Thermodynamics of the unfolding of β-lactoglobulin A in aqueous urea solutions between 5° and 55°C

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Thermodynamics of the Unfolding of β-Lactoglobulin A in Aqueous Urea Solutions between 5 and 55°*

N. C. Pace and Charles Tanford

ABSTRACT: This paper presents an experimental study of the denaturation of β-lactoglobulin by urea at pH 2.5–3.5, and at several temperatures. It is shown that the product of denaturation is probably completely disordered, without residual noncovalent structure. The transition between the native and unfolded states appears to be a reversible two-state process, without stable intermediates, at least between 10 and 55°. This permits the equilibrium data to be represented in terms of an equilibrium constant, K = (fraction of unfolded molecules)/(fraction of native molecules). It is found that K is strongly dependent on the urea concentration, being proportional to the 17th power of the urea concentration at 25°. The most interesting result, however, is its temperature dependence. K is largest at low and high temperature, and has a minimum value near 35°. This reflects a change in the ΔH for the reaction, which passes from negative values at low temperature (−40 kcal/mole at 15°) to zero near 35° to positive values at high temperature (+40 kcal/mole at 52°). The change in ΔH with temperature indicates that there is a large change in heat capacity associated with the unfolding reaction.

A value of approximately 2100 cal/deg per mole was observed over a wide range of temperature and urea concentrations. ΔC_p values of similar magnitude have been reported for unfolding reactions of other proteins, induced by heating as well as by the addition of urea, and they are probably a characteristic feature of all processes of this kind.

Considerable progress has been made in recent years toward an understanding of the process of protein denaturation. Work has been carried out to characterize the product of denaturation (Tanford *et al.*, 1967a,b; Nozaki and Tanford, 1967b; Aune *et al.*, 1967), to study the thermodynamics and kinetics of the transition from native to denatured states (Hermans and Scheraga, 1961; Scott and Scheraga, 1963; Brandts, 1964a; Hermans and Acampora, 1967), and to provide a theoretical basis for the results obtained (Tanford, 1964; Brandts, 1964b). In addition, studies have been made with model compounds which aim to correlate the behavior of proteins with that of smaller molecules bearing similar kinds of chemical groups (Nozaki and Tanford, 1963; Robinson and Jencks, 1965).

Most of the studies which we have cited have focussed on isothermal denaturation by urea or guanidine hydrochloride at room temperature or on thermal denaturation in the absence of a denaturing agent. The purpose of the present study, which considers the combined effects of urea and temperature on β-lactoglobulin, is to provide a link between these two kinds of denaturation processes. Parallel studies using guanidine hydrochloride are in progress, and a few preliminary results are quoted in this paper.

Several previous studies of the combined effects of temperature and of urea have been reported. The most noteworthy of them is the investigation of the urea denaturation of ovalbumin by Simpson and Kauzmann (1953). Unfortunately, the reaction turned out to be slow and irreversible, so that kinetic data and not thermodynamic data were obtained. Nevertheless, some parallels with the thermodynamic data reported in this paper will be shown to exist. Other previous work includes the study of Foss and Schellman (1959) with ribonuclease, and an earlier study by Christensen (1952) of β-lactoglobulin. Schellman (1958b) has investigated the effect of temperature on the product of the urea denaturation of β-lactoglobulin, but not the effect of temperature on the transition from native to denatured states. A preliminary study of the denaturation of β-lactoglobulin by urea at a single temperature has been reported by one of us (Tanford and De, 1961).

Experimental Procedure

Materials. Crystalline β-lactoglobulin A was generously provided by Drs. R. Townend and S. N. Timasheff of the Eastern Regional Research Laboratory, U. S. Department of Agriculture, Philadelphia, Pa. The protein was recrystallized before use.

Calbiochem A grade urea was recrystallized from 80% ethanol. During recrystallization, the temperature was not permitted to exceed 50°. The urea was dried in a vacuum desiccator over P_2O_5. Aqueous stock solutions were prepared on a weight basis, and their molar concentrations were calculated with the aid of densities given by Kawahara and Tanford (1966). Stock solutions

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were stored frozen, at −20°. No detectable decomposition occurred during storage at this temperature.

Guanidine hydrochloride was prepared as described previously (Nozaki and Tanford, 1967a). Solutions of HCl were prepared from constant-boiling HCl provided for us by Dr. Y. Nozaki. Potassium chloride was analytical reagent grade. Water was glass redistilled.

Methods. Protein stock solutions were obtained by dissolving the recrystallized protein in aqueous KCl solutions. Protein concentrations were determined by evaporating to dry weight at 107° and correcting for the known KCl content. Solutions for measurement were prepared on a volume basis from stock solutions of protein and the other reagents. The solutions were always adjusted to a final ionic strength of 0.15.

All pH measurements were made at room temperature, with a Radiometer Model 22 pH meter. The pH generally changed by less than 0.10 pH unit in the course of the transition from native to denatured protein. The values reported are those for the midpoints of the transition.

Optical rotation measurements were made with a Cary Model 60 spectropolarimeter. The results are reported as reduced specific rotations, $[\alpha']$, defined in terms of the observed specific rotation, $[\alpha']$, and the refractive index, n, by the relation $[\alpha'] = 3[\alpha]/(n^2 + 2)$. Refractive index values were based on the measurements of Foss *et al.* (cited by Fasman, 1963), and, for guanidine hydrochloride solutions, on those of Kielley and Harrington (1960), corrected for wavelength as described by Hooker (1966). Virtually all measurements reported were carried out at a single wavelength, 365 mμ. Protein concentration was usually about 0.5 g/100 ml. Quartz cells with a path length of 1 cm were used.

Temperatures were generally maintained within ±0.1° of nominal values. In isothermal experiments (Figure 1) each experimental point represents a freshly prepared individual solution. In experiments at constant urea concentration (Figure 10) a single solution was carried from the lowest to the highest temperature, as rapidly as possible, by using a circulating bath of small volume to supply the constant-temperature block of the spectropolarimeter. The longer accumulated exposure to high temperature in the latter type of experiment led to no significant irreversible denaturation.

Results

Figure 1 shows a typical isothermal denaturation curve, as measured by following the optical rotation at 365 mμ. The dominant feature is the more than threefold increase in the magnitude of $[\alpha']$ between 3 and 7 M urea, which represents the transition from the native to the unfolded state. This is the reaction which this paper will analyze in some detail.

The Native State. Native β-lactoglobulin does not represent a unique conformation of the protein molecule. It is known that the protein can exist in different states of aggregation (Townend *et al.*, 1960; Timasheff and Townend, 1961; Herskovits *et al.*, 1964). It is also known that there are two pH-dependent conformational

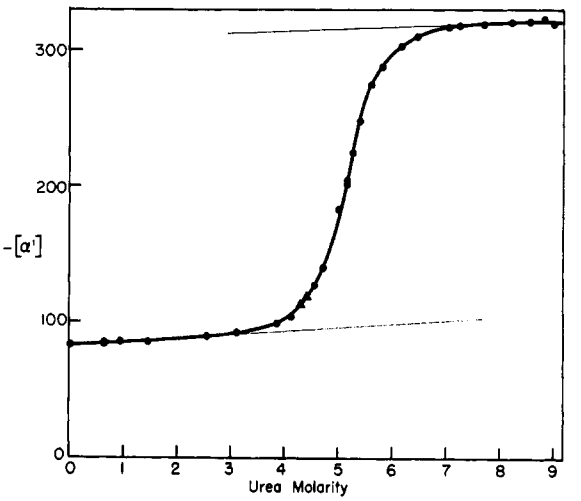

FIGURE 1: Typical isothermal denaturation curve. Temperature 24.8°, pH 2.77, ionic strength 0.15. The triangles represent solutions which were exposed to 8 M urea for about 10 min and then diluted to the final urea concentration. Optical rotations were measured at 365 mμ.

changes, a minor one near pH 5 (Timasheff *et al.*, 1966), and a more extensive one near pH 8 (Tanford and Taggart, 1961). The experiments described in this paper were carried out with β-lactoglobulin solutions initially between pH 2.5 and 3.5, *i.e.*, outside the range of the pH-dependent conformational changes. The effect of temperature on these solutions, as well as the initial addition of urea (Figure 1), produced only a small monotonic increase in levorotation preceding the onset of the major transition. The observed changes were much smaller than those associated with the N–R transition (Tanford and Taggart, 1961), *i.e.*, this transition can evidently not be induced at low pH, prior to denaturation, by changes in temperature or urea concentration. No attempt was made to determine whether the observed change in optical rotation might reflect in part the pH 5 transition of Timasheff *et al.* (1966), shifted by urea or temperature to lower pH. The change in rotation which accompanies this transition is insignificantly small in comparison with the change which accompanies the major transition under study here.

Sedimentation velocity experiments were carried out to determine the state of aggregation. At pH 3, at room temperature, in the absence of urea, β-lactoglobulin consists of a mixture of monomer (molecular weight 18,000) and dimer (Townend *et al.*, 1960). The effect of urea was found to promote dissociation to the monomer. Dissociation was complete at urea concentrations of 3 M and above. Since virtually all the data of this paper pertain to the transition from native to denatured state between 4 and 6 M urea, we are evidently dealing with monomeric native protein.

The native state, as defined here, may therefore be taken to be monomeric β-lactoglobulin, existing either

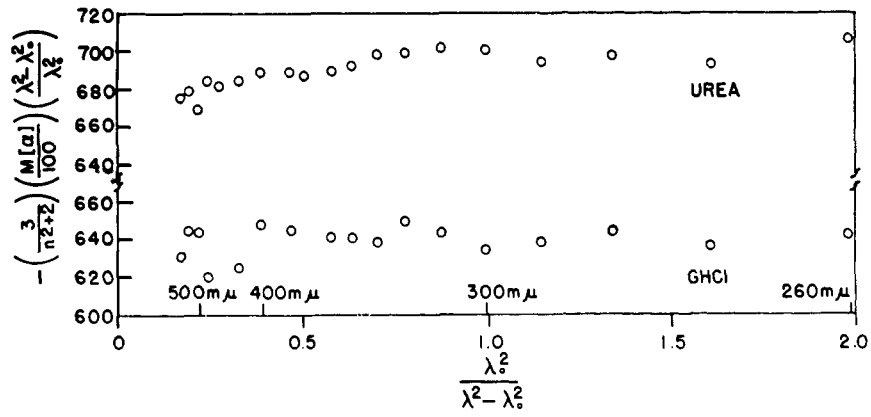

FIGURE 2: Optical rotatory dispersion of β-lactoglobulin denatured by urea (upper points) and guanidine hydrochloride (lower points.) The data are shown in the form of Moffitt–Yang (1956) plots.

in a uniquely folded conformation, or possibly in an equilibrium mixture of two or more closely related compact conformations. To determine the optical rotation of the native state in the transition region we have simply extrapolated the initial monotonic change, as shown by Figure 1. Extrapolations of this kind were performed at several temperatures and pH values so that the value of $[\alpha']$ which characterizes the native state is known as a function of pH and temperature at any concentration of urea.

Denatured State. Previous studies (Tanford *et al.*, 1967a,b; Nozaki and Tanford, 1967b; Lapanje and Tanford, 1967) have shown that proteins which have been denatured by guanidine hydrochloride behave as fully disordered random coils, without residual noncovalent structure. This conclusion was shown to hold true for proteins which possess disulfide bonds, as well as for reduced proteins, which are linear chains without cross-links. The disulfide bonds, when present, restrict the freedom of expansion of the polypeptide coil, but appear to have no other influence.

It is probable that β-lactoglobulin which has been denatured by urea is also randomly coiled, or very close to it. The intrinsic viscosity, for example, is essentially the same in 8 M urea as in 6 M guanidine hydrochloride.[1] Optical rotatory dispersion data obtained as part of the present study provide additional evidence. Figure 2 shows optical rotation measurements in both solvents, plotted according to the equation of Moffitt and Yang (1956). The slope represents the Moffitt–Yang parameter b_0, which is seen to be close to zero for both plots. The intercept, representing the Moffitt–Yang parameter a_0, is a few per cent more negative in the urea solution than in the guanidine hydrochloride solution. This difference does not imply a difference in conformation, but would appear to reflect an effect of the solvent on the optical rotation. Figure 3 shows this effect as measured at 365 mμ. Whereas urea increases the magnitude of the rotation, guanidine hydrochloride decreases it. Extrapolation to zero concentration yields values of $[\alpha']$ which are identical within experimental error.

An additional indication that β-lactoglobulin is as disordered after urea denaturation as after guanidine hydrochloride denaturation is provided by the temperature dependence of $[\alpha']$. For a randomly coiled polymer, the magnitude of $[\alpha']$ should diminish with increasing temperature (Kauzmann and Eyring, 1941; Schellman, 1958a) because the freedom of rotation about single bonds becomes greater at higher temperature and the polymer chain accordingly becomes less stiff. As Figure 4 shows, this effect is observed for β-lactoglobulin both in concentrated urea and in concentrated guanidine hydrochloride solution. The slope $d[\alpha']/dT$ is actually somewhat greater in urea than in guanidine hydrochloride. Both the larger value of $[\alpha']$ itself (Figures 2 and 3) and the larger temperature dependence may be explained by the fact that these studies are being carried out at low pH, where the protein carries a considerable excess of positive over negative charges. Mutual repulsion between these charges will lead to stiffening of the disordered polypeptide chain in urea solutions. In guanidine hydrochloride solutions, on the other hand, the very high ionic strength will largely eliminate this effect. The polypeptide chain will be less rigid, with a smaller $[\alpha']$, and a reduced dependence on temperature. The values of $[\alpha']$ which characterize the fully denatured protein in the transition region, at any pH, temperature, and urea concentration, were obtained, as for the native protein, by extrapolation of data such as those of Figures 1, 3, and 4.

Reversibility of the Transition. The equilibrium data presented in this paper represent true thermodynamic equilibria. Solutions exposed to high urea concentrations, and then diluted, gave rotations which agreed with those obtained at the final urea concentration

[1] S. Lapanje (unpublished data) obtained $[\eta] = 21.6$ cc/g for β-lactoglobulin in urea + β-mercaptoethanol and $[\eta] = 16.2$ cc/g in urea alone (disulfide bonds intact). The corresponding values in 6 M guanidine hydrochloride (Tanford *et al.*, 1967a) are 22.8 and 17.1 cc/g, respectively. The measurements were made near the isoelectric point of the protein, so that the expansion of the random coil which (as discussed in the text) is expected at low pH does not occur.

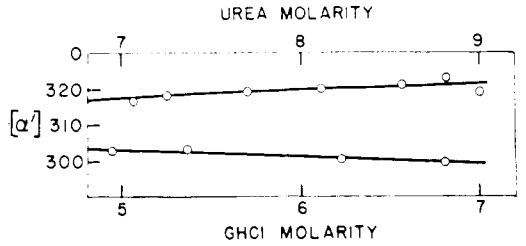

FIGURE 3: Solvent effects on the optical rotation (at 365 mμ) of β-lactoglobulin after complete denaturation by urea (upper line) and guanidine hydrochloride (lower line).

without previous exposure to a higher concentration. Two experimental points which illustrate this finding are shown in Figure 1. Similarly, denaturation by heating or cooling at constant urea concentration was found to be completely reversible, except that long exposure to temperatures near 50° produced a trace of irreversibility. The much more marked irreversibility which attends the urea denaturation of β-lactoglobulin at higher pH (Christensen, 1952; Kauzmann and Simpson, 1953) was not observed by us. This effect of pH suggests that the irreversible reaction involves interchange between the free SH group and the disulfide bond of β-lactoglobulin.

The Possible Existence of Stable Intermediate States. The transition under consideration here is a transition between native, globular β-lactoglobulin, characterized by an $[\alpha']_{365}$ value near −90°, and a denatured state, which is completely unfolded by all available criteria, and is characterized by an $[\alpha']_{365}$ value, which, at 25°, is near −310°. It is obvious that the protein must pass through many intermediate states in the transition from the native to the unfolded form, as successive portions of the polypeptide chain are unravelled. Such intermediate forms of the protein may be of two kinds. They can be unstable transition states which never make a significant contribution to any measured physical parameter, or they can be states which have some stability over a limited range of temperature or urea concentration.

We have obtained evidence concerning this question by analysis of the kinetics of the transition, as was done in a previous paper from this laboratory (Tanford *et al.*, 1966) for the unfolding of lysozyme by guanidine hydrochloride. Studies were carried out at several urea concentrations, at 15.5°. Measurements of [α'] were made as a function of time, and were plotted in the form of first-order kinetic plots, as shown in Figure 5. The final value of [α'], designated as $[\alpha']_\infty$, was taken from equilibrium curves such as Figure 1, and the value of $[\alpha']_N$ is the rotation of the native protein at the appropriate urea concentration.

As Figure 5 shows, straight-line plots were obtained, as in the previous study with lysozyme, indicating that the transition obeys first-order kinetics. The fact that the ordinate extrapolates to zero at zero time demonstrates that [α'] at zero time is equal to $[\alpha']_N$, or, in other words, that the first-order kinetics govern the

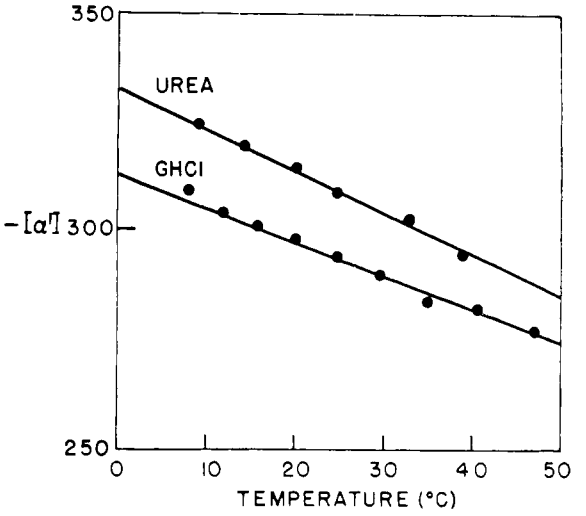

FIGURE 4: Effect of temperature on the optical rotation (at 365 mμ) of β-lactoglobulin denatured by urea (upper line) and guanidine hydrochloride (lower line).

entire transition from the native state to the equilibrium state, and not just a portion thereof. Such a result is possible only if all intermediate states are unstable. If stable intermediates occur, then the kinetics would follow the laws for successive (and, necessarily, successively slower) first-order reactions, and a first-order plot would be curved rather than linear, as was found, for example, by Simpson and Kauzmann (1943) for the urea denaturation of ovalbumin.

These data of course show only that molecules in intermediate states of unfolding do not represent a significant fraction of all protein molecules, as one passes from the native to the unfolded state, as a function of time, at constant urea concentration. One might expect, however, that the conclusions would also apply as one proceeds from the same initial to the same final state, at equilibrium, by progressively increasing the urea concentration. In any event, we have taken Figure 5 as evidence that no intermediate states make a contribu-

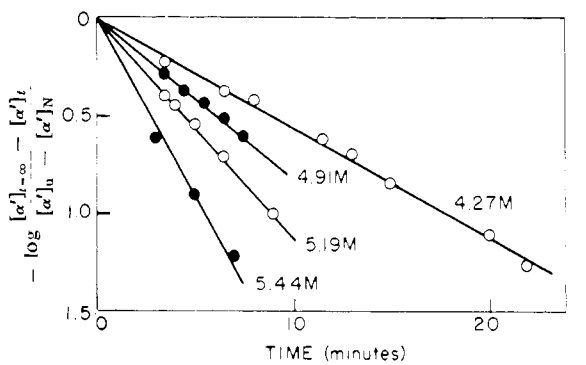

FIGURE 5: First-order kinetic plots for the unfolding reaction at pH 2.8, 15.5°.

tion to equilibrium data such as those of Figure 1 and other such data to be cited subsequently. It is recognized, however, that the results do not represent absolute proof of this assertion.[2]

Dependence of the Equilibrium Constant on the Concentration of Urea and on pH. In the absence of stable intermediates, the unfolding reaction becomes a two-state transition, in which all molecules under any experimental conditions exist either in the native (N) or completely unfolded (U) state. The observed rotation under any conditions is then a direct measure of the fraction of molecules (f_u) in the unfolded state, *i.e.*

$$[\alpha'] = (1 - f_u)[\alpha']_N + f_u[\alpha']_u \quad (1)$$

where $[\alpha']_N$ and $[\alpha']_u$ represent the rotations of the native and unfolded states at any urea concentration, determined by extrapolation, as described earlier. A value for the equilibrium constant (eq 2) is thus determined

$$K = f_u/(1 - f_u) \quad (2)$$

directly from each measured value of $[\alpha']$.

As in previous studies of this kind (Tanford, 1964), log K is found to be a linear function of the logarithm of the urea concentration over the major portion of the transition region. We can therefore describe the reaction (approximately between $f_u = 0.1$ and 0.9) by the relation

$$K = K_0C^n \quad (3)$$

where C is the urea concentration in moles per liter, and K_0 is a constant which has no physical significance since eq 3 will not hold true to $C = 1$. In tabulating the data it is sometimes convenient to use the concentration, $C_{1/2}$, at the midpoint of the transition, *i.e.*, where $K = 1$. This parameter is equal to $(1/K_0)^{1/n}$.

Typical plots of log K *vs.* log C are shown in Figure 6. Tabulated values of the parameters $C_{1/2}$ and n, at 25°, are given in Table I. The effect of pH on $C_{1/2}$, at several temperatures, is shown in Figure 7.

It is evident from Table I and Figure 7 that the effect of pH on the transition, within the narrow range of pH studied here, is very small. This proved to be useful in the investigation of the effect of temperature on the transition. Results obtained at slightly different pH values could be adjusted by use of these data to a common pH. It may be noted that ionic strength was found to have a more pronounced effect on the equilibrium than pH, but no detailed studies were carried out. All the results presented here were obtained at ionic strength 0.15.

TABLE I: Values of $C_{1/2}$ and n as a Function of pH at 24.8°.

pH	$C_{1/2}$ (moles/l.)	n of Eq 3
2.66	5.10	17.0
2.77	5.14	15.9
2.82	5.18	16.8
2.86	5.21	16.7
3.14	5.43	16.9
3.43	5.72	17.8

Dependence of Rate Constants on the Urea Concentration. A two-state first-order transition is described completely by two rate constants, k_1 and k_2, for the forward and reverse directions of the reaction $N \rightleftharpoons U$, respectively. The slopes of Figure 5 yield values of $k_1 + k_2$ (Tanford *et al.*, 1966), and k_1 and k_2 may be evaluated separately by combining these results with the equilibrium constant, $K = k_1/k_2$. Values of k_1 and k_2, at the single temperature at which kinetic measurements were made, are shown as a function of C in Figure 8. It is seen that k_1 is much more strongly dependent on C than k_2. This is the opposite result from that observed for the unfolding of lysozyme by GuHCl (Tanford *et al.*, 1966).

Effect of Temperature on the Equilibrium Constant. The effect of temperature on the reaction was determined in two ways: (1) by determining isothermal unfolding curves (Figure 1) at several temperatures, and (2) by determining the effect of temperature on the extent of unfolding at constant urea concentration. Results of the first kind are summarized in Figure 6. The results show that the unfolding reaction (between 2 and 35°) is shifted to higher urea concentration by an increase in temperature. Between 2 and 35°, the reaction is clearly exothermic. The data show that temperature affects the slope of logarithmic plots as well as the value of $C_{1/2}$, *i.e.*, the parameter n of eq 3 is evidently temperature dependent. A plot of n as a function of temperature is shown in Figure 9.

Figure 10 shows experiments of the second kind, *i.e.*, heating curves at constant urea concentration. The rotations for native and denatured protein as given in the figure apply to a urea concentration of 5.09 M. Slightly different values apply to the other two urea concentrations. Figure 11 is a plot of $C_{1/2}$ *vs.* temperature, using data from both Figures 6 and 10 after adjustment to a common pH of 3.0, to show that the results from the two kinds of experiments are self-consistent. Figure 12 shows the results of Figure 10 in the form of van't Hoff

[2] The data of Figure 5 are of poorer quality than our earlier data for the unfolding of lysozyme by guanidine hydrochloride (Tanford *et al.*, 1966). This is because the β-lactoglobulin transition is a faster one and difficult to study by optical rotation measurements. For the same reason, no kinetic data at all are reported for temperatures above 15.5°, as the rate increases with increasing temperature. Measurement of difference spectra can be made more quickly than measurement of optical rotation, and kinetic data for the unfolding of β-lactoglobulin (by guanidine hydrochloride rather than urea) are currently being obtained by Dr. T. Takagi. He has shown that the reaction is strictly first order in both forward and reverse directions at 25°, over a wide range of guanidine hydrochloride concentrations. The first-order behavior breaks down under certain conditions, however, notably in the study of renaturation at very low concentrations of denaturant.

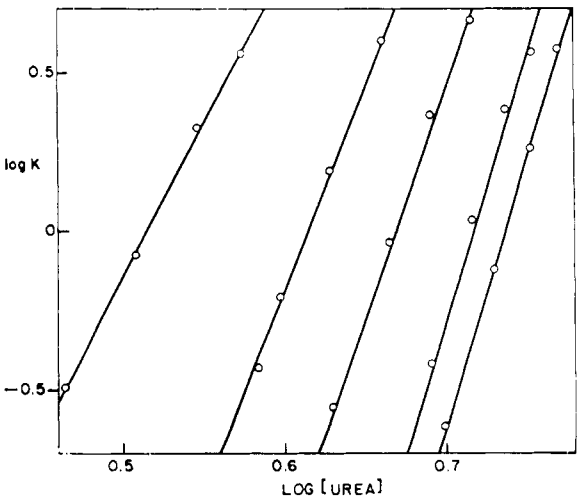

FIGURE 6: The equilibrium constant for the reaction as a function of urea concentration. The five lines represent, from left to right: 2.0°, pH 2.66; 9.3°, pH 2.80; 15.5°, pH 2.79; 24.8°, pH 2.82; and 34.7°, pH 2.95.

plots of the logarithm of the equilibrium constant *vs.* the reciprocal of the temperature.

These data clearly show that the increased stability of the native protein with an increase in temperature is reversed above about 35°, *i.e.*, the unfolding reaction is evidently exothermic below 35° and endothermic above that temperature. Since we are assuming that the reaction is a two-state process at all temperatures, we can determine the enthalpy of the transition, $\Delta H = -R\mathrm{d}(\ln K)/\mathrm{d}(1/T)$, from the slopes of the curves of Figure 12. Results for one experiment (at 4.48 M urea, pH 2.50) are shown in Figure 13. Similar plots were obtained from all three curves of Figure 10 and from a number of similar curves at other urea concentrations.

Figure 13 shows that ΔH for the transition is strongly temperature dependent, increasing linearly with temperature between about 10 and 55°. There is a break in the

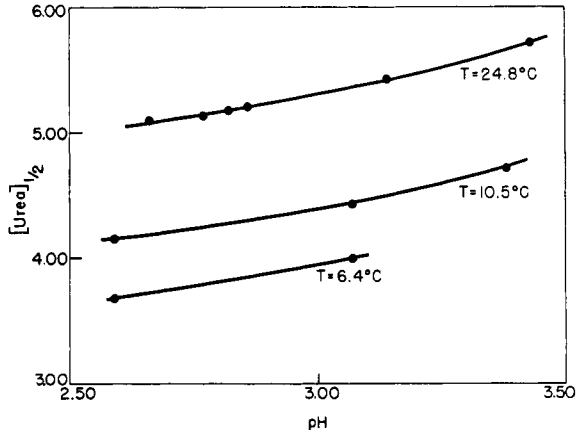

FIGURE 7: Effect of pH on the midpoint of the transition. These data were used to adjust results from different experiments to a common pH, as in Figure 11.

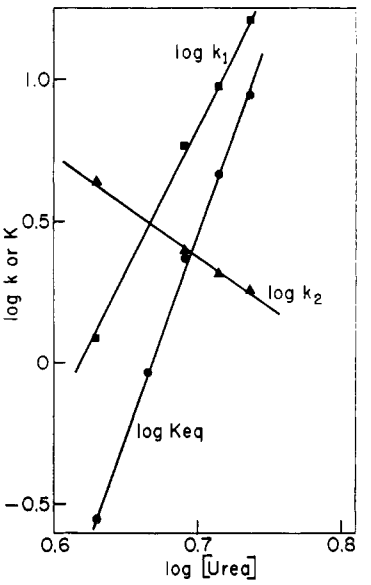

FIGURE 8: Comparison of the effects of urea concentration on the equilibrium constant and on the rate constants for the forward and reverse reaction, at 15.5°, pH 2.8.

curve below 10°, which is seen in all curves of this kind. A similar break in the dependence of ΔH on temperature results from direct analysis of the data of Figure 11. The experimental uncertainty in the determination of ΔH becomes quite large at the extremes of the temperature range, and we cannot be quite certain that the change in the slope of Figure 13 is experimentally significant. If it is, it may signify that the transition ceases to be a two-state transition below 10°.

Since $\mathrm{d}(\Delta H)/\mathrm{d}T$ is equal to ΔC_p, the data presented here indicate that there is a large difference in heat capacity between the unfolded and native proteins. Between 10 and 55°, ΔC_p is approximately independent of temperature. The value obtained from the data of Figure 13 is 2160 cal/mole per degree.

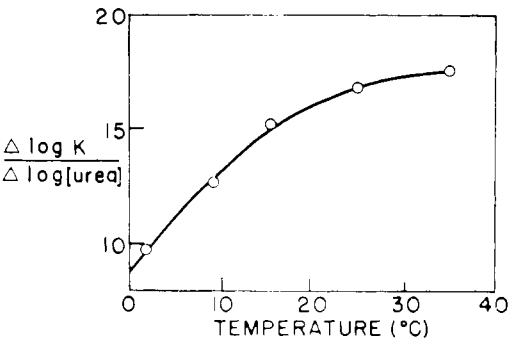

FIGURE 9: Effect of temperature on the parameter n of eq 3. Values of n were obtained from Figure 6 and adjusted to pH 3.0.

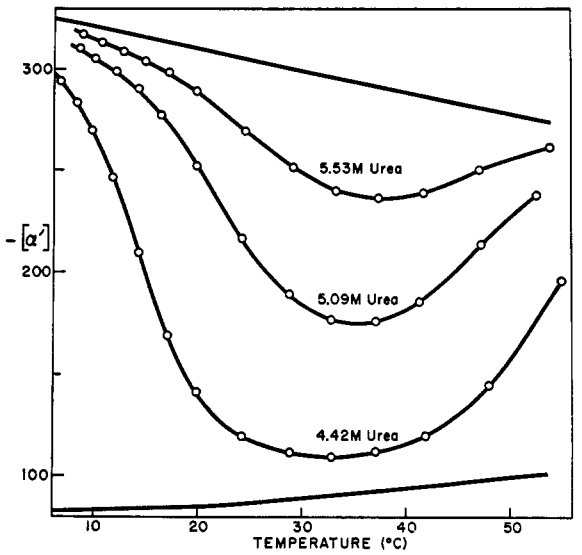

FIGURE 10: Typical heating curves at constant urea concentration. Rotations were measured at 365 mμ. The pH values differed slightly, but were all between pH 2.5 and 3.5. The upper and lower curves represent the [α'] values for completely unfolded and completely native protein at 5.09 M urea. The reference rotations at the other urea concentrations would be slightly different.

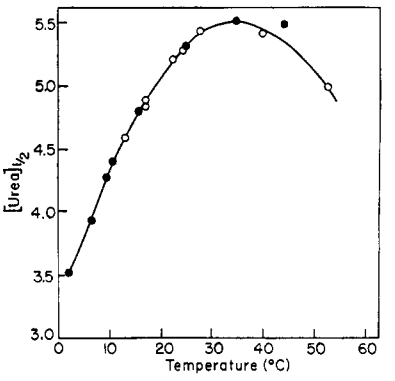

FIGURE 11: Test for consistency between isothermal denaturation curves, as in Figure 1 (filled circles), and heating curves, as in Figure 10 (open circles). The urea concentrations for 50% completion of the reaction were adjusted to a common pH of 3.0 with the aid of Figure 7.

The results shown in Figure 13 are typical of the results of a number of experiments of this kind, at different pH values and urea concentrations. A similar result was obtained from one heating experiment carried out in guanidine hydrochloride. All results are summarized in Table II. The differences between the individual experiments are probably within experimental error, and the data indicate that the negative ΔH value (at 25°) and the very large ΔC_p are characteristic of the unfolding reaction, and do not depend in any marked degree on the precise conditions under which the transition occurs.

TABLE II: Enthalpy and Heat Capacity Change.[a]

Urea Concn (moles/l.)	pH	ΔH at 25° (kcal/mole)	ΔC_p (cal/deg mole)
4.42	2.50	−18	2130
4.48	2.50	−19	2160
5.01	2.71	−21	2030
5.03	3.00	−21	2150
5.09	2.57	−23	2330
5.27	2.78	−21	1940
5.53	2.55	−20	1860
3.37 M guanidine hydrochloride	3.20	−21	2100

[a] Summary of all heating curves of the type shown in Figure 10.

Discussion

A number of recent studies have shown that there are remarkable similarities between the thermodynamic parameters which characterize the unfolding of several of the smaller globular proteins to predominantly disordered states. These similarities apply to unfolding by guanidine hydrochloride and urea, the products of which appear to be random coils without residual noncovalent structure, as well as to thermal transitions in aqueous salt solutions at low pH, although the products of the latter process are only partially disordered and may contain about 25% of residual globular structure. It is interesting to note that these same characteristics are shared by the activation parameters determined from kinetic studies of the irreversible unfolding of ovalbumin by urea by Simpson and Kauzmann (1953). The similarity between the latter data and those of the present paper are particularly striking, despite the fact that they represent the thermodynamics of transition to the activated complex for unfolding, rather than to the final product.

These similarities suggest that the results of the present paper should be discussed in the light of all the results available for unfolding of all globular proteins, with a view to formulating a unified general theory of the unfolding reaction. Such an analysis would however become too lengthy for a journal article such as this one, because there is not yet a consensus on the underlying causes of the observed phenomena, *i.e.*, several possible explanations would have to be considered and discussed for each aspect of the experimental results. Accordingly, a general analysis will be postponed to a later date, and the discussion presented here will be confined to "remarks" which pertain directly to the data presented in this paper.

Two-State Transitions. It is now evident that most

transitions of the type considered here are two-state transitions without stable intermediates within the range of conditions under which the transition occurs. The evidence comes not only from kinetic data, such as we have reported, but from a variety of other sources (Lumry *et al.*, 1966). There are of course some unfolding transitions in which stable intermediates do occur, such as the urea denaturation of ovalbumin, which has already been mentioned, and the denaturation of paramyosin by guanidine hydrochloride (Riddiford, 1966). As was pointed out earlier, the urea denaturation of β-lactoglobulin may become separable into distinct stages below 10°. Preliminary kinetic data (*cf.* footnote 2) suggest that the reaction U → N at low concentrations of denaturant may also be separable into two or more successive stages.

The Value of n. For a two-state transition, the value of n is related to the free-energy difference ($\Delta G°_u$) between native and unfolded states by the relation (Tanford, 1964)

$$RTn = -[\partial(\Delta G°_u)/\partial \ln C]_{C=C_{1/2}} \qquad (4)$$

This relation is true regardless of the mechanism by which a change in the concentration of urea affects $\Delta G°_u$.

Most mechanisms which have been suggested for the action of urea or guanidine hydrochloride[3] involve many weak interactions along the entire length of a polypeptide chain, rather than a small number of strong interactions (such as binding at a few specific residues). This would tend to make the effect of the denaturating agent on $\Delta G°_u$ (at constant temperature) proportional to the length of polypeptide chain involved. Thus the quantity $\delta\Delta G°_u$ defined previously (Tanford, 1964) or derivatives such as $\partial(\Delta G°_u)/\partial \ln C$ would become proportional to the length of polypeptide chain involved in the transition, and so would the parameter n of eq 4.[4] This permits an approximate interpretation of the values of n for the rate constants k_1 and k_2 (Figure 8) and for the differences noted in the text between the present data for the β-lactoglobulin–urea reaction and our earlier result for the lysozyme–guanidine hydrochloride reaction (Tanford *et al.*, 1966). Since the n value for k_1 is much larger than that for k_2, the activated complex of the N ⇌ U reaction for β-lactoglobulin would appear to be quite close to form U in its extent of unfolding. For lysozyme the reverse must be true, the activated complex being close to N, *i.e.*, quite highly folded.

The value of n obtained from the equilibrium constant (eq 3) for the over-all transition from N to U has been

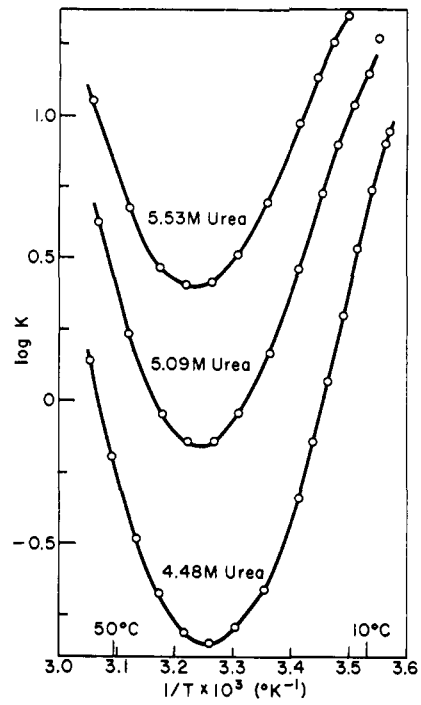

FIGURE 12: Typical van't Hoff plots. The data are derived from the heating curves of Figure 10.

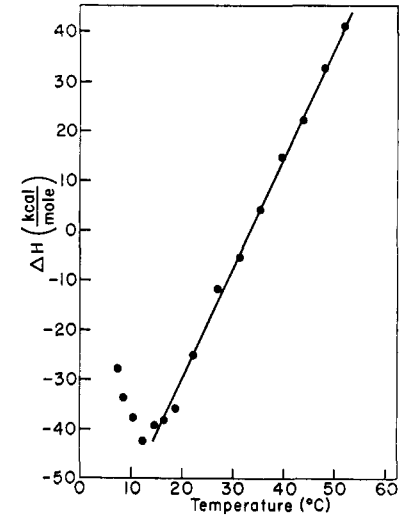

FIGURE 13: Typical plot of ΔH *vs.* temperature. These results come from the plot of Figure 12 for 4.48 M urea. Very similar results were obtained from all other plots of this kind, as is seen in the summarized results of Table II.

[3] Among the mechanisms suggested have been nonspecific solvent effects on hydrophobic groups, amide groups, and peptide links which are exposed to the solvent by the unfolding process (Tanford, 1964), and binding of the denaturing agent to sites on the unfolded chain (Tanford *et al.*, 1966), with particular emphasis on binding to peptide groups (Robinson and Jencks, 1965). To some degree all such mechanisms represent essentially the same idea: that urea and guanidine hydrochloride act on sites which are available in the unfolded state, but not in the native state. The older proposal (Simpson and Kauzmann, 1953) that urea reacts with the native protein to *destabilize* it is no longer considered likely.

[4] $\Delta G°_u$ itself, however, contains a major term which represents the intrinsic stability of the native protein (Tanford, 1964), and this will presumably be a unique property of each individual protein.

TABLE III: ΔH and ΔC_p for Unfolding of Proteins.

Protein	Type of Denaturation	Method of Measurement	ΔH, 25° (kcal/mole)	ΔC_p (cal/deg)[g] per Mole	ΔC_p (cal/deg)[g] per 10^4 g
Ribonuclease[a]	Acid + heat	van't Hoff	+50	2600	1900
Ribonuclease[b]	Acid + heat	Calorimetry	+43	2300	1700
Chymotrypsinogen[c]	Acid + heat	van't Hoff	+36[f]	3500	1400
Myoglobin[d]	Acid + heat	van't Hoff	+42	1400	800
β-Lactoglobulin[e]	Urea	van't Hoff	−20	2100	1100
	Guanidine hydrochloride	van't Hoff	−21	2100	1100

[a] Brandt (1965). [b] Danforth *et al.* (1967). [c] Brandts (1964a). [d] Hermans and Acampara (1967). [e] This paper, Table II. [f] Brandts has carried out some experiments in the presence of urea, and reports that ΔH is increased in the presence of urea. [g] Brandts found it necessary to assign a temperature dependency to ΔC_p for the low pH thermal unfolding of ribonuclease and chymotrypsinogen. The ΔC_p values given for these proteins are for 45°. For the unfolding of β-lactoglobulin by urea and guanidine hydrochloride (Figure 13 and similar data), ΔC_p is evidently independent of temperature between 10 and 55°. The data of Hermans and Acampara for myoglobin are too scattered to permit a definite conclusion regarding the temperature dependence of ΔC_p.

discussed before, on the assumption that the action of urea depends on local solvent effects which can be calculated from measurements on suitable model compounds (Tanford, 1964). The theory was applied specifically to the unfolding of β-lactoglobulin by urea, and it was shown that the experimental value of n was smaller than anticipated on the basis that perhaps 50–75% of all hydrophobic and peptide groups should be freshly exposed to solvent in the course of the transition. The value of n at 25° which was used in that discussion came from an earlier and less exact study of the unfolding reaction (Tanford and De, 1961), and it is larger ($n = 22$) than the value found in the present work ($n = 17$). However, some of the theoretical parameters which go into the calculation have also undergone revision since the publication of the previous paper, so that the theoretically expected value of n (on the same basis as before) is now $n \simeq 25 \pm 5$, compared to $n \simeq 42 \pm 8$ (Table II of Tanford, 1964). The discrepancy is therefore actually reduced. It was shown in the previous paper that a discrepancy of this kind can arise if the reaction being studied and treated as a two-state process is in fact not a two-state process. This possibility has been discounted by Brandts (1965), and, on the basis of the discussion presented above, we too would now consider this explanation unlikely. The most likely explanation is perhaps that transfer free energies of groups attached to polypeptide chains are smaller than for similar groups attached to small model compounds, because of the steric interference by neighboring parts of the polypeptide chain. An alternative possibility is that fewer than 50% of the hydrophobic and peptide groups of β-lactoglobulin are in fact exposed during the unfolding process, perhaps because the native structure is a relatively open one.

At this time we have no explanation for the effect of temperature on n, as shown in Figure 9. The opposite result was obtained in the kinetic study of ovalbumin by Simpson and Kauzmann (1953), *i.e.*, n for the activation processes decreases with increasing temperature. The two results are however not comparable because the ovalbumin reaction occurs in partially separable successive stages, and a change in the value of n can result from a change in the separation (in terms of urea concentration) between successive stages.

The Value of ΔC_p. The most interesting aspect of the results of this paper is the sharp minimum in the temperature dependence of the equilibrium constant (Figure 12) and the large value of ΔC_p which is the thermodynamic factor responsible for it. A strikingly similar minimum is found for the mean rate constant of the urea denaturation of ovalbumin (Simpson and Kauzmann, 1953), except that it occurs at a lower temperature, 20°, as against the value of 35° observed in this study. A similar minimum (for the equilibrium constant) occurs in the acid thermal denaturation of chymotrypsinogen (Brandts, 1964a), near 10°, in the acid thermal denaturation of ribonuclease (Brandts, 1965), near 0°, and in the neutral thermal denaturation of myoglobin (Hermans and Acampora, 1967), somewhat below 0°. There are also indications of it in the urea denaturation of ribonuclease (Foss and Schellman, 1959). ΔC_p values were calculated for most of these data, with the same assumptions used in this paper, *i.e.*, assuming a two-state transition and basing ΔC_p on a van't Hoff plot. For the low pH thermal denaturation of ribonuclease, ΔC_p has also been determined calorimerically. Actual values obtained in these experiments are summarized in Table III.

As Table III shows, ΔC_p values for all of these reactions are very similar when expressed on the basis of the same weight of protein, despite the fact that the ΔH values vary widely. The data of Brandts (1964a) for chymotrypsinogen indicate that the differences in ΔH between β-lactoglobulin and the other proteins are not due to the use of urea or guanidine hydrochloride as denaturing agent. He reports that the unfolding of

chymotrypsinogen is characterized by a larger ΔH in the presence of urea than in its absence.

As has been pointed out by Brandts (1964a), the large values of ΔC_p, which are clearly a characteristic feature of protein unfolding, are probably best explained as due to the exposure of nonpolar groups to the solvent in the unfolded state. The greater freedom of internal rotation of the unfolded polypeptide chain may also make a contribution, though this contribution may be balanced by vibrational contributions to the heat capacity of the native protein.

Quantitatively, the exposure of hydrophobic groups alone will account for the order of magnitude of the observed ΔC_p. The very marked difference between the partial molal heat capacities of aliphatic organic compounds in aqueous solutions, as compared to their solutions in organic solvents, was first pointed out by Edsall (1935). The effect has since been shown (Frank and Evans, 1945) to result from creation of temperature-labile-ordered water structures in the vicinity of nonpolar groups in aqueous solutions. Edsall (1935) showed that this effect, for aliphatic groups of several kinds of organic molecules, contributes about 18 cal/deg per mole for each CH_2 group in the molecule to the heat capacity in an aqueous medium. Bohon and Claussen (1951) have obtained values near 70 cal/deg per mole for aromatic compounds. The amount of heat capacity attributable to these effects is probably less in an aqueous urea solution than in pure water (Kresheck and Benjamin, 1964). However, even if these figures have to be reduced by one-half, they are still able to account for the observed ΔC_p for β-lactoglobulin with reasonable values (*i.e.*, less than 100%) for the fraction of aliphatic and aromatic residues which are exposed to the solvent during the unfolding process.

It should be noted that the data of Table III compare ΔC_p values for low pH thermal denaturation, which is a reaction leading to incomplete unfolding of polypeptide chain (estimated by Aune *et al.* (1967) to be about 75%), with ΔC_p values for the reaction studied in this paper, which leads to essentially completely unfolded polypeptide chains. Thus fewer hydrophobic groups are exposed in one kind of process than the other, but the difference in ΔC_p which would result is clearly insignificant when compared to uncertainties in the expected effects of temperature and urea or guanidine hydrochloride concentration on ΔC_p and the uncertainties in the data themselves. The discrepancy in the reported effects of temperature on ΔC_p cited in footnote *g* of Table III remains to be explained.

The excellent agreement between the calorimetric and van't Hoff values of ΔC_p for ribonuclease (Table III) is noteworthy. It is evident that the two-state assumption made in the van't Hoff treatment is, at least for this protein, correct.

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Physicochemical Studies of Bovine Fibrinogen. IV. Ultraviolet Absorption and Its Relation to the Structure of the Molecule*

Elemer Mihalyi

ABSTRACT: The specific extinction of bovine fibrinogen was determined under various conditions. From the alkaline spectrum the tyrosine and tryptophan content of the molecule was calculated. The neutral and alkaline spectra are in good agreement with spectra calculated on the basis of the number of aromatic amino acid residues found. The spectrophotometric titration of the tyrosine residues was performed in the native, alkali-, urea-, and guanidine hydrochloride denatured molecule. The titration curves were analyzed with respect to electrostatic interaction and intrinsic pK. There was no indication of more than one class of dissociating tyrosine groups, or of groups not ionizing at all in the native molecule. Perturbation spectra showed that only about 33% of both tyrosine and tryptophan residues are in contact with the solvent. Acid and alkali denaturation raised this figure; however, it did not bring about complete exposure of the buried residues. The perturbing power of the protein fabric was estimated from the difference spectra.

The study of the absorption of ultraviolet radiation by proteins provides answers on several different levels of inquiry into the protein structure. As an analytical method it is useful in determining the amounts of aromatic amino acids, which, because of their lability, are difficult to estimate even with the present day techniques (Beaven and Holiday, 1952). Furthermore, it can be utilized to investigate the ionizing ability of the tyrosine residues and also the nature of the environment in which these and the other aromatic amino acid residues are located (Wetlaufer, 1962).

Some of the tyrosyl residues in certain proteins are unable to ionize in the native molecule, but their ionization becomes normal after disrupture of the secondary and tertiary structure of the protein. The ionization of the phenolic hydroxyl groups results in a large increase in the ultraviolet absorption; therefore, ultraviolet spectral changes, in the pH range where these groups ionize, can be used to follow the denaturation of these particular proteins.

Whereas the above method is applicable only to the alkaline denaturation of the protein, the method of difference spectroscopy, which detects small perturbances in the absorbing properties of the chromophores, is of more general usefulness. It is not restricted to the tyrosyl groups either, but is applicable to all the chromophoric residues. In the native state some of these are buried in the hydrophobic regions of the molecule and become accessible to solvent only after disruption of the secondary structure. The change in environment accompanying this process is reflected in the absorbing properties of the molecule. These changes in absorptivity are one order of magnitude smaller than the ones caused by the phenoxide ionization; nevertheless, they can be used to great advantage in the study of denaturation kinetics of the protein molecule.

The difference spectrum, besides its use as an indicator of a change in the conformation of the molecule, can provide also information on the number and nature of buried chromophores, since each of these has a characteristic perturbation spectrum. By the use of perturbants of various molecular sizes one may find that some of the chromophores are located in crevices accessible to smaller but not to larger perturbants (Herskovits and Laskowski, 1960, 1962a,b). This method can furnish, therefore, valuable data on the surface topography of the native molecule.

Schauenstein and Hochenegger (1953) have shown

* From the Laboratory of Biochemistry, Section on Cellular Physiology, National Heart Institute, National Institutes of Health, Public Health Service, U. S. Department of Health, Education, and Welfare, Bethesda, Maryland 20014. *Received May 26, 1967.*