[CONTRIBUTION FROM THE DEPARTMENT OF CHEMISTRY, CORNELL UNIVERSITY]

Effect of Deuterium on the Strength of Hydrogen Bonds[1]

BY MELVIN CALVIN,[2] JAN HERMANS, JR., AND HAROLD A. SCHERAGA

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The substitution of deuterium for hydrogen will affect the strength of a hydrogen bond. As a consequence one would expect a deuterated helical macromolecule to have a different stability from a hydrogen-containing one, using the random coil as a reference state. This altered stability could manifest itself in a change of about 5° in the transition temperature between a helix and a random coil at room temperature. The effect of deuterium–hydrogen substitution was investigated using poly-γ-benzyl-L-glutamate in a mixture of dichloroethane and dichloroacetic acid. Because of specific solvent effects this transition is a reverse one,[3] the helix being the form stable at high temperature. The magnitude and direction of the change in the transition temperature in this system, when deuterium is substituted for hydrogen, depends on the relative strengths of the various types of hydrogen bonds involved and cannot be predicted from *a priori* considerations. The demonstrated decrease of 11° in the transition temperature upon deuteration has implications for the stability of helical macromolecules (*e.g.*, proteins and nucleic acids) in living systems.

Introduction

Experiments in which D_2O was fed to mice and other organisms have suggested that the hydrogen-bonded structures of nucleic acids are rendered less stable with respect to the randomly coiled form if deuterium is substituted for hydrogen.[4-7] Since the thermodynamics of the equilibrium between the helical and randomly coiled forms of biological macromolecules is a problem of general interest, it was decided to investigate the effect of substituting deuterium for hydrogen in a synthetic polypeptide.

Poly-γ-benzyl-L-glutamate (PBG) was selected as a model compound, since it has been shown[3] to undergo a relatively sharp transition from a random coil to a helix in an accessible temperature range in a 20:80 (v:v) mixture of dichloroethane and dichloroacetic acid (hereafter referred to as DEDA). The transition can be followed conveniently by measuring the optical rotation of the solution as a function of temperature.

Experimental

Materials.—1,2-Dichloroethane (DCE) and dichloroacetic acid (DCA) were Eastman Kodak products. The DCE was dried with $CaCl_2$ and decanted. The DCA was distilled under reduced pressure. Heavy water, containing at least 99.5% D_2O was obtained from the Stuart Oxygen Co., San Francisco. Two samples of poly-γ-benzyl-L-glutamate (PBG) were a gift from Dr. L. de Vries of the California Research Corporation. They were prepared according to the procedure of Blout and Karlson[8] and were reported to have molecular weights of 1–2 × 10^5 (No. 1) and 2–3 × 10^5 (No. 2), respectively.

Solutions.—Approximately 40 mg. of hydrogen-containing PBG was dissolved in hydrogen-containing DEDA to give a final volume of 3 ml. for each experiment. In this solvent PBG exists in the randomly-coiled form at room temperature.[3]

Elliott and Hanby[9] have shown that PBG can be deuterated essentially instantaneously by dissolving it in a solvent containing enough DCA (deuterated) to ensure that the polypeptide is in the randomly-coiled form.[10] Deuterated DCA was prepared by mixing 10 ml. of the hydrogen-containing acid with 10 ml. of D_2O and the excess D_2O and H_2O removed by vacuum distillation at 20° to a cold trap at −80°. The resulting product was treated with a second 10-ml. sample of D_2O and the vacuum distillation repeated to achieve essentially complete deuteration. Deuterated PBG was prepared by dissolving *ca.* 40 mg. of the hydrogen-containing polypeptide in deuterated DEDA to give a final volume of 3 ml. for each experiment. The contribution of the hydrogen from the original PBG reduced the D:H ratio of the solution to about 50:1, which was sufficiently high for our purpose.[10]

Optical Rotation.—Use was made of a model 80 Rudolph polarimeter equipped with a sodium lamp. The tube used had a length of 2 dm., a diameter of 2 mm., and water from a thermostat was run through a jacket around it to ensure temperature control. The tube contained between 1.5 and 2 ml. of liquid and was filled with the aid of a syringe with a long needle. A precision of ±0.01° was achieved in the readings of the extinction position. Since the total change in rotation in the transition is 0.7° at this concentration, this assures an accurate determination of the transition temperature, T_{tr}. In fact, the precision with which T_{tr} can be determined from a single experiment is much greater than the agreement between duplicate experiments (see Table I).

Since the solution expands more rapidly with temperature than the tube, liquid is forced out of the tube on heating. Upon cooling, air is drawn in, forming a bubble and obstructing the light path. Therefore, experiments were performed only with increasing temperature, since the reversibility of the transition has been demonstrated by Doty and Yang.[3]

Results

Figure 1 shows the specific rotation $[\alpha]_D$ as a function of temperature for a hydrogen-containing (H) and a deuterated (D) solution of sample 1. Other experiments gave curves of very much the same aspect, though not quite identical. Table I gives the relevant parameters for these curves: $\Delta[\alpha]$, the total change in specific rotation for the complete transition; T_{tr}, the temperature at which $[\alpha]$ has changed by half its total change; and the reciprocal of the slope at T_{tr}, $\Delta[\alpha]\{dT/d[\alpha]\}_{T_{tr}}$. It is seen that one cannot distinguish between samples 1 and 2. Although the reproducibility is not as high as would be desired, the effect of D–H substitution is evident. The fact most clearly emerging from these data is that T_{tr} is lowered by about 11° when H is replaced by D. Furthermore, the transition occurs in a smaller

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[1] This investigation was supported by research grant No. E-1473 from the National Institute of Allergy and Infectious Diseases, of the National Institutes of Health, Public Health Service.

[2] George F. Baker non-resident lecturer at Cornell University, 1958.

[3] P. Doty and J. T. Yang, THIS JOURNAL, **78**, 498 (1956).

[4] A. M. Hughes, B. M. Tolbert, K. Lonberg-Holm and M. Calvin, *Biochim. Biophys. Acta*, **28**, 58 (1958).

[5] V. Moses, O. Holm-Hansen and M. Calvin, *ibid.*, **28**, 62 (1958).

[6] A. M. Hughes and M. Calvin, *Science*, **127**, 1445 (1958).

[7] A. M. Hughes, E. L. Bennett and M. Calvin, *Proc. Nat. Acad. Sci.*, **45**, 581 (1959).

[8] E. R. Blout and R. H. Karlson, THIS JOURNAL, **78**, 941 (1956).

[9] A. Elliott and W. E. Hanby, *Nature*, **182**, 654 (1958).

[10] Since Elliott and Hanby[9] found no evidence for an N–H band in the infrared spectrum of deuterated PBG, we may assume that no complications arise from an exchange reaction involving the carbon-bound hydrogen of DCA. This remark is made since Potter and Ritter[11] did find evidence for such exchange in CH_3COOD at 100°.

[11] A. E. Potter, Jr., and H. L. Ritter, *J. Phys. Chem.*, **58**, 1040 (1954).

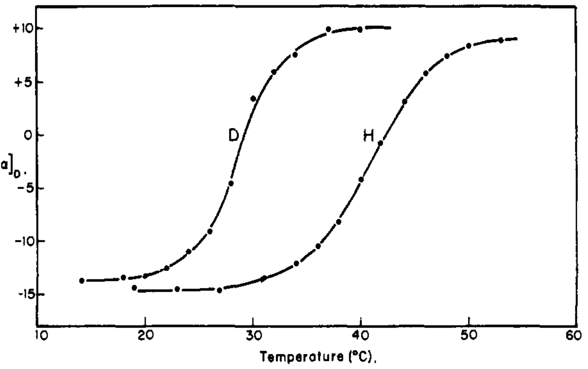

Fig. 1.—Specific rotation (NaD line) as a function of temperature for hydrogen-containing (H) and deuterated (D) solutions of PBG in DEDA (Sample 1, expts. 4 and 6). A large $[\alpha]_D$ corresponds to a high ratio (helix)/(random coil).

temperature range in the deuterated system. It is useful to point out here that the form occurring at *high* temperatures is the helix, as was shown by Doty and Yang.[3]

TABLE I

TRANSITION DATA FOR HYDROGEN-CONTAINING AND DEUTERATED SOLUTIONS

(For clarity the subscript D has been omitted from $[\alpha]_D$)

Sample	Expt.	$\Delta[\alpha]$, degree	T_{tr}, °C.	$\Delta[\alpha]\{dT/d[\alpha]\}_{T_{tr}}$
1 (H)	4	25	41	12
(H)	9	30	40	13
(H)	1	22	39	13
(D)	6	25	29	7
(D)	8	29	28	11
2 (H)	3	24	40	10
(H)	10	24	40	13
(D)	5	(33)	28	9
(D)	7	26	31	8

Discussion

Regarding the values of $[\alpha]_D$ as a measure of the relative concentrations of helical and randomly-coiled forms at any temperature, the transition temperature, T_{tr}, as defined above, may also be considered as the temperature at which $\Delta F = 0$, where ΔF is the free energy change for the formation of an n-membered helix from the random coil. Following Schellman,[12,13] ΔF may be approximated by $n\Delta F_{pep}$ for sufficiently large n (*i.e.*, neglecting the effect of the chain ends), where ΔF_{pep} is the free energy change when one peptide hydrogen bond is formed next to a bond already present. Therefore, ΔF_{pep} is also zero at T_{tr}, giving

$$T_{tr} = \Delta H_{pep}/\Delta S_{pep} \qquad (1)$$

where ΔH_{pep} and ΔS_{pep} are the enthalpy and entropy of formation of a single peptide hydrogen bond. Thus, the relative change in T_{tr} for the deuterium–hydrogen substitution will be determined by the relative change in ΔH_{pep} and ΔS_{pep}. Writing δF_{pep}, δH_{pep} and δS_{pep} for the differences in ΔF_{pep}, ΔH_{pep} and ΔS_{pep} when the molecule contains deuterium and when it contains hydrogen, and also δT_{tr} for the accompanying change in T_{tr}, it follows from equation 1, neglecting second and higher powers of δ, that

$$\frac{\delta T_{tr}}{T_{tr}} = \frac{\delta H_{pep}}{\Delta H_{pep}} - \frac{T_{tr}\delta S_{pep}}{T_{tr}\Delta S_{pep}} = \frac{\delta F_{pep}}{\Delta H_{pep}} \qquad (2)$$

since $\Delta H_{pep} = T_{tr}\Delta S_{pep}$.

If the transition under consideration here involved only the rupture of peptide hydrogen bonds, *i.e.*, a transformation from helix to random coil as the temperature is raised, then the computation of δF_{pep} would be straightforward in principle. The value of δF_{pep} would arise essentially from the difference between the vibrational zero-point energies (for stretching and bending) of the hydrogen bonded and non-hydrogen bonded ND and NH groups, and also from the effect of replacement of H by D on the rotational and vibrational degrees of freedom (of the whole molecule) which are lost or gained when the hydrogen bond is formed.[14]

As was already pointed out by Doty and Yang[3] and by Schellman,[13] the reverse transition (in which the random coil is transformed to a helix as the temperature is raised) arises from selective binding of DCA by PBG in the mixed solvent. As shown in Fig. 2, the binding of DCA breaks the peptide hydrogen bonds and stabilizes the randomly coiled form. Raising the temperature decreases the extent of this binding, shifting the equilibrium in favor of the helical form. Figure 2, which shows one of many possibilities, illustrates the fact that in first approximation there is no net change in the number of hydrogen bonds. In such a case, deuterium substitution will affect T_{tr} only if the hydrogen bonds are of unequal strength.

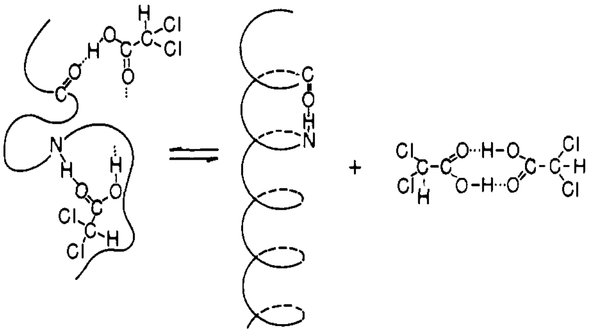

Fig. 2.—A schematic representation of the equilibrium between helical and randomly-coiled forms of PBG in DEDA. The binding of DCA to the random coil is represented in terms of hydrogen bonding to the peptide C=O and N—H groups.

According to Schellman[13]

$$\{1/\Delta[\alpha]\}\{d[\alpha]/dT\}_{T_{tr}} = -\Delta S/4RT \qquad (3)$$

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[12] J. A. Schellman, *Compt. rend. Lab. Carlsberg, Sér. chim.*, **29**, 230 (1955).

[13] J. A. Schellman, *J. Phys. Chem.*, **62**, 1485 (1958).

[14] It is of interest that experimental data are available on the effect of deuterium substitution on the OH...O bonds of the acetic acid dimer in the gas phase.[15] Values of $\Delta H = -7$ kcal./mole and (at 350°K.) $\delta F = -70$ cal./mole per hydrogen bond are reported. By analogy, an expected value of δF_{pep} between +100 and −100 cal./mole for the peptide hydrogen bond in water seems like a reasonable estimate. From eq. 2, using as an estimate $\Delta H_{pep} = -6$ kcal./mole,[16] this would mean a change δT_{tr} between +5 and −5° for $T_{tr} = 300°$.

[15] A. E. Potter, Jr., P. Bender and H. L. Ritter, *J. Phys. Chem.*, **59**, 250 (1955).

[16] For a discussion of the value of ΔH_{pep}, see H. A. Scheraga *Ann. Rev. Phys. Chem.*, **10**, in press (1959).

where ΔS is the entropy of formation of the form stable at high temperature. From the experimental data we can, therefore, compute the following values, assuming a degree of polymerization $n = 700$: $\Delta S/n = -0.30$ e.u. and $\Delta H/n = -94$ cal./mole for the hydrogen-containing molecule, and $\delta S/n = -0.09$ e.u., $\delta H/n = -24$ cal./mole, $\delta F/n = 4$ cal./mole for the changes accompanying deuterium–hydrogen substitution. The values of $\Delta H/n$ and $\Delta S/n$ are extremely small compared with those usually encountered,[16] and this is, of course, in agreement with the conclusion drawn in the preceding paragraph, *i.e.*, the values $\Delta H/n$ and $\Delta S/n$ obtained here are values for the over-all reaction (Fig. 2) involving ΔH and ΔS of several different species of hydrogen-bonds, and are necessarily small.[17]

In order to provide a quantitative argument to account for the magnitude and direction of the change in T_{tr}, the enthalpies and entropies of formation of each of these kinds of hydrogen bonds would have to be estimated. However, in the present state of our knowledge, it is not possible to make these estimates with sufficient accuracy, so that the observed decrease of 11° in T_{tr} must be reconciled qualitatively in terms of the explanation provided above, *viz.*, deuterium substitution affects all the hydrogen bonds indicated in Fig. 2 and the net effect on T_{tr} can be either an increase or a decrease depending on the relative stability of the various kinds of hydrogen bonds involved.

From an experimental point of view, a measurable change in T_{tr} upon deuterium–hydrogen substitution has been demonstrated. The reconciliation with theory must await a more accurate estimate of the thermodynamic parameters for the formation of various kinds of hydrogen bonds.

It is of interest to note from Fig. 1 that, at a temperature of *ca.* 35°, the fraction of molecules in the helical form is very small for the hydrogen-containing sample and very large for the deuterated sample, showing the very large effect of deuterium substitution in a small temperature range. It is thus very interesting to speculate that the ratio of the concentrations of helices to random coils in a biological system (*e.g.*, in genes) can be markedly affected by deuteration. The effect of deuteration on the helix/random coil ratio also has implications for the interpretation of data from experiments on the kinetics of deuterium–hydrogen exchange.[18] In the latter technique, it is assumed that the substitution of deuterium for hydrogen does not change the helix/random coil ratio. However, if such a study were carried out at a temperature near the transition region (*e.g.*, 35° in the case considered here), deuteration would affect this ratio. Of course, if the kinetics experiment is carried out at a temperature sufficiently removed from the transition region, then this ratio will presumably not be affected. It seems probable that most of the experiments heretofore carried out on the kinetics of deuterium–hydrogen exchange have not been performed near the transition region. The effects of D–H substitution on the stability of helical structures in proteins is at present under investigation and will be reported shortly.

Acknowledgment.—We are indebted to Prof. S. H. Bauer for helpful discussions of this problem.

ITHACA, N.Y.

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[17] It may be noticed that the ratio of the *experimental* quantities $\delta H/\Delta H$ is ~1/4, which is rather large. This large ratio probably arises because small changes in the degree of hydrogen bonding in each of the species of Fig. 2 (produced here by D–H substitution) can produce relatively large changes in the small over-all ΔH.

[18] See, for example, K. Linderstrøm-Lang, "Symposium on Peptide Chemistry," The Chemical Society, London, 1955, Special Publication No. 2, p. 1.

[CONTRIBUTION FROM THE DEPARTMENTS OF CHEMISTRY, UNIVERSITY OF KANSAS AND PRINCETON UNIVERSITY]

Recoil Tritium Reactions at an Asymmetric Carbon: L(+)-Alanine[1]

BY JACK G. KAY, R. P. MALSAN AND F. S. ROWLAND

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Recoil tritium reactions with L(+)-alanine have been investigated in both crystalline form and in aqueous solution. The T for H substitution at the asymmetric carbon atom occurs with complete retention of configuration in the crystal and occurs for only 0.01% of the total tritium in the liquid solution. More than 85% of the T for H substitutions in the methyl group in both cases proceed with retention of optical configuration.

Introduction

Recoil tritium atoms from the $Li^6(n,\alpha)H^3$ reaction have been shown in previous studies to replace protium in C–H bonds in a variety of molecules without affecting the molecule involved in any other permanent manner.[2] Isolation and intramolecular degradation of tritium labeled glucose and galactose molecules have demonstrated that substantial percentages of this radioactivity are bound in non-labile positions at asymmetric carbon atoms.[3,4] Experiments in both gas phase,[5] and condensed phases[6] indicate that the substitution of T for H in other molecules takes place through an intermediate complex rather than through a free radical mechanism. Considerable information can be obtained about the nature of such a complex by study of the reactions of recoil

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[1] Research supported in part by A. E. C. Contract No. AT-(11-1)-407. Presented in part at the 130th Meeting of the American Chemical Society, Atlantic City, September, 1956.

[2] See, for example, F. S. Rowland and R. Wolfgang, *Nucleonics*, **14**, No. 8, 58 (1956).

[3] F. S. Rowland, C. N. Turton and R. Wolfgang, THIS JOURNAL, **78**, 2354 (1956).

[4] H. Keller and F. S. Rowland, *J. Phys. Chem.*, **62**, 1373 (1958).

[5] M. El-Sayed, P. Estrup and R. Wolfgang, *ibid.*, **62**, 1356 (1958).

[6] W. J. Hoff, Jr., and F. S. Rowland, THIS JOURNAL, **79**, 4867 (1957).