Effect of Deuteration on the Strength of the Amide Hydrogen Bond

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Abstract: The self-association of *N*-deuterio-*N*-methylacetamide in carbon tetrachloride solution was studied at 25° by equilibrium centrifugation and the data were fit to an indefinite self-association model. The apparent weight-average molecular weight, $M_{wa,c}$, and solute concentration, c, were related to the monomer molecular weight, indefinite self-association constant, and second and third virial coefficients (M_1, k, B, and C) by the equation[2] $M_1/M_{wa,c} = 1(1 + 4kc)^{1/2} + BM_1c + 2CM_1c^2$. The quantities k, BM_1, and CM_1 were found to be equal to $4.10 \pm 0.35 \times 10^4$ ml/g, $4.0 \pm 1.8 \times 10^{-2}$ ml/g, and $6.7 \pm 0.8 \times 10^{-1}$ ml^2/g^2, respectively, with c expressed in units of grams per milliliter. Slightly revised values of the same quantities for protiated *N*-methylacetamide were $2.36 \pm 0.12 \times 10^4$ ml/g, $2.3 \pm 0.23 \times 10^{-1}$ ml/g, and $6.3 \pm 1.2 \times 10^{-1}$ ml^2/g^2, respectively. It is shown that the effect of deuteration on previously observed thermal transition curves for poly-γ-benzyl-L-glutamate is consistent with earlier interpretations which emphasized the importance of hydrogen bonds.

It has been observed for various macromolecules that their thermal transition curves are different in deuterated and protiated solutions,[3-8] although recent calorimetric measurements have shown that the enthalpy of denaturation for ribonuclease[9] and unfolding polypeptides[7] is similar in the two solvents. Attempts[3,10] have been made to assess the role of hydrogen bonds in these processes for proteins and polypeptides, but such efforts suffer from the lack of suitably precise data with model compounds. Therefore, the present investigation was undertaken with *N*-deuterio-*N*-methylacetamide to provide data for comparison with our previous results[11] with the protiated derivative. *N*-Methylacetamide was chosen as a model compound because of its ability to undergo indefinite self-association, which can be conveniently studied with the analytical ultracentrifuge with sufficient precision to allow a quantitative determination of the isotope effect.

Experimental Section

Materials. The sample of *N*-methylacetamide used in this study was the same material previously used.[11] It was distilled at about 70° (0.24 mm) after overnight drying with $CaSO_4$, and stored in a desiccator over $CaCl_2$. A deuterated sample was prepared by exchanging a protiated sample three times with a 20-fold excess of D_2O, followed each time by extraction with chloroform. The final material was vacuum distilled without drying under the same conditions as described above. The sample used for these studies was 97 ± 2% exchanged as determined by nmr analysis with a Varian A60-A spectrometer under ambient conditions of a neat sample using the methyl group spectra as an internal standard for integration of a barely detectable NH peak.

Methods. The experimental procedures employed in this study were the same as previously reported.[11] All experiments were performed at 25°. The partial specific volume and refractive index of the deuterated material were found to be the same as observed for the protiated sample within the limits of experimental error. To ensure that solvent evaporation did not occur during the runs, the column length at the start of each experiment was compared with the column length at the conclusion of the run. In all but a very few cases, they were the same within the limits of experimental error. The data were discarded in the event a leak occurred in the cell. The solute concentration at a given distance from the center of rotation was determined by standard procedures.[12] The integrals involved were evaluated numerically using Simpson's rule. These calculations as well as all curve fitting employed the IBM System 360/67 at the Northern Illinois University Computer Center. All programs were written by R. J. Albers. Also, our previous sedimentation equilibrium results for protiated *N*-methylacetamide were verified at the conclusion of the studies with the deuterated sample to ensure that some unidentified systematic error had not entered into our procedures.

Results

The Schlieren patterns from individual centrifugation experiments were similar to those observed with *N*-methylacetamide, *viz.*, a typical flotation pattern at low concentrations, a typical sedimentation pattern at high concentrations, and a pattern exhibiting a maximum for intermediate concentrations. Also, a separation of the solvent and solution base lines was observed as before when the rotor was initially brought to the speed used for the equilibrium run. However, quantitative differences were observed when the weight-average molecular weight, $M_{wa,c}$, was calculated[11] at various concentrations, c, from the equation

$$M_{wa,c} = \frac{2RT}{(1 - \bar{v}\rho)\omega^2} \frac{\mathrm{d} \ln c}{\mathrm{d}(r^2)} \qquad (1)$$

where the quantities $\bar{v}$, ρ, ω, r, R, and T have their usual meaning in the equilibrium centrifugation equation, corresponding to the partial specific volume, solution density, angular velocity, distance from the center

(1) National Science Foundation undergraduate research participant, Summer 1971.
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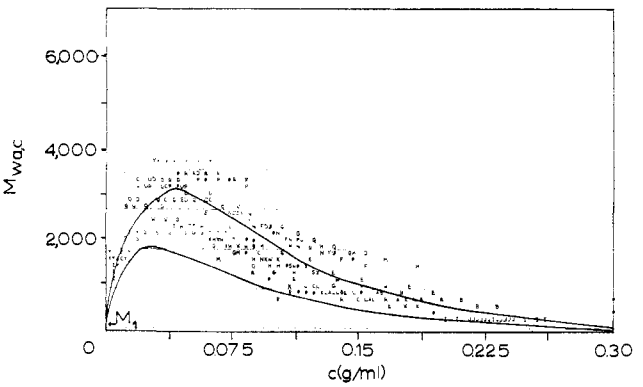

Figure 1. Combined results of 26 experiments at different initial concentrations and equilibrium speeds of the concentration dependence of the apparent weight-average molecular weight for *N*-deuterio-*N*-methylacetamide in carbon tetrachloride solutions at 25°. The upper line drawn through the points is the curve of best fit of the data to M_1 times the reciprocal of eq 2 according to a least-squares criterion, and was obtained using values for k, BM_1, and CM_1 of 41,000 ml/g, 0.040 ml/g, and 0.67 ml²/g², respectively. The lower line represents the data obtained for protiated *N*-methylacetamide and was obtained using values of k, BM_1, and CM_1 of 23,600 ml/g, 0.23 ml/g, and 0.63 ml²/g², respectively.

of rotation, universal gas constant, and abolute temperature, respectively.

A summary of results obtained is presented in Figure 1. The curve which gave the best fit of the data for protiated *N*-methylacetamide is shown for comparison. It may be seen that the initial slopes are quite different and the maximum molecular weight obtained for the deuterated sample is about twice that of the protiated material. The data represent the results of 26 equilibrium runs. The results were fitted according to an unweighted least-squares criterion[13] to eq 2 which relates the apparent weight-average molecular weight and concentration, expressed in units of grams per milliliter, with the indefinite self-association constant, k, monomer molecular weight, M_1, and second and third virial coefficients, B and C, as[14]

$$\frac{M_1}{M_{w,a,c}} = \frac{1}{(1 + 4kc)^{1/2}} + BM_1c + 2CM_1c_2 \qquad (2)$$

The results obtained along with values for the undeuterated sample are given in Table I. It may be seen that the effect of deuteration is to approximately double k and to decrease the second virial coefficient. The effect on C is within the limits of experimental error.

Discussion

It is clear that deuteration of *N*-methylacetamide produces a pronounced effect on the weight-average molecular weight in carbon tetrachloride solution as determined by equilibrium centrifugation. This increased molecular weight of the deuterated sample is accompanied by an increased self-association constant and decreased second virial coefficient. It should be emphasized that although our data are entirely consistent with an indefinite self-association model, it is most likely that other models could be developed that would also be consistent with the data. However, such attempts suffer from their lack of mathematical tractability. In addition, it is not certain that it would be possible to distinguish the physical significance of other models, which invoke four or more constants, from the one used here with the existing precision of the data. While the uncertainties reported in Table I for the quantities k, B, and C properly reflect the *precision* of our data, it is not possible to assess the *accuracy* of the constants. It is imagined that this may come from related studies in other laboratories. However, one of the most prominent features of the results should be kept in mind, *viz.*, the relative ratio of the initial slopes in Figure 1 for the deuterated and nondeuterated samples is by inspection about 2:1. Since the initial slope primarily determines the value of the association constant in our analysis, it is then understandable that the calculated values of k are also in a nearly 2:1 ratio. It is this relative difference in k which appears to be clearly established, and serves as the basis for our later discussions.

A quantitative interpretation of the effects of deuteration on the virial coefficients cannot be given for lack of a theoretical treatment of polydisperse condensed systems. However, it would appear that the second virial coefficient is most sensitive to the size of the aggregate, and reflects the increased solute–solute interaction which accompanies deuteration. Also, it would appear that the third virial coefficient is less sensitive to molecular weight and is primarily influenced by solute–solvent and/or solvent–solvent interactions, which would be assumed to be similar for the protiated and deuterated molecules. This interpretation is limited by a lack of fundamental knowledge of the number of virial coefficients beyond the second that one is justified in using for curve fitting. For this reason, no attempt was made, for example, to calculate a fourth virial coefficient from the data to determine the extent this would alter k, and in particular, B and C.

Accepting the experimentally determined ratio of the indefinite self-association constants at 25° for the deuterated and protiated samples, it is found that deuteration of the amide hydrogen bond would decrease the standard free energy of this interaction in carbon tetrachloride solution by about 300 cal/mol. Although other determinations of the effect of the deuterium isotope effect on hydrogen bonding systems have been made,[15,16] this investigation appears to be

Table I. Comparison of the Indefinite Association Parameters for Protiated and Deuterated *N*-Methylacetamide in Carbon Tetrachloride Solution at 25°

Sample	k, ml/g	BM_1, ml/g	CM_1, (ml/g)²
Protiated[a]	2.3×10^4	0.23	0.63
	$\pm 0.12 \times 10^4$	±0.023	±0.12
Deuterated	4.10×10^4	0.040	0.67
	$\pm 0.35 \times 10^4$	±0.018	±0.081

[a] These values are slightly different than those previously reported due to the inclusion of eight additional data sets for calculation of the association parameters. The standard deviation of fitting the molecular weight for the protiated and deuterated samples was ±400 g/mol and ±650 g/mol, respectively. In each case, a small percentage of the data (about 5%) was not used for final curve fitting because they deviated by more than two standard deviations from the initial curve of best fit. Possible reasons for scattering of the data at the cell extremities were discussed in our previous report.

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the only direct thermodynamic study. Conclusions based upon the effect of deuteration on a single stretching frequency may be questioned as noted previously.[10,15]

A straightforward application of the information gained in this study to conformational transitions of proteins and polypeptides in aqueous solutions is not possible because several types of interactions contribute to maintaining the structural integrity of these macromolecules, and some of them are compensating. This situation was recognized by Appel and Yang[6] when they noted that the transition curves for poly-L-glutamic acid and poly-L-lysine in H_2O and D_2O were superimposable, taking into consideration the state of ionization of the side chain. They indicated that this did not imply a necessary identity in strength of the N–H *vs.* the N–D hydrogen bond, but rather the possible existence of compensation for breaking the peptide hydrogen bond by solvation effects. Although the free energy of the amide hydrogen bond in a nonpolar environment is lowered by deuteration, the free energy of the aqueous species may be lowered by an equal amount so that the net difference in free energy, as developed by Klotz and Farnharm,[17] is the same. Therefore, when one is considering the effects of deuteration on proteins, at least three factors need to be taken into consideration, *viz.*, hydrogen bonding,[3,10] hydrophobic bonding,[18] and effects on ionizable groups.[6] In addition, there is the possible importance of preferential binding of buffer components to a particular conformation, which would effect the nature of a transition curve. The possible complexity of this situation is demonstrated by phycocyanin[6,19] where both increased and decreased thermal stability are noted upon changing the solvent from H_2O to D_2O. This behavior may be found to be typical of other proteins as well.

The thermal transition of poly-γ-benzyl-L-glutamate (PBG) would appear to offer promise of being a simple system where the effects of deuteration could be understood. This transition is an example of a reverse one[20] in which a presumed helical form is favored at high temperatures and a less ordered random form is found at low temperatures. An explanation for this behavior is based upon the preferential binding of the acidic component of the mixed solvent to the random form by means of hydrogen bonds. This can be represented by the following mechanism for a solvent system containing dichloroacetic acid (DCA) as

$$(DCA)_2 \rightleftharpoons 2DCA \tag{3a}$$

$$N\text{—}H\cdots O{=}C \rightleftharpoons N\text{—}H,O\text{—}C \tag{3b}$$

$$N\text{—}H,O\text{—}C + 2DCA \rightleftharpoons N\text{—}H\cdots DCA + C{=}O\cdots DCA \tag{3c}$$

where the commas and dots represent ruptured and intact hydrogen bonds, respectively. Since the dimerization of DCA in a nonaqueous solvent is quite favored,[21] the concentration of monomeric DCA in 80% DCA solutions of PBG as determined by eq 3a is essentially constant in spite of the existence of eq 3c. Therefore, it is the reactions represented by eq 3b and 3c that are of importance for the free-energy change for the conformational transition of PBG. The effect of these latter two reactions is to produce two mixed amide–carboxylic acid hydrogen bonds at the expense of one backbone amide hydrogen bond as PBG is unfolded.

If use is made of the theoretical treatment of the helix–coil transition of Schellman[22] employed by Calvin, *et al.*,[3] the change in transition temperature due to unfolding, δT_{Tr}, can be calculated from the equation

$$\delta T_{Tr} = \frac{\delta F_{pep} T_{Tr}}{\Delta H_{pep}} \tag{4}$$

where T_{Tr}, ΔH_{pep}, and δF_{pep} correspond to the transition temperature of the protiated sample, enthalpy change for the formation of a single peptide hydrogen bond, and difference in free energy of hydrogen bond formation which accompanies deuteration, respectively. A 17° change in transition temperature is obtained for which the observed value is 11°. The value of δF_{pep} determined from this study, a transition temperature of 298°K, and an enthalpy change of 5100 cal/mol as determined by Kresheck and Klotz[23] from solution calorimetry were employed to evaluate eq 4. This treatment can only be considered approximate since the thermodynamic parameters employed are based upon model compound data in carbon tetrachloride, which was not the solvent used for the PBG studies. It is known from related model compound data that the solvent has an effect on the thermodynamics of amide hydrogen bond formation.[24–26]

It is also possible to employ the more recent theory of Zimm and Bragg[27] and Applequist,[28] which has been shown to reasonably account for the experimental thermodynamics of the PBG helix–coil transition, *e.g.*[29] This theory defines a quantity s, the equilibrium constant for the addition of one hydrogen bond to the end of an existing sequence of bonds. The effect of changing solvent composition on the PBG transition is to alter s, and in that way determine the value of the transition temperature. Another parameter which is given by this theory is σ, which is primarily an entropic term, and is related to the steepness of the transition. Karasz and O'Reilly[7] have shown that the transition temperatue of PBG rather than σ and the enthalpy change *per se* are altered by deuteration. Therefore, it appears as though the parameter s is mostly affected by deuteration since it is the parameter related to the transition temperature. If we make use of Applequist's eq 24 which relates σ and s to the fraction of

$$f = \tfrac{1}{2} + (s - 1)/2[(1 - s)^2 + 4\sigma s]^{1/2} \tag{5}$$

helix, f,[28] it is possible to evaluate f at a given temperature assuming the effect of deuteration on the mixed amide hydrogen bonds given by eq 3c is the same as on the backbone amide bond given by eq 3b for the situation where $s = 1.0$ and $s = 1.75$ (the ratio of the

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indefinite self-association constants for deuterated and protiated *N*-methylacetamide) using the experimental values of Karasz and O'Reilly[7] for σ. In the former case $f = 0.5$ and in the latter case $f \simeq 1.0$. Upon examination of Figure 1 of Calvin, *et al.*,[3] which gives the variation of the specific rotation, $[\alpha]_D$, with temperature for hydrogen- and deuterium-containing solutions of PBG, it is seen that the deuterated sample is essentially all in the helical form at the transition temperature of the protiated sample (which corresponds to the temperature where $f = 0.5$). Similarly, the protiated sample is nearly all in the low-temperature random configuration at the temperature which corresponds to the transition temperature of the deuterated material. These results are consistent with mechanisms 3a–3c.

Thus, subject to other detailed interpretations of the thermally induced transition of PBG,[30] it would appear that the effect of deuteration on the transition temperature can be semiquantitatively understood in terms of an effect on peptide hydrogen bonds if use is made of the theory of Zimm and Bragg[27] and Applequist[28] and the effect of deuteration on the self-association of *N*-methylacetamide.

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Effects of Sulfur Substituents on Base Stacking and Hydrogen Bonding. The Crystal Structure of 6-Thioguanosine Monohydrate[1]

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Abstract: Crystals of 6-thioguanosine monohydrate are orthorhombic, space group $C222_1$, with $a = 12.272$ (1), $b = 6.904$ (1), and $c = 32.466$ (6) Å. X-Ray diffraction data were collected with an automated diffractometer. The crystal structure was solved by the heavy-atom method and refined by least squares to $R = 0.07$. The pattern of hydrogen bonding between bases is the same as that found in the crystal structures of guanine monohydrate and guanosine dihydrate. The bases form planar ribbons wherein adjacent thioguanine residues are related by screw axes and are joined by N(1)–H···N(7) and N(2)–H···S hydrogen bonds that have lengths of 2.96 and 3.27 Å, respectively. The parallel ribbons of bases are stacked with an interplanar separation of 3.4 Å. The base-stacking pattern involves a slight base overlap, with the sulfur atoms in close contact with purine rings of adjacent bases. The hydrogen-bonding and the base-stacking properties of thiopurines and thiopyrimidines are reviewed. The crystal structures of almost all other thio bases also involve hydrogen bonding to the sulfur substituents, and display stacking patterns in which the sulfur substituents are positioned in close contact with the ring systems of adjacent bases. It appears that replacement of carbonyl oxygen atoms by sulfur substituents affects hydrogen bond lengths, but has little additional effect on the solid state hydrogen-bonding patterns of purines and pyrimidines.

Sulfur derivatives of purines and pyrimidines occur as minor components of transfer ribonucleic acids (tRNA's).[2] Little is known about the function of these thio derivatives, but they probably play an important role in controlling the conformation of tRNA. Therefore, there has been considerable interest in the structural properties of thiopurines and thiopyrimidines.[3–20] Since secondary and tertiary structures of nucleic acids are largely controlled by base-stacking and hydrogen-bonding interactions, it is particularly important to understand the effects that sulfur substituents exert on these interactions.

In addition to the naturally occurring thio bases, a synthetic sulfur derivative, 6-thioguanine, can be incorporated into nucleic acids,[21–26] probably by sub-

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