An Equilibrium Centrifugation Study of the Self-Association of *N*-Methylacetamide in Carbon Tetrachloride Solutions at 25°[1]

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Abstract: The self-association of *N*-methylacetamide in carbon tetrachloride solution at 25° was found to be described by the relationship $M_1/M_{\mathrm{wa},c} = M_1/(1 + 4kc)^{1/2} + BM_1c + 2CM_1c^2$, where $M_{\mathrm{wa},c}$ and c are the apparent weight-average molecular weight and solute concentration (grams per milliliter) and the constants M_1, k, B, and C, corresponding to the monomer molecular weight, indefinite association constant, and second and third virial coefficients, had values of 73 g/mol, $2.68 \pm 0.15 \times 10^4$ ml/g, 0.0312 ± 0.0036 mol/ml, and 0.0970 ± 0.0201 mol/ml^2, respectively. The average aggregation number of a 0.3 *m* solution was 66, and pure *N*-methylacetamide was estimated to contain 0.39% monomer. It is shown that peptide hydrogen bonds would tend to stabilize polypeptides and helical proteins if an indefinite association is used as a model system.

The self-association of *N*-methylacetamide in aqueous and nonaqueous solutions has been studied by a variety of physical methods; *e.g.*, vapor-pressure lowering,[3–5] near-infrared spectroscopy,[6,7] nmr spectroscopy,[8,9] viscosity and diffusion studies,[10] and calorimetry.[11,12] In general, association has been found to increase with solute concentration, and decrease with solvent polarity and increasing temperature. The mechanism of self-association has been consistently ascribed to linear hydrogen bonding, although a dipole-aligned hydrophobic interaction was recently suggested[12] for dilute aqueous solutions.

In spite of all that is known about the *N*-methylacetamide association, the "degree" of self-association in nonaqueous solutions is not known with certainty. For this reason, centrifugation studies were undertaken to directly determine the molecular weight of the associated species. From a knowledge of the variation of the apparent molecular weight with concentration, it was possible to calculate the weight-average molecular weight, the indefinite self-association constant, and the second and third virial coefficients which quantitatively reflect the nonideal nature of the solutions. Thus, a more complete description of the nature of the self-association process for *N*-methylacetamide is provided. The possible relevence of these results to the importance of peptide hydrogen bonds in proteins is discussed.

Experimental Section

Materials. The sample of *N*-methylacetamide used in this study was distilled after drying overnight over $CaSO_4$ at about 0.24 mm at 70°, and stored in a desiccator over $CaCl_2$. It was the same material used previously.[11,12] Analyzed reagent grade carbon tetrachloride (Matheson) was used directly from the shipping bottle. All solutions were freshly prepared by weight using a Mettler B6 semimicroanalytical balance.

Partial Specific Volume. The density of solutions of *N*-methylacetamide in carbon tetrachloride was determined at 25.00 ± 0.05° using 25-ml pycnometers as previously described.[13] A value of $1.04_2 \pm 0.01_0$ cm^3/g was found to represent the partial specific volume of *N*-methylacetamide over the concentration range used in this study.

Equilibrium Centrifugation. Equilibrium centrifugation studies were performed with a Beckman-Spinco Model E analytical ultracentrifuge. The temperature was maintained at 25.00 ± 0.05° by means of the standard RTIC temperature control unit. Photographic plates were obtained with a schlieren optical system for the determination of molecular weight. The latter were measured with a Nikon Model 6C microcomparator equipped with an Elms Systems digital readout attachment.

A double sector cell containing a Kel-F centerpiece with sapphire windows was used with column lengths of about 3 mm. Rotor speeds varied from 13,400 to 44,770 rpm, although the speeds used most often were 27,690, 35,600, and 44,770 rpm. It was shown that equilibrium generally took place within 8 hr by allowing some solutions to run as long as 24 hr. It was observed that the base line of the sample solution was below the base line of the solvent when the rotor reached operating speed in most of our experiments. The magnitude of this difference was found to increase with increasing solute concentration and rotor speed. To allow for this effect when calculating the refractive index increment of the solution at equilibrium, a positive correction in the refractive index increment was applied to the observed differences between the solution and solvent at all points in the cell. The concentration distribution was then calculated,[14] and a concentration dependent correction then was applied at various positions in the cell. This process was reiterated ten times in order to obtain values used in calculating the final equilibrium molecular weight distribution.

Several synthetic boundary experiments were performed with different concentrations of solutions in order to relate the initial concentration of solute to the area on the photographic plates. A linear relationship between solute concentration and area was observed.

Theory

It was expected from previous studies that *N*-methylacetamide would undergo an indefinite self-association in carbon tetrachloride solutions, which could be represented with the following scheme

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[2] National Science Foundation undergraduate research participant, Summer 1969.

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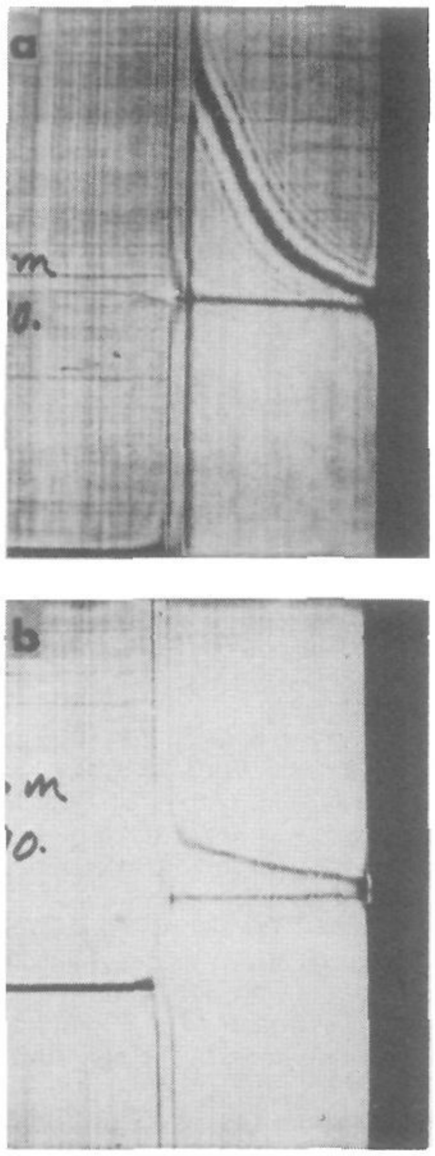

Figure 1. Equilibrium centrifugation patterns observed for 0.216 *m* solutions of *N*-methylacetamide in carbon tetrachloride at 25°: (a) upper curve at 44,770 rpm and (b) lower curve at 27,690 rpm.

$$\left.\begin{aligned} M_1 + M_1 &= M_2;\; K_2 = \frac{(M_2)}{(M_1)^2} \\ M_1 + M_2 &= M_3;\; K_3 = \frac{(M_3)}{(M_2)(M_1)} \\ &\text{- - - - - - - - - - - - - - - -} \\ M_1 + M_{n-1} &= M_n;\; K_n = \frac{(M_n)}{(M_1)(M_{n-1})} \end{aligned}\right\} \qquad (1)$$

where (M_1), $(M_2)\ldots(M_n)$ represent the concentration of monomer, dimer...*n*-mer, respectively. The equilibrium constants defined according to eq 1 are expressed in terms of concentrations of the various chemical species. It has been shown by Adams and Lewis[15] that the basic equilibrium centrifugation equation for indefinite self-associating systems which relates the total solute concentration, *c*, and the weight-average apparent molecular weight at concentration *c*, $M_{\mathrm{wa},c}$, is

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

where $M_{\mathrm{wa},c}$ is defined as

$$\frac{M_1}{M_{\mathrm{wa},c}} = \frac{M_1}{M_{\mathrm{w},c}} + BM_1c + 2CM_1c^2 \qquad (3)$$

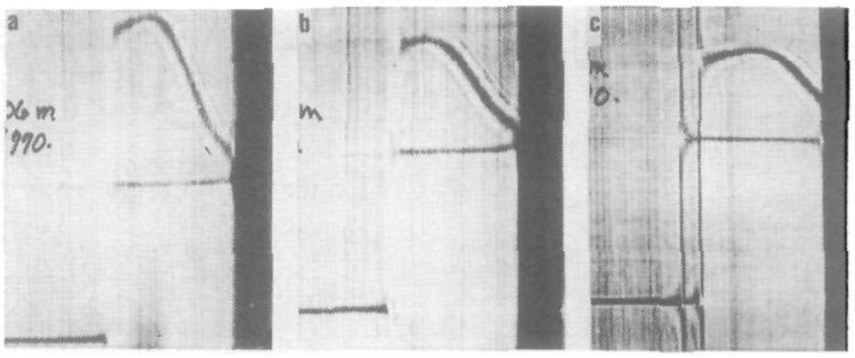

Figure 2. Equilibrium centrifugation patterns observed at 44,770 rpm for solutions of *N*-methylacetamide in carbon tetrachloride at 25°: (a) 0.465 *m*; (b) 0.565 *m*; (c) 0.606 *m*.

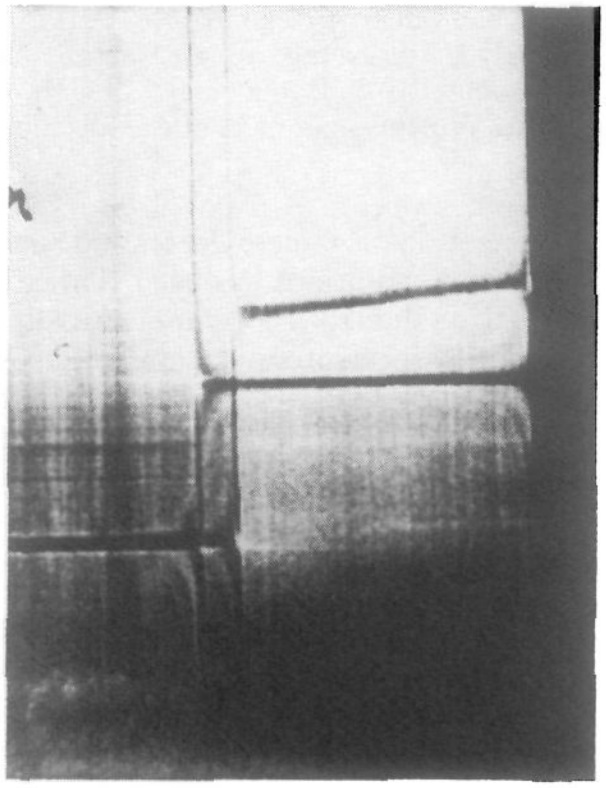

Figure 3. Centrifugation equilibrium pattern observed at 35,600 rpm for 1.100 *m* solution of *N*-methylacetamide in carbon tetrachloride solution at 25°.

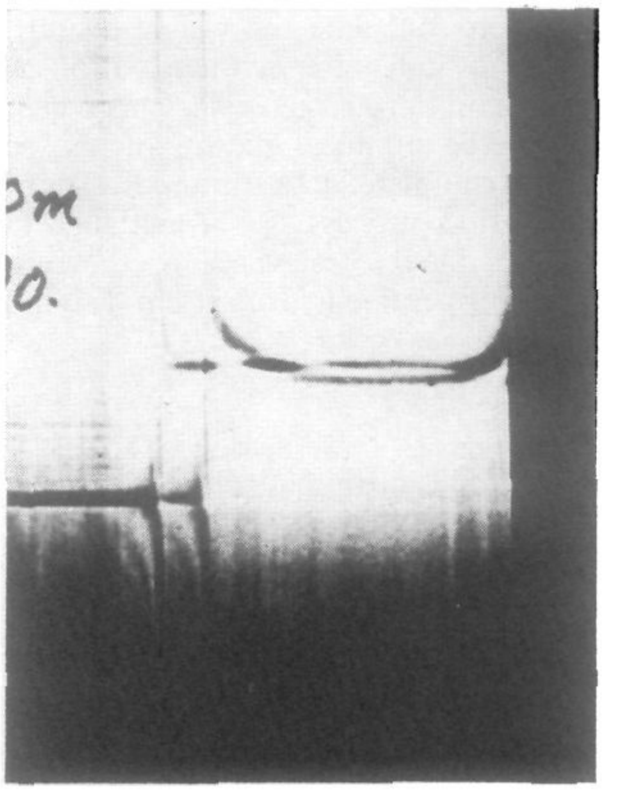

Figure 4. Centrifugation pattern observed when the rotor first reached a speed of 27,690 rpm for a 1.100 *m* solution of *N*-methylacetamide in carbon tetrachloride solution at 25°.

The quantities $\bar{v}$, r, ρ, R, T, and ω have their usual meaning in the equilibrium centrifugation equation, representing the partial specific volume of the solute, distance from the center of rotation, solution density at the distance equal to *r*, the universal gas constant, absolute temperature, and angular velocity, respectively. The quantity $M_{\mathrm{w},c}$ is the weight-average molec-

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[15] E. T. Adams, Jr., and M. S. Lewis, *Biochemistry*, 7, 1044 (1968).

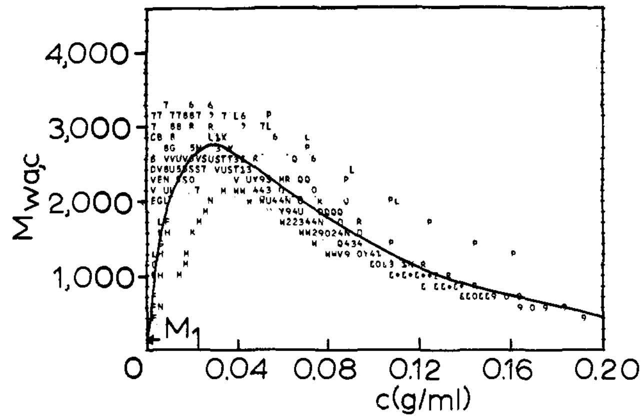

Figure 5. Combined results from 35 experiments at different initial concentrations and equilibrium speeds of the concentration dependence of the apparent weight-average molecular weight for *N*-methylacetamide in carbon tetrachloride solutions at 25°. The line drawn represents the curve of best fit of the data to M_1 times the reciprocal of eq 5 according to a least-squares criterion, and was drawn using values for k, BM_1, and CM_1 of 26,826, 0.2279, and 0.7080, respectively.

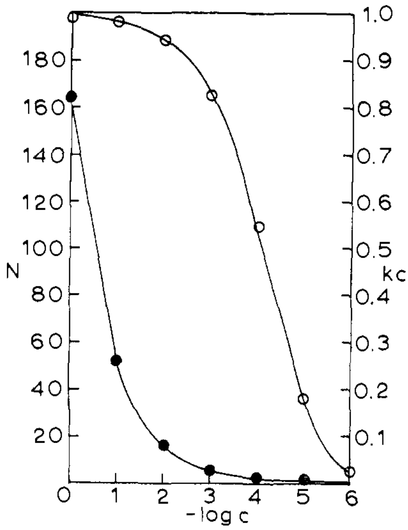

Figure 6. Variation of the most probable oligomer species, *N* (filled circles), and the product kc_1 (open circles) with *N*-methylacetamide concentration, *c* (grams/milliliter).

ular weight at the concentration equal to c, and B and C represent the second and third virial coefficients, which are defined for each species, i, in terms of the natural logarithm of the activity coefficient for each species γ_i as

$$\ln \gamma_i = iB_iM_1c + iB_iM_1c^2 + \ldots \quad (4)$$

where terms higher than the order c^2 are considered negligible. When all the association constants defined according to eq 1 are assumed to be equal to an equilibrium constant defined on a grams per milliliter scale, k, and the product of the monomer concentration, c_1, times k is less than one, eq 3 can be rewritten as

$$\frac{M_1}{M_{\mathrm{wa},c}} = \frac{M_1}{(1 + 4kc)^{1/2}} + BM_1c + 2CM_1c^2 \quad (5)$$

In principle, the quantities k, B, and C can be determined from a knowledge of the concentration dependency of the apparent weight-average molecular weight. The latter is determined from experimental quantities according to eq 2, where d ln c/d(r^2) is related to the phase-boundary image displacement and the photographic plate area. The partial specific volume and refractive index increments of all forms of the associating species are assumed to be equal in the derivation of the above equations.

Results

The results from several individual centrifugation experiments which illustrate the effects observed in this study are presented in Figures 1–4. From the data in Figure 1, it may be noted that a typical centrifugation flotation pattern was observed for a relatively low concentration of *N*-methylacetamide. In addition, the apparent molecular weight distribution in the cell increases with increasing speed of centrifugation (as reflected by the relative differences in concentration at the cell meniscus and bottom for the two different speeds). As the concentration of *N*-methylacetamide is increased, the centrifugation pattern begins to deviate from a typical flotation pattern until a distinct maximum is observed with a 0.606 *m* solution at 44,770 rpm (Figure 2). Finally, a pattern which is typical of sedimentation behavior was found with a 1.100 *m* solution at 35,600 rpm (Figure 3). In general, at low concentrations a typical flotation pattern was found, whereas at high concentration a typical sedimentation pattern emerged. A pattern is also included as Figure 4 which illustrates the base-line displacement, where the solution base line was found to fall below the base line of the solvent at the time the rotor reached operating speed. This difference between the two base lines increased with concentration and rotor speed.[16]

The value of $M_{\mathrm{wa},c}$ was found to depend on solute concentration as expected, and these results are presented in Figure 5. The overlap of data from the large number of experiments gives confidence to the shape of the apparent molecular weight curve. It may be seen that the molecular weight increases rapidly above the monomer molecular weight of 73 g/mol to a value of about 3000 g/mol at 0.032 g/ml and then decreases in a steady manner. The large increase in molecular weight reflects the large degree of self-association of *N*-methylacetamide at rather low concentrations, indicative of a large self-association constant. The distinct downward curvature denotes that thermodynamic nonideality exists in the solutions and reflects the importance of the virial coefficients. It then remains to be determined whether one or two virial coefficients are required to represent the data. Therefore, the results represented in Figure 5 were fitted according to a least-squares criterion[17] to an equation which was the recriprocal form of eq 5. The values found for k, B, and C are given in Table I. It may be noted that a slightly better fit on k is obtained with the use of a single virial coefficient; however, the uncertainty in the second virial coefficient is much improved with the use of a third virial coefficient. Therefore, it is con-

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[16] A linear relationship was found between initial solute concentration and the base-line separation, which was proportional to the rotor speed. The correction factor applied in calculating molecular weights is described in the Experimental Section.

[17] H. Kim, *J. Chem. Educ.*, **47**, 120 (1970).

Table I. Indefinite Association Parameters for *N*-Methylacetamide in Carbon Tetrachloride Solution at 25°

k, (g/ml)$^{-1}$	BM_1, g/ml	CM_1, (g/ml)^2
2.6826×10^4	0.2279	0.7080
$\pm 0.1467 \times 10^4$	±0.0264	±0.1467
3.2166×10^4	0.3546	0[a]
$\pm 0.1449 \times 10^4$	±0.0908	

[a] Value of third virial coefficient set equal to zero for curve-fitting procedure.

sidered that the use of two virial coefficients to describe the data for *N*-methylacetamide is to be preferred.

From the values of k, B, and C determined in this investigation, it is possible to evaluate the relative distribution of oligomers at various concentrations using the definition of the indefinite association constant. These results are presented in Figure 6. It may be noted that the average oligomer size, N, rapidly falls off over the concentration range of 1.0–10^{-6} g/ml. Also shown is the product of the monomer concentration and indefinite association constant, and the restriction introduced in obtaining eq 5 is seen to be justified. The monomer concentration is found to level off with increasing solute concentration, and indicates that the concentration of monomer in pure *N*-methylacetamide is about 3.9×10^{-3} g/ml, or 0.39%.

Discussion

The present study represents an application of the recent theoretical treatment of indefinite associating systems by Adams and Lewis[15] for a system which clearly fits the model. The good agreement between theory and experiment corroborates both the theory and the experimental observations. The fact that two virial coefficients provide the best overall fit of the data emphasizes the importance of taking the thermodynamic nonideality of the system into account. Although the work of Faucher and Kegeles[18] predicts a maximum in the relationship between the refractive index gradient and radius under conditions of flotation equilibrium, it can be easily shown that this maximum would occur much closer to the center of rotation than at the position observed for the maximum in this investigation. A recent ultrasonic absorption investigation of *N*-methylacetamide in carbon tetrachloride[19] presented evidence that the thermodynamic properties of the system were concentration dependent. The results of this investigation lend quantitative support to this conclusion. This use of a virial coefficient was also required in a recent centrifugation study of the self-association of purine in aqueous solutions.[20]

It is considered that the base-line separation between solvent and solutions observed at the beginning of the equilibrium runs results from the concentration-dependent compressibility of the solutions, rather than from differences between the apparent molar refraction and apparent molar volume of the associated and non-associated solute species,[21] since the partial molal volume was independent of concentration and the area under the schlieren curve was proportional to concentration within the limits of experimental error. It should also be noted that values near the extremes of the cell were not used in some cases for lack of precision as experienced by Albright and Williams.[22]

The average degree of association may be estimated from the true weight-average molecular weight, and a value of 66 is found at 0.3 m in fair agreement with the estimate of 140 by Longsworth from diffusion studies.[10]

It is possible to compare the indefinite self-association constant determined in this study with previous estimates of the indefinite association constant.[33] When the present value of k is converted to units of moles/liter, it is found that the indefinite association constant obtained in this study is about 3–20 times higher than previous values. It can be shown that the effect of not explicitly introducing a dimerization step with an association constant less than the indefinite association constant is to give a slightly lower value for the indefinite association constant than what would have been found with the inclusion of the dimerization step. Therefore, the difference between our indefinite association constant and previous values is considered to reflect the fact that reliable data could be collected over a broader concentration range with the ultracentrifuge, and that corrections for thermodynamic nonideality were only made in this investigation.

Finally, a significantly higher value for the indefinite association constant raises the question as to which association constant is most relevant for use in evaluating the contribution of peptide hydrogen bonds to protein and polypeptide stability. The previous work of Klotz and Franzen,[7] Klotz and Farnham,[24] and Kresheck and Klotz[11] indicated that peptide hydrogen bonds contribute little to protein stability if the dimerization reaction is selected as a model for the peptide hydrogen bond. However, it could be argued that indefinite self-association is as good if not better a model as dimerization for peptide hydrogen-bond formation in polypeptides and largely helical proteins where a cooperative sequence of intact hydrogen bonds exists. The use of the indefinite self-association constant to estimate the hydrogen bond strength in a nonaqueous environment would reverse the conclusion of Klotz and Franzen[7] with respect to the free energy of peptide hydrogen-bond formation, and suggest that the peptide bond would have a stability of about 0.2 kcal/mol in a nonpolar environment as opposed to the aqueous nonbonded state. This would appear to be more consistent with the generally accepted role of hydrogen bonds in maintaining polypeptide stability. However, the dimerization reaction may still be the preferred model for hydrogen bonds in globular proteins which do not contain long sequences of hydrogen bonds. This could be debated. The conclusions of Kresheck and Klotz[11] with respect to the enthalpy of peptide hydrogen bond formation would appear to be unchanged for either model.

Therefore, a knowledge of the indefinite association coefficient for *N*-methylacetamide in CCl_4 solutions permits a reassessment of the role of peptide bonds in maintaining protein structure, and provides a more complete picture of the thermodynamics of self-associating nonaqueous systems.

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