THE JOURNAL OF BIOLOGICAL CHEMISTRY
Vol. 248, No. 11, Issue of June 10, pp. 4047-4051, 1973
Printed in U.S.A.

Thermodynamics of Unfolding of Lysozyme in Aqueous Alcohol Solutions

(Received for publication, November 31, 1972)

ROSA MARIA PARODI, ESTELLA BIANCHI, AND ALBERTO CIFERRI
From the Chemistry Department, University of Genoa, Genoa, Italy

SUMMARY

The thermal denaturation of lysozyme in aqueous alcohol solutions has been investigated through ultraviolet difference spectrophotometry and optical rotation measurements. Alcohols used were CH_3OH, C_2H_5OH, and C_3H_7OH; concentrations up to 50% (v/v) of alcohol in water were employed. It was found that upon increasing the size or the concentration of the aliphatic alcohol, the denaturation temperature is gradually depressed. At low alcohol concentration, the α helix content of the protein decreases through the thermal transition. However, on increasing alcohol size or concentration, the α helix content goes through a maximum during denaturation. Apparent equilibrium constants and corresponding van't Hoff heats of denaturation were determined. The data indicate that the depression of the denaturation temperature due to addition of alcohols is essentially entropy dependent. It is suggested that these results may be explained in terms of a theoretical expression containing a binding constant for the alcohol to the nonpolar sites of the protein plus a diluent term accounting for changes in the dilution parameters of different conformers.

Systematic investigations of the role of aliphatic alcohols on the stability of a fibrous protein (1) (collagen) and on the helix → coil transformation of poly(L-ornithine) and poly(L-glutamic acid) (2) were recently presented. It was found that the series

$$C_2H_4ClOH > C_3H_7OH > C_2H_5OH > CH_3OH \quad (1)$$

characterizes the effectiveness of alcohols for the stabilization of the α helical form of the syntetic polypeptides and of the randomly coiled form of collagen at low alcohol concentration, *i.e.* alcohols raise the helix → coil transformation temperature of poly(L-ornithine) and poly(L-glutamic acid) and lower the denaturation temperature of collagen and tropocollagen. These opposite effects were justified assuming (1) that Series 1 characterizes diluents which possess increasing solvating power toward the apolar side chains which become exposed during the conversion of the helical or crystalline forms of collagen to the random coil. This suggestion is similar to that advanced by Schrier *et al.* (3) who studied the effect of several alcohols on the denaturation of ribonuclease. For poly(L-ornithine) and poly(L-glutamic acid), when side chains are exposed to the solvent in both conformations, a reduction of the entropy of mixing of the peptide groups in the random coiled conformation, due to increased organization of the solvent according to Series 1 was suggested to occur (2).

The above results lead to the expectation that for globular proteins capable of assuming extended α helical conformations, Series 1 should still characterize alcohols of increasing denaturating power. However, thermal denaturations involving the occurrence of intermediate α helical conformations (between the globular and random coiled extremes) should occur on increasing the size of the alcohol or alcohol concentration. Although there is scattered evidence indicating that this is indeed the case (4–7), we have considered useful a more systematic investigation of the thermodynamics of denaturation of lysozyme in alcohol solutions. In particular, we have aimed at an evaluation of the thermodynamic components of the unfolding process and to their interpretation in terms of binding and solvent organization effects.

EXPERIMENTAL PROCEDURE

Materials—B.D.H. lysozyme (Lots 0744670 and 0811520) was used without further purification. The concentration of stock solutions was determined by optical density using an extinction coefficient ϵ at 280 nm of 43860 cm^2 per mg (8). Alcohols employed were methanol, ethanol, and 1-propanol of reagent grade. Lysozyme solutions, obtained by mixing suitable aliquots of stock solution with alcohol, were buffered at pH 3 using a mixture of 0.1 M glycocoll and 0.1 M HCl (the ionic strength was kept constant at 0.1 by adding suitable amounts of NaCl). This pH was chosen because of the convenient occurrence of denaturation on the temperature scale.

Optical Density—Difference spectra at λ = 294 nm (at which the optical density of lysozyme exhibits a maximum) were obtained using a Carl Zeiss PMQ II Spectrophotometer. The concentration of protein was about 0.8 to 1.2 mg per cm^3. To obtain denaturation curves, the temperature of sample and solvent cells was raised taking measurements at about 1° intervals. At each temperature about 10 min were allowed for thermal equilibrium. Samples were allowed to stay one night at 4° before determinations.

Optical Rotation—Optical rotation data were obtained with a JASCO model ORD/UV spectropolarimeter, equipped with 10-mm path length cells. To determine denaturation profiles, the reduced mean residue rotation at 233 nm (in degrees square centimeter per dmole) (9)

$$[m']_{233} = \frac{3}{n^2+2}\frac{M_0[\alpha]_{233}}{100}$$

was measured as a function of temperature. Here n is the refractive index of solvent, $[\alpha]$ the specific rotation, and M_0 is the mean residue molecular weight (equal to 110). Experimental conditions similar to those employed for obtaining the optical density data were used. Refractive index corrected to 233 nm was used.

In same cases we also determined the optical rotatory dispersion curves. The data were analyzed using the Moffitt-Yang equation (10)

$$[m']_\lambda = \frac{a_0\lambda_0^2}{\lambda^2-\lambda_0^2} + \frac{b_0\lambda_0^4}{(\lambda^2-\lambda_0^2)^2}$$

The parameter b_0 was obtained from the slope of $[m'][(\lambda^2 - \lambda_0^2)/\lambda_0^2]$ versus $\lambda_0^2/(\lambda^2 - \lambda_0^2)$ plots, taking (9, 10) λ_0 equal to 212 nm. For α-helical poly(L-amino acids), b_0 is generally between -600 and -650, whereas for randomly coiled polypeptides, b_0 is between 0 and $+50$ (9).

RESULTS

Temperature variations of optical density and optical rotation for lysozyme solutions containing various amounts of methanol, ethanol, and propanol are reported in Figs. 1 and 2.

In the case of C_3H_7OH (*cf.* Fig. 1), both optical density and optical rotation decrease with increasing temperature at low alcohol concentration. Thus, the α helical content present in globular lysozyme decreases (9) with increasing temperature during denaturation. However, when C_3H_7OH concentration is greater than 10%, the behavior of the optical rotation is quite different from that of the optical density. The value of $[m']_{233}$ increases with increasing temperature, indicating (9) an increase of the α helix content until a maximum which, in some cases, approximately corresponds to the end of the optical transition curve. The trend of b_0 supports (10) the initial increase of α helix content with increasing temperature. Upon further increase of temperature, the α helix content decreases and eventually becomes smaller than that of the native protein. We also note a vertical displacement of the $[m']_{233}$ *versus* T curves reported in Fig. 1. In particular, the data seem to imply that native lysozyme, in the low T limit, is more helical in concentrated propanol solutions. An alternative interpretation is that alcohol causes a wave length shift of the absorption bands which affects the ORD curve. Although the origin of this effect remains unclear, we note that a similar behavior has also been reported by others (7).

In the case of CH_3OH (% <50%) and C_2H_5OH (% <40%), the behavior of optical density is similar to that of the optical rotation, both quantities decreasing continuously with increasing temperature, just as in the case observed with the 10% C_3H_7OH solution. The similarity of the behavior of the two quantities in CH_3OH (% <50%), C_2H_5OH (% <40%), and C_3H_7OH (% = 10%) solutions is well documented in Fig. 2. The decrements $\Delta\epsilon_T$ and $\Delta[m']_T$ of optical density and optical rotation, obtained from subtracting values observed at any temperature T from values observed at low temperature (native state), are divided by the total decrements $\Delta\epsilon_{TOT}$ and $\Delta[m']_{TOT}$ of the corresponding quantities observed at low and high temperature (denaturated state). The corresponding ratios (y and y'), reported in Fig. 2 as a function of temperature, define a single denaturation curve. Only when CH_3OH and C_2H_5OH concentrations are greater than 50 and 40%, respectively, differences between the trends of optical density and optical rotation (similar to those observed when C_3H_7OH concentration is greater than 10%) were noticed. Thus, upon increasing alcohol size or concentration, the thermal denaturation of lysozyme (which in water or at low alcohol

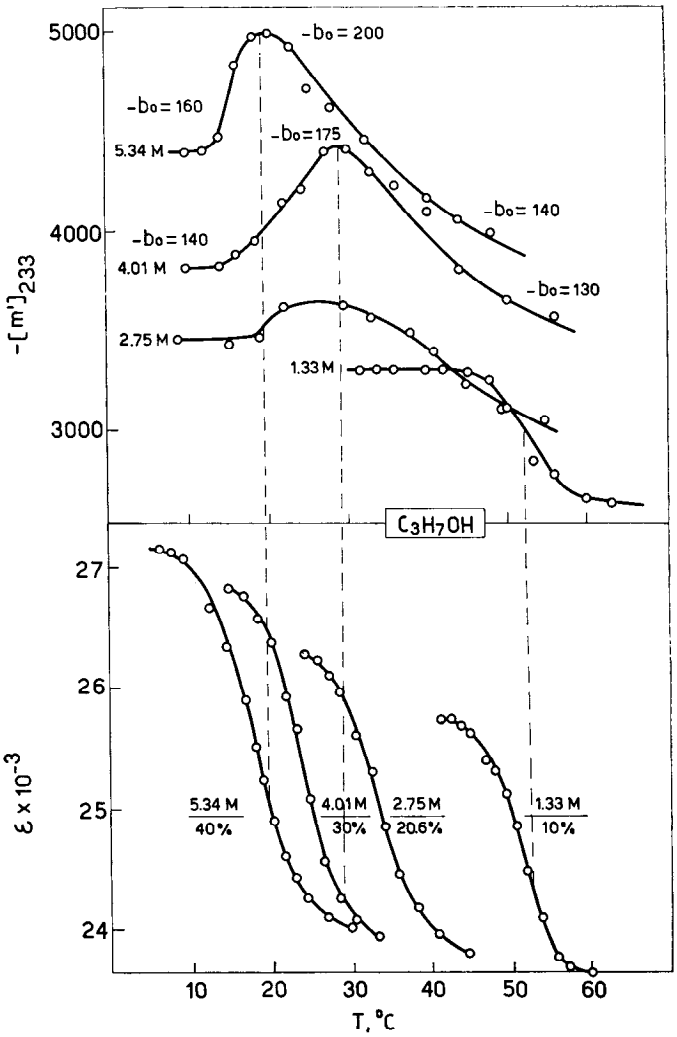

FIG. 1. Temperature variation of optical density and optical rotation for lysozyme solutions containing propanol of the indicated molarity (or % v/v); pH 3. b_0 values at different temperatures are indicated.

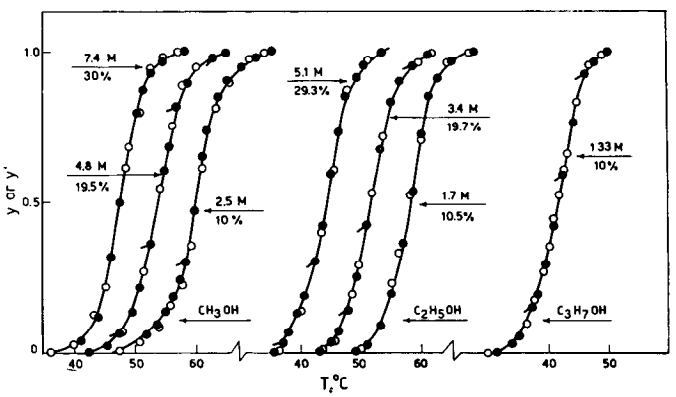

FIG. 2. Temperature variation of the ratios $y = \Delta/\Delta\epsilon_T\epsilon_{TOT}$ (○) and $y' = \Delta[m']_T/\Delta[m']_{TOT}$ (●) for lysozyme solutions containing methanol, ethanol and propanol of the indicated molarity (or % v/v); pH 3. ◪, points obtained on decreasing temperature to assess reversibility.

concentration is a transformation of the globular → random coil type) tends, at least for some portion of molecules, to become a transition of the globular → α helix → random coil type. As indicated in Fig. 2, the denaturation processes investigated were found to be reversible ones (providing comparable rates of heating and cooling were used, and retention at the highest temperature for more than 1 hour was avoided).

The variation of denaturation temperature, T_t, with alcohol concentration is reported in Fig. 3. The midpoints of the optical density curves in Figs. 1 and 2 were taken as denaturation temperatures. Accordingly, in these pseudo phase diagrams, the final state is the pure random coiled form only for methanol and ethanol. In the concentration range investigated, the plots for methanol and ethanol are nearly linear, while a curvature is exhibited in the case of C_3H_7OH, as found by Schrier *et al.* (3) in the case of ribonuclease.

Apparent equilibrium constants were calculated from denaturated form to native form ratios deduced from the data in Figs. 1 and 2. Typical plots ln k *versus* $1/T$ are reported in Fig. 4. The plots were found to be linear for all solutions for which the denaturation was of the globular → random coil type. The plots exhibited a curvature when C_3H_7OH concentration was greater than 10% and coincidence of the degree of transformation obtained from optical density and optical rotation was not verified. Accordingly, enthalpies of denaturation were obtained from the slopes of the ln k *versus* $1/T$ straight lines (11) in the case in which the transition appeared to be a two-step process. These denaturation enthalpies are plotted in Fig. 5 as a function of alcohol concentration.

DISCUSSION

The data in Fig. 3 confirm that Series 1 characterizes alcohols of increasing effectiveness toward destabilization of the compact, tertiary structure of proteins. On the other hand, the optical rotation data at high alcohol concentration confirm that Series 1 characterizes the order of effectiveness of alcohol toward stabilization of the α helical, secondary structure of polypeptides.

In the search of a coherent interpretation of these effects, we consider in more detail the role of enthalpy and entropy contributions to the denaturation process. The value of ΔH obtained in water (78 Cal per mole) appears to be in line with recent independent determination of ΔH, indicating a value of ~60 Cal per mole for lysozyme at pH 1 (12), and of ~100 Cal per mole for lysozyme at pH ~5 (13). The peculiar variation of ΔH with alcohol content is unexpected (3). Since it is known (12, 14) that ΔH increases with increasing pH, a small electrostatic component due to alteration of the relevant pK values by the alcohol is likely present. In any event, since ΔH is consistently higher than its value in water, and the denaturation temperature is continuously depressed (*cf.* Fig. 3) on increasing alcohol concentration, the latter depression seems to be associated to a prevalence of an increased entropy of the globular → random coil transformation. This result is surprising in view of

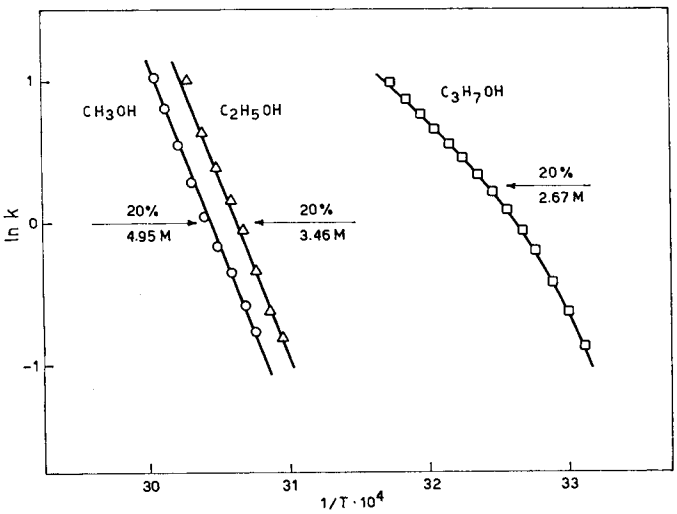

FIG. 4. Apparent equilibrium constants for the denaturation of lysozyme in alcohol solutions (containing 20% alcohol) plotted as a function of reciprocal temperature.

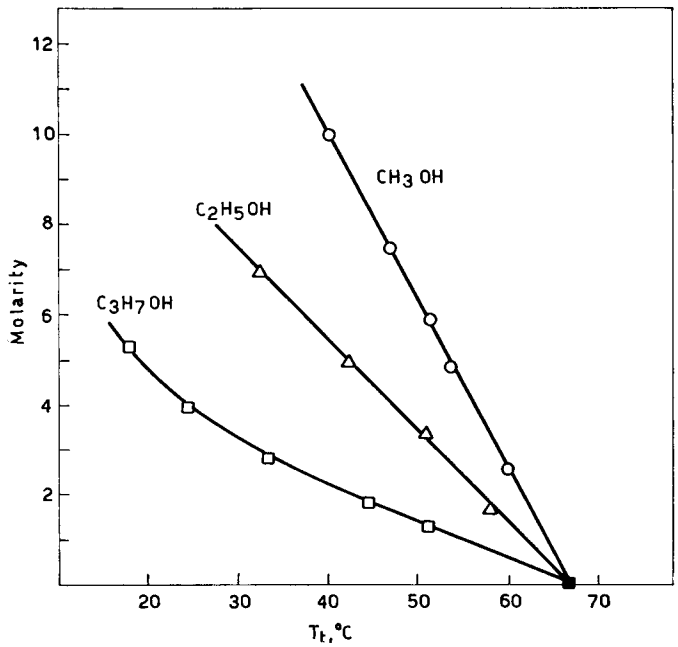

FIG. 3. Variation of the denaturation temperature of lysozyme with alcohol concentration. Denaturation temperatures taken as midpoints of optical density curves in Fig. 1.

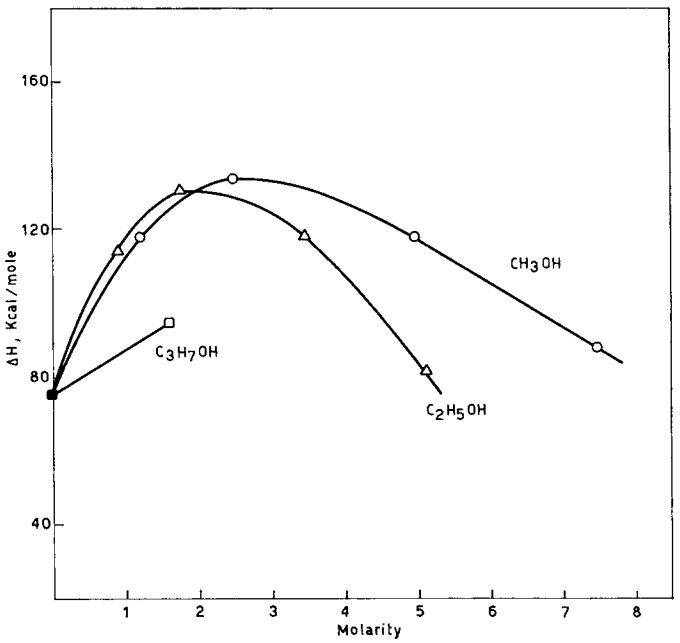

FIG. 5. Van't Hoff heat of denaturation for lysozyme in alcohol solutions when the transition is of the globular → random coil type.

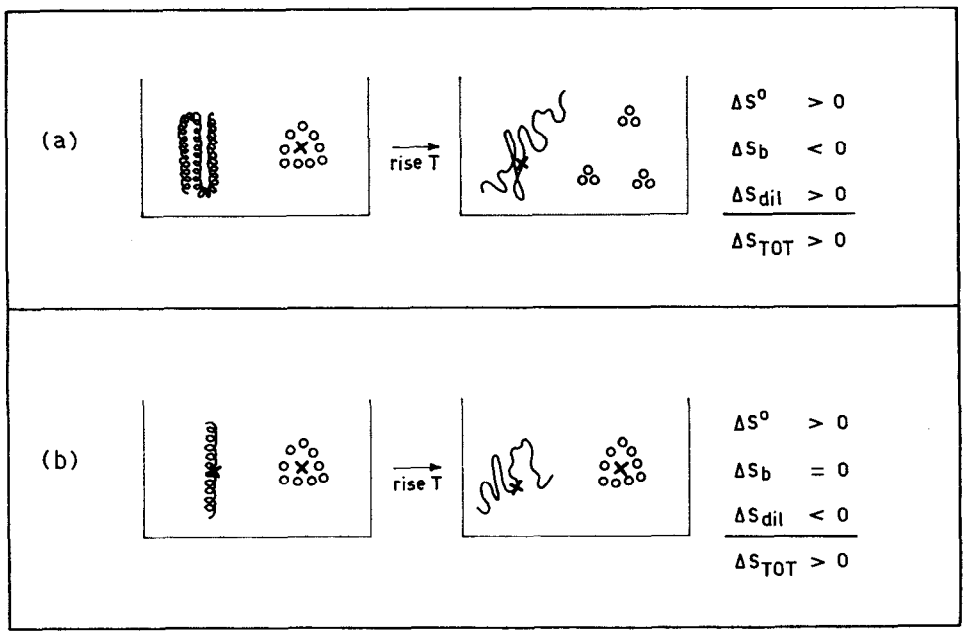

FIG. 6. Schematization of globular → random coil (*a*) and helix → random coil (*b*) transformations in aqueous alcohol solutions. × represents alcohol and ○ represents water molecules.

the data of Conio *et al.* (2), indicating a prevailing role of the reduction of the entropy for the helix-coil transition in the stabilization of the secondary structure of poly(L-ornithine) and poly(L-glutamic acid) by alcohols. The present results are, however, in agreement with the suggestion of Schrier *et al.* (3) suggesting that the lowering of the transition temperature of ribonuclease by the alcohols is largely entropy dependent. Moreover, recent results obtained by Nozaki and Tanford (15), for the free energy of transfer of several amino acids from water to aqueous ethanol solution, indicate that the alcohol favors the solubilization of apolar side chain groups, and the insolubilization of the peptide group. Conio *et al.*[1] have recently reported that the free energy of transfer, from water to aqueous alcohol solution, for the peptide group is positive, and that the decreased solubility of the peptide group is due to an unfavorable entropy of transfer. They indicated that this effect accounts for the stabilization of the α helix in water-alcohol solutions.

Thus, one is faced with the seemingly conflicting conclusion that, upon increasing alcohol concentration, the entropy of the globular → random coil transformation increases while, simultaneously, the entropy of the helix → random coil transformation decreases. Binding of alcohol to the nonpolar side chains which become exposed during the former transition, coupled with water organization effects, satisfactorily explains the above findings. The model is qualitatively schematized in Fig. 6. Following a previously adopted formalism (16, 17), the total entropy of denaturation may be written as the sum

$$\Delta S_{TOT} = \Delta S^0 + \Delta S_b + \Delta S_{dil} \qquad (2)$$

where ΔS° is the conformational entropy change (always positive, the superscript refers to globular → random coil or helix → random coil transformations ipotetically induced by increasing temperature in absence of solvent); ΔS_b is the entropy of binding of alcohol to the nonpolar, exposed groups of the polymer (generally a negative quantity); and ΔS_{dil} is the entropy of dilution (which may be positive or negative) representing alterations in the organization of the solvent and in the conformational adaptation of the bound polymer within the solution (diluent effect (16)). An increased organization of water due to addition of alcohols (according to Series 1) has been indicated in the literature (18). On the other hand, selective absorption, or binding of alcohols to nonpolar groups of proteins has been experimentally evidenced (1, 6, 19). Thus, during the globular → random coil transition, alcohol molecules are competitively absorbed by the nonpolar groups, simultaneously reducing the organization of the binary diluent. ΔS_{TOT} may thus be positive (Fig. 6*a*), and increase with increasing alcohol size or concentration, leading to the observed depression of denaturation temperature.

On the other hand, ΔS_b changes little during the helix → coil transition (Fig. 6*b*) due to the similar exposition of nonpolar sites for the two conformations. Consequently, the contribution to ΔS_{dil} due to alterations in solvent organization is negligible. As suggested by Patrone *et al.* (2), ΔS_{dil} may nevertheless be negative due to the difficulty for a diluent of increasing organization to be a good diluent for the peptide group in the random coil form. ΔS_{TOT} may thus be positive (Fig. 6*b*), and decrease with increasing alcohol size or concentration, leading to the corresponding stabilization of the α helix.

The interpretation of the globular → random coil transformation for lysozyme schematized in Fig. 6*a* is essentially similar to that suggested by Schrier *et al.* (3) in their study of the thermal transition of ribonuclease (the occurrence of multistate transitions was not considered in the latter study). However, our approach tends to draw attention to a separation of binding effects associated to the liquid nature of the diluent. The advantages and the limitations of this approach, which have already been successfully used to explain the melting temperature-salt concentration dependence of insoluble collagen (20, 21), and the concentration dependence of the helix → coil transformation of poly(γ-benzyl-L-glutamate) (17), have been discussed elsewhere (16). The theory, based on the recognition of separate contributions to the free energy of transformation, as exemplified in Equation 2, leads, in the case of a transformation between two soluble conformers, to the following limiting equation for the depression of the transformation temperature (17)

[1] G. Conio, L. Curletto, E. Patrone, submitted for publication.

$$T_t^0 - T_t \simeq \frac{R(T_t^0)^2}{\Delta H^0} pK_a + \left(\frac{V_p}{V_s}\right)(1 - v)\,\Delta\chi \qquad (3)$$

where the term pK_a may, in the present case, be assumed to represent the contribution due to binding of alcohol to the nonpolar sites (p, number of sites per residue; K, binding constant; a, activity of alcohol), and the term in $\Delta\chi$ contains contributions related to alterations of the water structure (V_p and V_s, molar volumes of a residue and solvent, respectively; v, volume fraction of polymer; $\Delta\chi$, difference in the thermodynamic parameters of dilution for the two conformers). The analysis of Schrier *et al.* (3) is instead based on the Peller (22) equation

$$T_t^0 - T_t \simeq \frac{R(T_t^0)^2}{\Delta H^0} k\,a \qquad (4)$$

which does not contain an explicit expression for the diluent effect. Accordingly, Schrier *et al.* include in k both the entropy loss due to binding and the free energy of hydrophobic bond formation, calculated on the basis of the theory of Nemethy and Sheraga (23).

The $\Delta\chi$ term may be amenable to independent, experimental evaluation (17, 24). When this is done, a quantitative analysis may be made concerning the generalization of the theory based on Equation 3 to the case of binding processes involving hydrophobic interactions.

REFERENCES

1. Bianchi, E., Rampone, R., Tealdi, A., and Ciferri, A. (1970) *J. Biol. Chem.* **245,** 3341
2. Conio, G., Patrone, E., and Brighetti, S. (1970) *J. Biol. Chem.* **245,** 3335
3. Schrier, E. E., Ingwall, R. T., and Scheraga, H. A. (1965) *J. Phys. Chem.* **69,** 298
4. Weber, R. E., and Tanford, C. (1959) *J. Amer. Chem. Soc.* **81,** 3255
5. Inoue, H., and Timasheff, S. N. (1968) *J. Amer. Chem. Soc.* **90,** 1890
6. Timasheff, S. N., and Inoue, H. (1968) *Biochemistry* **7,** 2501
7. Hermans, J., Jr., Puett, D., and Acampora, G. (1969) *Biochemistry* **8,** 22
8. Fromageot, C., and Schnek, G. (1950) *Biochim. Biophys. Acta* **6,** 113
9. Fasman, G. D. (1963) *Methods Enzymol.* **6,** 928
10. Moffitt, W., and Yang, J. J. (1956) *Proc. Nat. Acad. Sci. U. S. A.* **42,** 596
11. Foss, J. G., and Schellman (1959) *J. Phys. Chem.* **63,** 2007
12. O'Reilly, J. M., and Karasz, F. E. (1970) *Biopolymers* **9,** 1429
13. Delben, F., and Crescenzi, V. (1969) *Biochim. Biophys. Acta* **194,** 615
14. Ogasahara, K., and Hamaguchi, K. (1967) *J. Biochem.* **61,** 199
15. Nozaki, J., and Tanford, C. (1971) *J. Biol. Chem.* **246,** 2211
16. Orofino, T. A., Ciferri, A., and Hermans, J. J. (1967) *Biopolymers* **5,** 773
17. Puett, D., and Ciferri, A. (1971) *Bipolymers* **10,** 547
18. Franks, F., and Ives, D. J. G. (1966) *Quart. Rev. Chem. Soc.* **20,** 1
19. Inoue, H., and Timasheff, S. N. (1972) *Biopolymers* **11,** 737
20. Ciferri, A., Garmon, R., and Puett, D. (1967) *Biopolymers* **5,** 439
21. Puett, D., and Rajagh, L. V. (1968) *J. Macromol. Sci. Chem.* **A2,** 111
22. Peller, L. (1962) *J. Phys. Chem.* **66,** 1773
23. Nemethy, G., and Scheraga, H. A. (1962) *J. Phys. Chem.* **66,** 1773
24. Rajagh, L. V., Puett, D., and Ciferri, A. (1965) *Biopolymers* **3,** 421