[CONTRIBUTION FROM THE CHEMICAL LABORATORY OF NORTHWESTERN UNIVERSITY, EVANSTON, ILLINOIS]

Hydrogen Bonds between Model Peptide Groups in Solution

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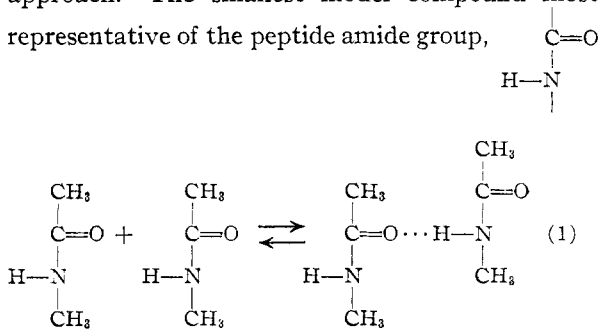
The aggregation of N-methylacetamide through the formation of inter-amide hydrogen bonds has been studied in three solvents: water, dioxane and carbon tetrachloride. Stability constants, enthalpies, entropies of hydrogen-bond formation have been evaluated. In aqueous solution the interamide hydrogen bond of this model for a peptide has essentially no intrinsic strength. This bond strength is slight in dioxane solution, moderately strong in carbon tetrachloride. It seems unlikely, therefore, that interpeptide hydrogen bonds contribute significantly to the stabilization of macromolecular configuration in aqueous solution.

Introduction

Among the forces that contribute to the stabilization of configuration of protein molecules in solution, intramolecular hydrogen bonds have often been assigned a key role. Hydrogen bonding between peptide groups has indeed been definitely demonstrated in proteins and polypeptides, as well as in model amino acids and small peptides, in the solid state.[1,2] In aqueous solution, however, the situation is not clear. Even for model amides there has been no reliable information on the intrinsic strength and stability of interamide hydrogen bonds under exposure to competing bonds from water.

It has appeared essential, therefore, to examine hydrogen bonding in an appropriate solute dissolved in water, by some direct experimental approach. The smallest model compound most representative of the peptide amide group,



is N-methylacetamide which at least in principle could form interamide hydrogen bonds as indicated in the equilibrium in equation 1. In nonaqueous solution one would expect this equilibrium to lie far to the right, but in water the monomers on the left-hand side of equation 1 would form hydrogen bonds with the solvent which would tend to draw the equilibrium toward the left.

In any solvent the environment of the N–H group of an N-methylacetamide molecule changes if it forms a hydrogen bond with another such molecule. One might expect, therefore, to be able to follow the equilibrium of equation 1 either by infrared or nuclear magnetic resonance methods. Both of these have indeed been found useful, but infrared, in the overtone range, has proved more suitable for quantitative investigations.

(1) G. Pimentel and A. L. McClellan, "The Hydrogen Bond," W. H. Freeman and Co., San Francisco, 1960.

(2) J. C. Kendrew, R. E. Dickerson, B. E. Strandberg, R. G. Hart, D. R. Davies, D. C. Philips and V. G. Shore, *Nature*, **185**, 422 (1960).

Infrared studies of absorption in the 3μ region due to N–H stretching, or in the 6μ region due to C=O vibrations, have indeed been carried out in the past.[1,3] For quantitative investigations in aqueous solution, however, these regions are not suitable, for the absorption of water is so strong that formidable experimental difficulties are encountered in attempts to obtain reproducible small thicknesses between faces of the absorption cell. In the overtone range of 1.5 μ, however, extinction coefficients are much smaller, so that optical path lengths of about 1 mm. are appropriate. Furthermore glass is transparent in this range and hence absorption cells with precision and stable optical path lengths are obtainable. Absorption measurements in the near infrared have proved very fruitful for investigations of the hydrogen-bond equilibrium of equation 1.

In connection with any attempts to transpose conclusions from model amides to interpeptide hydrogen bonds in proteins, it is necessary to know not only the over-all stability of the bond, as measured by ΔF° or the equilibrium constant, but also the intrinsic strength of the bond, as given by ΔH. The equilibrium of equation 1 was studied, therefore, at two temperatures. In addition hydrogen bonding in N-methylacetamide was examined in organic solvents so that an appraisal might be made of the effects of an apolar environment within a protein on the stability of such bonds.

Experimental

Materials.—N-Methylacetamide and N-dimethylacetamide, purchased from Eastman Organic Chemicals, were distilled at atmospheric pressure through a 38-cm. Vigreux column, fractions boiling at 205–206° and 164–165°, respectively, being collected. Carbon tetrachloride and dioxane were Fisher Spectranalyzed Certified Reagent grade liquids.

Preparation of Sample and Reference Solutions.—Particularly in aqueous solvents, special care is necessary to compensate precisely for the absorption of water in the near infrared. The stratagem finally adopted maintained the water concentrations in sample and reference cell essentially identical by placing dimethylacetamide in the reference cell, corresponding to the solute methylacetamide in the sample cell. The former, $CH_3CON(CH_3)_2$, shows very little absorption in the region 1.4–1.6 μ since it lacks the NH group of $CH_3CONHCH_3$.

In actual practice the preparation of solutions was preceded by a series of determinations of densities of aqueous solutions of N-methylacetamide of accurately-known composition by weight, as well as of solutions of dimethylacetamide. With these data one could readily prepare sample and reference solutions containing equal molar concentrations of water.

(3) M. Tsuboi, *Bull. Chem. Soc. Japan*, **22**, 215 (1949).

A similar procedure was used with dioxane as the solvent except that carbon tetrachloride was used as the transparent solute for the reference cell.

For studies with carbon tetrachloride as solvent, pure solvent was used in the reference cell. The optical density of this solvent in the 1.4–1.6 μ range is very small. Furthermore aggregation of N-methylacetamide in carbon tetrachloride occurs at low concentrations of solute, in which solutions the concentration of solvent is nearly equal to that in the reference cell.

Spectroscopic Measurements.—Infrared spectra in the region of 1.3–1.7 μ were recorded with both the Beckman *DK*-2 and Cary 14 spectrophotometers. The latter was used at a late stage in our investigations to obtain the best equilibrium constants at 25°. Since the Beckman instrument contained a cell holder mounted in a water-heated block, it was used for parallel measurements at 25 and 60° to provide data for the computation of the enthalpy change for the reaction in equation 1. Temperatures were measured with a thermistor sealed into the stopper of the absorption cell and connected to a Wheatstone bridge.

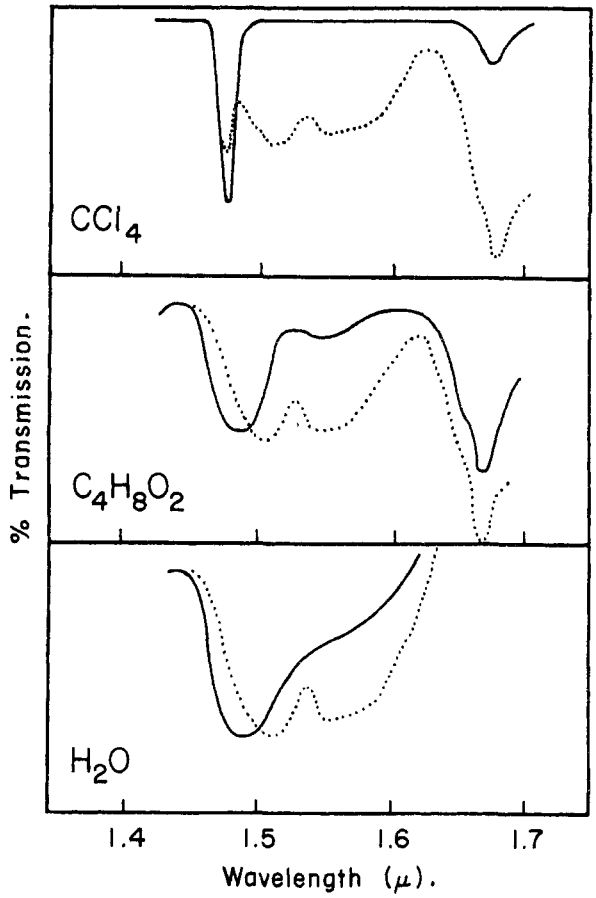


Fig. 1.—Absorption spectra of N-methylacetamide in carbon tetrachloride, dioxane and water, respectively: solute concentrations in CCl_4, ———, 0.01 *M*; ....., 1 *M*; $C_4H_8O_2$, ——— 0.2 *M*; ..... 3 *M*; and in H_2O, ———, 7 *M*; ....., 12.5 *M*.

Cells with optical path lengths of 0.05–10 cm. were used with different solvents. For water the 0.2 cm. cell was most convenient. One must realize that with pure water, even in such a small cell, 99% of the incident light at 1.44 μ is absorbed by the solvent, *i.e.*, the optical density is 2. (At 1.6 μ the optical density drops to 0.5.) In essence then one must follow changes in absorption at some wave lengths above optical densities of 2, where artifacts sometimes arise due to stray light, and where if there are any small perturbations in the spectrum of water induced by the solute, the large optical density of water could lead to appreciable uncertainty as to the contribution of the solute to the observed spectra. Before a Cary spectrophotometer was available to us, we tested for such possible artifacts by recording some spectra with cells of 0.05 cm. optical path in which water transmits 20–30% of the incident light even at its point of maximum absorption. These spectra, although not as precise, were essentially the same as those in the 0.2 cm. cells. Subsequently when a Cary spectrophotometer was available, which is designed to permit measurements appreciably above optical densities of 2, all aqueous solutions were run again; there were small differences in extinction coefficients, but in general the spectra were similar to those from the Beckman instrument. We feel, therefore, that artifacts due to stray light have not been encountered.

In actual practice, furthermore, all measurements for the determination of extent of hydrogen bonding were made under more favorable conditions of optical transmission, for as soon as any solute is dissolved in the water, the concentration of the latter decreases and so does its contribution to the optical density. In concentrated amide solutions where the important changes in spectra are observed, the contribution of water to the absorption drops decidedly. For example an 11 *M* solution of N-methylacetamide contains only about 10 *M* water. Hence even at 1.44 μ the optical density of the water should be only about 0.36, instead of 2, and of course its optical density is markedly less at surrounding wave lengths.

It is apparent that the contribution of water to the total optical density decreases and that of the solute increases, with increasing concentration of the latter. In contrast, any perturbation by the solute of the spectrum of water, if it occurs at all, would become more pronounced at high concentrations of solute, but at such high concentrations water's contribution to the absorption declines markedly and hence the uncertainty due to this factor must also decrease sharply. In addition, any ambiguity due to a spectroscopic perturbation should be minimized even further by our technique of using dimethylacetamide, in the reference cell. This solute in the reference cell is very similar in structure to that of the amide in the sample cell.

We feel, therefore, that ambiguities attributable to the high optical density of pure water are negligible in magnitude.

Results and Discussion

Comparison of Amide Spectra in Different Solvents.—In carbon tetrachloride solutions, N-methylacetamide at low concentrations (~0.01 *M*) shows a single sharp peak (Fig. 1) at 1.47 μ (6800 cm.$^{-1}$), corresponding to the first overtone of the N-H stretching vibration.[4] In dilute dioxane or water solutions, this peak (Fig. 1) appears at 1.48 μ (6750 cm.$^{-1}$), but it is markedly broadened and its extinction coefficient is reduced. Nevertheless, computed integrated extinction coefficients are nearly equal in all three solvents (about 88,000 cm. mole^{-1}).

As the concentration of N-methylacetamide is increased in each solvent, the extinction coefficient at 1.47–1.48 μ decreases and a new double-peaked absorption band appears between 1.5–1.6 μ. The new band is attributed to N–H groups hydrogen-bonded to O=C. As the solute concentration increases, the spectra in the three solvents approach a common shape, that of liquid N-methylacetamide.

The similarity in position of the peaks in the three solvents seems to justify the assumption that corresponding monomer–oligomer equilibria are being observed in each case. The concentration range in which aggregation of solute molecules

(4) M. Davies, J. C. Evans and L. Jones, *Trans. Faraday Soc.*, **51**, 761 (1955).

becomes observable, however, differs greatly from solvent to solvent.

Computation of Extent of Aggregation.—Previous investigations[5,6] show pretty conclusively that N-methylacetamide in its aggregation phenomena forms dimers, trimers and higher oligomers. Such associations would be expected for a *trans*—C(=O)—N(H)— structure. For our quantitative calculations we must recognize that each n-mer has one free (terminal) N–H group. Furthermore, we have assumed that each such terminal N–H group, in a specific solvent, absorbs at the same frequency and to the same extent in the 1.47–1.48μ range. Such an assumption seems reasonable on *a priori* grounds since aggregation perturbs atoms at least two atoms removed from the terminal N–H group. The assumption also seems justified by previous examinations of the corresponding question for hydroxyl groups. Hammaker's[7] investigations of methanol aggregation in carbon tetrachloride showed only one high-frequency peak due to free O–H groups and revealed no evidence for a separate O–H band due to terminal O–H groups on oligomers. Similarly Kuhn's[8] studies of the infrared spectra of a number of glycols containing one intramolecular hydrogen bond and one free O–H showed that the non-hydrogen-bonded O–H band appeared at essentially the same frequency as that of the free O–H group of the corresponding monohydric alcohol.

The concentration of associated N–H groups may then be calculated by the following procedure. If we assume that aggregated and non-aggregated species obey Beer's law, then the observed optical density, O. D., at any concentration is given by

$$\text{O. D.} = C_f E_f l + C_b E_b l = C_t E_{app.} l \qquad (2)$$

where C_f = concentration of free N—H groups *not* hydrogen-bonded to O=C
C_b = concentration of N—H groups hydrogen-bonded to O=C
C_t = total concentration of N-methylacetamide residues
E_f = extinction coefficient of free N—H
E_b = extinction coefficient of N—H bonded to O=C
$E_{app.}$ = apparent extinction coefficient
l = optical path length of the cell

Since conservation of mass requires that

$$C_t = C_f + C_b \qquad (3)$$

we find by simple algebraic manipulation that

$$C_f = \frac{E_{app.} - E_b}{E_f - E_b} C_t \qquad (4)$$

To compute C_f we must know $E_{app.}$, which is calculated from the observed O. D. at a particular C_t and known l, and E_f and E_b. The former, E_f, is obtained by extrapolating a graph of $E_{app.}$ *versus* C_t to zero concentration, at which point all of the solute should be in the monomeric state, and the latter, E_b, is obtained from an extrapolation of $E_{app.}$ to unit mole fraction of solute, at which point essentially all the N–H groups should be hydrogen-bonded to O=C. At 1.48 μ E_f differed substantially in the three solvents (being about 2×10^3 cm.^{2} mole^{-1} in CCl_4 but only about 2×10^2 in dioxane or water), whereas in all cases E_b was essentially the same (about 60) as that for liquid N-methylacetamide.

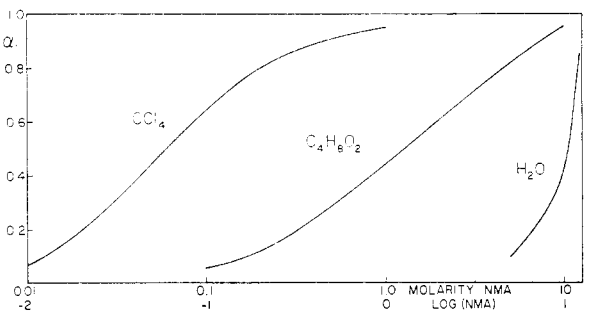


Fig. 2.—Variation of degree of association, α, with concentration of N-methylacetamide in carbon tetrachloride, dioxane and water, respectively.

Knowing C_f from equation 4 one can obtain C_b immediately from equation 3. It has proved convenient thereafter to summarize the experiments in terms of α

$$\alpha = \frac{C_b}{C_t} \qquad (5)$$

and to plot α as a function of total solute concentration. α is thus the degree of association, or the fraction of N–H groups bonded to carbonyl groups. Figure 2 shows the results for the three solvents studied.

As one might have anticipated, interamide hydrogen bonds are most stable in carbon tetrachloride and least stable in water. At the outset we did not know which extreme dioxane would approach, for its dielectric constant is almost identical with that of CCl_4, but it does have electron donor groups in its ether oxygens which could participate in hydrogen bonding. The results in Fig. 2 indicate that the latter factor rather than the dielectric constant is the dominant one. The crucial role of competitive hydrogen bonding by solvent molecules is also demonstrated by the results in water; interamide hydrogen bonds do not form until the solute concentration is very high.

Multiple Equilibria in Amide Aggregation.—To evaluate the thermodynamic stability of the interamide hydrogen bond, we must start with an analysis of the multiple equilibria present in a reversible monomer-oligomer aggregation. For this purpose we shall represent each monomer residue as —A·, the dot indicating the free N–H group. Assuming a *trans* structure for the CONH group, the successive equilibria may then be written as

(5) S. Mizushima, T. Simanouti, S. Nagakura, K. Kuratani, M. Tsuboi, H. Baba and O. Fujioka, *J. Am. Chem. Soc.*, **72**, 3490 (1950).
(6) M. Davies and D. K. Thomas, *J. Phys. Chem.*, **60**, 767 (1956).
(7) R. Hammaker, Ph.D. Dissertation, Northwestern University, 1960.
(8) L. Kuhn, *J. Am. Chem. Soc.*, **74**, 2492 (1952).

$$-A\cdot + -A\cdot = -A\cdot\cdot A- \qquad k_2 = \frac{(A_2)}{(A)^2} \qquad (6)$$

$$A + A_2 = A_3 \qquad k_3 = \frac{(A_3)}{(A_2)(A)} \qquad (7)$$

$$\vdots \qquad\qquad \vdots$$

$$A + A_{n-1} = A_n \qquad k_n = \frac{(A_n)}{(A_{n-1})(A)} \qquad (8)$$

The fraction of bonded N–H groups,[9] α, is given by

$$\alpha = \frac{(A_2) + 2(A_3) + 3(A_4) + \ldots}{(A) + 2(A_2) + 3(A_3) + \ldots} \qquad (9)$$

and of non-bonded N–H groups, $1 - \alpha$, by

$$1 - \alpha = \frac{(A) + (A_2) + (A_3) + \ldots}{(A) + 2(A_2) + 3(A_3) + \ldots} \qquad (10)$$

Consequently

$$\frac{\alpha}{1 - \alpha} = \frac{(A_2) + 2(A_3) + 3(A_4) + \ldots}{(A) + (A_2) + (A_3) + \ldots} \qquad (11)$$

The concentrations of various oligomer species in equation 11 may be replaced[9] by corresponding association equilibrium constants $k_2, \ldots, k_n$, from equations 6-8, and the concentration of monomer, A. Thereby we obtain

$$\frac{\alpha}{1 - \alpha} = \frac{k_2A + 2k_2k_3A^2 + \ldots}{1 + k_2A + k_2k_3A^2 + \ldots} \qquad (12)$$

from which it follows that

$$\lim_{A \to 0}\left(\frac{\alpha}{1 - \alpha}\frac{1}{A}\right) = k_2 \qquad (13)$$

Thus in principle the stabilities of interamide hydrogen bonds of N-methylacetamide in different solvents can be evaluated when k_2's are known.

In practice our infrared measurements do not give us values of monomer concentration (A) but rather of C_f, the concentration of terminal free N–H groups contributed by each of the oligomers. In the limit of zero concentration, however, as $(A) \to 0$

$$\frac{(A)}{C_f} \longrightarrow 1$$

and (14)

$$\alpha \longrightarrow 0$$

and hence equation 13 may be replaced by

$$\lim_{\alpha \to 0}\left(\frac{\alpha}{1 - \alpha}\frac{1}{C_f}\right) = k_2 \qquad (15)$$

Since α and C_f are directly obtainable from the experimental observations, it is the limiting value of a graph of $\frac{\alpha}{1 - \alpha}\frac{1}{C_f}$ *versus* α that has been used in practice to evaluate k_2 in each solvent. Such extrapolations have been carried out, and the values of the association constants for dimer formation (in molar concentration units) are assembled in Table I.

TABLE I

THERMODYNAMICS OF INTERAMIDE HYDROGEN BOND FORMATION BY N-METHYLACETAMIDE AT 25°

Solvent	Association constant for dimerization, k_2	$\Delta F°$, kcal. mole^{-1}	$\Delta H°$, kcal. mole^{-1}	$\Delta S°$, gibbs mole^{-1}
Carbon tetrachloride	4.7 (5.8)	−0.92	−4.2	−11
Dioxane	0.52 (0.58)	0.39	−0.8	− 4
Water	0.005 (0.005)	3.1	0.0	−10

Relatively large positive slopes have been observed in the graphs of $\frac{\alpha}{1 - \alpha}\frac{1}{C_f}$ *versus* α. These indicate that there are strong positive interactions in the higher aggregation steps, that is, that once the dimer is formed, a trimer is formed even more readily. We have, therefore, also turned to a previous mathematical analysis of aggregation of N-methylacetamide, in benzene as solvent, that of Davies and Thomas,[6] which assumes explicitly that

$$k_2 < k_3 = k_4 = \ldots = k_n \qquad (16)$$

On this basis Davies and Thomas have shown that an equation of the following form provides the basis of a linear graphical determination of k_2 and k_3

$$\frac{C_b^{1/2}}{C_f - C_b} = k_2 + k_3\frac{C_fC_b^{1/2}}{C_f - C_b} \qquad (17)$$

We have found that our data fit equation 17 well in each solvent. The values obtained for k_2 by this procedure are shown in parentheses in Table I and are in reasonable agreement with the results of the first procedure.

For water as solvent, the Davies–Thomas graph gives a more reliable extrapolation than is obtained with our function. Determined by either procedure, however, the precise value of the equilibrium constant for N-methylacetamide dimerization in water is uncertain because it is so small. Aggregation is not appreciable until the concentration C_t is above 5 molar. On this basis alone there is no doubt that k_2 is a very small number, substantially below 0.01, and our best estimate is 0.005.

A few measurements also were carried out in a mixed solvent, dioxane–water (45:55 by weight), with a dielectric constant[10] of 39.1 at 25°. The variation of molar extinction coefficient with concentration of N-methylacetamide was very nearly the same as for pure water as solvent. Not enough data were accumulated to estimate an association constant, but it is evident that the lower dielectric constant had little effect on the aggregation in

(9) For details compare the analysis of corresponding multiple equilibria in protein systems in I. M. Klotz, *Arch. Biochem.*, **9**, 109 (1946), and "The Proteins," Vol. IB, Chapter 8, edited by H. Neurath and K. Bailey, Academic Press, Inc., New York, N. Y., 1953.

(10) J. B. Hasted, G. H. Haggis and P. Hutton, *Trans. Faraday Soc.*, **47**, 577 (1951).

this solvent in which the mole ratio of water to dioxane is 6:1.

Energetics of Hydrogen-bond Formation.—The standard free energies of dimerization may be readily computed from k_2 by straight forward thermodynamic methods and the results are summarized in Table I. On this basis, too, it is obvious that the stability of interamide hydrogen bonds in an aqueous milieu is very weak indeed. In fact under standard conditions the free energy change is positive and interamide bonds would be almost completely dissociated. Even in dioxane, a solvent with a dielectric constant near 2, the stability of inter-solute hydrogen bonds is not great. Only in a non-hydrogen-bonding solvent such as carbon tetrachloride do solute–solute hydrogen bonds exist to a large extent in moderately dilute solutions.

Since the stability constant k_2 could not be evaluated with high precision in water, it did not seem promising to attempt to evaluate ΔH°, the enthalpy of dimerization from the temperature coefficient of k_2. Instead we used the following analysis. At any stage in the aggregation process the equilibrium may be represented as

$$\mathrm{NH_{free}} + \mathrm{O{=}C_{free}} \rightleftarrows \mathrm{N{-}H\cdots O{=}C} \qquad (18)$$

For this reaction we may write

$$\Delta F^\circ = -RT \ln \left[\frac{(\mathrm{N{-}H\cdots O{=}C})}{(\mathrm{N{-}H_{free}})(\mathrm{O{=}C_{free}})}\right]_{\mathrm{equil.}} \qquad (19)$$

$$= -RT \ln \left[\frac{(\mathrm{N{-}H\cdots O{=}C})}{(\mathrm{N{-}H_{free}})}\right]_{\mathrm{equil.}} + RT \ln (\mathrm{O{=}C_{free}})_{\mathrm{equil.}} \qquad (20)$$

In view of our previous definition of α, and since for each free O=C group there exists a corresponding free N-H group, we may transform equation 20 into

$$\Delta F^\circ = -RT \ln \left(\frac{\alpha}{1-\alpha}\right) + RT \ln (\mathrm{N{-}H_{free}})_{\mathrm{equil.}} \qquad (21)$$

From this relation it follows that

$$\left(\frac{\partial \frac{\Delta F^\circ}{T}}{\partial T}\right)_{P,\alpha} = R\left(\frac{\partial \ln (\mathrm{N{-}H_{free}})_{\mathrm{equil.}}}{\partial T}\right)_{P,\alpha} = -\frac{\Delta H^\circ}{RT^2} \qquad (22)$$

the right-hand equality following from the classical thermodynamic relationship for the temperature coefficient of $\Delta F/T$. Thus in practice what we have done is to carry out measurements of α and $(\mathrm{N{-}H_{free}})_{\mathrm{equil.}}$ at two temperatures, 25° and 60°, and to plot α as a function of log $(\mathrm{N{-}H_{free}})$. At any α we read Δ log $(\mathrm{N{-}H_{free}})$ for the temperature interval ΔT. Then assuming that ΔH° is independent of temperature we compute its value. Slightly different values of ΔH° are obtained at different α's. The results entered in Table I are for $\alpha = 0.5$. Values at other α's deviate by a few tenths of a kilocalorie.

Given ΔF° and ΔH°, the entropies of dimerization, ΔS°, may be computed from the equation

$$\Delta F^\circ = \Delta H^\circ - T\Delta S^\circ \qquad (23)$$

These thermodynamic quantities are also shown in Table I.

A comparison of thermodynamic quantities among our three solvents shows that the value of ΔF°, and hence the stability of the interamide hydrogen bond, is determined largely by ΔH°. This is particularly evident in comparing CCl_4 and H_2O, for which ΔS°'s are essentially identical.

As has been mentioned, a detailed thermodynamic study of the association equilibria of N-methylacetamide has been made in benzene as solvent by Davies and Thomas.[6] Their equilibrium constant is expressed in units of mole fraction, but if converted to molarity their k_2 becomes 6.1. Thus ΔF° is -1.07 kcal. mole^{-1}. Correspondingly they find a ΔH° of -3.6 kcal. mole^{-1} and, in terms of a standard state based on molarity, a ΔS° of about -8 e.u. These results for benzene as solvent agree very well with ours in carbon tetrachloride, as might reasonably be expected in view of the comparable apolar nature of these solvents. It should also be pointed out, however, that neither of these studies agrees well with earlier investigations of the equilibrium constant in carbon tetrachloride by Davies, Evans and Jones,[4] but we believe that this last work was much more approximate in nature and certainly the data were not subjected to a detailed multiple-equilibria analysis.

The only previous estimate of the stability and strength of an N–H···O=C bond dissolved in water is that of Schellman[11] and Kauzmann[12] for aqueous urea solutions. Making the assumption that deviations from ideal behavior in urea–water solutions are due entirely to the formation of aggregated species of urea, ΔF° and ΔH° of dimerization were found to be 1.9 and -2.1 kcal. mole^{-1}, respectively. Correspondingly the equilibrium constant for association was computed as 0.041. These values are appreciably different from those determined in this paper for N-methylacetamide (Table I).

Since the differences in thermodynamic properties might be ascribed to differences in chemical nature and structure of urea *versus* N-methylacetamide, we have also examined the infrared absorption in the overtone region of the N–H group of urea in aqueous solutions. A strong free N–H band was found at 1.48 μ, just as in aqueous solutions of N-methylacetamide. However, the molecular extinction coefficient for urea (approximately 550 cm.^{2} mole^{-1}) remained essentially unchanged over a concentration range of 1 to 8 molar, in water. If the urea aggregation had an equilibrium constant of 0.04, appreciable quantities (about 8%) should be in the dimeric state even in a 1 molar solution, and the fraction dimerized should be very great in an 8 molar solution. We conclude, therefore, that at least infrared spectra indicate no significant dimerization of urea in aqueous solution.

(11) J. A. Schellman, *Compt. rend. trav. lab. Carlsberg, Ser. chim.*, **29**, 223 (1955).

(12) W. Kauzmann, in "The Mechanism of Enzyme Action," W. D. McElroy and B. Glass, Editors, Johns Hopkins University Press, Baltimore, Md., 1954, pp. 70–120; *Advances in Protein Chem.* **14**, 1 (1959).

Conclusion.—It seems clear from these experiments that the intrinsic stability of interpeptide hydrogen bonds in aqueous solution is small. That this is to be expected in water has been pointed out previously,[11,12] but the actual experimental evaluation of the stability and strength of these bonds in a model amide leads to values of $-\Delta F$ and ΔH much below those that had been expected. Furthermore, experiments with nonaqueous solvents indicate that dielectric constant *per se* does not exert a dominant influence on the stability of amide hydrogen bonds. Rather the crucial feature seems to be the chemical nature of the solvent. If it has nucleophilic sites, they can compete successfully for the N–H group. We would presume, although we carried out no appropriate experiments, that solvents with H-donor groups would also disrupt interamide H-bonds by forming bonds between C=O and solvent.

The results with this model system indicate that for protein molecules in aqueous solution, interpeptide hydrogen bonds cannot contribute significantly to the stabilization of macromolecular organization, except perhaps in a few regions with a very low local dielectric constant due to a specific high concentration of hydrocarbon-like residues. Other forces must be primarily responsible, therefore, for the maintenance of protein configuration in aqueous solution.[13]

Acknowledgments.—This investigation was assisted by a research grant (H-2910) from the National Heart Institute, United States Health Service. J.S.F. is indebted to the United States Public Health Service for a Postdoctoral Fellowship.

(13) I. M. Klotz, *Brookhaven Symposia in Biology*, **13**, 25 (1960).