The Effect of Hydrogen-Deuterium Substitution on the Association of *N*-Methylacetamide

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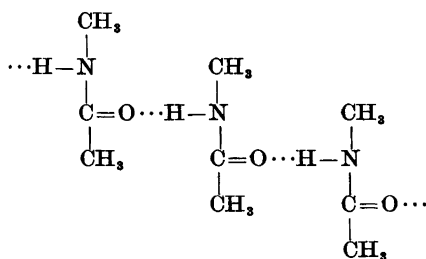
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The self-association of *N*-methylacetamide (NMA-H) and of deuterated *N*-methylacetamide (NMA-D) dissolved in carbon tetrachloride was measured by infrared spectroscopy at three different temperatures. Assuming that the amides form chain aggregates through hydrogen bonding, values of the dimerization equilibrium constant, K_0, and of the equilibrium constant, K, for the formation of higher aggregates, were calculated. The values of K for NMA-D, ranging from 19 l mole^{-1} at 50°C to 32 l mole^{-1} at 25°C, were found to be 10—20 % smaller than the corresponding values for NMA-H. This result was ascribed to a difference of 0.5 kcal mole^{-1} in $\Delta H°$ for the association reaction. The experiments do not permit any quantitative estimate of the isotope effect on K_0; but since it was observed that the transition from free to associated amide with increasing concentration is steeper for NMA-H than for NMA-D, it was concluded that the ratio K_0/K is smaller for NMA-H than it is for NMA-D.

I t is well-known [1-8] that *N*-methylacetamide in the pure state and in solution forms aggregates through hydrogen bonding in the following way:

$$
\cdots\text{H}-\overset{\displaystyle\overset{\textstyle\text{CH}_3}{|}}{\text{N}}
$$

CH₃
 |
⋯H—N   CH₃
 | |
 C=O⋯H—N   CH₃
 | | |
 CH₃   C=O⋯H—N
              | |
              CH₃   C=O⋯
                          |
                          CH₃

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Measurements of the extent of this association, and, thereby, estimates of the strength of the hydrogen bonds formed, are of particular interest,[2,5] because similar hydrogen bonds formed between the peptide groups of the polypeptide chains of proteins and of synthetic polypeptides are thought to contribute to the stability of the conformation of these macromolecules.

In the present investigation the degree of association of N-methylacetamide (NMA-H) and of deuterated N-methylacetamide, $CH_3CONDCH_3$, (NMA-D) dissolved in carbon tetrachloride has been measured at three different temperatures. The equilibrium constants and other thermodynamic parameters for the dimerization and for the formation of higher associates of the amides are presented.

The thermodynamic parameters of the dimerization of NMA-H were determined by Klotz and Franzen,[5] and our measurements are in fair agreement with these earlier results, when the considerable experimental error of both investigations is taken into consideration. The main purpose of the present investigation has been, however, to estimate the equilibrium constants for the formation of the higher associates of the amides, and to study the effect of hydrogen-deuterium substitution on the strength of the hydrogen bonds formed. We find, as it has also been shown to be the case in other amide solutions,[4-6] that the association is a cooperative phenomenon, the equilibrium constant of the dimerization, K_0, being an order of magnitude smaller than the equilibrium constant, K, of the formation of higher associates. The values of K measured for NMA-H are about 20 % larger than the values of K measured for NMA-D, whereas K_0 for NMA-H is comparable to or smaller than K_0 for NMA-D.

EXPERIMENTAL

Materials. N-Methylacetamide was BDH Laboratory Reagent. Before use it was dried *in vacuo* over P_4O_5. M.p. 27—30°C.

Deuterated .N-methylacetamide was prepared in the following way. Equal volumes of 99.7 % D_2O (Norsk Hydro, Norway) and of N-methylacetamide were mixed at 40°C in a closed vessel, and then cooled to −60°C. Vacuum was applied, and most of the water was removed by gradually raising the temperature to 40°C under continuous pumping by an oil pump for about 2 h. After a fourfold repetition of this procedure the sample was left at 20°C under continuous pumping, until crystals of deuterated N-methylacetamide were formed.

The infrared spectrum in the region 1300—4000 cm^{-1}, and the NMR spectrum at 60 Mcps of the crystals dissolved in carbon tetrachloride were recorded, and showed no evidence of water, $CH_3CONHCH_3$, or of any other impurities. M.p. 28—31°C.

Carbon tetrachloride was BDH Laboratory Reagent.

The spectrophotometric measurements. Infrared spectra in the region 1300—1800 cm^{-1} of NMA-H and of NMA-D dissolved in carbon tetrachloride were recorded on a Beckman IR 9 spectrophotometer. The photometer was used in double beam operation with pure solvent in the reference beam. The optical pathlength was 0.0105 cm, measured according to Smith and Miller.[9] The optical cells were thermostated by circulating water in jackets surrounding the cells; the temperature was measured by means of a thermocouple placed directly in the solution within the cell.

Fig. 1 shows spectra of NMA-H and of NMA-D recorded at 25°C. The so called amide I absorption, ascribed to a $C=O$ stretching frequency of the amide group,[10] has been utilized to measure the degree of association in the solutions. In dilute solutions of the amides at 25°C the amide I absorption appears as a single band at 1690 cm^{-1}; this band is attributed to $C=O$ groups which do not act as acceptors of any hydrogen bonds
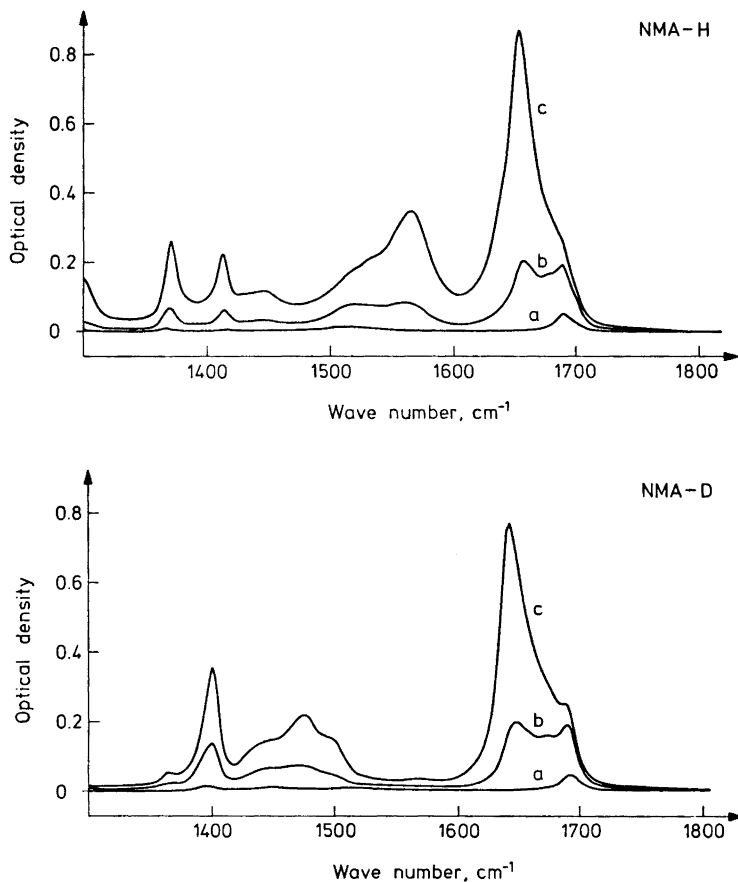
Fig. 1. Infrared absorption spectra of *N*-methylacetamide (NMA-H) and of deuterated
N-methylacetamide (NMA-D) dissolved in carbon tetrachloride at 25°C.
NMA-H: *a* 8.53×10^{-3} mole 1^{-1}, *b* 6.82×10^{-2} mole 1^{-1}, *c* 2.05×10^{-1} mole 1^{-1}. NMA-D: *a*
7.83×10^{-3} mole 1^{-1}, *b* 6.26×10^{-2} mole 1^{-1}, *c* 1.57×10^{-1} mole 1^{-1}.

("free" C=O groups). At higher concentrations of the amides another peak of the amide
I absorption appears at 1655 cm^{-1} for NMA-H, and at 1641 cm^{-1} for NMA-D. This band
is attributed to C=O groups acting as hydrogen bond acceptors ("associated" C=O
groups). A weaker band observed at 1680 cm^{-1} for NMA-H, and at 1670 cm^{-1} for NMA-D
is tentatively ascribed to the associated group of dimers of the amides.

The extinction coefficients, ε_{free} and ε_{ass}, of the two major bands of the amide I
absorption were estimated from the limiting slope of plots of the optical density, OD_{free}
and OD_{ass}, *vs.* the molar concentration of the amide, $[A_{tot}]$:

$$\left.\begin{array}{l} \varepsilon_{free} = \dfrac{1}{l}\dfrac{dOD_{free}}{d[A_{tot}]} \quad \text{for } [A_{tot}] \to 0, \\[3mm] \varepsilon_{ass} = \dfrac{1}{l}\dfrac{dOD_{ass}}{d[A_{tot}]} \quad \text{for } \dfrac{dOD_{free}}{d[A_{tot}]} \ll \dfrac{dOD_{ass}}{d[A_{tot}]} \end{array}\right\} \quad (1)$$

Table 1. Extinction coefficient of the two major bands of the amide I absorption in units of 1 mole^{-1} cm^{-1}.
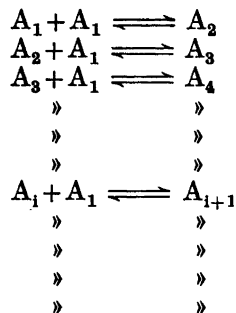
Temperature °C	NMA-H		NMA-D	
	ε_{free}	ε_{ass}	ε_{free}	ε_{ass}
25	620	500	650	650
35	590	490	620	600
50	540	470	600	550

where $l = 0.0105$ cm is the optical pathlength. The extinction coefficients measured at three different temperatures are summarized in Table 1.

The concentrations $[A_{free}]$ and $[A_{ass}]$ of free and associated C=O groups were estimated by measuring the optical density at the corresponding band maxima. In order to minimize the error due to overlapping of the bands only measurements of OD_{free} for which $OD_{ass} < \frac{1}{2}OD_{free}$, and measurements of OD_{ass} for which $OD_{free} \leq 2OD_{ass}$ were considered.

THEORY

Analysis of multiple association equilibria. The association of N-methyl-acetamide is represented by the following reaction scheme in which A_i denotes an aggregate consisting of i monomer units, *i.e.* an i-mer:

$$A_1 + A_1 \rightleftharpoons A_2$$
$$A_2 + A_1 \rightleftharpoons A_3$$
$$A_3 + A_1 \rightleftharpoons A_4$$
$$\text{»} \qquad \text{»}$$
$$\text{»} \qquad \text{»}$$
$$\text{»} \qquad \text{»}$$
$$A_i + A_1 \rightleftharpoons A_{i+1}$$
$$\text{»} \qquad \text{»}$$
$$\text{»} \qquad \text{»}$$
$$\text{»} \qquad \text{»}$$
$$\text{»} \qquad \text{»}$$

$[A_i]$ is the molar concentration of i-mers, $K_{i,\,i+1} = [A_{i+1}]/[A_1][A_i]$ is the equilibrium constant of the i'th equilibrium of the reaction scheme above, and we assume, as it is usually done,[3,5] that the equilibrium constants of the second and following steps of the association are identical, and larger than or equal to the equilibrium constants of the dimerization,

$$K_{i,\,i+1} = \begin{cases} K_0 \text{ for } i = 1 \\ K \text{ for } i \geq 2 \end{cases}, \; K_0 \leq K$$

The concentrations of the various i-mers present are expressed by

$$[A_i] = K_0 K^{i-2}[A_1]^i, \; i \geq 2 \tag{2}$$

so the total concentration of i-mers is given by

$$\sum_{i=1}^{\infty} [A_i] = [A_1] + K_0 \sum_{i=2}^{\infty} K^{i-2}[A_1]^i = [A_1] + K_0[A_1]^2/(1 - K[A_1]), \ (K[A_1]<1). \quad (3)$$

The total concentration of N-methylacetamide, $[A_{tot}]$, expressed as the molar concentration of units of N-methylacetamide present, can be calculated as

$$[A_{tot}] = \sum_{i=1}^{\infty} i[A_i] = [A_1] + K_0[A_1]^2\{1/(1 - K[A_1])^2 + 1/(1 - K[A_1])\} \quad (4)$$

(The formulae used in the summations of eqns. (3) and (4) can be found in many mathematical handbooks, e.g. in Ref. 11).

By the spectrophotometric method, described in the preceding paragraph, we estimate the concentrations of free $C = O$ groups, $[A_{free}]$, and of associated $C = O$ groups, $[A_{ass}]$, and since each i-mer contains one free group and $(i-1)$ associated groups, we have

$$[A_{free}] = \sum_{i=1}^{\infty} [A_i] = [A_1] + K_0[A_1]^2/(1 - K[A_1]) \quad (5)$$

and

$$[A_{ass}] = [A_{tot}] - [A_{free}] = K_0[A_1]^2/(1 - K[A_1])^2 \quad (6)$$

Eqn. (6) can be rewritten as

$$[A_1] = 1/(K + \sqrt{K_0/[A_{ass}]}) \quad (7)$$

which introduced into (4) and (5) leads to expressions relating the measured quantities $[A_{free}]$, $[A_{ass}]$, and $[A_{tot}]$ with the equilibrium constants K_0 and K.
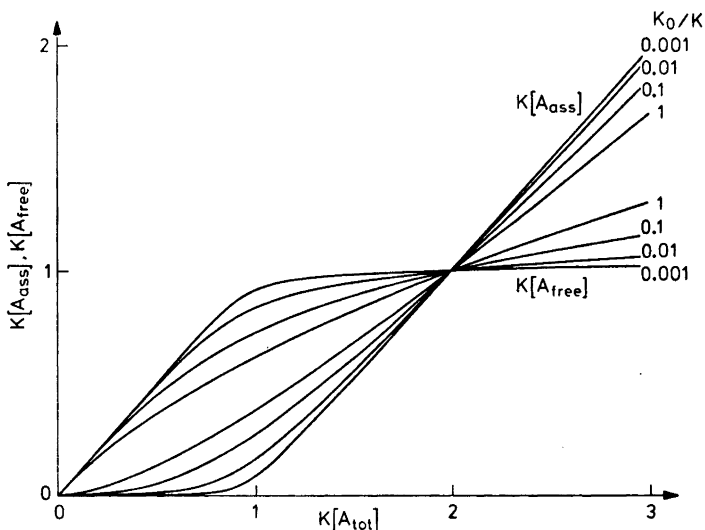

Fig. 2. $K[A_{free}]$ and $K[A_{ass}]$ as functions of $K[A_{tot}]$, calculated according to eqns. (4), (5), and (6) for various values of the ratio K_0/K.
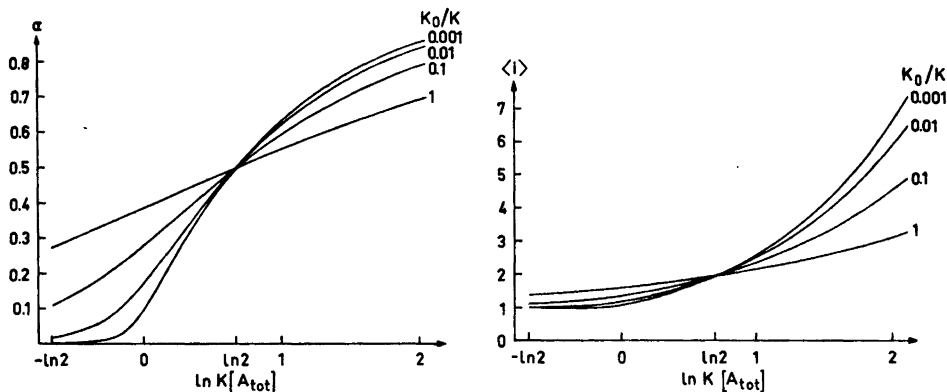
Fig. 3. The degree of association α, and the average chain length $\langle i \rangle$ as functions of $\ln K[A_{tot}]$ calculated according to eqns. (4), (5), and (6) for various values of the ratio K_0/K.

A graphical representation of $K[A_{free}]$ and $K[A_{ass}]$ as functions of $K[A_{tot}]$ is given in Fig. 2, and on Fig. 3 the degree of association $\alpha = [A_{ass}]/([A_{ass}] + [A_{free}])$, and the average chain length $\langle i \rangle$ are plotted as functions of $\ln K[A_{tot}]$. The curves on these figures are theoretical curves, calculated according to the formulae given by eqns. (5) and (6). It appears from eqns. (4), (5), and (6) that $K[A_{free}]$, $K[A_{ass}]$, and $K[A_{tot}]$ can be regarded as functions of K_0/K and $K[A_1]$, and therefore (by eliminating $K[A_1]$) $K[A_{free}]$, α and $\langle i \rangle$ can be expressed as functions of $K[A_{tot}]$ and K_0/K. The ratio K_0/K thus determines the shape of the curves in Figs. 2 and 3.

From eqns. (5) and (6) one gets that

and

$$\left.\begin{array}{l} [A_{free}] = [A_{ass}] = \tfrac{1}{2}[A_{tot}] = 1/K \\[2mm] \dfrac{d\alpha}{d\ln[A_{tot}]} = \tfrac{1}{2}\dfrac{1}{1 + 2\sqrt{K_0/K}} \end{array}\right\} \text{ for } [A_1] = 1/(K + \sqrt{K_0 K}) \qquad (8)$$

This means that, independently of the value of K_0, K can be estimated by measuring the concentration of N-methylacetamide at which the concentrations of free and associated molecules are equal, $i.e.$ at which $\alpha = \tfrac{1}{2}$ or $\langle i \rangle = 2$ (see Figs. 2 and 3). The steepness of the transition from free to associated amide, measured as $d\alpha/d\ln[A_{tot}]$ at the transition point $\alpha = \tfrac{1}{2}$, determines the ratio K_0/K; the smaller this ratio, the steeper the transition.

The values of K_0 and K reported in Table 2 were estimated on the basis of the expressions (8), considering only experimental data for which $\alpha \simeq \tfrac{1}{2}$, as well as by numerical fitting to the complete set of experimental data, using the procedure described in the following paragraph.

The numerical method. The equations obtained from eqns. (4), (5), and (6), relating the measured quantities [A_{free}], [A_{ass}], and [A_{tot}] with the equilibrium constants, K and K_0, are rather complicated mathematical expressions.

Table 2. Equilibrium constants K_0 for the dimerization and K for the formation of higher associates in units of l mole^{-1}.

	Temperature °C	K	K_0/K	K_0
NMA-H	25	38	0.03	1
	35	30	0.03	1
	50	21	0.05	1
NMA-D	25	32	0.08	3
	35	26	0.06	2
	50	19	0.05	1

We, therefore, estimated K_0 and K by using a computer program which 1) solves eqn. (4) with respect to $[A_1]$ using a Newton-Raphson method,[12] 2) introduces the value of $[A_1]$ in eqns. (5) and (6), and then 3) minimizes the expression

$$\sum \left(\frac{[A_{free}]^{mea} - [A_{free}]^{fit}}{[A_{tot}]} \right)^2 + \sum \left(\frac{[A_{ass}]^{mea} - [A_{ass}]^{fit}}{[A_{tot}]} \right)^2 \qquad (9)$$

The superscripts mea and fit in (9) indicate measured and fitted values, respectively. Minimizing (9) means estimating the values of K_0 and K for which the agreement between measured and calculated values of α is optimal over the entire range of experimental data.

The program used is written in GIER-ALGOL IV,[13] and is available on request.

RESULTS

The association of NMA-H and of NMA-D dissolved in carbon tetrachloride was measured at the temperatures 25°C, 35°C, and 50°C by the method described in the preceding sections. Fig. 4 shows a plot of $[A_{free}] = OD_{free}/\varepsilon_{free}$ and $[A_{ass}] = OD_{ass}/\varepsilon_{ass}$ vs. the molar concentration of the amide, $[A_{tot}]$, measured for NMA-H at 25°C.

The experimental data for NMA-H and NMA-D measured at 25°C are presented in Fig. 5 as plots of the degree of association $\alpha = [A_{ass}]/([A_{ass}] + [A_{free}])$ vs. $\log_{10}[A_{tot}]$. The curves drawn on these figures are calculated according to (4), (5), and (6) using the value of K_0 and K reported in Table 2. The corresponding values of the thermodynamic parameters $\Delta G^\circ = -RT \ln K$, $\Delta H^\circ = -R \frac{\delta \ln K}{\delta T^{-1}}$, and $\Delta S^\circ = (\Delta H^\circ - \Delta G^\circ)/T$ calculated for $T = 298°K$ are summarized in Table 3.

DISCUSSION

The isotope effect on the strength of hydrogen bonds formed between amide groups has been the subject of several discussions.[14-18] One of the difficulties in the interpretation of available data is that in most cases the
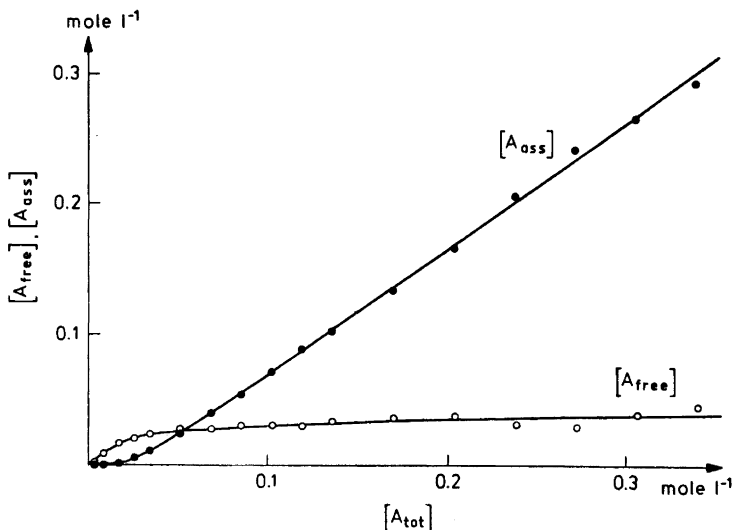
Fig. 4. The concentrations of free and associated amide groups, $[A_{free}]$ and $[A_{ass}]$, measured as functions of the total amide concentration $[A_{tot}]$ for N-methylacetamide dissolved in carbon tetrachloride at 25°C.
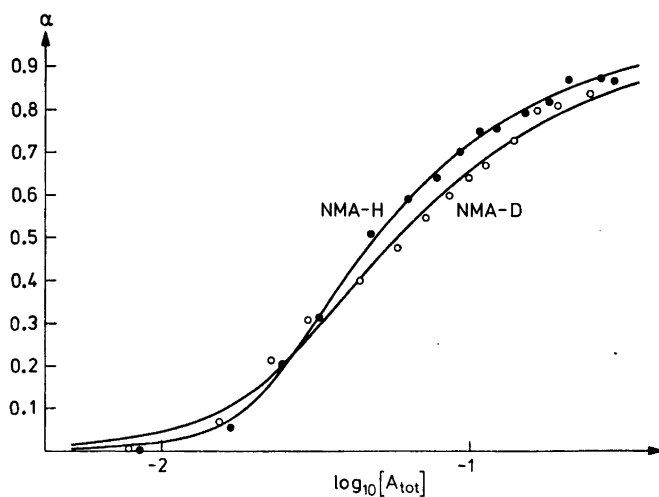

Fig. 5. The degree of association as a function of $\log_{10}[A_{tot}]$ ($[A_{tot}]$ mole l^{-1}) measured for N-methylacetamide (NMA-H) and for deuterated N-methylacetamide (NMA-D) dissolved in carbon tetrachloride at 25°C.

effect of the hydrogen isotope substitution has been measured in solvents which themselves contain exchangeable hydrogen atoms. If $-CONH-$ compounds are studied in solvents with a normal isotopic composition, and

Table 3. Thermodynamic parameters for the formation of higher associates.

	ΔG° kcal/mole (25°C)	ΔH° kcal/mole	ΔS° cal/mole°K
NMA-H	-2.16	-4.7	-9
NMA-D	-2.06	-4.2	-7

$-$COND$-$ compounds are studied in deuterated solvents, differences observed in the strength of hydrogen bonds formed can be ascribed partly to differences in the solvents.

In the present investigation a solvent is chosen (carbon tetrachloride) which has no exchangeable hydrogen atoms, and which can, therefore, serve for both NMA-H and NMA-D. The effect of hydrogen deuterium substitution on the association of NMA in carbon tetrachloride at 25°C is illustrated in Fig. 5, showing the degree of association α measured as a function of $\log_{10}[A_{tot}]$. For $\alpha \geq \frac{1}{2}$ the curve obtained for NMA-D is displaced towards higher amide concentrations relative to the curve obtained for NMA-H, indicating that according to eqn. (8) the equilibrium constant K for NMA-D is smaller than K for NMA-H. The curve of NMA-H is slightly steeper than the curve of NMA-D, which means that the ratio K_0/K for NMA-H is smaller than it is for NMA-D. The numerical treatment of the data indicates that K_0 for NMA-H is comparable to or even smaller than K_0 for NMA-D (see Table 2). This implies the fact that, in very dilute solutions where the accuracy of the spectrophotometric measurements is poor, the degree of association for a given amide concentration may be larger for NMA-D than for NMA-H; at higher concentrations the isotope effect is the opposite.

The value of $K_0 = 1$, measured for NMA-H, is lower than the values of this constant reported by Klotz and Franzen.[5] Extrapolating measurements of the degree of association in solutions of NMA-H in carbon tetrachloride to zero concentration, these latter authors obtained the values $K_0 = 4.7$ and $K_0 = 5.8$. The extrapolation procedure applied requires measurements of the association in very dilute solutions ($[A_{tot}] \ll 1/K \cong 0.03$ 1 mole^{-1}), where the spectrophotometric method used by us is not sufficiently accurate. If, however, we apply the relations given by eqn. (8) to the plot of α *vs.* $\log_{10}[A_{tot}]$ shown in Ref. 5, we find the values $K = 34$, $K_0/K = 0.1$, $K_0 = 3$ to be consistent with the experimental data reported. This latter set of values agrees satisfactorily with the equilibrium constant of Table 2.

The accuracy of the numerical calculation of the equilibrium constants of Table 2 is ± 1 1 mole^{-1}, so the isotope effect on K_0 is too small to be measured by our method. The effect on K, however, is significant; and, as shown in Table 3, it may be ascribed to a difference of 0.5 kcal/mole in ΔH° for the formation of a hydrogen bond, $\Delta H^\circ = -4.7$ kcal/mole for NMA-H, and $\Delta H^\circ = -4.2$ kcal/mole for NMA-D.

The problem of the isotope effect on the strength of hydrogen bonds formed between peptide groups may be of particular interest in connection with

studies of protein conformations by measurements of the rates of hydrogen isotope exchange in aqueous protein solution.[18] During measurements of these exchange rates the isotopic composition of the hydrogen atoms of the proteins gradually changes, and it is important for the interpretation of the rate constants to know, whether the exchange influences the stability of the protein conformations. The results of the present investigation confirms the prevalent point of view, that if the stability of protein conformations is influenced by the exchange, the effect most likely to occur is smaller than the accuracy at which the stability can be measured by the exchange methods.

REFERENCES

1. Mizushima, S. *Structure of Molecules and Internal Rotation*, Academic, New York 1954, p. 117.
2. Davies, M., Evans, J. C. and Jones, R. L. *Trans. Faraday Soc.* **51** (1955) 761.
3. Davies, M. and Thomas, D. K. *J. Phys. Chem.* **60** (1956) 763.
4. Davies, M. and Thomas, D. K. *J. Phys. Chem.* **60** (1956) 767.
5. Klotz, I. M. and Franzen, J. S. *J. Am. Chem. Soc.* **84** (1962) 3461.
6. La Planche, L. A. Thompson, H. B. and Rogers, M. T. *J. Phys. Chem.* **69** (1965) 1482.
7. Longsworth, L. G. *J. Coll. and Interface Science* **22** (1966) 3.
8. Rasing, J. and Østerberg, O. *Acta Chem. Scand.* **23** (1969) 693.
9. Smith, D. C. and Miller, E. C. *J. Opt. Soc. Am.* **34** (1944) 130.
10. Fraser, R. D. B. and Price, W. C. *Nature* **170** (1952) 490.
11. Jolley, L. B. W. *Summation of Series*, Dover Publications, New York 1961.
12. Ralston, A. *A First Course in Numerical Analysis*, McGraw, New York 1965.
13. Naur, P., Ed., *A Manual of GIER-Algol IV*, Regnecentralen, Copenhagen 1967.
14. Calvin, M., Hermans, I., Jr. and Scheraga, H. A. *J. Am. Chem. Soc.* **81** (1959) 5048.
15. Hermans, I., Jr. and Scheraga, H. A. *Biochim. Biophys. Acta* **36** (1959) 534.
16. Scheraga, H. A. *Ann. N. Y. Acad. Sci.* **84** (1960) 608.
17. Englander, S. W. *Biochemistry* **2** (1963) 798.
18. Hvidt, Aa. and Nielsen, S. O. *Advan. Protein Chem.* **21** (1966) 287.