = 2 | References |
| Ribonuclease | 7.6 | 9.4 | 9.1 (T = 43.5°C) | 2, 44 |
| Cytochrome *c* | 14.9 | 14.6 | 22.5 (T = 25°C) | 49, 136, 137 |

Figure 5 shows the urea denaturation curve for phosphorylase *b*.[41] Denaturation occurs in three steps, a clear indication that denaturation is not a two-state process. Most denaturation curves, however, show a single step, but this is no guarantee that denaturation follows a two-state mechanism. One method of detecting the presence of intermediate states is to show the non-coincidence of denaturation curves obtained by using different techniques for following denaturation.[42] A dramatic illustration of this approach is provided by the results of Wong and Tanford[43] in Figure 6. The fraction of unfolded carbonic anhydrase B observed depends on the parameter used for following denaturation. At 2*M* GdnHCl, the value of $f_u$ (see Equation 5, below) varies from 1.0 to 0.6 to 0.55, depending on the parameter used in the analysis. These results indicate a marked departure from a two-state mechanism, much larger than is usually observed. (The acid denaturation of carbonic anhydrase B also has a complex mechanism.[32]) As a contrast, consider similar results from a study of the thermal denaturation of ribonuclease by Ginsburg and Carroll.[44] As shown in Figure 7, identical results are obtained when different techniques are used for following denaturation. This is the expected result for a two-state mechanism.

The technique of greatest potential for this type of a study is NMR. Using NMR, it is possible to follow unfolding by observing many different residues simultaneously. Ribonuclease,[45] lysozyme,[46] and staphyloccocal nuclease[47] have all been studied in some detail with NMR. For ribonuclease, intermediate states have been observed with NMR that were not detectable with other techniques. These intermediates appear to involve small conformational changes in the native state of the protein. In Brandts' terminology,[4] many such states would be microscopic states within the macroscopic state we refer to as the native state of the protein.

The non-coincidence of denaturation curves has also been used to show the presence of inter-

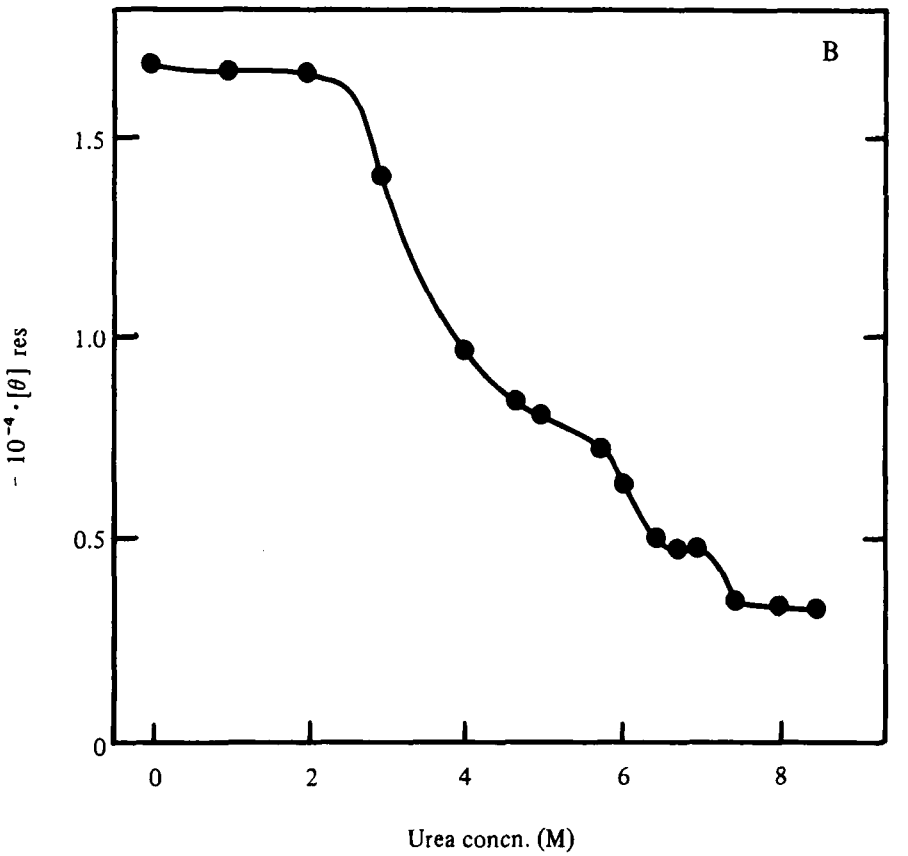



FIGURE 5. Urea denaturation curve of reduced phosphorylase *b*. $[\theta]$ is the molar residue ellipticity at 220 nm. (Reproduced from Chignell, D. et al., *Eur. J. Biochem.*, 26, 37, 1972. With permission.)

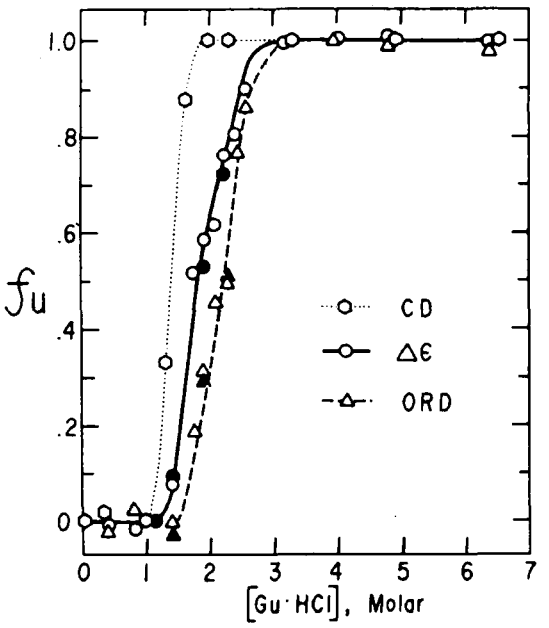



FIGURE 6. Fraction of unfolded carbonic anhydrase B, $f_u$, as a function of GdnHCl molarity. $f_u$ was calculated from circular dichroism measurements (hexagons), difference spectral measurements (○), or optical rotation measurements (●), using Equation 5. (Reproduced from Wong, K. P. and Tanford, C., *J. Biol. Chem.*, 248, 8518, 1973. With permission.)

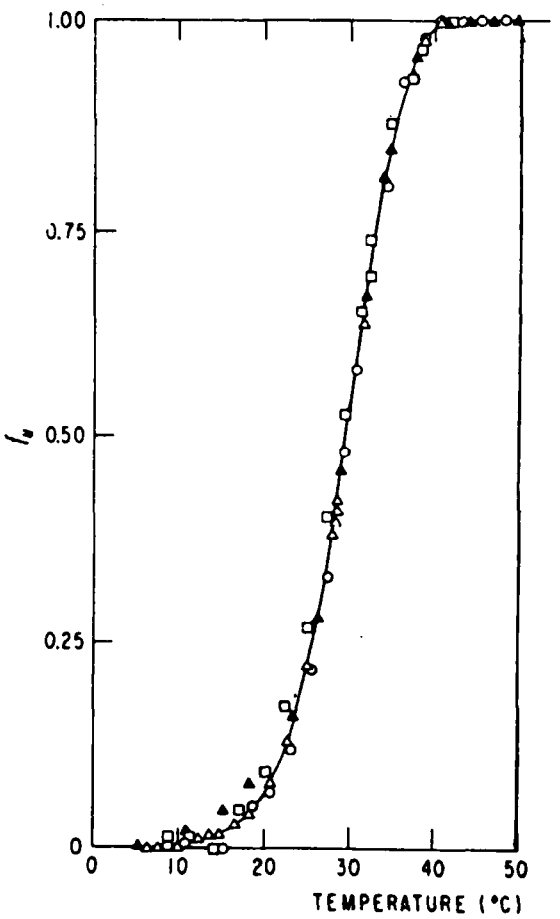



FIGURE 7. Fraction of unfolded ribonuclease, $f_u$, as a function of temperature at pH 2.19, 0.019 ionic strength. $f_u$ was calculated from intrinsic viscosity (□), optical rotation at 365 nm (○), difference absorption at 287 nm (△), and reversal of difference absorption measurements (▲) with Equation 5. (Reprinted from Ginsburg, A. and Carroll, W. R., *Biochemistry*, 4, 2159, 1965, © 1965 by the American Chemical Society. Reprinted by permission of the copyright owner.)

mediate states in the unfolding of growth hormone[48] and of cytochrome *c*.[33] If intermediates are detected by this approach, it probably indicates that they are present at significant concentrations. This approach will probably be of some use in characterizing the intermediate states present.

In recent years there have been a number of kinetic studies that have helped clarify the mechanism of protein denaturation.[49-55] It has been shown, for example, that the intermediate states present are not always on the pathway of unfolding from the native state. For cytochrome *c*, a mechanism consistent with most of the kinetic data is

$$N \rightleftharpoons X_1 \rightleftharpoons D \rightleftharpoons X_2 \qquad (2)$$

where $X_2$ is a substantially folded state, which must unfold before refolding to form N.[49] At the midpoint of the transition, the kinetic data suggest that the equilibrium composition may be 20% N, 25% D, 15% $X_1$, and 40% $X_2$. This is clearly not a two-state composition. In studies of the acid denaturation of myoglobin, the reaction follows a two-state mechanism in the transition region, but when it is studied outside the transition region, intermediates similar to those found for cytochrome *c* are observed.[55] For other proteins, denaturation appears to follow a sequential mechanism in which all of the intermediates lie on the pathway of unfolding from N to D.[51] For equilibrium studies, it is the existence of the intermediates, and not their location, which is of importance.

Another interesting finding is that urea or GdnHCl denaturation is generally closer to a two-state mechanism than thermal or acid

denaturation. For lysozyme, the GdnHCl denaturation is very close to a two-state mechanism in the transition region, but intermediate states can be detected at low denaturant concentrations.[50] For ribonuclease, intermediates are very difficult to detect in the urea[56] or GdnHCl[57,58] denaturation, but they are readily detected in acid-thermal denaturation.[51] Similarly, for cytochrome *c*, the mechanism is more complex for acid or thermal denaturation[52] than for GdnHCl denaturation.[49] The increased concentration of intermediates in the absence of urea and GdnHCl is in accord with the ideas presented in Section II.

As the results above suggest, the degree of departure from a two-state mechanism depends on the protein and on the denaturant. Thus, the mechanism must be investigated for each protein and each set of conditions. For some proteins, denaturation approaches a two-state mechanism very closely, e.g., the GdnHCl denaturation of lysozyme,[50] or the acid denaturation of myoglobin.[55] Nevertheless, it is certain that intermediates will be detected when more sensitive techniques are used to look for them. This need not concern us here, however, for intermediates present in such low concentrations will not lead to large errors in our equilibrium analysis of unfolding. Cases such as cytochrome *c*, where the GdnHCl denaturation is known to depart substantially from a two-state mechanism, are of more concern. It is difficult to establish the mechanism with certainty, and it will be even more difficult to characterize the intermediate states that exist. We will see below that, even in cases such as this, analysis in terms of a two-state mechanism can lead to some useful conclusions.

In one interesting case it does appear possible to analyze the denaturation curve even though the mechanism is more complex than a two-state mechanism. Rowe and Tanford[59] have studied the GdnHCl denaturation of an immunoglobulin light chain. Their data are inconsistent with a two-state mechanism, but support a mechanism in which the two domains of the molecule unfold independently. This leads to a three-state mechanism that can be analyzed. The authors show that the two domains have identical $\Delta G_D^{H_2O}$ values of about 5.5 kcal/mole. For other light chains the stabilities of the domains differ substantially, and separable stages are observed in the denaturation curve.[116]

## IV. METHODS OF ESTIMATING $\Delta G_D^{H_2O}$

There are three distinctly different approaches that have been used to estimate $\Delta G_D^{H_2O}$. Most of the available estimates are derived from an analysis of denaturation curves, such as those shown in Figures 1 and 4. In this approach, $\Delta G_D$ is measured under denaturing conditions, and an attempt is made to extrapolate back to physiological conditions. This generally requires an extrapolation over several pH units, over a temperature interval of 15 to 40°C, or back from 2 to 7 *M* urea or GdnHCl concentration. The data of Hermans and Scheraga[60] in Figure 8 illustrate the problem. Here you have a choice: use the data pH 7 and extrapolate from 65°C to 25°C, or use the data at 25°C and extrapolate from pH 1 to pH 7. The resulting $\Delta G_D^{H_2O}$ estimates may correspond to a reaction that occurs to only a very small extent under physiological conditions. For example, the $\Delta G_D^{H_2O}$ estimates from urea or GdnHCl denaturation are applicable to unfolding to a random coil, but in water the denatured state would contain only a small proportion of randomly coiled molecules. Nevertheless, estimates of $\Delta G_D^{H_2O}$ for unfolding to a random coil under physiological conditions are of considerable interest.

A few estimates of $\Delta G_D^{H_2O}$ have been based on measurements of the rate of hydrogen exchange.[11] We will see below that under certain conditions the rate of hydrogen exchange is directly proportional to the concentration of an unfolded state and, consequently, can be used to measure the concentration. This approach looks directly at the unfolding equilibria that exist under native-state conditions. In fact, any reaction with the protein in which the rate depends on the concentration of D might be used in the same way. Proteolytic cleavage is an obvious possibility that has been investigated and generally found to be too complicated to be useful. (For an exception, see Matthyssens et al.[61])

In a few cases, chemical modification might be applicable. For example, if all the sulfhydryl groups in a protein were inaccessible to chemical modification in the native state, then it might be possible to estimate the equilibrium constant for unfolding to an accessible conformation by measuring the rate of modification under native-state conditions. Vas and Boross[62] used just such an approach to measure $\Delta G$ for a small conforma-

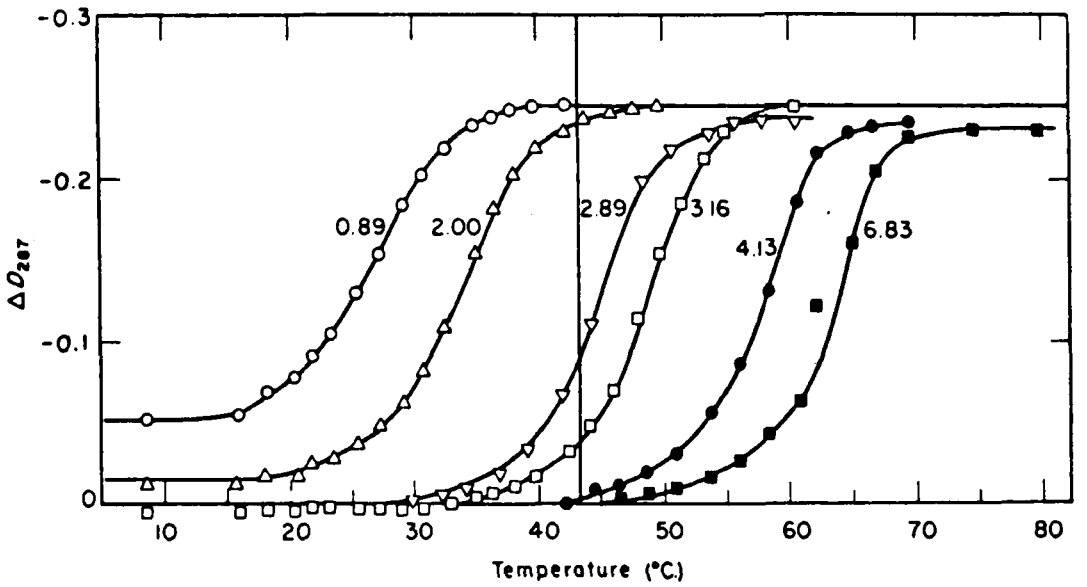



FIGURE 8. The thermal denaturation of ribonuclease at several pH values at 0.16 ionic strength, followed by difference absorption measurement. (Reprinted from Hermans, J., Jr. and Scheraga, H. A., *J. Am. Chem. Soc.*, 83, 3283, 1961, © 1961 by the American Chemical Society. Reprinted by permission of the copyright owner.)

tional change in glyceraldehyde-3-phosphate dehydrogenase. In a related approach, Weber et al.[63] recently measured the rate of exchange of the water molecules trapped inside chymotrypsin. Unfortunately, the internal water molecules exchanged completely in less than the 20 min required to complete the measurements. Why the exchange is this rapid is still not clear.

Sachs et al.[64] have devised an interesting immunological approach for studying a similar equilibrium, in which the native conformation is the state present at low concentration. Fragment 99-149 of staphylococcal nuclease is largely devoid of ordered structure free in solution. Using antibodies to the structured form of this peptide, the authors were able to estimate the equilibrium constant ($2 \times 10^{-4}$) for the folding of the fragment. Using a similar approach to study the N $\rightleftharpoons$ D equilibrium under native-state conditions is likely to be considerably more difficult.

Very recently, the results of calorimetric studies of protein-unfolding became sufficiently accurate to allow their use in estimating $\Delta G_D^{H_2O}$.

### A. Denaturation Curve Analysis

Typical denaturation curves are shown in Figures 1 and 4. In these cases unfolding was followed by measuring the optical rotation, but any observable parameter y can be used. (Hermans[65] has discussed many of the experimental aspects of studying protein denaturation.) The usual method of analysis is to assume a two-state mechanism (Equation 1). Values of y characteristic of the native state, $y_N$, and of the denatured state, $y_D$, can be obtained at any point in the transition region by extrapolation from the linear portions of the denaturation curve at high and low denaturant concentration. At any point in the transition

$$y = f_N y_N + f_D y_D \tag{3}$$

where $f_N$ and $f_D$ represent the fraction of the protein present in the N and D states respectively. Since $f_N + f_D = 1$,

$$y = y_N + f_D (y_D - y_N) \tag{4}$$

and

$$f_D = (y - y_N)/(y_D - y_N) \tag{5}$$

Thus, an equilibrium constant, $K_D$, and a free energy of unfolding, $\Delta G_D$, can be calculated using

$$e^{-\Delta G_D/RT} = K_D = \frac{f_D}{1 - f_D} = \frac{y_N - y}{y - y_D} \tag{6}$$

We will see below that analysis in terms of a two-state mechanism can still be useful even in cases where denaturation is known to deviate

substantially from a two-state mechanism. In such cases we will use the terms $K_{app}$ and $\Delta G_{app}$ rather than $K_D$ and $\Delta G_D$. These terms will also be used when the mechanism of denaturation is uncertain.

Since the mechanism of denaturation is obviously important in analyzing denaturation curves, it will be useful to consider the effect of the presence of intermediate states on a two-state analysis. Tanford[1] has shown that if stable intermediate states, $X_i$, each characterized by the property $y_i$ and concentration $f_i$, are present, then y at any point will now be given by

$$y = y_N + f_D (y_D - y_N) + \sum_i f_i (y_i - y_N) \quad (7)$$

and the observed extent of unfolding, $f_{obs} = (y - y_N)/(y_D - y_N)$, becomes

$$f_{obs} = f_D + \sum_i f_i z_i \quad (8)$$

where $z_i = (y_i - y_N)/(y_D - y_N)$. Thus, $f_{obs}$ will differ from $f_D$ by an amount that depends on the concentration of the intermediates weighted by their $z_i$ values. The $z_i$ value for an intermediate is likely to be between 0 and 1, because $y_i$ will generally fall between $y_N$ and $y_D$. For some techniques, such as optical rotation measurements above 300 nm or viscosity, the $z_i$ value will be roughly proportional to the extent to which the intermediate is unfolded. For other techniques, such as enzyme activity measurements or measurements dependent on only a small number of residues, there may be little correlation between $z_i$ and the extent of unfolding.

The relationship between $K_{app}$ ($= f_{obs}/1 - f_{obs}$) and $K_D$ ($= f_D/f_N$) is

$$K_{app} = K_D \left( \frac{1 + \Sigma z_i K_i/K_D}{1 + \Sigma (1 - z_i) K_i} \right) \quad (9)$$

where $K_i = f_i/f_N$. For reasonable values of $z_i$ ($z_i = 0.1$ to 0.9), $K_D$ and $K_{app}$ will be equal somewhere near the midpoint of the transition ($K_{app} = 1$), but $K_D$ will be less than $K_{app}$ below the midpoint and greater than $K_{app}$ above the midpoint of the transition. Thus, the presence of intermediates will cause the slope of plots such as those in Figure 9 (see below) to be less than the slope would be for a plot of $\Delta G_D$ versus denaturant concentration. This means that estimates of $\Delta G_D^{H_2O}$ derived from two-state analysis of a system with stable intermediates will at least serve as lower limits for the true value of $\Delta G_D^{H_2O}$.

It is clear that the mechanism of denaturation is a problem that must be considered in analyzing a denaturation curve. A second problem is extrapolation of the results back from the denaturing conditions used for unfolding the protein to native-state conditions. This problem is discussed individually for different types of denaturation in the next three sections.

### B. Urea and GdnHCl Denaturation

Studies using urea and GdnHCl as denaturants offer advantages over studies using acid or heat as denaturants. The randomly coiled product of urea or GdnHCl denaturation is a useful reference state, since all of the non-covalent interactions that stabilize the native state are disrupted. Acid and thermal denaturation often do not bring about complete unfolding, and the denatured state is difficult to characterize. In addition, the mechanism of denaturation is generally less complex for urea and GdnHCl denaturation than for acid and thermal denaturation. Thus, it would be useful to have available a reliable method of obtaining $\Delta G_D^{H_2O}$ from a study of the urea or GdnHCl denaturation.

Three different methods have been suggested for obtaining $\Delta G_D^{H_2O}$ from data such as those shown in Figure 1. These methods are discussed separately below, and then compared.

#### *1. Linear Extrapolation*

When the denaturation curves for lysozyme (Figure 1) are analyzed as described above, the results shown in Figure 9 are obtained. It can be seen that $\Delta G_D$ varies linearly with denaturant concentration. Aune and Tanford[66] have shown that linearity is observed over an even wider GdnHCl concentration range (see Figure 11, below). Similar results are obtained in studies of the urea and GdnHCl denaturation of other proteins. The simplest method of estimating $\Delta G_D^{H_2O}$ is to assume that this linear dependence of $\Delta G_D$ on denaturant concentration continues to zero concentration of denaturant. This allows $\Delta G_D^{H_2O}$ to be determined from the intercept of plots such as those shown in Figure 9.

In an attempt to evaluate the methods that have been used to estimate $\Delta G_D^{H_2O}$, we recently studied the urea and GdnHCl denaturation, under

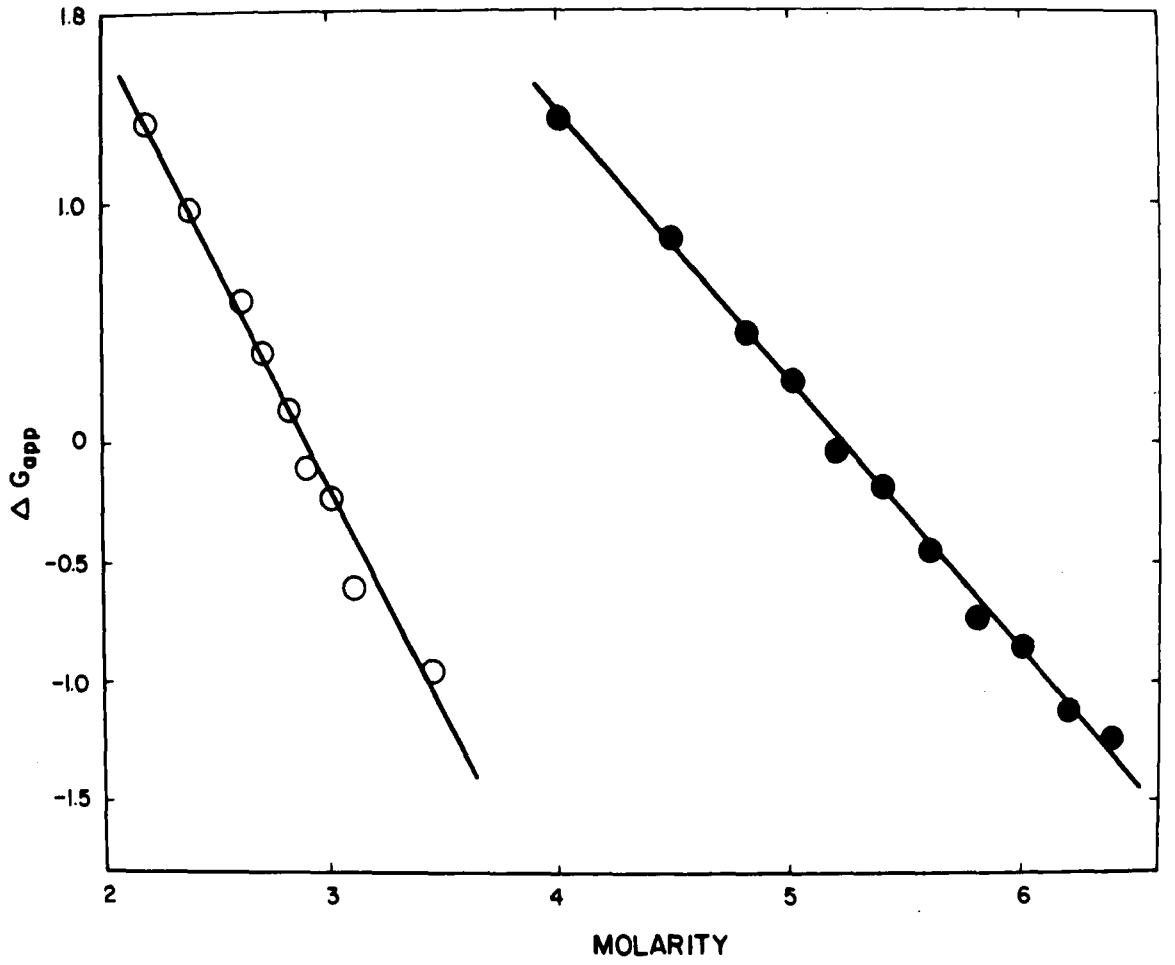



FIGURE 9. $\Delta G_D$ for the denaturation of lysozyme as a function of urea (•) and GdnHCl (○) molarity. $\Delta G_D$ was calculated with Equation 6, using the data in Figure 1. (Reproduced from Greene, R. F., Jr. and Pace, C. N., *J. Biol. Chem.*, 249, 5388, 1974. With permission.)

identical conditions, of four proteins. The denaturation curves in Figure 1 were taken from this study. A least-squares analysis was used to fit data such as those in Figure 9 to the equation

$$\Delta G_D = \Delta G_D^{H_2O} + m\,(\text{denaturant}) \qquad (10)$$

and the results are listed in Table 2. Note that m measures the dependence of $\Delta G_D$ on denaturant concentration. These same data will be analyzed by the other extrapolation procedures, and the $\Delta G_D^{H_2O}$ values obtained will be compared below.

Included in Table 2 is the denaturant concentration at the midpoint of the transition, denoted $(\text{denaturant})_{1/2}$ or $(D)_{1/2}$. In reporting the results of a denaturation study, this reviewer strongly recommends that values of m and $(\text{denaturant})_{1/2}$ be given whenever possible. In the past, the slopes of plots of log $(K_D)$ vs log (denaturant), generally denoted n, have been used as measures of the steepness of the transition. The m values are more useful and meaningful parameters for this purpose than n values. At the midpoint of a transition, the n and m values are related by the following equation[67]

$$\left(\frac{d\,(\Delta G_D)}{d\,(\text{denaturant})}\right)_{\text{denaturant} = D_{1/2}} = m = \frac{nRT}{(D)_{1/2}} \qquad (11)$$

*2. Denaturant Binding Model*

One of the first attempts to extract an estimate of $\Delta G_D^{H_2O}$ from measurements of $\Delta G_D$ as a function of denaturant concentration was described in a paper by Aune and Tanford.[66] These authors analyzed their data on the GdnHCl

TABLE 2

**Parameters Characterizing the GdnHCl and Urea Denaturation of Four Proteins[15]**

| Protein | pH | GdnHCl | | | Urea | | |
|---|---|---|---|---|---|---|---|
| | | $(GdnHCl)_{1/2}$,* *M* | $\Delta G_{app}^{H_2O}$,† kcal/mole | m,† kcal/mole/*M* | $(Urea)_{1/2}$,* *M* | $\Delta G_{app}^{H_2O}$,† kcal/mole | m,† kcal/mole/*M* |
| Ribonuclease | 6.6 | 3.01 | 9.3 | 3.10 | 6.96 | 7.7 | 1.10 |
| Lysozyme | 2.9 | 3.07 | 5.8 | 1.88 | 5.21 | 5.8 | 1.12 |
| α-Chymotrypsin | 4.3 | 1.90 | 7.8 | 4.10 | 4.04 | 8.4 | 2.07 |
| Goat β-lactoglobulin | 3.2 | 3.23 | 12.5 | 3.87 | 5.01 | 10.5 | 2.10 |

*The midpoint ($G_{app} = 0$) of the denaturation curve.
†From Equation 10.

denaturation of lysozyme by assuming that denaturation results because there are a greater number of binding sites for denaturant molecules in the denatured state than in the native state. If the sites in both states are identical and non-interacting, then it can be shown that

$$\Delta G_D = \Delta G_D^{H_2O} - \Delta nRT \ln (1 + ka) \quad (12)$$

where $\Delta n$ is the difference in the number of binding sites between D and N, k is the equilibrium constant for binding at each site, and a is the activity of the denaturant.[2] Values of $\Delta G_D^{H_2O}$, $\Delta n$, and k are obtained by a least-squares fit of measurements of $\Delta G_D$ as a function of a. In the case of GdnHCl denaturation, the mean ion activity is generally used as an approximation of the activity of the guanidinium ion.[c] It is more likely that the binding of guanidinium ion causes denaturation than the binding of neutral GdnHCl molecules. Typical results for lysozyme are shown in Figure 10. A value of k = 1.2 led to the best fit of the data in this study of lysozyme and has been used to fit similar data from studies on ribonuclease[69] and some of its derivatives,[70] myoglobins,[71] cytochromes *c*,[33] and α-lactalbumin[72] (see Table 8).

We have analyzed the data presented in Table 2 using Equation 12. For GdnHCl denaturation, calculations were made using k = 1.2 and k = 0.1; for urea, calculations were made using k = 1.0 and 0.1. The results are presented in Table 3. The lower k values provide fits to the experimental data that are comparable to the fits obtained with the larger k values.

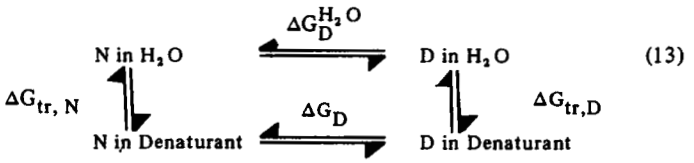
Urea and GdnHCl increase the solubility of peptide groups and almost all of the component parts of a protein. In several cases it is possible to account for this enhanced solubility by assuming that the denaturant binds to the model compound.[2] This gives rise to an equation similar in form to Equation 12. For GdnHCl, the values of k needed to account for the model compound data are in the range from 0.5 to 1.1, and the values for urea are in the range from 0.08 to 0.28. Thus, calculations using a value of k = 0.1 are perhaps not too far-fetched. The values of $\Delta G_D^{H_2O}$ obtained when k = 0.1 is used are similar to the estimates obtained by linear extrapolation.

### *3. Tanford's Model*

Tanford[67] has shown that the denaturation of proteins by urea and GdnHCl can be accounted for quantitatively by using model compound data. His approach provides another means of estimating $\Delta G_D^{H_2O}$ and is also useful for answering other questions that arise in connection with denaturation studies.

Denaturation can be represented as

[c]The activity of GdnHCl, a, can be calculated from the molar concentrations, *M*, using $\log a = -0.5191 + 1.4839 \log M - 0.2562(\log M)^2 + 0.5884(\log M)^3$. This equation is based on experiments of E. P. K. Hade, Jr.[66] The mean ion activity is the square root of a. Urea activities in aqueous solutions are given by Ellerton and Dunlop.[68]




(13)

where $\Delta G_{tr,N}$ and $\Delta G_{tr,D}$ represent the free-energy changes accompanying the transfer of N and D, respectively, from water to a solution containing the denaturant. It can be seen from the diagram that

$$\Delta G_D - \Delta G_D^{H_2O} = \Delta G_{tr,D} - \Delta G_{tr,N} \quad (14)$$

It is easy to see that the difference between $\Delta G_{tr,D}$ and $\Delta G_{tr,N}$ depends only on the groups that are exposed to solvent in D but not in N. The contribution of groups that are exposed in both states cancels out. If we let $\alpha_i$ represent the average fractional change in the degree of exposure to solvent of groups of type i, then it follows that

$$\Delta G_{tr,D} - \Delta G_{tr,N} = \sum_i \alpha_i n_i^o \delta g_{tr,i} \quad (15)$$

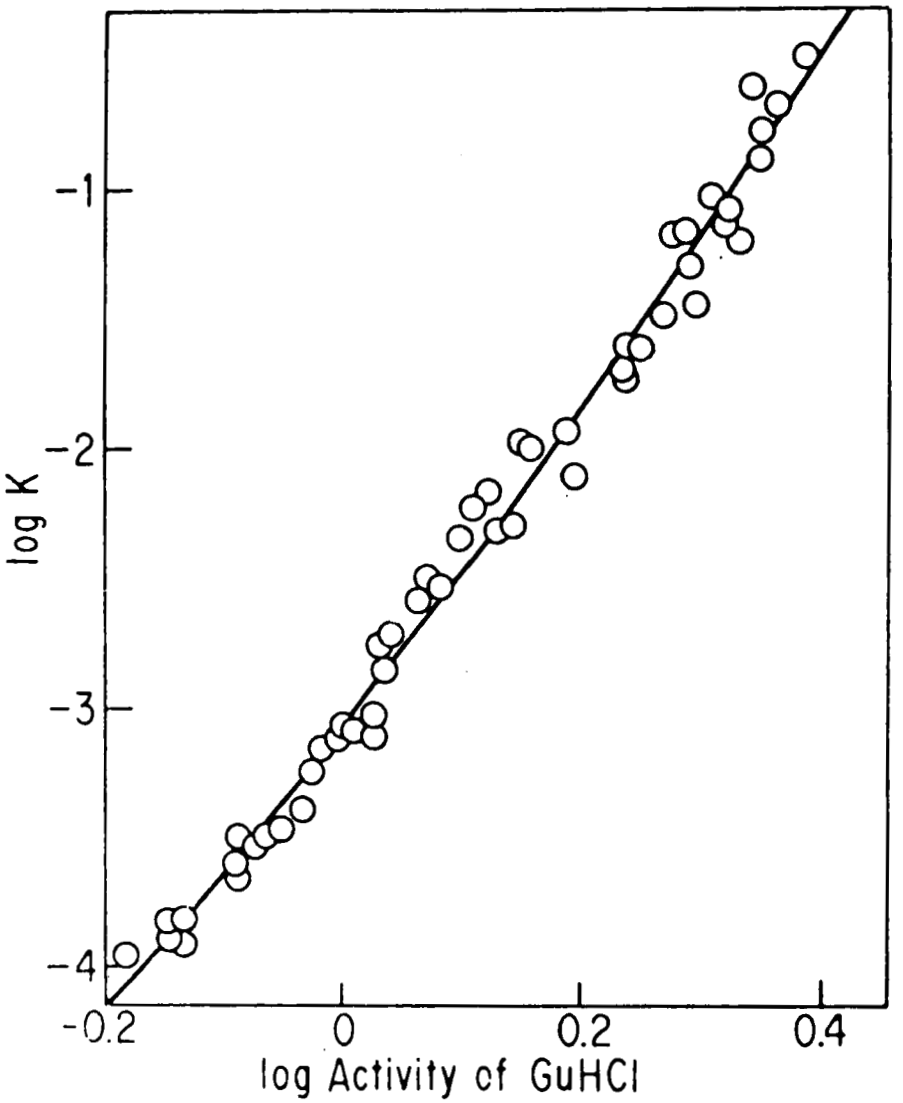



FIGURE 10. Aune and Tanford's[66] data for the GdnHCl denaturation of lysozyme. The curve is drawn according to Equation 12, with k = 1.2, $\Delta n$ = 21.5, and log $K_D^{H_2O}$ = -10.44. (Reproduced from Tanford, C., *Adv. Protein Chem.*, 24, 1, 1970. With permission.)

TABLE 3

**Parameters Characterizing the Urea and GdnHCl Denaturation, Using Equation 12**

| Protein | GdnHCl k | GdnHCl Δn | GdnHCl $\Delta G_D^{H_2O}$ (kcal/mole) | Urea k | Urea Δn | Urea $\Delta G_D^{H_2O}$ (kcal/mole) |
|---|---|---|---|---|---|---|
| Ribonuclease | 0.1 | 170 | 11.9 | 0.1 | 30 | 8.8 |
| | 1.2 | 32 | 17.3 | 1.0 | 13 | 15.7 |
| Lysozyme | 0.1 | 104 | 7.1 | 0.1 | 30 | 6.9 |
| | 1.2 | 19 | 10.0 | 1.0 | 12 | 12.1 |
| α-Chymotrypsin | 0.1 | 220 | 10.7 | 0.1 | 53 | 9.9 |
| | 1.2 | 34 | 14.3 | 1.0 | 18 | 16.7 |
| Goat β-lactoglobulin | 0.1 | 214 | 15.8 | 0.1 | 57 | 12.3 |
| | 1.2 | 41 | 23.2 | 1.0 | 21 | 21.4 |

TABLE 4

**Free Energies of Transfer, $-\delta g_{tr}$, from Water to Aqueous Urea or GdnHCl Solutions for Peptide Group and Amino Acid Side Chains**

$-\delta g_{tr}$ (cal/mole)

| | (Urea)[138] | | | | (GdnHCl)[139] | | | |
|---|---|---|---|---|---|---|---|---|
| | *2M* | *4M* | *6M* | *8M* | *1M* | *2M* | *4M* | *6M* |
| Peptide* | 49 | 86 | 118 | 130 | 83 | 134 | 207 | 245 |
| Ala | 0 | −15 | −10 | −10 | 10 | 20 | 30 | 45 |
| Val† | 60 | 85 | 125 | 160 | 85 | 115 | 195 | 265 |
| Leu | 110 | 155 | 225 | 295 | 150 | 210 | 355 | 480 |
| Ile† | 100 | 140 | 205 | 265 | 135 | 190 | 320 | 430 |
| Met | 115 | 225 | 325 | 415 | 150 | 245 | 400 | 535 |
| ½Cys† | 115 | 225 | 325 | 415 | 150 | 245 | 400 | 535 |
| Phe | 180 | 330 | 470 | 600 | 215 | 355 | 580 | 775 |
| Tyr | 225 | 395 | 580 | 735 | 235 | 385 | 605 | 770 |
| Trp | 270 | 505 | 730 | 920 | 400 | 630 | 980 | 1,235 |
| Pro† | 75 | 105 | 155 | 200 | 100 | 140 | 240 | 320 |
| Thr | 40 | 60 | 90 | 115 | 65 | 90 | 120 | 125 |
| His | 100 | 160 | 205 | 255 | 180 | 285 | 385 | 420 |
| Asn | 135 | 225 | 330 | 430 | 200 | 320 | 490 | 645 |
| Gln | 80 | 130 | 190 | 230 | 135 | 215 | 315 | 360 |

*Based on solubility studies of N-acetyltetraglycine ethyl ester and ethyl acetate by Robinson and Jencks.[140]

†The $\delta g_{tr,i}$ values for these side chains are estimates based on results for the other side chains and on results at a single denaturant concentration given in a paper by Wetlaufer et al.[141]

where $\delta g_{tr,i}$ is the free energy of transfer of a group of type i from water to denaturant, and $n_i^o$ is the total number of groups of type i present in the protein. Values of $\delta g_{tr,i}$ for most of the component parts of a protein have been determined from solubility studies on amino acids and their derivatives. The values so determined for both urea and GdnHCl are given in Table 4.

Calculations with Equation 15 can be simplified in various ways. In calculations presented in his review article[2] and elsewhere,[59] Tanford has included only the contribution of peptide groups

TABLE 5

**Results Calculated Using Tanford's Procedure and Some Physical Properties of the Proteins**

| Protein | GdnHCl $\bar{\alpha}$* | GdnHCl $\Delta G_{app}^{H_2O}$,* kcal/mole | Urea $\bar{\alpha}$* | Urea $\Delta G_{app}^{H_2O}$,* kcal/mole | Molecular weight | Disulfides intact, $[\eta]$,† cc/g | Disulfides broken, $[\eta]$,† cc/g |
|---|---|---|---|---|---|---|---|
| Ribonuclease | 0.35 | 14.8 | 0.34 | 12.1 | 13,700 | 9.4 | 16.3 |
| Lysozyme | 0.20 | 9.1 | 0.21 | 6.6 | 14,300 | 6.5 | 17.1 |
| α-Chymotrypsin | 0.17 | 10.4 | 0.21 | 8.8 | 25,000 | (11.0)‡ | (26.8)‡ |
| β-Lactoglobulin | 0.33 | 18.4 | 0.34 | 12.1 | 18,400 | 19.1 | 22.8 |

*From Equation 16.
†Intrinsic viscosity in 6*M* GdnHCl.[1]
‡Data for chymotrypsinogen.



and hydrophobic side chains (Trp, Phe, Tyr, Leu, Ile, Val, Pro, Met) and has assigned a single average value of $\alpha_i$ for all of these groups. We will show below that we obtain more satisfactory results if we follow Tanford and use a single $\alpha_i$ value but include all of the side chains given in Table 4 in our calculations.[d] Combining Equations 14 and 15 and using $\bar{\alpha}$ to represent the single average value of $\alpha_i$ leads to

$$\Delta G_D = \Delta G_D^{H_2O} + \bar{\alpha} \sum_i n_i^o \delta g_{tr,i} \qquad (16)$$

This equation allows the calculations of $\Delta G_D$ as a function of denaturant concentration, using only the $\delta g_{tr,i}$ values from model compound studies and an $\bar{\alpha}$ value. Generally, a value of $\bar{\alpha}$ is chosen to give a dependence of $\Delta G_D$ on denaturant concentration equal to that determined experimentally, i.e., equal to the *m* value in Equation 10.

The data in Table 2 characterizing the urea and GdnHCl denaturation of four proteins provide a means of testing the reliability of Tanford's procedure. The $\bar{\alpha}$ values that led to calculated m values identical to those determined experimentally are given in Table 5. The values of $\bar{\alpha}$ from the two denaturants are in remarkably good agreement, considering the approximate approach used in the calculations. This agreement would not have been observed if the extent of unfolding or the mechanism of denaturation differed appreciably in the two denaturants for a given protein.

As noted, less satisfactory agreement between the $\bar{\alpha}$ values from urea and GdnHCl is obtained when only the peptide groups and the hydrophobic side chains are included in the calculation. The $\bar{\alpha}$ values differ by 0.08 for ribonuclease, by 0.06 for α-chymotrypsin, and by 0.04 for the other two proteins when the polar side chains are not included in the calculations. The deviation is expected to be greatest for ribonuclease, since this is the least hydrophobic of the four proteins. While this agreement is still reasonable, it does appear that better results are obtained if the polar side chains are included in the calculations. Very similar $\Delta G_D^{H_2O}$ values are obtained from the two approaches.

One aspect of the $\bar{\alpha}$ values is puzzling. The $\bar{\alpha}$ values should generally increase with increasing molecular weight, because the fraction of buried residues should increase. This is not observed. The largest protein, α-chymotrypsin, has the smallest value of $\bar{\alpha}$, and the two proteins with similar molecular weights, ribonuclease and lysozyme, have significantly different $\bar{\alpha}$ values. The most likely explanations for this behavior are these: (a) the relative accessibilities of the native states differ; (b) the relative accessibilities of the denatured states differ; (c) our method of analysis,

[d]Values of $\delta g_{tr,i}$ for the side chains of Asp, Glu, Lys, Arg, and Ser have not been determined. The four charged side chains will generally make a small contribution to the calculated $\Delta G_D$ values because their $\alpha_i$ values will be small. The contribution of the seryl side chain should also be small, since the $\delta g_{tr,i}$ values are expected to be small.

where all of the groups are given the same $\bar{\alpha}$ value, is at fault; or (d) the use of a two-state analysis introduces significant errors.

Lee and Richards[73] have analyzed the accessibility of atoms in native lysozyme and in ribonuclease S. Their results allow us to gain insight into the possible explanation. When $\alpha_i$ values based on the accessibilities are used for each type of side chain and the peptide groups, the disparity between the results for ribonuclease and lysozyme becomes even greater. Also, native ribonuclease S is more accessible to solvent than lysozyme. Thus, possibilities (a) and (c) do not offer an explanation for the discrepancy between the $\bar{\alpha}$ values for these two proteins.

The denaturation of lysozyme and ribonuclease is close enough to a two-state transition that possibility (d) seems an unlikely explanation for these two proteins. The mechanism of denaturation for the other two proteins is not as well characterized, and this may well be a contributing factor.

At least part of the difference in the $\bar{\alpha}$ values probably results from differences in the accessibility of the denatured states. The intrinsic viscosities of the four proteins in 6*M* GdnHCl with and without their disulfide bonds broken are given in Table 5. Data for chymotrypsinogen are used for $\alpha$-chymotrypsin, since Biltonen[74] has shown that the properties of the denatured states are similar. On breaking the disulfide bonds, the percentage increase in the intrinsic viscosity is 62% for lysozyme, 59% for chymotrypsinogen, 42% for ribonuclease, and 16% for $\beta$-lactoglobulin. This suggests that the denatured states of lysozyme and ribonuclease are considerably more compact and probably less accessible to solvent than the denatured state of $\beta$-lactoglobulin, whereas ribonuclease shows intermediate behavior. This probably accounts at least in part for the relatively low $\bar{\alpha}$ values observed for $\alpha$-chymotrypsin and lysozyme. Since ribonuclease contains relatively more disulfide bonds than the other three proteins, it is clear that these differences in compactness result in part from the location of the disulfide bonds.

The conclusion that proteins denatured by urea and GdnHCl may not be completely accessible to solvent is supported by the magnitude of the $\bar{\alpha}$ values. In addition to the analysis of accessibility to solvent of ribonuclease and lysozyme by Lee and Richards,[73] all of the side chains of $\alpha$-chymotrypsin have been classified as exposed, buried, or surface on the basis of the crystal structure by Birktoft and Blow.[75] The $\bar{\alpha}$ values expected on the basis of these analyses are larger than the experimental $\bar{\alpha}$ values given in Table 5. This is probably another indication that the denatured states of these proteins are not completely accessible to solvent.

As noted above, calculations using Tanford's procedure[2] are also of use in answering questions that arise in a denaturation study. The m value for the cytochrome *c* from *Candida krusei* is about 800 cal/mole/M larger than the value for the cytochrome *c* from horse.[33] The protein from *Candida* contains five more residues than the protein from horse. Using Tanford's procedure,[67] it can be shown that an increase in m of this magnitude is expected if the additional residues in *Candida* cytochrome *c* do nothing more than bury an additional five residues.

After $\bar{\alpha}$ has been determined, Equation 16 can be used to calculate $\Delta G_D^{H_2O}$. At the midpoint of the transition, $\Delta G_D = 0$; consequently,

$$\Delta G_D^{H_2O} = -\bar{\alpha} \sum_i n_i^0 \, \delta g_{tr,i} \qquad (17)$$

Thus, the experimentally determined m and $(D)_{1/2}$ values are used with the model compound data to obtain an estimate of $\Delta G_D^{H_2O}$. Figure 11 shows the same data presented in Figure 10 fitted to a curve calculated using Tanford's procedure.[67] Note that a considerably larger value of $\Delta G_D^{H_2O}$ is obtained than with a linear extrapolation. Values of $\Delta G_D^{H_2O}$ calculated using Tanford's procedure are given in Table 5.

*4. Comparison of the Extrapolation Procedures*

The values of $\Delta G_D^{H_2O}$ estimated by the methods described above are listed in Table 6. The values of $\Delta G_D^{H_2O}$ from urea and GdnHCl denaturation should be the same, since the reaction occurring is the same. Linear extrapolation leads to the lowest estimates of $\Delta G_D^{H_2O}$, and the results from urea and GdnHCl agree moderately well. When k values of 0.1 are used, the denaturant-binding model leads to $\Delta G_D^{H_2O}$ values slightly higher than those obtained by direct extrapolation. However, when the larger k values are used, the resulting $\Delta G_D^{H_2O}$ values are almost double those obtained by linear extrapolation. Except for ribonuclease, using Tanford's model for extrapolation leads to $\Delta G_D^{H_2O}$ values from the urea data in reasonable agreement with the values from direct

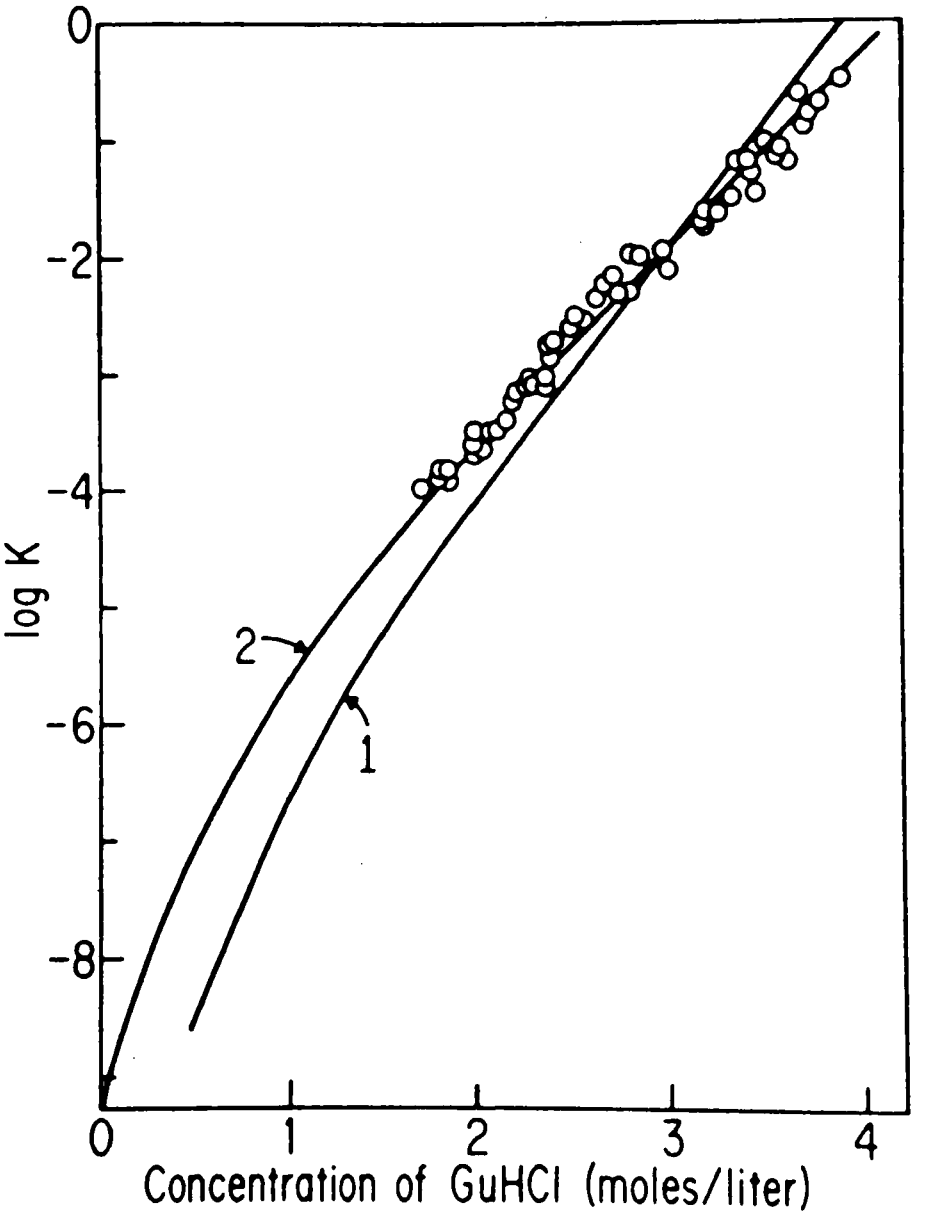



FIGURE 11. Aune and Tanford's[66] data for the GdnHCl denaturation of lysozyme. The curve is drawn according to Equation 16, with $\bar{\alpha} = 0.35$ for curve 1 and $\bar{\alpha} = 0.275$ for curve 2. (Reproduced from Tanford, C., *Advs Protein Chem.*, 24, 1, 1970. With permission.)

TABLE 6

Comparison of the $\Delta G_D^{H_2O}$ Values Obtained by Various Extrapolation Procedures

| Extrapolation procedure | $\Delta G_D^{H_2O}$ (kcal/mole) | | | | | | | |
|---|---|---|---|---|---|---|---|---|
| | Ribonuclease, pH = 6.6 | | Lysozyme, pH = 2.9 | | α-Chymotrypsin, pH = 4.3 | | β-Lactoglobulin, pH = 3.2 | |
| | Urea | GdnHCl | Urea | GdnHCl | Urea | GdnHCl | Urea | GdnHCl |
| Linear extrapolation | 7.7 | 9.3 | 5.8 | 5.8 | 8.4 | 7.8 | 10.5 | 12.5 |
| Tanford's method | 12.1 | 14.8 | 6.6 | 9.1 | 8.8 | 10.4 | 12.1 | 18.4 |
| Denaturant-binding | | | | | | | | |
| k = 0.1 | 8.8 | 11.9 | 6.9 | 7.1 | 9.9 | 10.7 | 12.3 | 15.8 |
| k = 1.0 (urea) or 1.2 (GdnHCl) | 15.7 | 17.3 | 12.1 | 10.0 | 16.7 | 14.3 | 21.4 | 23.2 |
| Average* | 10.0 ± 1.6 | | 6.4 ± 0.5 | | 9.1 ± 0.9 | | 12.6 ± 1.2 | |

*Excluding the GdnHCl results using Tanford's method and the urea and GdnHCl results using denaturant-binding and the larger k values.

extrapolation. However, the values from the GdnHCl data are substantially higher than those obtained by linear extrapolation.

The different methods of extrapolation are illustrated with data from the urea denaturation of lysozyme in Figure 12. The methods all fit the experimental data well, but they can lead to substantially different values of $\Delta G_D^{H_2O}$. Several recent studies have employed GdnHCl as the denaturant and have used either Tanford's method[2] or the denaturant-binding method (k = 1.2) for estimating $\Delta G_D^{H_2O}$.[33,58,59,66,69-72] As a consequence, the $\Delta G_D^{H_2O}$ values obtained were several kcal/mole higher than they would have been with linear extrapolation.

At present it is not clear which extrapolation procedure leads to the best estimate of $\Delta G_D^{H_2O}$. The estimates from linear extrapolation are probably too low. Denaturant-binding is likely to play at least some part in protein denaturation, and this would lead to $\Delta G_D^{H_2O}$ values larger than those obtained by linear extrapolation. The curvature in the plots of $\Delta G_D$ versus denaturant concentration obtained using Tanford's procedure[67] and the model data is consistent with a contribution from denaturant-binding.

While the $\Delta G_D^{H_2O}$ values from linear extrapolation are probably too low, the estimates from denaturant-binding with the larger k values and the estimates from GdnHCl data using Tanford's model[67] are probably too high. It is not surprising that the denaturant-binding models with the larger k values lead to disagreement. Using k = 1.0 for urea leads to a good fit of the experimental data, but this value is clearly too high, based on the results with model compounds cited in Section IV (Part B2). For GdnHCl, a k value of around 1.2 is reasonable, based on the model compounds that GdnHCl solubilizes best. However, other model compounds, which are solubilized to a lesser extent and whose enhanced solubility cannot be accounted for in terms of a binding mechanism, would tend to decrease the effective k value of GdnHCl. Thus, a lower value of k should probably be used.

The failure of Tanford's model[67] to lead to a consistent value of $\Delta G_D^{H_2O}$ when using data from urea and GdnHCl denaturation is puzzling and deserves further study. We previously suggested that an ionic-strength effect on the model compound data in GdnHCl might contribute to the problem.[15] The $\delta g_{tr}$ values for a peptide group are another possible source of error. These values are based on solubility studies of N-acetyltetraglycine ethyl ester and ethyl acetate. Other model compound combinations do not give comparable

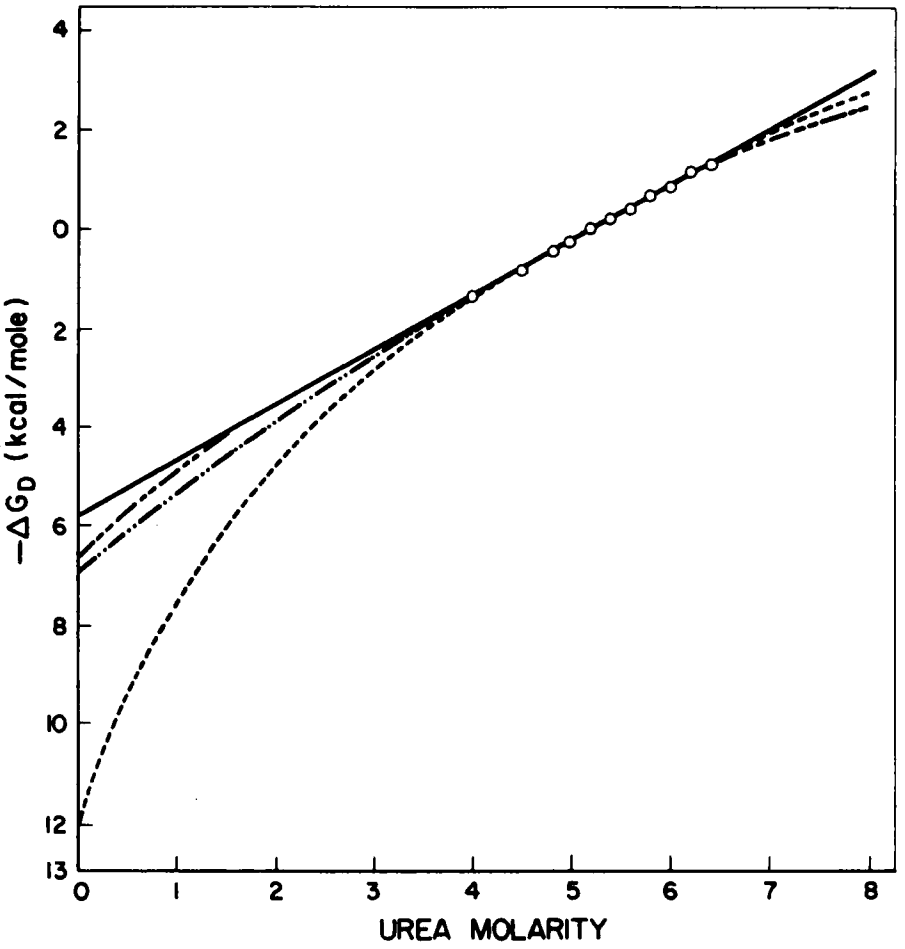



FIGURE 12. The data from Figure 9 with curves drawn according to Equation 10 (————), Equation 12 with k = 0.1 (—·—) or k = 1.0 (– – – – – – – –), or Equation 16 (— – —).

$\delta g_{tr}$ values,[2] but none appear to be as satisfactory as peptide group models. Since the contribution of the peptide groups dominates the calculations, it is of considerable importance to check the $\delta g_{tr}$ values, using a different model.

If we exclude the $\Delta G_D^{H_2O}$ values from GdnHCl using Tanford's procedure,[2] and from denaturant-binding using the larger k values, the average $\Delta G_D^{H_2O}$ values at the bottom of Table 6 are obtained. It is clear that at present $\Delta G_D^{H_2O}$ cannot be estimated to better than about ± 15% from urea and GdnHCl denaturation studies.

## C. Acid Denaturation

As the pH of a protein solution is lowered, most proteins unfold. The unfolding generally occurs between pH 5 and pH 1, but a few proteins remain folded even at pH 1. Proteins can also be unfolded by increasing the pH, but alkaline denaturation is found to be irreversible more often than acid denaturation. Covalent changes in the structure of proteins are known to occur at high pH, and these changes probably cause the irreversibility in most cases.[76]

At higher ionic strengths, the electrostatic interactions among the charged groups of an unfolded protein will be largely eliminated, and the intrinsic dissociation constants of the ionizable groups can be used to account for the acid–base equilibria of the protein.[e] In the native state, the

[e]While there appears to be very little electrostatic interaction between ionizable groups in urea-or GdnHCl-denatured proteins, in acid-or thermally denatured proteins significant interactions may still exist. For myoglobin, for example, the electrostatic interaction factor[77] is reduced from 0.050 in N to 0.034 for the acid-denatured state.[78] For GdnHCl-denatured lysozyme[79] and ribonuclease,[14] the elctrostatic interaction factor is near zero.

dissociation constants of these same groups may be quite different for any of several reasons. The groups may not be capable of ionizing in N because they are buried, or their pK values may be altered by the overall charge on the protein or by their local environment on the surface of the protein. The net effect is that there are generally a different number of protons bound by D and N; as a result, $K_D$ depends on pH. Although the pK values of all of the ionizable groups probably change on unfolding, it is generally possible to describe the pH dependence of $K_D$ by considering the contribution of only a small number of ionizable groups. Tanford[2] has shown that, if these groups titrate independently of each other, the dependence of $K_D$ on pH is given by

$$K_D = (K_{pH=\infty}) \frac{\prod_{i=1}^{n} (1 + K_{i,D}/a_{H^+})}{\prod_{i=1}^{n} (1 + K_{i,N}/a_{H^+})} \quad (18)$$

where $K_{pH=\infty}$ represents $K_D$ for the fully protonated form of the protein, $K_{i,D}$ and $K_{i,N}$ are the acid dissociation constants of group i in D and N respectively, and $a_{H^+}$ is the hydrogen ion activity.

To obtain $K_D^{H_2O}$ at pH 7, Equation 18 is used to fit data over a range where $K_D$ can be measured, and then the resulting equation is used to calculate $K_D^{H_2O}$ at pH 7. The difficulty with this approach is that there will be little confidence in the value of $K_D^{H_2O}$ at pH 7 unless measurements of $K_D$ are extended to within a pH unit or so of pH 7. To do this, using another denaturant in concert with acid is generally required and this introduces a different extrapolation problem.

The pH dependence of $K_D$ for the unfolding of lysozyme was studied between pH 1 and about 3.5 by Aune and Tanford,[66] and between pH 3 and 8 by Ogashara and Hamaguchi.[80] Some of the latter's results are shown in Figure 13. Curves 1 and 2 gave good fits to Aune and Tanford's data at low pH, but it is clear that they would have led to

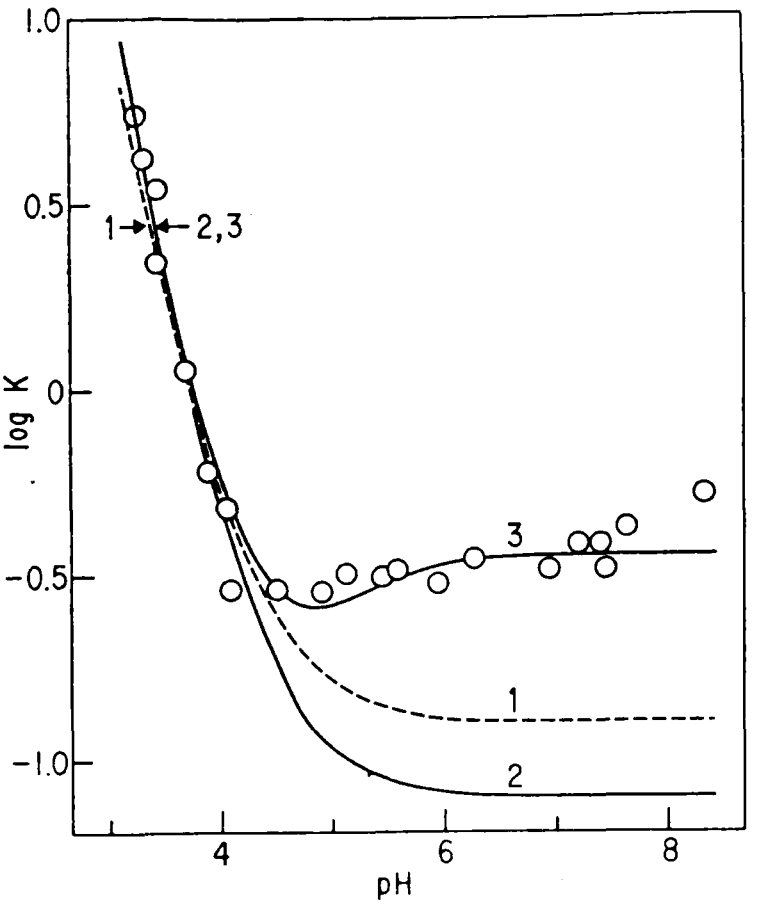



FIGURE 13. The effect of pH on the equilibrium constant for unfolding of lysozyme at 25°C in 3.84*M* GdnHCl.[80] Curves 2 and 3 are drawn according to Equation 19 and 20 respectively. (Reproduced from Tanford, C., *Adv. Protein Chem.*, 24, 1, 1970. With permission.)

an incorrect value of $K_D^{H_2O}$ at pH 7. The equation describing curve 2 is

$$K_D = (K_{pH=\infty}) \frac{(a_{H^+} + 10^{-3.9})\ (a_{H^+} + 10^{-4.4})}{(a_{H^+} + 10^{-1.9})\ (a_{H^+} + 10^{-1.9})} \quad (19)$$

To fit the data at higher pH values, it was necessary to take into account the participation of another group. Curve 3 represents the following equation:

$$K_D = (K_{pH=\infty}) \frac{(a_{H^+} + 10^{-3.9})\ (a_{H^+} + 10^{-4.4})\ (a_{H^+} + 10^{-4.4})}{(a_{H^+} + 10^{-1.9})\ (a_{H^+} + 10^{-1.9})\ (a_{H^+} + 10^{-5.1})} \quad (20)$$

Tanford[2] has discussed which groups in native lysozyme are likely to be giving rise to the terms in Equation 20. For example, the carboxyl group of the glutamic acid at residue 7 lies near positive charges in the native protein and should have an abnormally low pK, such as 1.9. In D, on the other hand, a pK of 4.4 is expected for all of the glutamic acid residues.

As a guide to the number of groups to include in Equation 18, the difference in the number of protons bound to D and N can be determined, using

$$\frac{\partial \ln K_D}{\partial \ln a_{H^+}} = \bar{Z}_D - \bar{Z}_N \quad (21)$$

where $\bar{Z}_D$ and $\bar{Z}_N$ represent the number of protons bound to the denatured and native states of the protein at a given pH.[2] This is illustrated for myoglobin by the data in the inset in Figure 14; the slope of the plot of log K versus pH is six. Exactly the same value for $\bar{Z}_D - \bar{Z}_N$ is obtained from the difference between titration curves for native and denatured myoglobin. Hermans and Acampora[81] have shown that these titration data on myoglobin can be used with Equation 21 to obtain $\Delta G_D^{H_2O}$ for myoglobin. The excess six protons taken up on unfolding combine with the imidazole side chains of six histidine residues, which are known to be buried in the interior of native myoglobin.

### D. Thermal Denaturation

One of the simplest and most frequently used methods of unfolding a protein is to increase the

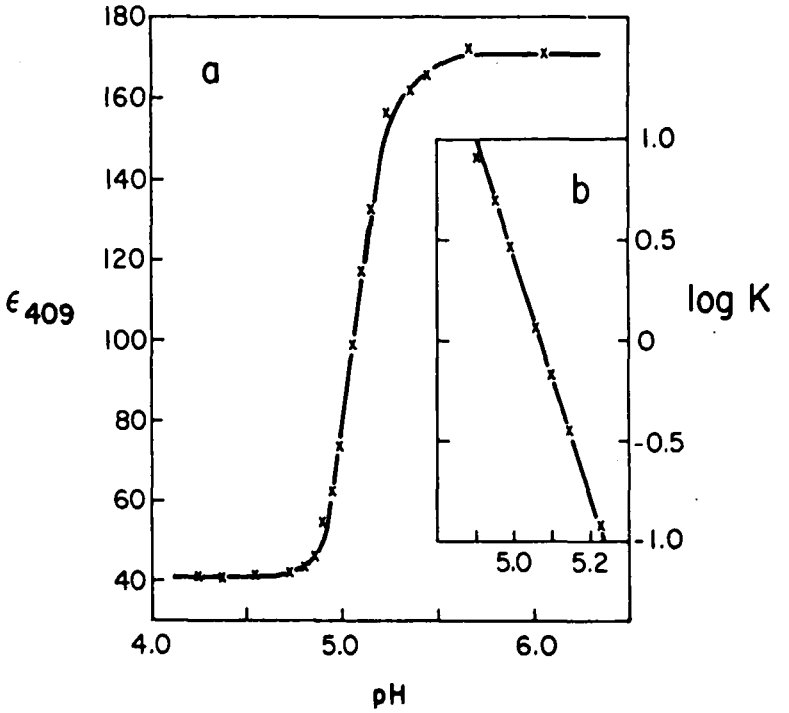



FIGURE 14. (a) The acid denaturation curve for horse ferrimyoglobin in 2*M* urea, 0.1*M* KCl, 0.01*M* acetate buffer. $\epsilon_{409}$ = millimolar extinction coefficient at 409 nm. (b) The data from (a) analyzed, using Equation 21. (Reproduced from Puett, D., *J. Biol. Chem.*, 248, 4623, 1973. With permission.)

temperature. Thermal-denaturation curves for ribonuclease over a wide pH range were shown in Figure 8. The midpoint of the transition is shifted to higher temperatures as the pH approaches neutrality. Thermal denaturation is often studied in the presence of another denaturant, such as acid or urea. Irreversible effects generally become more pronounced at higher temperatures, and using another denaturant allows studies of the thermal denaturation at more moderate temperatures. Also, the denatured state may be insoluble in the absence of another denaturant, as has been observed for myoglobin[81] and β-lactoglobulin.[82]

Studies of thermal denaturation have led to a much better understanding of the thermodynamics of protein denaturation. It has been found that denatured proteins generally have heat capacities from 1 to 4 kcal/deg/mole larger than native proteins.[1] This difference in heat capacity gives rise to a strong temperature dependence for the enthalpy, ΔH, and entropy, ΔS, of denaturation. Typical results from a study of the denaturation of ribonuclease are shown in Figure 15.[83] Note that ΔH increases by more than 65 kcal/mole between 20 and 50°C. The small ΔG values observed in the transition region, ± 2 kcal/mole, generally represent a difference between large values of ΔH and ΔS.

In theory, ΔH and ΔS could be calculated at any temperature, given the $\Delta C_p$ value and one accurate determination of ΔH and ΔS. Unfortunately, $\Delta C_p$ is a second derivative of the original experimental data and cannot be determined accurately; even worse, $\Delta C_p$ is often found to be temperature-dependent.[83] As a consequence, these thermodynamic parameters are of little use in determining $\Delta G_D^{H_2O}$ at 25°C.

The experimental data that led to the results in Figure 15 are shown in Figure 16, where $\Delta G_D$ is plotted as a function of temperature. The solid lines in the figure were drawn according to an equation of the form

$$\Delta G = E + FT + GT^2 (1 + PT) \qquad (22)$$

where E, F, and G are empirical parameters

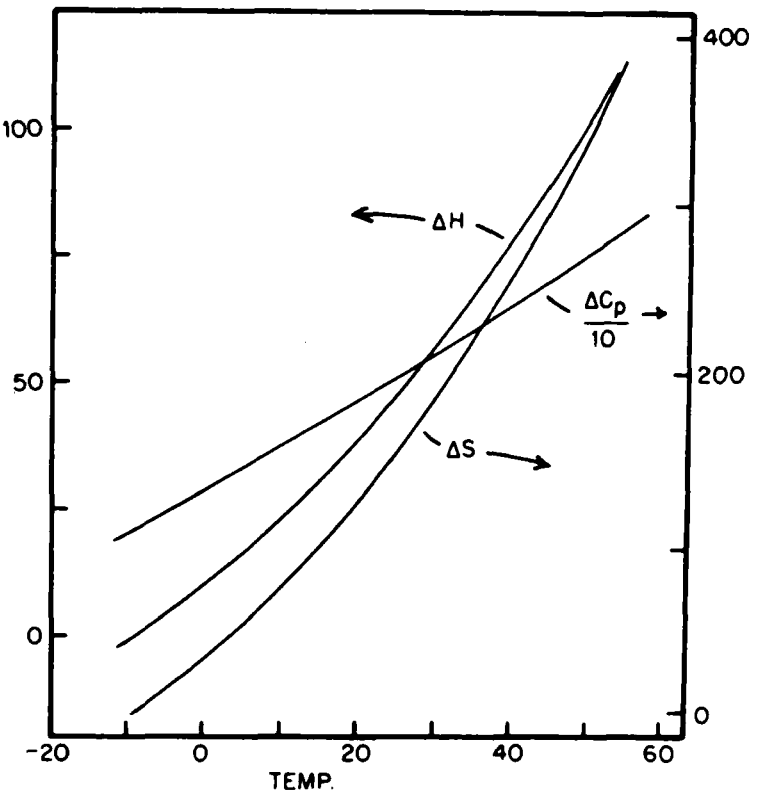



FIGURE 15. Values of ΔH, ΔS, and $\Delta C_p$ for the thermal denaturation of ribonuclease at pH 2.5. (Reprinted from Brandts, J. F. and Hunt, L., *J. Am. Chem. Soc.*, 89, 4826, 1967, © 1967 by the American Chemical Society. Reprinted by permission of the copyright owner.)

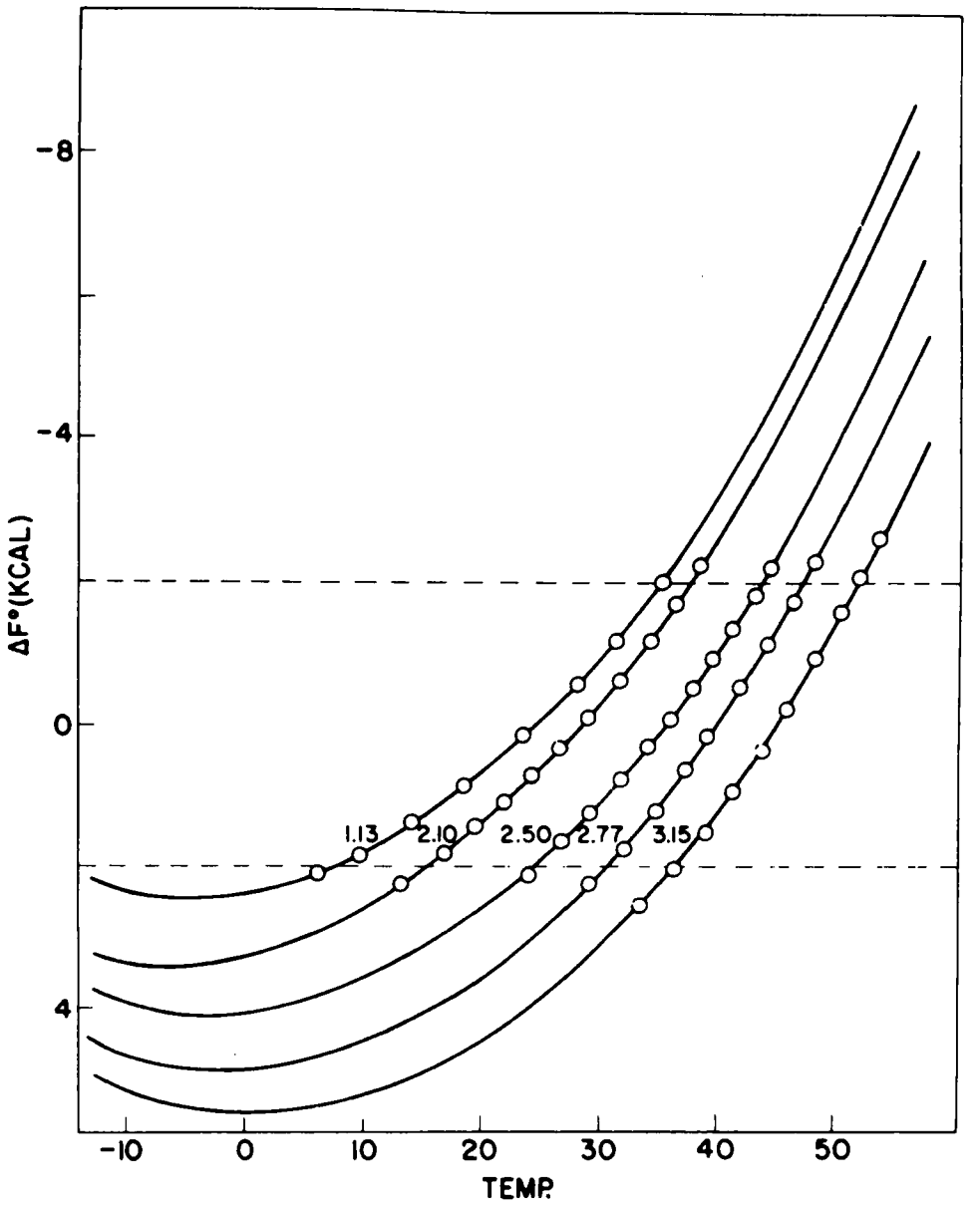



FIGURE 16. The temperature dependence of $\Delta G_D$ for the thermal denaturation of ribonuclease at several pH values. The curves were drawn according to Equation 22. (Reprinted from Brandts, J. F. and Hunt, L., *J. Am. Chem. Soc.*, 89, 4826, 1967, © 1967 by the American Chemical Society. Reprinted by permission of the copyright owner.)

determined by the experimental data. The form of the equation and the value of P are based on the results of solubility studies of small hydrophobic model compounds. This equation leads to a value of $\Delta G_D^{H_2O}$ = 5.5 kcal/mole at 25°C, which is in good agreement with the value obtained by a pH extrapolation (see Section V, Part A).

The solid lines in Figure 16 indicate that ribonuclease should be most stable at −10 to 0°C and that the stability should decrease at both higher and lower temperatures. A direct demonstration of this type of behavior is provided by studies of the denaturation of β-lactoglobulin, shown in Figure 17. The maximum stability of this protein occurs at 35°C, and the protein can be unfolded further by either heating or cooling.[84] Similar results have now been obtained on several proteins; the maximum stability occurs at the

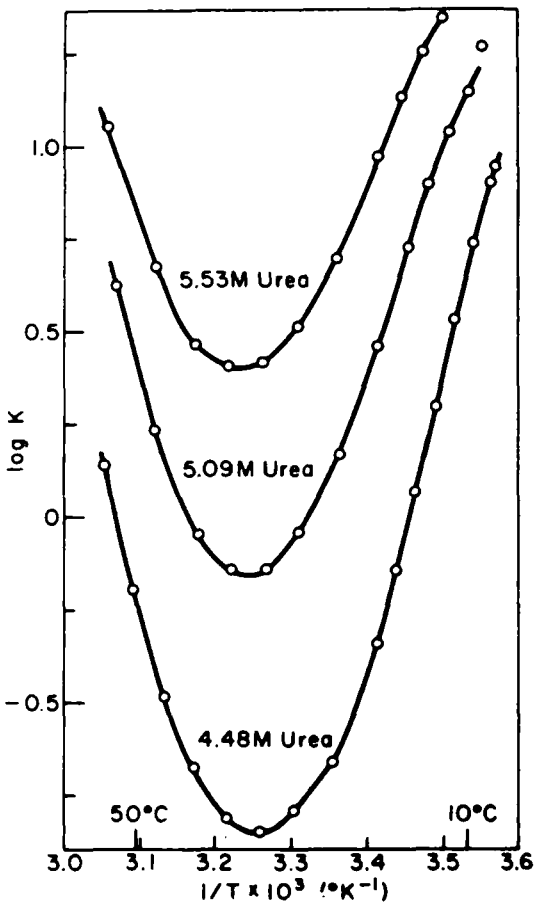



FIGURE 17. Van't Hoff plots for the denaturation of β-lactoglobulin A at pH 3 at the indicated urea molarities. (Reprinted from Pace, C. N. and Tanford, C., *Biochemistry*, 7, 198, 1968, © 1968 by the American Chemical Society. Reprinted by permission of the copyright owner.)

temperature where $\Delta H = 0$. The large value of $\Delta C_p$ for denaturation results because many non-polar groups are exposed to solvent by unfolding. These non-polar groups have a much larger heat capacity when they are in contact with water.[85]

An important paper on thermal denaturation appeared in 1974 and was brought to my attention by the reviewer. Privalov and Khechinashvili[143] have studied the thermal denaturation of five proteins using a calorimeter that is considerably more accurate than those used previously for similar measurements. Typical results for lysozyme are shown in Figure 18, where the partial specific heat capacity is plotted as a function of temperature. These experiments provide a measure of the heat capacity of N and D and can be used to measure the $\Delta H$ of denaturation in two different ways. By integrating curves such as those in Figure 18, a calorimetric $\Delta H$ can be determined. Alternatively, by using the measurements of heat absorption as an observable parameter, $\Delta G$ can be calculated with Equation 6 and $\Delta H$ can be determined by using the van't Hoff equation. It has been shown that for a non-two-state mechanism $\Delta H_{van't\ Hoff} \leq \Delta H_{calorimetric}$. For the five proteins studied by Privalov and Khechinashvili,[143] $\Delta H_{calorimetric}/\Delta H_{van't\ Hoff} = 1.05 \pm 0.03$. Thus, this is further evidence that, for these proteins, denaturation approaches a two-state mechanism quite closely.

Privalov and Khechinashvili[143] show that the temperature dependence of $\Delta C_p$ is considerably smaller than previously thought. Consequently, they are able to use their measured values of $\Delta H$, $\Delta C_p$, and $T_d$, the temperature at the midpoint of the transition, to calculate $\Delta G_D$ as a function of temperature. The results are shown in Figure 19. We will see that these results are in good agreement with estimates of $\Delta G_D^{H_2O}$ obtained in other ways.

### E. Hydrogen Exchange Rates

In globular proteins, the amide hydrogens of the peptide groups exchange with solvent hydrogens at markedly different rates. Some hydrogens exchange in seconds, whereas others exchange only over a period of days or longer. The quickly exchanging hydrogens are those on the surface of the molecule exposed to solvent. The slowly exchanging hydrogens are those hydrogen-bonded[86] or buried in the interior of the mole-

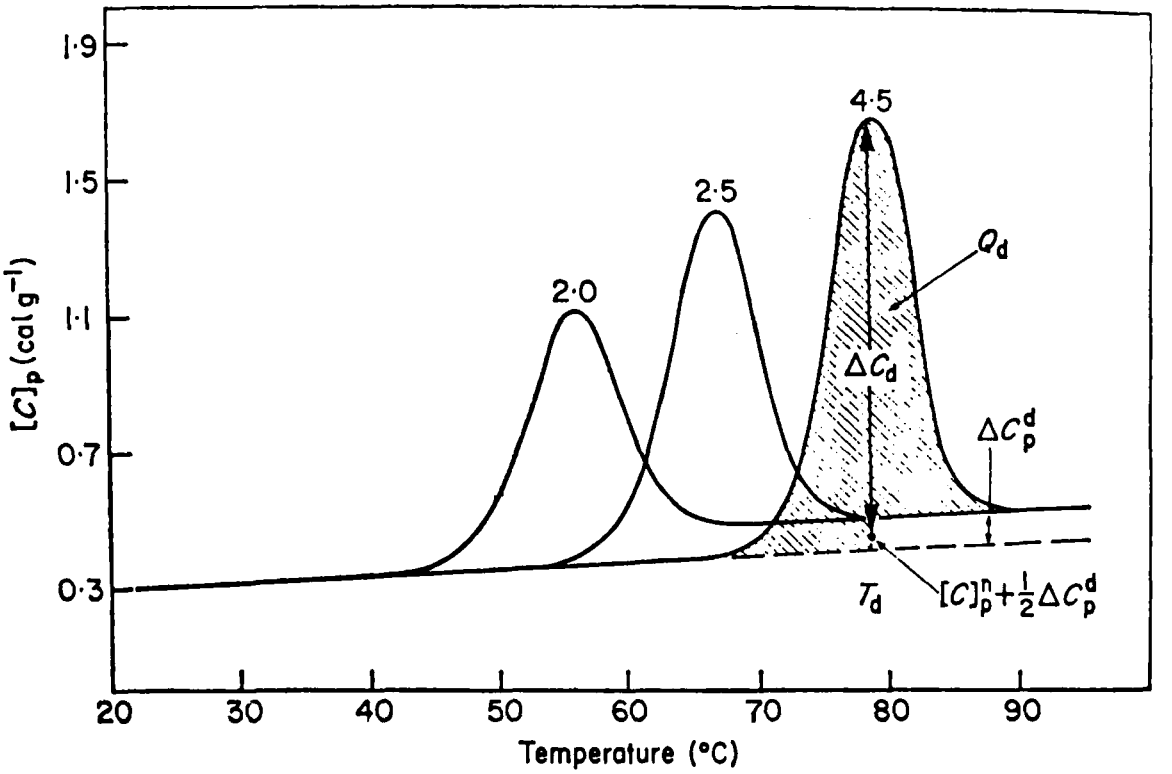



FIGURE 18. Temperature dependence of the partial heat capacity of lysozyme at pH values 2.0, 2.5, and 4.5. (Reproduced from Privalov, P. L. and Khechinashvili, N. N., *J. Mol. Biol.*, 86, 665, 1974. With permission.)

cule. They are thought to exchange by the following mechanism:[11]

$$N \underset{k_2}{\overset{k_1}{\rightleftharpoons}} U \xrightarrow{k_{ex}} U_{ex} \qquad (23)$$

where U is an unfolded form of N in which some previously buried hydrogens are now in contact with solvent, and $U_{ex}$ is the same state of the molecule with the hydrogens exchanged. (Woodward and Rosenberg[87] have suggested another branch for this mechanism, in which exchange occurs from the folded protein.) For this mechanism it has been shown[11] that the observed rate of exchange of a given hydrogen, $k_{obs}$, is given by

$$k_{obs} = \frac{k_1 \, k_{ex}}{k_1 + k_2 + k_{ex}} \qquad (24)$$

Exchange is usually measured under native-state conditions, where $k_2 >> k_1$. As we will see below, conditions can be chosen so that $k_2 >> k_{ex}$; this leads to

$$k_{obs} = \frac{k_1}{k_2} k_{ex} \qquad (25)$$

Since $k_{ex}$ can be estimated from independent experiments, the equilibrium constant for the unfolding, $k_u = k_1/k_2$, can be determined under native-state conditions by measuring $k_{obs}$.

There are a variety of techniques used for following exchange.[88] Some techniques follow the replacement of hydrogen by deuterium or tritium; others follow the replacement of tritium or deuterium by hydrogen. Equilibrium and kinetic isotope effects can usually be taken into account.[86] However, it is difficult to estimate the effect of these substitutions on the stability of the protein. For example, the thermal denaturation curves for lysozyme are identical in $H_2O$ and $D_2O$,[89] but the midpoints of the thermal denaturation curves for α-lactalbumin differ by about 4° in $H_2O$ and $D_2O$.[90] Thus, the stability of a protein can change during the course of an exchange experiment. It will be difficult to correct for such an effect, but exchange experiments have not reached this degree of refinement.

Typical results from a study of the tritium exchange of ribonuclease are shown in Figure 20.[40] The slope of these plots at any point gives

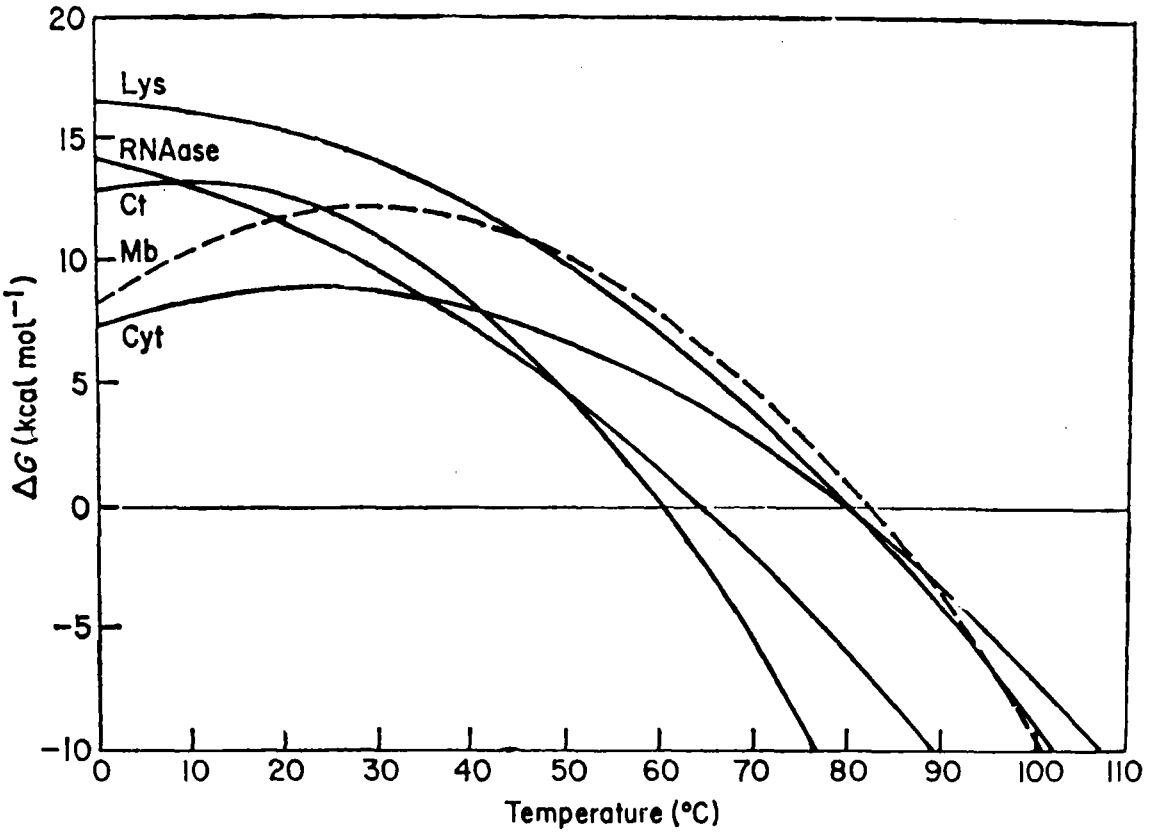



FIGURE 19. Temperature dependence of $\Delta G_D$ calculated from calorimetric data. Lys = lysozyme; RNAase = ribonuclease; Mb = metmyoglobin; Ct = α-chymotrypsin, Cyt = cytochrome *c*. (Reproduced from Privalov, P. L. and Khechinashvili, N. N., *J. Mol. Biol.*, 86, 665, 1974. With permission.)

the rate constant for exchange. It can be seen that there is a range of exchange rates. For lysozyme, for example, at least six classes of hydrogens with different exchange rates are needed to account for the observed hydrogen exchange.[11] This range of exchange rates results in part from a nearest-neighbor effect dependent on amino acid sequence (see below).[91] In addition, departures from a two-state mechanism (Section III) and the existence of partially folded denatured states under these conditions (Section II) undoubtedly contribute to the range of exchange rates. For considering the equilibrium between N and the most completely unfolded denatured states, only the rates of the most slowly exchanging hydrogens should be used; in Figure 20, these would be the rates observed at longer times.

It is not always a simple matter to determine the most slowly exchanging class of hydrogens. Nakanishi et al.[89] find a group of 44 slowly exchanging hydrogens for lysozyme, using an infrared technique for following exchange. However, using a more sensitive technique for measuring exchange, it is difficult to resolve a distinct group of slowly exchanging hydrogens for lysozyme.[92] Ottesen[88] has proposed arbitrarily dividing the exchangeable hydrogens into ten equal groups and estimating the stability of each group.

There are also difficulties in deciding on a value for $k_{ex}$. The experimental points in Figure 21 show the tritium for hydrogen exchange of oxidized ribonuclease, a randomly coiled form of ribonuclease.[91] The dashed line shows the exchange of randomly coiled poly-D,L-alanine, the model most often used for calculating $k_{ex}$. All of the amide hydrogens of poly-D,L-alanine exchange at the same rate, but the non-linearity observed with ribonuclease indicates that the hydrogens exchange at different rates. Under conditions where the $k_{ex}$ for poly-D,L-alanine is $1.3 \times 10^{-2}$ $min^{-1}$, the $k_{ex}$ for oxidized ribonuclease varies from 0.9 to $34.5 \times 10^{-2}$ $min^{-1}$.[93] The range of rates observed for ribonuclease results mainly from

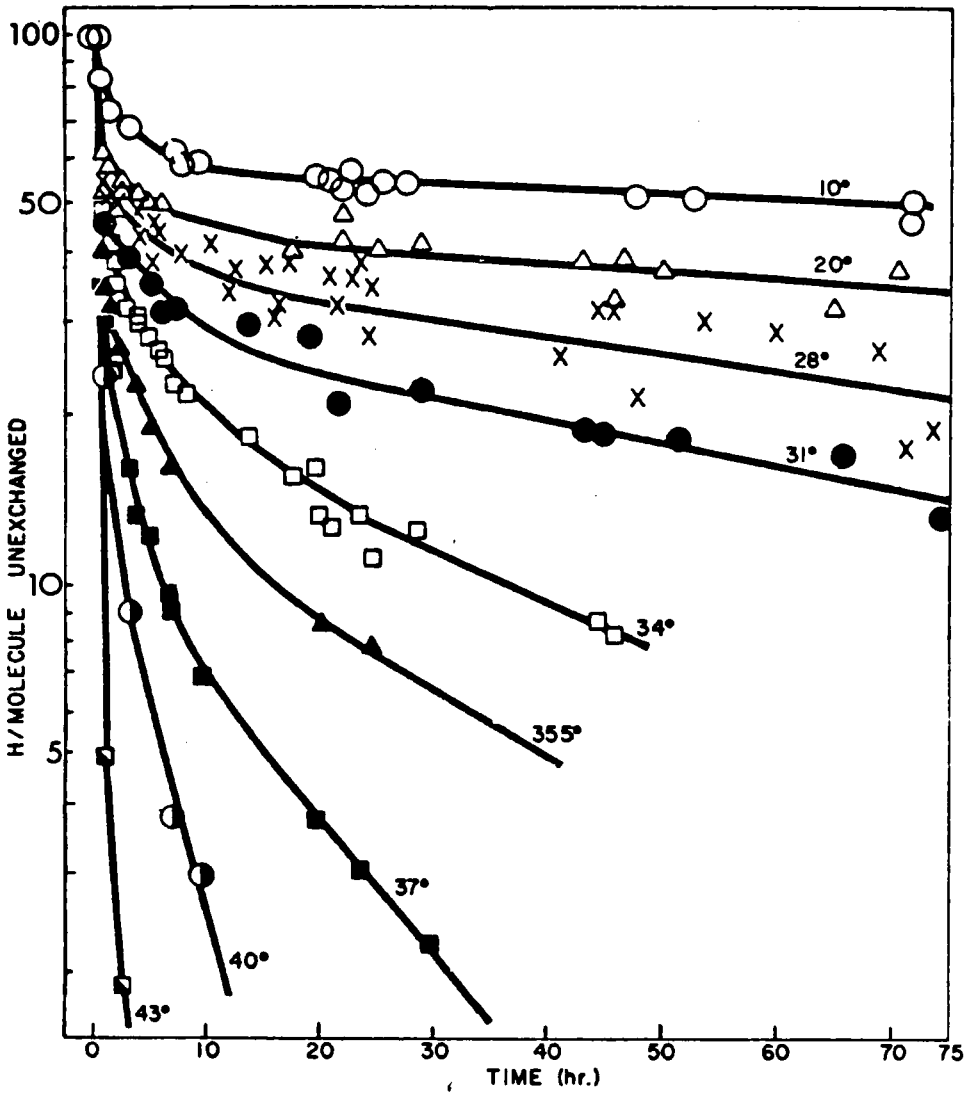



FIGURE 20. The exchange of hydrogen for tritium in fully tritiated ribonuclease at pH 3.15 at several temperatures. H/molecule = hydrogens per molecule. (Reproduced from Woodward, C. K. and Rosenberg, A., *J. Biol. Chem.*, 246, 4105, 1971. With permission.)

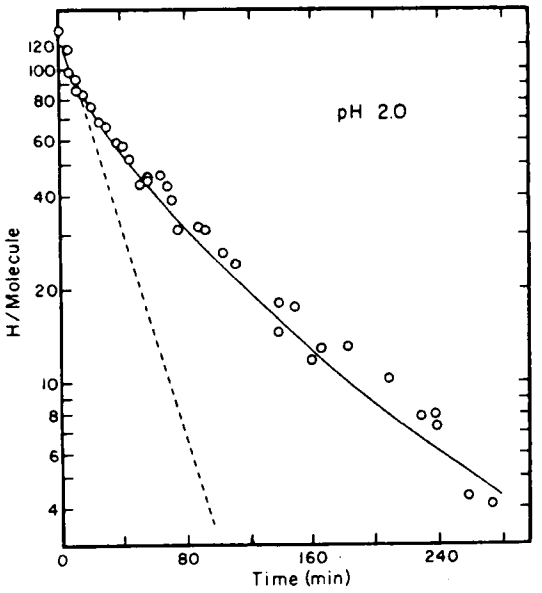



FIGURE 21. Tritium-hydrogen exchange of oxidized ribonuclease at pH 2.0 and 0°C (– – – – – –). The exchange of randomly coiled poly-D,L-alanine under similar conditions (————). The exchange curve predicted for oxidized ribonuclease, taking into account effects of neighboring side chains. (Reprinted from Molday, R. S. et al., *Biochemistry*, 11, 150, 1972, © 1972 by the American Chemical Society. Reprinted by permission of the copyright owner.)

nearest-neighbor effects in the amino acid sequence.[91] The solid line in Figure 21 is the exchange curve predicted when such nearest-neighbor effects are taken into account.

To determine $k_u$ from an exchange study requires that $k_2 >> k_{ex}$. The rate constant for H–T exchange of poly-D,L-alanine in $min^{-1}$ at any pH or temperature in °C is given by[94]

$$k_{ex} = 3.47 \times 10^{-3} \ (10^{+0.05T}) \ (10^{pH-3} + 10^{3-pH}) \quad (26)$$

Thus, the minimum rate of exchange occurs at pH 3, and the rate increases approximately 10-fold for each unit change in pH above about pH 4. Tanford[2] has shown that for lysozyme and β-lactoglobulin $k_2 >> k_{ex}$ up to at least pH 5. However, no general rule is possible, and the assumption must be checked for individual proteins.

It is clear from the above discussion that reaching an estimate of $\Delta G_D^{H_2O}$ from a study of the hydrogen exchange is not a straightforward procedure. As a consequence, in most hydrogen-exchange studies the authors do not hazard a guess at $\Delta G_D^{H_2O}$. In Table 7, estimates of $\Delta G_D^{H_2O}$ from hydrogen exchange are listed for three of the proteins that have been most thoroughly studied using denaturation-curve analysis. In general, the estimates from hydrogen exchange are comparable to those obtained through denaturation-curve analysis. Perhaps this will encourage those studying hydrogen exchange to attempt to estimate $\Delta G_D^{H_2O}$ in future studies.

## V. RESULTS FROM INDIVIDUAL PROTEINS

In this section, we present the results of denaturation studies on individual proteins. In many papers a denaturation curve is presented, but the data are not analyzed; only the midpoint of the denaturation curve can be obtained from these studies. In other studies only one method of analysis is used, and it is often difficult to determine reliably what the results would be if another method of analysis were used; nevertheless, we have attempted to do so in a few cases. For urea and GdnHCl denaturation we recommend that the results always be analyzed assuming a two-state mechanism and the $(denaturant)_{1/2}$ and m values (Equation 10) given. This is the most direct and useful way of presenting such data.

TABLE 7

**Estimates of $\Delta G_D^{H_2O}$ from Hydrogen Exchange Studies***

| Protein | pH | T | $\Delta G_D^{H_2O}$ | Reference |
|---|---|---|---|---|
| Lysozyme | 5.4 | 20°C | 7.4 kcal/mole | 89 |
| Ribonuclease | 4.7 | 38°C | 6.5 kcal/mole | 11 |
| Myoglobin | 7 | 21°C | 13.0 kcal/mole | 88 |

*These estimates are based on $k_{ex}$ values from poly-(D,L)-alanine, and on $k_{obs}$ values for the most slowly exchanging hydrogens.

TABLE 8

**$\Delta G_D^{H_2O}$ and $\Delta n$ Values Characterizing GdnHCl Denaturation in Terms of Denaturant Binding, using Equation 12 with k = 1.2***

| Protein | $\Delta n$ | $\Delta G_D^{H_2O}$ | Reference |
|---|---|---|---|
| RNase A | 24.4 | 13.0 | 58 |
| RNase A | 32 | 17.3 | 15 |
| RNase A | 27 | 14.6 | 69 |
| RNase B′ | 27 | 15.2 | 98 |
| RNase-(1–120) | 27 | 10.5 | 69 |
| Lysozyme | 21.5 | 14.2 | 66 |
| Lysozyme | 19 | 10.0 | 15 |
| Cytochrome *c* | | | |
| Cow | 30.4 | 15.4 | 33 |
| Horse | 26.4 | 12.7 | 33 |
| *Candida* | 32.9 | 14.0 | 33 |
| Myoglobin | | | |
| Horse | 30 | 11.8 | 71 |
| Whale | 30 | 14.3 | 71 |
| α-Lactalbumin | 13.5 | 6.8 | 72 |
| α-Chymotrypsin (pH = 4.3) | 29.3 | 12.3 | 15 |
| Goat β-lactoglobulin (pH = 3.2) | 41 | 23.2 | 15 |

*The conditions are 25°C and neutral pH, unless otherwise indicated.

In Section IV, B4, we concluded that estimates of $\Delta G_D^{H_2O}$ based on GdnHCl-binding, with k = 1.2 (Equation 12), are too large, probably by at least a few kcal/mole. Nevertheless, Equation 12 has been used to analyze GdnHCl denaturation curves for a wide variety of proteins, and the results (Table 8) provide a useful comparison of the differences in stability between globular proteins. It is clear that the results from different laboratories can differ substantially (c.f. ribonuclease and lysozyme). Many factors surely contribute to these differences, but one of the more important is probably protein purity. As with intermediates, the presence of impurities will broaden denaturation curves, leading to lower m values and lower values of $\Delta G_D^{H_2O}$.

### A. Ribonuclease and Derivatives

The acid-thermal denaturation of ribonuclease was studied in detail by Hermans and Scheraga,[60] and by Brandts and Hunt.[83] On the basis of their data, $\Delta G_D^{H_2O}$ = 3.8 kcal/mole at pH = 3.15, 25°C. At 40 to 50°C, raising the pH from 3.15 to 7.0 increases $\Delta G_D^{H_2O}$ by about 2.2 kcal/mole.[60] In 3.2*M* GdnHCl, raising the pH from 3.15 to 7.0 increases $\Delta G_D^{H_2O}$ by about 2.5 kcal/mole.[58] Assuming that a similar increase in $\Delta G_D^{H_2O}$ will occur in 0.15*M* KCl at 25°C leads to an estimate of $\Delta G_D^{H_2O}$ = 6.2 kcal/mole.[f] This is in good agreement with an estimate of $\Delta G_D^{H_2O}$ = 5.5 ± 1 kcal/mole, reached by Brandts (personal communication) on the basis of a temperature extrapolation, and with an estimate of 5.7 kcal/mole given by Hermans and Scheraga.[60] Recall that these $\Delta G_D^{H_2O}$ values characterize unfolding to a denatured state that is not completely unfolded. The denaturation of ribonuclease is probably close enough to a two-state mechanism under most conditions, so that a two-state analysis will not lead to large errors.

The results of studies of the isothermal urea and GdnHCl denaturation of ribonuclease are summarized in Table 9. All of the estimates of $\Delta G_D^{H_2O}$ are based on the linear extrapolation of

[f] The effects of pH on $K_D^{H_2O}$ should be largely temperature-independent below about pH 5. At low pH, the difference in ionization state between D and N will depend mainly on the pK values of carboxyl groups; these pK values will not vary much with temperature, since $\Delta H$ for the ionization of carboxyl groups is small. This has been confirmed for ribonuclease[83] and chymotrypsinogen.[95] Similarly, the presence of 3.2 *M* GdnHCl should not significantly alter the effect of pH on $K_D^{H_2O}$. An increase in the ionic strength from 0.15 to 3.2 *M* is not expected to cause a large change in the pK of a carboxyl group.[96]

TABLE 9

**Parameters Characterizing the Urea and GdnHCl Denaturation of Ribonuclease at pH 7**

| Denaturant | $(D)_{½}$, *M* | m, cal/mole/*M* | $\Delta G_D^{H_2O}$, kcal/mole | Reference |
|---|---|---|---|---|
| GdnHCl | 3.01 | 3,100 | 9.3 | 15 |
| GdnHCl | 2.90 | 3,000 | 8.7 | 69 |
| GdnHCl | 2.93 | 2,600 | 7.6 | 58 |
| Urea | 6.96 | 1,100 | 7.7 | 15 |
| Urea | 6.70 | 1,400 | 9.4 | 97 |

$\Delta G_D$ to zero concentration of denaturant. Since both urea and GdnHCl lead to a randomly coiled denatured state, the values of $\Delta G_D^{H_2O}$ from the two denaturants should be the same. It can be seen that there is reasonable agreement among the results from different laboratories and between the results from the two denaturants. The average of the values of $\Delta G_D^{H_2O}$ given in Table 9 is 8.5 kcal/mole.

These results suggest that $\Delta G$ for unfolding to a random coil is 2 to 3 kcal/mole greater than for unfolding to the denatured state produced by acid-thermal denaturation. This is substantiated by the results of Aune et al.,[27] who studied the GdnHCl denaturation of the acid-thermal-denatured protein directly at 56°C. Their denaturation curve was characterized by $(GdnHCl)_{½}$ = 2.7*M* and m = 770 ± 100 cal/mole/*M* again indicating a difference in stability between the two unfolded states of about 2 kcal/mole.

Puett[98] has studied the GdnHCl denaturation of ribonuclease B′, a derivative of ribonuclease A that contains 1 mole of N-acetylglucosamine and 2 to 3 moles of mannose covalently attached as an oligosaccharide to an asparagine residue at position 34. The carbohydrate has little, if any, effect on the conformation of the protein or on the mechanism of denaturation. It does appear to stabilize the protein, $(GdnHCl)_{½}$ = 3.16*M*, but not by more than one kcal/mole. This is not surprising. If a sugar residue did nothing more than bury the $-CH_3$ group of an alanine side chain, it could increase the stability of the protein by 0.5 kcal/mole.

Denaturation studies have also been carried out on several of the proteolytic-cleavage products of ribonuclease. The results obtained are summarized in Table 10. By assuming that $\Delta G$ varies with temperature or denaturant, as it does in intact ribonuclease, we can get a rough idea of how much less stable these derivatives are than intact ribonuclease. Ribonuclease (1–120) is 3 ± 0.6 kcal/mole, and ribonuclease (1–119) is 5 ± 1 kcal/mole less stable than intact ribonuclease. These results are remarkable. Removing just five residues leads to more than a halving of the stability of the protein. This is especially interesting since Taniuchi[99] has shown that reduced and unfolded ribonuclease (1–120) will not spontaneously refold under native-state conditions, whereas intact ribonuclease will. Ribonuclease (1–115) and (1–114) are found to be largely unfolded at 25°C. These findings should be kept in mind when one considers the folding of the polypeptide chain during or after biosynthesis. The C-terminal portion of the polypeptide chain is also very important to the stability of staphylococcal nuclease[103] and barnase.[104]

Ribonuclease can be specifically cleaved between residues 20 and 21, giving rise to the S-protein (21–124) and the S-peptide (1–20) or, when they are combined, ribonuclease S. The data of Sherwood and Potts[102] suggest that the S-protein is 5.5 ± 1.2 kcal/mole less stable than ribonuclease A. Thus, the N-terminal portion of the molecule is also of considerable importance to the stability of ribonuclease A. The reviewer has pointed out that the denaturation curve of ribonuclease S will depend on the total protein concentration. Unpublished data from his laboratory (D. M. Pederson and J. Hermans, Jr., personal communication) show that at 35°C the midpoint of the acid denaturation curve is shifted to lower pH by almost 0.5 pH units by an increase in the protein concentration from 0.83 to 9.6 mg/ml. This complication makes it difficult to determine

TABLE 10

**Midpoints of Denaturation Curves for Several Ribonuclease Derivatives**

| RNase derivative | $(D)_{1/2}$ | $(T)_{1/2}$ | Conditions | Reference |
|---|---|---|---|---|
| RNase-(1–120) | | 44.5°C | 0.02*M* Tris, pH 7.5, | 100 |
| RNase-(1–119) | | 34.4°C | RNase A $(T)_{1/2}$ = 61.0°C | 100 |
| RNase-(1–118) | | 32.5°C | | 100 |
| RNase-(1–118) | | 33°C | 0.02*M* Tris, pH 7.5 | 101 |
| RNase-(1–115) + peptide-(116–124) | | 38°C | | 101 |
| RNase-(1–120) | 1.7*M* GdnHCl | | 0.01*M* $NaPO_4$, pH 7, 0.1*M* KCl | 69 |
| RNase-(1–119) | 0.9*M* GdnHCl | | | 70 |
| RNase-(1–118) | 0.65*M* GdnHCl | 32°C | | 70 |
| RNase S | 4.8*M* urea | 42°C | 0.01*M* $NaPO_4$, 0.9% NaCl, pH 6.8 | 102 |
| RNase S protein | 2.1*M* urea | 34°C | RNase A $(urea)_{1/2}$ = 8.3*M* $(T)_{1/2}$ = 62°C | 102 |
| RNase S | | 47.7°C | 0.2*M* NaCl, pH 7.0 | 144 |
| RNase S protein | | 37.6°C | RNase A $(T)_{1/2}$ = 61.3 | 144 |

the difference in stability between ribonuclease S and ribonuclease A.

## B. Cytochromes *c*

At present, 60 different amino acid sequences have been determined for the cytochromes *c* from 67 different species.[105] High-resolution three-dimensional structures are available for the reduced and oxidized forms of the cytochromes *c* from two species. Thus, the cytochromes *c* offer an excellent system for investigating the relationship between structure and globular-protein stability. Unfortunately, the mechanism of all forms of denaturation for horse ferricytochrome *c* appears to be complex,[49,52] and this makes it difficult to determine the $\Delta G_D^{H_2O}$ values. (This may, of course, prove a boon to those interested in the mechanism of folding and unfolding proteins.)

Horse and cow cytochrome *c* differ in amino acid sequence by three residues, as shown in Table 11. The cytochrome *c* from *Candida krusei* contains five more residues than horse cytochrome *c* and differs in sequence at 45 of the 103 residues common to both proteins.[105] We have studied the GdnHCl and acid denaturation of the oxidized form of these three proteins.[33] An analysis of the GdnHCl denaturation in terms of a two-state mechanism led to the results shown in Figure 22.

TABLE 11

**Differences in Sequence between Horse and Cow Cytochrome *c***

| | Sequence position | | |
|---|---|---|---|
| Cytochrome *c* | 47 | 60 | 89 |
| Horse | Thr | Lys | Thr |
| Cow | Ser | Gly | Gly |

The parameters characterizing these results are given in Table 12. It is clear that cow ferricytochrome *c* is the most stable of the three proteins. Cow ferricytochrome *c* is found to be more stable than horse ferricytochrome *c* by 650 cal/mole at the midpoint of GdnHCl denaturation curve (pH 6.5, 2.5*M* GdnHCl), by 720 cal/mole near the midpoint of the acid denaturation curve (pH 2.5, $3.16 \times 10^{-3}$ *M* $Cl^-$), and by 1,100 cal/mole when the GdnHCl data are extrapolated to zero concentration of denaturant. Thus, even though the $\Delta G_{app}^{H_2O}$ values in Table 12 are uncertain because of the presence of intermediate states, we are quite certain that cow ferricytochrome *c* is from 600 to 1,100 cal/mole more stable than horse ferricytochrome *c*.

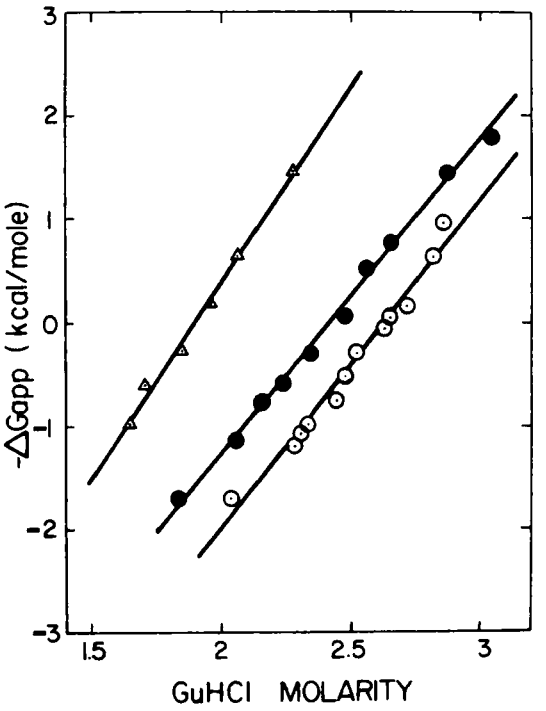



FIGURE 22. $\Delta G_{app}$ for the denaturation of *Candida* (△), horse (●), and cow (○) ferricytochrome *c* as a function of GdnHCl molarity at 25°C, pH 6.5. (Reprinted from Knapp, J. A. and Pace, C. N., *Biochemistry*, 13, 1289, 1974, © 1974 by the American Chemical Society. Reprinted by permission of the copyright owner.)

TABLE 12

**Parameters Characterizing the GdnHCl Denaturation of Ferricytochromes *c* at pH 6.5, 25°C**

| Protein | $(GdnHCl)_{½}$,* *M* | $\Delta G^{H_2O}_{app}$,† kcal/mole | m,† kcal/mole/*M* |
|---|---|---|---|
| Cow | 2.63 | 8.38 | 3,190 |
| Horse | 2.42 | 7.27 | 3,010 |
| *Candida* | 1.89 | 7.24 | 3,820 |

*The midpoint of the denaturation curve ($\Delta G_{app} = 0$).
†From Equation 10.

It is difficult to account for this difference in stability on the basis of the differences in amino acid sequence. We considered the following: (1) steric effects in the native proteins; (2) steric effects in the denatured proteins (e.g., the two Gly residues in cow cytochrome *c* will increase the flexibility and, hence, the conformational entropy of the denatured state); (3) hydrogen bonding; and (4) electrostatic interactions involving the ε-amino of Lys 60. All of the factors considered either suggest that horse ferricytochrome *c* should be more stable than cow ferricytochrome *c* or that they will have no effect on the stability.

Two of the differences in sequence between horse and cow cytochrome *c* occur at hypervariable positions; nine different amino acids have been found at each position.[105] Consequently, it is unlikely that these residues are involved in the function of the protein, but it appears that substitutions at these positions can influence the stability of cytochrome *c*.

The m value for *Candida* ferricytochrome *c* is considerably higher than that for the other two proteins. It was pointed out in Section IV (Part B3) that this is expected, due to the additional amino acid residues it contains. As a consequence, even though *Candida* ferricytochrome *c* is less stable than the other two proteins in the presence of GdnHCl, it appears to have a stability comparable to that of horse ferricytochrome *c* in the absence of GdnHCl. When data from both acid and GdnHCl denaturation are taken into account, they suggest that, if any difference exists, horse ferricytochrome *c* will be somewhat more stable than *Candida* ferricytochrome *c*. Thus, these two proteins have very similar stabilities, despite the large difference in amino acid sequence between them. It will be interesting to see if the stabilities of all of the cytochromes *c* fall in a reasonably narrow range.

Three observations suggest that the $\Delta G^{H_2O}_{app}$ and m values obtained are probably close to the values expected for a two-state mechanism, i.e., the values would not be appreciably changed if we could correct for the presence of intermediate states. First, the studies of Privalov and Khechinashvili[143] show that the calorimetric and van't Hoff enthalpies of denaturation are in good agreement. Second, an $\bar{\alpha}$ value of around 0.42[g] is needed to account for the observed m values using Tanford's procedure.[67] This is considerably greater than the $\bar{\alpha}$ values required to account for the unfolding of any of the four proteins in Table 5. Third, the $\Delta G^{H_2O}_{app}$ values for the cytochromes *c* are comparable to those obtained for other

[g]The contribution of the heme group has been neglected in calculating $\bar{\alpha} = 0.42$. Including the $-CH_2-$ groups of the heme would decrease $\bar{\alpha}$ to about 0.40, still considerably greater than the values in Table 5.

proteins (Table 8). On the basis of these observations, the concentrations of intermediates suggested by the kinetic studies (see Section III) may well be too large.

When the iron is removed from the heme in horse cytochrome *c*, the protein is still globular, but the stability to thermal denaturation is markedly decreased.[106] When the heme is entirely removed, the protein is no longer globular.[106,107] Thus, the presence of the covalently bound heme in cytochrome *c* is essential to the stability of the native state.

### C. β-Lactoglobulins

The A and B genetic variants of bovine β-lactoglobulin differ at only two residues in amino acid sequence.[108] At position 68, a Val is found in A and an Ala in B; at position 121, an Asp is found in A and a Gly in B. The physical properties of the two proteins are very similar, suggesting that they have very similar conformations.[109] Nevertheless, these substitutions give rise to a significant difference in the stability.[110]

Urea and GdnHCl denaturation have been investigated for the A variant and the β-lactoglobulin from goat's milk, and urea denaturation has been studied for the B variant. Parameters characterizing the denaturation are listed in Table 13. The m values differ substantially for the A and B variants. This is surprising and cannot be accounted for on the basis of the small difference in amino acid sequence. Kinetic studies of the GdnHCl denaturation of β-lactoglobulin A have shown the existence of intermediate states.[111] The m value for A may be lower than that for the B variant because it deviates further from a two-state mechanism. We feel that this is a more likely explanation than postulating a difference in the conformation or accessibility of either the N or D states for the two proteins.

Near the midpoints of the urea denaturation curves, β-lactoglobulin A is 250 ± 50 cal/mole more stable than the B variant and 950 ± 100 cal/mole more stable than goat β-lactoglobulin. The differences in stability in the absence of denaturant will probably be quite similar.

The pH dependence of $\Delta G_{app}$ has not been investigated over a wide enough range to allow an estimation of $\Delta G_{app}^{H_2O}$ at neutral pH.

### D. Immunoglobulin Light Chains

The light chains from immunoglobulins fold up to give a dumbbell-type structure with two domains, each containing about one half of the amino acid sequence.[112] One domain has an amino acid sequence that varies from antibody to antibody and is referred to as the variable or V portion of the molecule; the amino acid sequence of the other domain is referred to as the constant or C portion of the molecule.[113] Rowe and Tanford[59] have studied the GdnHCl denaturation of an intact light chain. Kinetic studies and a low $\overline{\alpha}$ value both indicate that the unfolding does not follow a two-state mechanism. However, all of their data are consistent with a mechanism in which the two domains of the molecule unfold independently. Using Tanford's method,[67] they show that $\Delta G_{app}^{H_2O} = 5.5$ kcal/mole for the unfolding of each domain at pH 7, 25°C.

A light chain can be cleaved with proteolytic enzymes to give two distinct globular subunits, each with a molecular weight of about 11,000. Karlsson et al.[114] have studied the thermal and GdnHCl denaturation of an intact light chain and the separated domains. It is interesting that, although they observed substantial differences in

TABLE 13

**Parameters Characterizing the Urea and GdnHCl Denaturation of Three β-Lactoglobulins at pH 3.15, 25°C**

| | Denaturant | $(D)_{½}$, *M* | m, cal/mole/*M* | $\Delta G_{app}^{H_2O}$, kcal/mole |
|---|---|---|---|---|
| β-Lactoglobulin A | Urea | 5.43 | 1,870 | 10.2 |
| | GdnHCl | 3.45 | 3,830 | 13.2 |
| Goat β-lactoglobulin | Urea | 4.98 | 2,100 | 10.5 |
| | GdnHCl | 3.20 | 3,870 | 12.4 |
| β-Lactoglobulin B | Urea | 5.34 | 2,200 | 11.7 |

the GdnHCl denaturation curves, the midpoints of the thermal denaturation curves fell between 57 and 58°C. This is a good illustration of the fact that proteins with distinctly different stabilities in water at pH 7 may give rise to identical midpoints for a certain type of denaturation. The midpoint of a denaturation curve depends on the $\Delta G_D^{H_2O}$ value of the protein and on how $\Delta G_D$ varies with denaturant.

The midpoints of the GdnHCl denaturation curves are 1.05, 1.39, and 1.61*M* for the C domain, intact light chain, and V domain respectively. Thus, in this case, the domains have different stabilities, but not so much so that the denaturation curve for the intact light chain shows separate stages.

Azuma, Hamaguchi and Migita have studied the acid[115] and GdnHCl[116] denaturation of several different light chains. Most of their denaturation curves show two distinct stages, which indicates that for some light chains the domains differ sufficiently in stability to give rise to separable stages in the transition. From an analysis of the circular dichroism spectra at intermediate stages of unfolding, they show that the C domain is generally less stable than the V domain.

In every case studied to date, then, the V domain is as stable or more stable than the C domain. This is a surprising result. It is also interesting that light chains are considerably less stable than most globular proteins.

### E. Myoglobins

The acid-thermal denaturation of sperm whale myoglobin was first studied in detail by Hermans and Acampora.[81] More recently, Puett[71] has investigated the urea, GdnHCl, and acid denaturation of horse and sperm whale myoglobin, and Puett et al.[117] have studied the GdnHCl denaturation of bovine, human, and turtle myoglobin.

From acid denaturation curves and titration data, Hermans and Acampora[81] showed that $\Delta G_D^{H_2O}$ = 15.0 kcal/mole for whale myoglobin at 25°C, pH 7. Also from acid denaturation, but in solutions containing from 0 to 1.5*M* GdnHCl, Puett[71] obtained $\Delta G_D^{H_2O}$ values of 13.0 and 10.6 kcal/mole for whale and horse myoglobin respectively.

Analysis of the urea and GdnHCl denaturation of horse and whale myoglobin gives results quite different from those obtained with the four proteins in Table 2. The dependence of $\Delta G_D$ on urea and GdnHCl concentration for the two proteins is shown in Figure 23, and parameters characterizing these data are given in Table 14. In this case the $\Delta G_D^{H_2O}$ values obtained by linear extrapolation differ by more than 4 kcal/mole. Also, when $\bar{\alpha}$ values for the two denaturants are

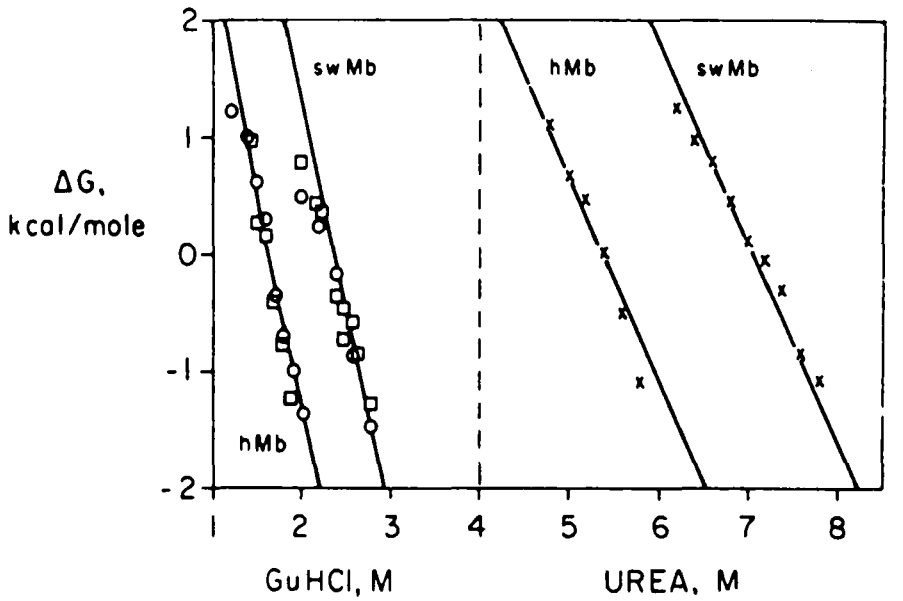



FIGURE 23. ΔG as a function of the urea (x) and GdnHCl (○,□) molarity for the denaturation of horse myoglobin (hMb) and sperm whale myoglobin (swMb). 0.1*M* KCl, 0.01*M* sodium phosphate. (Reproduced from Puett, D., *J. Biol. Chem.*, 248, 4623, 1973. With permission.)

TABLE 14

**Parameters Characterizing the Urea or GdnHCl Denaturation of Five Myoglobins at Neutral pH at 25°C**

| Myoglobin | $(D)_{½}$,*M | | m,* cal/mole/M | $G_D^{H_2O}$,* kcal/mole | $G_D^{H_2O}$† kcal/mole |
|---|---|---|---|---|---|
| Whale | 2.36 | GdnHCl | 2,600 | 6.1 | 13.2 |
| Horse | 1.63 | | 3,710 | 6.0 | 10.4 |
| Cow | 1.98 | | 3,190 | 6.3 | 11.3 |
| Human | 1.24 | | 4,990 | 6.2 | 8.8 |
| Turtle‡ | 1.13 | | 3,590 | 4.1 | 7.2 |
| Whale | 7.10 | urea | 1,460 | 10.4 | 13.0 |
| Horse | 5.39 | | 2,140 | 11.5 | 10.0 |

*Equation 10. These values were supplied by Dr. David Puett (personal communication). The experimental data are presented in References 71 and 117.
†Estimated using Tanford's Procedure[67] as described in text.
‡Data obtained at 5°C.

calculated using Tanford's method[2] (Section IV, Part B3), they differ by 0.13 for horse myoglobin and by 0.16 for whale myoglobin. Puett[71] has also applied Tanford's method[67] to these data, but he used some different $\delta g_{tr}$ values and a slightly different approach than outlined above. The most significant difference is in the $\delta g_{tr}$ values chosen for the peptide group. Puett[71] used one fourth of the $\delta G_{tr}$ values for N-acetyltetraglycine ethyl ester. We have used the same data, except that a correction was applied for the solubilizing effect of the non-peptide portions of the model. For myoglobin, Puett's approach[71] leads to much better agreement between the results from urea and GdnHCl, as can be seen in the last column of Table 14.

The GdnHCl results in Table 14 show clearly the importance of the method of extrapolation. Using linear extrapolation, the proteins appear to have similar stabilities, but using Tanford's procedure,[2] the stabilities differ by as much as 6 kcal/mole. In this case, the results based on linear extrapolation appear to be far out of line with results from urea denaturation, from acid denaturation, from calorimetry (Figure 19), and from hydrogen exchange (Table 7).

Schechter and Epstein[118] have shown that removing the heme from either horse or whale myoglobin shifts the midpoint of the urea denaturation curve to a 3.1*M* lower concentration. Thus, apomyoglobin is at least 4.5 kcal/mole less stable than myoglobin. On the basis of the hydrogen exchange of the last 10% of the hydrogens in these proteins, Ottesen[88] has estimated a $\Delta G_D^{H_2O}$ value of 13 kcal/mole for myoglobin and 9 kcal/mole for apomyoglobin, leading to a difference in reasonable agreement with the result from urea denaturation.

### F. Lysozyme

The denaturation of lysozyme appears to be closer to a two-state mechanism than the denaturation of any other protein. This, plus its low cost, has made it a favorite protein for denaturation studies. The acid-thermal denaturation has been studied by Sophianopoulos and Weiss,[119] and the combined effects of acid, temperature, and GdnHCl have been investigated by Ogashara and Hamaguchi,[80] and by Aune and Tanford,[66,120]

Sophianopoulos and Weiss[119] estimated $\Delta G_D^{H_2O}$ = 9.7 kcal/mole, using temperature extrapolation. Aune and Tanford[66,120] carried out a careful analysis of their data, in which they took into account the presence of the heat-denatured state along with the randomly coiled state. In the presence of 1*M* GdnHCl they showed that $\Delta G_D$ = 7.8 kcal/mole for unfolding to the heat-denatured state and $\Delta G_D$ = 7.9 kcal/mole for unfolding to the randomly coiled state. The m values for the two reactions are about 1,650 and 2,180 cal/mole/*M*, so, in the absence of GdnHCl, $\Delta G_D^{H_2O}$ values of about 9.5 and 10.1 kcal/mole are expected for unfolding to the heat-denatured and to the randomly coiled state respectively.

Imoto and Rupley[121] have shown that, when lysozyme is treated with iodine, tryptophan 108 is

covalently linked to the carboxyl of glutamic acid 35. This derivative of lysozyme has a thermal transition midpoint at least 20°C higher than that of native lysozyme. Consequently, they estimate that the ester cross-link adds more than 6 kcal/mole to the stability of lysozyme.

### G. α-Chymotrypsin

Biltonen and Lumry[122] have shown that $\Delta G_D^{H_2O}$ = 7.2 kcal/mole at pH 3, 25°C, for the acid-thermal unfolding of α-chymotrypsin. By unspecified procedures,[5] they estimate that $\Delta G_D^{H_2O}$ will be increased to 14 kcal/mole at pH 7. For unfolding to the random coil, we estimate $\Delta G_D^{H_2O}$ = 8.3 kcal/mole at pH 4.3, 25°C. The information needed to extrapolate this value to pH 7 is not available.

### H. Chymotrypsinogen

Brandts has analyzed the acid-thermal denaturation of chymotrypsinogen in detail.[95] His results suggest that $\Delta G_D^{H_2O}$ = 7.4 at 25°C, pH 3. Brandts' guess for the stability at neutral pH is 15 ± 5 kcal/mole for chymotrypsinogen (personal communication). Unfortunately, the denaturation of chymotrypsinogen cannot be studied above pH 3 because irreversible effects are observed.

### I. Other Proteins

The similarity of the amino acid sequences of α-lactalbumin and lysozyme led to a suggestion that these proteins evolved from a common ancestor.[123] This prompted a number of comparative studies of the physical properties of the two proteins. Sharma and Bigelow[124] have carried out studies on α-lactalbumin similar to those shown in Figure 3 for lysozyme. They show that the denatured states produced by the different denaturants are very similar for the two proteins. Sugai et al.[72] have studied the equilibrium and kinetics of the unfolding of α-lactalbumin by GdnHCl. The kinetics of folding and refolding differ quantitatively from those of lysozyme, but are consistent with a two-state mechanism. The authors analyzed their equilibrium data using Equation 12, and the results are given in Table 8. Despite the similarity in structure, lysozyme appears to be twice as stable as α-lactalbumin.

Growth hormone is a globular protein with a molecular weight of about 22,000. Holladay et al.[48] have studied the GdnHCl denaturation of rat, bovine, and ovine growth hormone. The midpoints of the denaturation curves are found to occur at lower GdnHCl concentrations when using difference absorption measurements at 290 nm than when using circular dichroism measurements at 222 nm. This indicates that denaturation is not a two-state process. By guessing at the mechanism and using Tanford's method[2] for extrapolation, they find $\Delta G_{app}^{H_2O}$ values of 14.0, 9.7, and 8.0 kcal/mole for bovine, ovine, and rat growth hormone respectively. While these values are uncertain, it does appear that there are sizeable differences in stability among these homologous proteins.

The denaturation of hemoglobin by acid,[125] urea,[126] or GdnHCl[117] is considerably more complex than the corresponding denaturation of myoglobin. This complexity is also found in the denaturation of isolated hemoglobin subunits[117] and in single-chain hemoglobins.[126] As a consequence, only qualitative data are available on the difference in stability between hemoglobins from different species[127] and on the effect of different ligands[126] on hemoglobin stability.

The unfolding of human serum albumin by GdnHCl is unique.[128] A low pH (below the N to F conformational change) the unfolding is continuous; the protein begins to unfold at 0*M* GdnHCl and is still unfolding at 8*M* GdnHCl. At higher pH values the transition becomes steeper, and distinct steps in the transition are evident. The low cooperativity observed and the complexity of the unfolding are consistent with the multidomain structure suggested for serum albumins as well as with the known microheterogeneity.[129]

The unfolding of enterotoxin B from *Staphylococcus aureus* by urea, GdnHCl, and acid has been investigated by Warren et al.[130] The midpoints of the urea and GdnHCl denaturation curves for enterotoxin B are typical of those found for other globular proteins; however, the time required for unfolding is much greater. In 7*M* urea the unfolding of this protein requires about 6 days to reach equilibrium. This indicates, of course, a large activation energy for unfolding. Kinetic studies of the folding and unfolding are consistent with a two-state mechanism.

Some bacteria grow best at temperatures where many of their proteins are expected to be thermally unfolded. Stellwagen and co-workers[131, 132] have characterized the enzyme enolase from two of these thermophilic bacteria. In both cases the native enzyme is a globular protein containing

eight apparently identical subunits. One of the enzymes has optimal activity at a temperature of 90°C. As anticipated, both enzymes are much more heat-stable than the enolase from rabbit and yeast. Despite this remarkable heat-stability, the enzymes are inactivated at relatively low GdnHCl concentrations. The authors suggested that increased hydrogen bonding is more important to enhanced thermal stability than increased hydrophobic bonding.

## VI. CONCLUDING REMARKS

Globular proteins are not very stable. The best available estimates of $\Delta G_D^{H_2O}$ are given in Table 15. On the basis of these and less reliable results, it appears that the native state of a globular protein will be from 5 to 15 kcal/mole more stable than unfolded states under physiological conditions. This stability is remarkably low when you consider that the dissociation energy of an aliphatic carbon-carbon bond is more than 80 kcal/mole or that burying a single tryptophan side chain in the interior of the globular protein contributes more than 3 kcal/mole to the stability of the protein.

The marginal stability of globular proteins may be important to the control of metabolism in the cell.[133] One important mechanism of metabolic control is the regulation of enzyme concentration. The concentration of an enzyme depends on both its rate of synthesis and its rate of degradation. Much more is known about the mechanism and control of synthesis than about degradation.[133] For most enzymes it seems likely that degradation is carried out by non-specific proteolytic enzymes, which act on unfolded conformations of the enzyme.[134] Berlin and Schimke[135] have shown that small changes in the rate of degradation can cause sizeable changes in the steady-state concentration of an enzyme. Thus, the steady-state level of an enzyme may depend in part on its stability. It is clear from this that changes in the stability of an enzyme can influence the metabolism of the cell even though the change in stability has no effect on the activity of the enzyme. In this same way, the differences in stability among homologous proteins may be important in connection with their evolution.

Edsall[142] has suggested that there may be very few amino acid sequences for which the globular conformation is most stable. The low stability observed for globular proteins and the fact that even small changes in structure, such as removing one terminal residue or cleaving one peptide bond, generally decrease the stability support this idea.

## ACKNOWLEDGMENTS

I am grateful to Sheryl Pace for her help in preparing this review. I thank Dr. John Brandts, Dr. Jan Hermans, and Dr. David Puett for sending me unpublished results. The research from my laboratory was supported by Grant #A-403 from the Robert A. Welch Foundation.

TABLE 15

**Estimates of the Stability of Globular Proteins under Physiological Conditions***

| Protein | Denaturant | $\Delta G_D^{H_2O}$, kcal/mole |
|---|---|---|
| Lysozyme | GdnHCl or Urea | 9.5 |
| | T-pH | 10 |
| Ribonuclease | GdnHCl or Urea | 8.5 |
| | T-pH | 6 |
| Myoglobin | pH | 14 |
| α-Chymotrypsin | T-pH | 14 |
| Immunoglobulin light chain (Wes) domain | GdnHCl | 5.5 |

*See Section V for references.

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